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Multiple Origins of Cholinergic Innervation of the Cochlear Nucleus

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Abstract

Acetylcholine affects a variety of cell types in the cochlear nucleus (CN) and is likely to play a role in numerous functions. Previous work in rats suggested that the acetylcholine arises from cells in the superior olivary complex, including cells that have axonal branches that innervate both the CN and the cochlea (i.e., olivocochlear cells) as well as cells that innervate only the CN. We combined retrograde tracing with immunohistochemistry for choline acetyltransferase to identify the source of ACh in the CN of guinea pigs. The results confirm a projection from cholinergic cells in the superior olivary complex to the CN. In addition, we identified a substantial number of cholinergic cells in the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT) that project to the CN. On average, the PPT and LDT together contained about 26% of the cholinergic cells that project to CN, whereas the superior olivary complex contained about 74%. A small number of additional cholinergic cells were located in other areas, including the parabrachial nuclei.

The results highlight a substantial cholinergic projection from the pontomesencephalic tegmentum (PPT and LDT) in addition to a larger projection from the superior olivary complex. These different sources of cholinergic projections to the CN are likely to serve different functions. Projections from the superior olivary complex are likely to serve a feedback role, and may be closely tied to olivocochlear functions. Projections from the pontomesencephalic tegmentum may play a role in such things as arousal and sensory gating. Projections from each of these areas, and perhaps even the smaller sources of cholinergic inputs, may be important in conditions such as tinnitus as well as in normal acoustic processing.

Keywords

arousal; sensory gating; tinnitus; olivocochlear; pedunculopontine tegmental nucleus; laterodorsal tegmental nucleus

Cholinergic innervation of the cochlear nucleus (CN) has been demonstrated by many methods, including physiology, receptor binding, enzyme assays, histochemistry and

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immunohistochemistry (Comis and Whitfield, 1968; Caspary et al., 1983; Osen et al., 1984; Godfrey et al., 1987, ²⁰⁰⁰; Moore, 1988; Henderson and Sherriff, 1991; Sherriff and Henderson, 1994; Happe and Morley, 1998; Fujino and Oertel, 2001; Oertel and Fujino, 2001; Gomez-Nieto et al., 2008). Acetylcholine (ACh) can excite or inhibit cells in both the ventral cochlear nucleus (VCN) and the dorsal cochlear nucleus (DCN) (Caspary et al., 1983; Fujino and Oertel et al., 2001; Oertel and Fujino, 2001). The affected cells include fusiform and cartwheel cells in the DCN and a subgroup of stellate (multipolar) cells in the VCN (T stellate cells; Fujino and Oertel, 2001; Oertel and Fujino, 2001). Other cells in the VCN, including D stellate cells, octopus cells and bushy cells, appear to be relatively unaffected by cholinergic inputs.

ACh can have a significant effect on spontaneous firing as well as sound -evoked firing (Chen et al, 1994, 1998; Zhang and Kaltenbach, 2000; Fujino and Oertel, 2001; Oertel and Fujino, 2001). This effect may be of particular significance in tinnitus, a perception of phantom sounds that may be associated with abnormally high spontaneous activity in the DCN (and elsewhere in the auditory pathways; Kaltenbach and Afman, 2000; Salvi et al., 2000; Brozoski et al., 2002; Seki and Eggermont, 2003; Kaltenbach and Godfrey, 2008). Manipulations that can cause tinnitus, such as cochlear trauma, also cause changes in ACh-related enzymes and receptors in the CN (Jin and Godfrey, 2006; Jin et al., 2006). An exact role of ACh in tinnitus has yet to be identified. Nevertheless, the evidence suggests that ACh in the CN plays a role both in normal processing and in some pathological conditions. In fact, ACh has been implicated in changes associated with development and aging as well as with acoustic trauma (e.g., Morley and Happe, 2000; Morley et al, 2004; Meidinger et al., 2006). Identifying all the sources of the cholinergic innervation is an essential step toward understanding the roles of ACh in the CN.

