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MULTIPLE ORIGINS OF CHOLINERGIC INNERVATION OF THE COCHLEAR NUCLEUS

J. G. MELLOTT,[a] S. D. MOTTS[a,b] AND B. R. SCHOFIELD[a,b]*

[a]*Department of Anatomy and Neurobiology, Northeastern Ohio Universities College of Medicine, Rootstown, OH 44272, USA*

[b]*School of Biomedical Sciences, Kent State University, Kent, OH, USA*

Abstract—Acetylcholine (Ach) 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.

Key words: 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 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; Gómez-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, 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

*Correspondence to: B. R. Schofield, Department of Anatomy and Neurobiology, NEOUCOM, PO Box 95, Rootstown, OH 44272, USA. Tel: +1-330-325-6655; fax: +1-330-325-5916.
E-mail address: bschofie@neoucom.edu (B. R. Schofield).

Abbreviations: ACh, acetylcholine; ChAT, choline acetyltransferase; CN, cochlear nucleus; DCN, dorsal cochlear nucleus; LDT, laterodorsal tegmental nucleus; PMT, pontomesencephalic tegmentum; PPT, pedunculopontine tegmental nucleus; SOC, superior olivary complex; VCN, ventral cochlear nucleus; VNTB, ventral nucleus of the trapezoid body.

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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 superior olivary complex (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 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—that is, 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 of 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 an 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 CN via stereotaxic coordinates (Table 1). Either a Hamilton microsyringe (1 μl or 10 μl; 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

Table 1. Locations of tracer injections and immunofluorescent tag used in each case

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

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.