Immunohistochemical studies suggest that there are very few cholinergic neurons within the CN (Godfrey, 1993; Motts et al., 2008). However, there has been substantial evidence for inputs originating from other brainstem areas. Many olivocochlear cells, which are known to be cholinergic, have axon collaterals that innervate the CN (Brown et al., 1988; Benson and Brown, 1990; Brown, 1993). These collaterals arise at least in part from medial olivocochlear cells. Some collaterals may arise from the lateral olivocochlear cells, although the prominence of such collaterals is less well established (Ryan et al., 1987). The likelihood of additional (i.e., non-olivocochlear) sources of cholinergic innervation of the CN was suggested by lesion studies in cats and rats (Godfrey et al., 1987, 1990). These studies confirmed a cholinergic pathway to the CN via the olivocochlear bundle. However, they also identified a substantial cholinergic projection that entered the CN via the trapezoid body (as opposed to the spatially segregated olivocochlear bundle) and appeared to originate in the SOC.

Sherriff and Henderson (1994) provide the only study in which retrograde tracers were injected into the CN and combined with immunohistochemistry to identify the source(s) of ACh. They identified cholinergic cells in the superior olivary complex as the source of ACh in the CN of rats. In addition to the expected labeling of olivocochlear cells, they identified a group of cholinergic cells in the ventral nucleus of the trapezoid body that were morphologically distinct from olivocochlear cells but that projected to the CN. These cells likely are the source of the cholinergic axons that enter the CN via the trapezoid body, as described by Godfrey et al. (1987). The results of Sherriff and Henderson (1994) provide a framework for interpreting cholinergic effects in the CN by establishing the SOC as the sole source of inputs.

We have been studying cholinergic innervation of other parts of the ascending auditory pathways. In an earlier study, we identified the sources of cholinergic input to the inferior

colliculus (Motts and Schofield, 2009). This auditory midbrain structure receives its cholinergic input from the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus. These two nuclei, referred to collectively as the pontomesencephalic tegmentum (PMT), are associated with many functions, including arousal, sleep-wake cycle, sensory gating, reward, and motor functions (e.g., Chen et al., 2006; Reese et al., 1995b; Rye, 1997; Vincent, 2000; Swerdlow et al., 2001; Diederich and Koch, 2005; Jenkinson et al., 2009; Garcia-Rill et al., 2010). The PMT nuclei are also the source of cholinergic innervation of the thalamus, including the medial geniculate body (Steriade et al., 1988; Motts and Schofield, 2010). The results suggested a clear distinction between the cholinergic innervation of the “middle” parts of the central auditory pathway – i.e., the auditory midbrain and thalamus – and cholinergic innervation of the CN, which constitutes the lowest part of the pathway. However, there were no data on the cholinergic innervation of the CN in guinea pigs (the species in which we had done our previous studies). In fact, the rat is the only species in which retrograde tracers have been used to identify the cholinergic inputs to the CN (Sherriff and Henderson, 1994). We sought to expand these observations to guinea pigs, and thus provide a basis for contrasting within a single species the two auditory-related cholinergic projection systems in the brainstem: those from the superior olivary complex vs. those from the pontomesencephalic tegmentum. The results demonstrate the expected cholinergic inputs from the superior olivary complex, and reveal an unexpected projection from the PMT. The latter nuclei contain, on average, about one quarter of the cholinergic cells that project to the CN. These results show 1) that the PMT provides a cholinergic projection to a wide expanse of the auditory pathways (from the CN to the thalamus), and, 2) that the CN receives dual cholinergic innervation, from the PMT and the superior olivary complex.

Experimental Procedures

All procedures were conducted in accordance with the Institutional Animal Care and Use Committee and NIH guidelines. Nine adult pigmented guinea pigs (Elm Hill Labs; Chelmsford, MA, USA) of either gender weighing 350-600 g were used. During all experiments, efforts were made to minimize the number of animals and their suffering.

Surgery

Each animal was anesthetized with halothane or isoflurane (4-5% for induction, 1.75-2.25% for maintenance) in oxygen. The head was shaved and disinfected with Betadine (Purdue Products L.P., Stamford, CT, USA). Atropine sulfate (0.08 mg/kg i.m.) was given to minimize respiratory secretions and Ketofen (ketoprofen, 3 mg/kg i.m.; Henry Schein, Melville, NY 11747, USA) was given for post-operative pain management. Moisture Eyes PM ophthalmic ointment (Bausch & Lomb, Rochester, NY, USA) was applied to each eye. The animal's head was positioned in a stereotaxic frame. Body temperature was maintained with a feedback-controlled heating pad. Sterile instruments and aseptic techniques were used for all surgical procedures. An incision was made in the scalp and the surrounding skin was injected with Marcaine (0.25% bupivacaine with epinephrine 1:200,000; Hospira, Inc., Lake Forest, IL, USA), a long-lasting local anesthetic. A craniotomy was made over the desired target coordinates using a dental drill. Following the tracer injection, Gelfoam (Harvard Apparatus, Holliston, MA, USA) was placed in the craniotomy site and the scalp was sutured. The animal was then removed from the stereotaxic frame and placed in a clean cage. The animal was monitored until it could walk, eat and drink without difficulty.

Retrograde tracers

Fluorescent tracers (red fluorescent microspheres [“red beads”], Luma-Fluor, Inc., Naples, FL, USA; Fast Blue, EMS-Chemi GmbH, Gross Umstadt, Germany) were deposited into the

cochlear nucleus (CN) via stereotaxic coordinates (Table 1). Either a Hamilton microsyringe (1 μ l or 10 μ l; Hamilton, Reno, NV, USA) or WPI Nanoliter Injector (World Precision Instruments, Sarasota, FL, USA) was used to deposit one of the tracers into the CN. Each syringe/ micropipette was dedicated to a single tracer. In order to include as much of the CN as possible, tracer was deposited at 1-3 locations in the nucleus (total volume = 0.09 - 0.4 μ l). Deposits were contained within the CN and did not invade the neighboring inferior cerebellar peduncle.

Perfusion and tissue processing

Five to seven days after surgery, the animal was perfused with Tyrode's solution followed by 250 ml of 4% paraformaldehyde in 0.1 phosphate buffer, pH 7.4, followed by 250 ml of the same fixative with 10% sucrose. The brain was removed and stored at 4 °C in fixative with 25-30% sucrose for cryoprotection. The following day the brain was prepared for processing by removing the cerebellum and blocking the remaining piece with transverse cuts posterior to the olive and anterior to the auditory cortex. Most cases were cut in the transverse plane. In some cases, the cortex was removed from the brainstem/ thalamus. The left and right sides of the brainstem/ thalamus were separated at the midline to be cut in the parasagittal plane. Each piece of tissue was frozen and cut on a sliding microtome into 50 μ m thick sections that were collected serially in six sets.

Putative cholinergic cells were identified with immunohistochemistry for choline acetyltransferase (ChAT). Details of the processing are described in Motts et al. (2008). 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 Laboratories, Burlingame, CA, USA: BA-5000) and labeled with streptavidin conjugated to a fluorescent marker (AlexaFluor 488 [AF488, green] or AlexaFluor 647 [AF 647; near-infrared, pseudocolored magenta], Invitrogen, Carlsbad, CA, USA) (see Table 1 for details). Stained sections were mounted on gelatin-coated slides, allowed to dry and coverslipped with DPX (Sigma). Control studies (Motts et al., 2008) included Western blot analysis of guinea pig brain tissue as well as elimination of tissue staining by pre-adsorption of the primary antibody with ChAT or by omission of primary or secondary antibody from the solutions.

Data analysis

Preliminary analyses showed that immunopositive, retrogradely-labeled cells (double-labeled cells) were present in substantial numbers in the superior olivary complex (SOC), the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT). Seven cases with robust immunostaining were used for quantitative analysis. We used a Neurolucida reconstruction system (MBF Bioscience, Williston, VT, USA) attached to a Zeiss Axioplan II microscope (Carl Zeiss MicroImaging, Inc., Thornwood, N Y, USA) to plot labeled cells in the SOC and PMT in a series of sections through these nuclei. Each type of label was plotted with a unique marker. The results of these plots were used for a quantitative summary of the distributions of the labeled cells. A small number of double-labeled cells were present in other nuclei in some cases; these will be described separately.

SOC nuclei were identified according to criteria published previously (Schofield and Cant, 1991; Schofield, 2005). In general, the SOC nuclei could be distinguished in the fluorescent sections. When necessary, borders were confirmed by comparison with adjacent thionin-stained sections or by removing the coverslips from the plotted sections, staining the sections with thionin and re-applying the coverslips. The LDT and PPT were identified as previously described (Leonard et al., 1995; Motts et al., 2008). For descriptive purposes, we

use the term “pontomesencephalic tegmentum (PMT)” to refer to the PPT and LDT collectively (Woolf and Butcher, 1989).

Figures showing the distribution of labeled cells were created with Neurolucida software (MBF Bioscience) and refined with Adobe Illustrator (Adobe Systems, Inc., San Jose, CA, USA). Photomicrographs were captured using either a Zeiss Imager Z1 fluorescence microscope and AxioCam HRm or HRc cameras (Zeiss) or a Zeiss Axioskop fluorescence microscope and Magnafire camera (Optronics, Goleta, CA, USA). Adobe Photoshop (Adobe Systems) was used to add scale bars, size and crop images, erase background around tissue sections, and adjust intensity levels. Images of ChAT positive cells immunolabeled with fluorescent marker AF647, which fluoresces at infrared wavelengths, were taken in a monochrome mode and pseudocolored with Adobe Photoshop.

Results

We identified sources of cholinergic input to the CN by combining fluorescent retrograde tracing and immunolabeling for ChAT. The results were consistent across all cases regardless of the tracer or the fluorescent marker used for ChAT. In each of the cases the principal sources of input were the PMT and SOC.

Injection sites and distribution of retrogradely-labeled cells

Injections of either Fast Blue (FB) or red beads (RB) were confined to the CN (Fig. 1). In two cases, the injection site was confined to DCN. In the remaining cases, the injection included parts of the DCN, the granule cell area and parts of both the anterior and posterior divisions of the VCN. While an ideal injection would have filled the entire CN, this was not accomplished in any single case. Few injections included the rostral extreme of the granule cell area or the spherical cell area (cf. Hackney et al., 1990), or the ventral-most extreme of the posterior VCN. As a result, it is unlikely that we labeled every cholinergic cell that projects to the CN. Significantly, all the tracer deposits were confined to the CN, so it is highly likely that those cells that were labeled have axons that terminate in the CN. The results were similar qualitatively across all the cases. It was not possible to identify differences in the distributions of labeled cells that could be related to the extent of the injections (of course, if such differences may exist, they would be revealed best by smaller injections). Retrogradely labeled cells were identified in numerous areas that contain cholinergic cells (Motts et al., 2008), including the LDT, PPT, SOC, ventral nucleus of the lateral lemniscus, lateral paragigantocellular reticular nucleus and parabrachial nucleus.

Retrogradely-labeled, immunopositive cells

ChAT-positive cells were identified throughout the brainstem, as previously described in detail (Motts et al., 2008). All areas that contained ChAT positive cells were examined for retrogradely labeled cells. Double-labeled cells, i.e., those that contained both tracer and immunofluorescence, were interpreted as cholinergic cells that project to the CN. Such cells were readily identified in the SOC and the PMT (Fig. 2) in every experiment. In each region, there were immunonegative as well as immunopositive retrogradely-labeled cells (e.g., Fig. 2 C, E, and F arrows). These cells may be non-cholinergic or the immunolabeling could have failed to label them (due perhaps to limited penetration of the antibodies). Incomplete ChAT penetration is unlikely to explain all the ChAT-negative cells because we observed tracer-labeled, immunonegative cells in close proximity to double-labeled cells.

Figure 3 illustrates the distribution of ChAT-immunopositive and double-labeled cells in the SOC and the PMT in a representative case. On average, the majority (74%) of the double-labeled cells were located in the SOC (n = 334 double labeled cells in 7 cases). Within the

SOC, the vast majority of the double-labeled cells (>90% of those in the SOC) were located in VNTB. The ipsilateral VNTB often, but not always, contained slightly more double labeled cells than the contralateral VNTB. On both sides, the cells were spread from the medial to the lateral extent of the nucleus. Outside the VNTB, most of the remaining olivary cells were located in the ipsilateral lateral superior olivary nucleus. Again, the cells were spread throughout the nucleus, with no apparent topography related to the injection site. In some cases, a few double-labeled cells could be found in the superior paraolivary nucleus (5 of 7 cases) and, rarely (2 cases), in the medial nucleus of the trapezoid body.

The double-labeled cells in the LDT and the PPT represent the major finding in the present study. On average, these cells constituted 26% of the double-labeled cells. Such cells were found bilaterally in both the PPT and the LDT, with more cells on the ipsilateral side. On each side, there were more cells in the PPT than the LDT on both sides. Within the PPT, double-labeled cells were more numerous in the middle and caudal parts of the nucleus, although cells were occasionally observed near the rostroventral border (e.g., Fig. 3, section R5).

Figure 4 provides a quantitative summary of the relative contributions of the major sources of cholinergic input to the CN. The majority of inputs arise from the SOC (Fig. 4, green arrows), which provides bilateral inputs with the ipsilateral side predominant. About one-quarter of the cholinergic input originates in the nuclei of the PMT (Fig. 4, red arrows). The PMT inputs are also bilateral, with an ipsilateral predominance.

A few other nuclei contained a small number of double-labeled cells. The most prominent was the parabrachial nucleus. Double-labeled cells were found in the medial and lateral subdivisions of this nucleus, but were not sufficiently numerous to comment further on the relative contributions of the subdivisions. Double-labeled cells were not present in the parabrachial nucleus in every case, but were numerous in others. For example, the case illustrated in Figure 3 contained double-labeled cells bilaterally in the parabrachial nucleus (Fig. 3, sections R2, R4, L1, L2). The only other nucleus that contained double-labeled cells was the lateral paragigantocellular nucleus. Retrogradely-labeled cells were present in this nucleus in all cases, but immunopositive cells were few and were present only in some cases. Double-labeled cells were not observed in other nuclei.

Discussion

The current study identifies the SOC and the PMT as major sources of cholinergic input to the CN (Fig. 4). The SOC input is similar to that reported in rats (Sherriff and Henderson, 1994). In contrast, this is the first report of projections from the PMT to the CN. These projections arise from both nuclei of the PMT - the LDT and the PPT. The results imply the existence of distinct cholinergic pathways that are likely to affect the CN in different ways.

Technical Considerations

Immunohistochemistry for ChAT is an established method for identifying cholinergic cells (Levey and Wainer, 1982; Armstrong et al., 1983; Maley et al., 1988). The antibody (Chemicon AB144P), fixation and processing have been validated for the guinea pig (Motts et al., 2008). We conclude that the immunolabeled cells are likely to be cholinergic. Interpreting the absence of immunolabeling can be more difficult, but the presence of immunonegative cells in close proximity to the immunopositive cells suggests that the former cells are non-cholinergic. In the PMT it is likely that the non-cholinergic cells use GABA or glutamate (Vincent et al., 1986; Lavoie and Parent, 1994; Ford et al., 1995; Leonard et al., 1995; Jia et al., 2003). In the SOC, the non-cholinergic cells are likely to include both glycinergic and GABAergic cells (Ostapoff et al., 1990, 1997).

Our injections did not spread beyond the CN, so it is likely that the retrogradely labeled cells have axons that terminate in the CN. Many olivocochlear cells have axon branches that innervate the CN (reviewed by Warr, 1992), so we expect that these cells were labeled in our experiments. The vestibular efferent cells are cholinergic cells located near the genu of the facial nerve (Strutz, 1982; Motts et al., 2008); they were immunopositive in our experiments but were not labeled by the retrograde tracers, suggesting that we did not label substantial numbers of cells that project to the inner ear but not to the CN.

Comparison with earlier studies

Sherriff and Henderson (1994) conducted the only previous study to combine retrograde tracers with a cholinergic marker to identify sources of cholinergic input to the CN. They identified two groups of cholinergic olivary cells that project to the CN. One group is readily identified as olivocochlear cells. The second group of olivary cells projects to the CN but not to the cochlea. In rats, these cells are located in the VNTB (like medial olivocochlear cells) but are smaller than the medial olivocochlear cells. We observed both large and small double-labeled cells in the VNTB in guinea pigs (compare Figs. 2A vs. 2B). The olivocochlear cells in guinea pigs are large (Robertson et al., 1987), similar to those in rats. We conclude that guinea pigs have olivocochlear and non-olivocochlear cholinergic cells in the SOC that project to the CN.

Functional Implications

Cholinergic effects have been seen throughout much of the CN, with evidence for direct cholinergic effects on fusiform and cartwheel cells in the DCN and on T stellate cells in the VCN (Caspary et al., 1983; Fujino and Oertel, 1991; Oertel and Fujino, 2001). The effects are often modulatory, affecting excitability and/ or spontaneous firing as well as responses to acoustic stimuli. The assumption that all cholinergic input originates in the SOC has provided the main context for interpreting the physiological results.

Functions associated with the SOC—Numerous functions have been associated with olivocochlear cells, including control of cochlear gain, reduction of masking, and selective attention (reviewed by Guinan, 1996). The collateral projections of olivocochlear cells to the CN have been proposed to allow a subset of CN neurons to encode sound intensity despite olivocochlear effects on cochlear gain (Benson and Brown, 1990). The olivocochlear branches terminate primarily in the granule cell areas of the CN, where the primary targets appear to be dendrites of stellate cells in the VCN (Benson and Brown, 1990). Fujino and Oertel (2001) showed that ACh increases the excitability of T stellate cells, supporting the idea that cholinergic inputs could compensate for the decreased afferent input resulting from olivocochlear effects on the cochlea. They suggested that these effects would increase the dynamic range of the system and would be advantageous for encoding spectral peaks in noise.

Very little is known about the cholinergic cells that project from the SOC to the CN but not to the cochlea. Since these cells are located in the ventral nucleus of the trapezoid body, they could provide feedback to the CN or relay signals from the contralateral ear or from higher centers (see discussion in Thompson and Schofield, 2000).

Functions associated with the PMT—The PMT has been implicated in many functions, including arousal, sleep/ wake cycles, motor control, sensory gating (including prepulse inhibition of the acoustic startle reflex), learning conditional avoidance and attention (e.g. Garcia-Rill, 1991; Steckler, 1994; Vincent, 2000; Steriade, 2004; Mena-Segovia et al., 2005; Winn, 2006; Yeomans et al., 2006; Jones, 2008). A close association between PMT cells and the auditory system is indicated by several facts. First, about half of

PMT cells respond to acoustic stimuli (Koyama et al., 1994; Reese et al., 1995a). Second, the PMT projects to a wide span of the auditory pathways: the present study documents projections to the CN and previous studies show projections to the auditory midbrain and thalamus (Hallanger et al., 1987; Steriade et al., 1988; Motts and Schofield, 2009; 2010).

A gating function may be associated with PMT projections to the CN. The PMT is essential for a form of sensory gating known as prepulse inhibition of the startle reflex (Yeomans et al., 2006; Li et al., 2009). Graham (1975) suggested that prepulse inhibition of startle might allow continued processing of sensory stimuli without the interruption that would be caused by the startle reflex. PMT projections to the CN may help to support this continued processing. A special form of gating may occur during sleep. PMT cells are known to change their firing in concert with changes in sleep state (e.g., from slow wave sleep to paradoxical sleep, or sleep to waking; Garcia-Rill, 1991; Jones, 1993; Vincent, 2000; Steriade, 2004). Many CN cells continue to respond to acoustic stimuli through all stages of sleep, but their responses can vary with the stage (Peña et al., 1992). It seems likely that the PMT projections to the CN could contribute to the modulation of these responses and, in so doing, may control the efficacy with which the signal is transmitted to higher levels.

The fact that the PMT projects to multiple centers in the ascending auditory pathways may reflect a gating role at each of these levels (i.e., CN, midbrain, thalamus). While a suppressive effect at any single location could stop the flow of information, getting a signal through a system that is broadly suppressed in a particular behavioral state may require enhancement (e.g., increased cellular excitability) at multiple stages in the pathway.

A cetylcholine and tinnitus—Acetylcholine has been associated with elevated spontaneous activity in the CN (Chen et al., 1998; Jin et al., 2005; Jin et al., 2006) and elevated spontaneous activity in the DCN has been associated with tinnitus (Kaltenbach, 2006; Kaltenbach and Godfrey, 2008). Several studies have revealed changes in ChAT activity and cholinergic receptors in the DCN in animal models of tinnitus (Jin et al., 2006; Kaltenbach and Zhang, 2007) or after cochlear damage (which can often lead to tinnitus) (Meidinger et al., 2006). The findings in the present study suggest that perhaps these “new” cholinergic sources – those outside the SOC – should be investigated for their potential role in tinnitus.

Functions associated with other nuclei—A small number of cholinergic cells project to the CN from areas outside the SOC and the PMT. The parabrachial nucleus is associated with interoception. It has connections with somatic and visceral sensory pathways as well as with numerous cortical and non-cortical limbic areas (e.g., see discussion in Yasui et al., 1985). It also has connections with motor centers, and may play a role in coordinating breathing and vocal control (Smotherman et al., 2006). Parabrachial projections to the CN could provide a means for interoceptive effects on auditory processing. Lenhardt et al., (2007) have pointed out an association between the parabrachial nucleus and tinnitus, and suggested that the nucleus plays a role in some of the negative bodily feelings that are often associated with tinnitus. Collectively, the cholinergic projections to the CN from the PMT and the parabrachial nucleus could have a very broad influence upon underlying mechanisms of tinnitus.

Descending systems and cholinergic innervation of the CN

All of the areas that we have identified as sources of cholinergic inputs to the CN are direct targets of descending projections from higher centers. The SOC receives extensive projections from the inferior colliculus (Thompson, 2005) and auditory cortex (Coomes and Schofield, 2004). The collicular projections target SOC cells that project to the CN

(Schofield and Cant, 1999), including olivocochlear cells (Thompson and Thompson, 1993; Vetter et al., 1993). The cortical projections also contact SOC cells that project to the CN, again including olivocochlear cells (Mulders and Robertson, 2000; Schofield and Coomes, 2006). The olivocochlear cells are cholinergic; whether any of the other targets of the cortical projections are cholinergic remains to be determined.

The PMT also receives projections from higher centers, including prefrontal cortex (Semba and Fibiger, 1992) and auditory cortex (Schofield and Motts, 2009). Preliminary evidence suggests that some of the auditory cortical projections make direct contact with PMT cholinergic cells that project to the CN (Schofield, 2011). We conclude that the cholinergic inputs to the CN are likely to be affected directly by many higher neural centers and, conversely, that many “higher” functions are in a position to affect processing in the CN via cholinergic intermediaries.

Conclusions

The CN in guinea pigs receives substantial cholinergic inputs from both the SOC and the PMT. The SOC projections are bilateral and similar to those described in rats. The nuclei of the PMT contain about a quarter of the cholinergic cells that innervate the CN. These projections originate bilaterally in the LDT and PPT, with the PPT providing the greater contribution. Other nuclei of the reticular formation provide smaller, but potentially significant, projections. The multiple sources of cholinergic innervation suggest distinct functions. The SOC projections have been implicated in intensity tuning, control of dynamic range and detection of spectral peaks in noise; some or all of these functions could rely on coordinated effects in the CN and the cochlea. The PMT projections, on the other hand, may be more related to arousal and sensory gating. An intriguing possibility is that one or more of the cholinergic projections may be closely associated to the plastic changes in the CN that are associated with tinnitus.

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Figure 1.

Photomicrographs showing a representative injection of Fast Blue (*FB*) in the right CN of case GP458. The left panel shows the fluorescence photomicrograph; the right panel shows the fluorescence overlaid on a brightfield image of the section after it was stained with thionin. The deposit site includes parts of both the dorsal and ventral cochlear nuclei, but does not spread beyond these regions. Note that some tissue from the center of the injection site fell loose during tissue processing, leaving a hole in the images. Scale bar = 1 mm. DCN – dorsal cochlear nucleus; icp – inferior cerebellar peduncle; VCN – ventral cochlear nucleus.

Figure 2.

Paired photomicrographs of labeled cells located in the SOC, PPT and LDT that project to the CN. The left image in each pair shows cells retrogradely labeled by either red beads (RB) or Fast Blue (FB). The right image shows cells that were immunopositive for the ChAT marker (visualized with AlexaFluor 488 [green] or AlexaFluor 647 [magenta]). Arrowheads indicate double-labeled cells. Arrows indicate cells that were retrogradely labeled but immunonegative for ChAT. **A, B**) cells in the ventral nucleus of the trapezoid body that project to the ipsilateral CN. **C, D**) cells in the PPT that project to the ipsilateral (C) or contralateral (D) CN. **E, F**) cells in the LDT that project to the contralateral (E) or ipsilateral (F) CN. GP442 (A,B,C,E); GP500 (D) GP639 (F). Sagittal sections (A,B,C,E); transverse sections (D,F). Scale bar = 10 μ m.

Figure 3.

Plots of parasagittal sections illustrating the distribution of ChAT-immunoreactive cells in the brainstem that project to the CN. A retrograde tracer (red beads) was injected into the right CN. Labeled cells are shown in the ipsilateral brainstem (left column) and the contralateral brainstem (right column). Double-labeled cells (red stars) contained red beads and were ChAT-immunoreactive. Additional immunoreactive cells that were not labeled by the retrograde tracer (green circles) are indicated in the superior olivary complex and in the nuclei of the pontomesencephalic tegmentum (pedunculopontine tegmental nucleus and laterodorsal tegmental nucleus, PPT and LDT, respectively). Each symbol represents one labeled cell. The extents of LDT and PPT are indicated by the distribution of ChAT+ cells. ChAT+ cells were present in other nuclei (e.g., in the cranial nerve nuclei) but are not shown here. The superior cerebellar peduncle (scp, shaded gray) serves as a useful landmark for the PPT. Sections are 300 μ m apart and are presented lateral to medial in each column. Scale bar = 1 mm. DCN - dorsal cochlear nucleus; IC - inferior colliculus; LDT - laterodorsal tegmental nucleus; LSO - lateral superior olivary nucleus; MNTB - medial nucleus of the trapezoid body; MSO - medial superior olive; PAG - periaqueductal gray; PB - parabrachial nucleus; Pn - pontine nucleus; PPT - pedunculopontine tegmental nucleus; SC - superior colliculus; SPN - superior paraolivary nucleus; VLL - ventral nucleus of lateral lemniscus; VNTB - ventral nucleus of the trapezoid body; 5 - motor trigeminal nucleus; 7 - facial nucleus; 7n - facial nerve. Case GP442.

Figure 4.

Quantitative schematic summary of the major sources of cholinergic input to the cochlear nucleus (CN). Arrows indicate projections from the superior olivary complex (SOC) and the two nuclei of the pontomesencephalic tegmentum, the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT). Numbers next to the origin of each arrow indicates the average contribution of each nucleus in terms of the number of ChAT-immunoreactive cells that were labeled by a retrograde tracer injected into the CN (results based on averages from 7 cases); arrow thickness is scaled to reflect the relative contributions of each area. The sections represent transverse sections through the CN and SOC (bottom section) and through the caudal midbrain (upper section), which contains both the LDT and the caudal part of the PPT. The superior cerebellar peduncle (scp) is shaded as a reference point for the PPT. IC – inferior colliculus.

Table 1

Locations of tracer injections and immunofluorescent tag used in each case. AF488 – AlexaFluor 488; AF647 – AlexaFluor 647; CN – cochlear nucleus; FB – Fast Blue; RB – red beads. Note that some of these animals included injections of other fluorescent tracers into other brain regions as part of separate investigations; these injections are not listed here.

Case	Tracer injected into left CN	Tracer injected into right CN	Immunofluorescent marker
GP400		FB	AF647
GP442		RB	AF488
GP444		FB	AF488
GP458		FB	AF488
GP462	RB		AF488
GP469		FB	AF488
GP493	FB		AF647
GP500	FB		AF647
GP639	FB		AF647