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PROJECTIONS FROM AUDITORY CORTEX TO MIDBRAIN CHOLINERGIC NEURONS THAT PROJECT TO THE INFERIOR COLLICULUS

B. R. SCHOFIELD*

Department of Anatomy and Neurobiology, Northeastern Ohio Universities College of Medicine, 4209 State Route 44, PO Box 95, Rootstown, OH 44272, USA

Abstract—We have shown that auditory cortex projects to cholinergic cells in the pedunculopontine tegmental nucleus (PPT) and laterodorsal tegmental nucleus (LDT). PPT and LDT are the sources of cholinergic projections to the inferior colliculus, but it is not known if the cortical inputs contact the cholinergic cells that project to the inferior colliculus. We injected FluoroRuby into auditory cortex in pigmented guinea pigs to label cortical projections to PPT and LDT. In the same animals, we injected Fast Blue into the left or right inferior colliculus to label PPT and LDT cells that project to the inferior colliculus. We processed the brain to identify cholinergic cells with an antibody to choline acetyltransferase, which was visualized with a green fluorescent marker distinguishable from both FluoroRuby and Fast Blue. We then examined the PPT and LDT to determine whether boutons of FluoroRuby-labeled cortical axons were in close contact with cells that were double-labeled with the retrograde tracer and the immunolabel. Apparent contacts were observed ipsilateral and, less often, contralateral to the injected cortex. On both sides, the contacts were more numerous in PPT than in LDT. The results indicate that auditory cortex projects directly to brainstem cholinergic cells that innervate the ipsilateral or contralateral inferior colliculus. This suggests that cortical projections could elicit cholinergic effects on both sides of the auditory midbrain. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: arousal, pedunculopontine nucleus, laterodorsal nucleus, sensory gating, acoustic startle, prepulse inhibition.

The inferior colliculus (IC) is a large midbrain nucleus that integrates auditory and other information from many brainstem nuclei and numerous cortical regions and serves as the primary source of auditory projections to the thalamus (Winer and Schreiner, 2005). Several types of data suggest that most or all of the IC cells could be affected by inputs from the brainstem cholinergic system. Acetylcholinesterase, the degradative enzyme for acetylcholine, as well as nicotinic and muscarinic receptors, the two major classes of cholinergic receptors, are distributed throughout the IC (Shute and Lewis, 1967; Schwartz, 1986; Glendenning and Baker, 1988; Henderson and Sherriff, 1991; Morley and Happe, 2000). Physiological studies have confirmed that most IC cells are affected by acetylcholine (Watanabe and Simada, 1973; Farley et al., 1983; Habbicht and Vater, 1996). These effects are considered modulatory in the sense that application of acetylcholine to IC cells has little effect on their firing at rest but can dramatically alter their responses to sounds. The effects vary across cells and include either enhancement or suppression of evoked responses. Finally, acetylcholine has been implicated in plasticity in the IC induced by fear conditioning (Ji et al., 2001). In this situation, pairing a leg shock with a tone can lead to changes in the frequency tuning of IC cells. This plasticity is blocked if the muscarinic antagonist atropine is applied to the IC prior to conditioning. It seems likely that acetylcholine plays multiple roles in the IC, but identifying these roles has been hindered by lack of information about the underlying circuitry.

The cholinergic inputs to the IC originate from two large tegmental nuclei—the pedunculopontine and laterodorsal tegmental nuclei (PPT and LDT; Motts and Schofield, 2009). These nuclei are well known as the primary sources of cholinergic projections to much of the brainstem and spinal cord as well as to the thalamus (e.g. Rye et al., 1987; Hallanger et al., 1987; Hallanger and Wainer, 1988; Woolf and Butcher, 1989). Their widespread projections are associated with a wide range of functions, including arousal, the sleep–wake cycle, motor control and sensorimotor gating (e.g. Diederich and Koch, 2005; Mena-Segovia et al., 2005; Winn, 2006; Jones, 2008; Takakusaki, 2008; Jenkinson et al., 2009).

Recently, we identified direct projections from primary auditory cortex to the PPT and LDT (Schofield and Motts, 2009). At least some of the cortical axons appear to terminate on the cholinergic cells. This finding was unexpected in that there are no other reports of auditory cortex, or other primary sensory cortical areas, projecting to the PPT or LDT. The functions of these projections would presumably be reflected in the projections of the target cells. We speculated that the auditory cortical projections contact PPT and LDT cells that project to other auditory nuclei. However, the PPT and LDT project to a number of auditory nuclei, including the medial geniculate body, IC, and cochlear nucleus, raising the question of which (if any) of these output pathways may be the targets of the auditory cortex (AC) projections (Hallanger et al., 1987; Shute and Lewis, 1967; Steriade et al., 1988; Tebecis, 1972; Woolf and Butcher, 1986; Motts and Schofield, 2005, 2009). For the present study, we combined anterograde and retrograde fluorescent tracers with fluorescent immunohistochemistry to determine whether AC axons are likely to contact cholinergic cells in the PPT and LDT that project to the

*Tel: +1-330-325-6655; fax: +1-330-325-5916.
E-mail address: bschofie@neoucom.edu (B. R. Schofield).
Abbreviations: AC, auditory cortex; ACh, acetylcholine; Aq, cerebral aqueduct; ChAT, choline acetyltransferase; FR, FluoroRuby; FB, Fast Blue; IC, inferior colliculus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus.

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inferior colliculus. The purpose of the present report was to determine whether the targets of the auditory cortical axons include cells that project to the inferior colliculus.

EXPERIMENTAL PROCEDURES

All procedures were performed in accordance with the Institutional Animal Care and Use Committee and NIH guidelines. Six adult pigmented guinea pigs (Elm Hill; Chelmsford, MA, USA) of either gender weighing 400–900 g were used. During all experiments, efforts were made to minimize suffering and the number of animals used.

Surgery

Each guinea pig was anesthetized with isoflurane (4%–5% for induction, 1.75%–2.25% for maintenance) in oxygen. The animal was given atropine sulfate (0.05 mg/kg i.m.) to minimize respiratory secretions during anesthesia and Ketofen (ketoprofen, 3 mg/kg i.m.; Henry Schein, Melville, NY 11747, USA) for post-operative pain control. The animal's head was shaved and disinfected. 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 technique 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 small hole was drilled in the skull 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.

Table 1 summarizes the locations of tracer injections. Injections into the auditory cortex were made as described previously (Schofield and Motts, 2009). Briefly, FluoroRuby (tetramethylrhodamine dextran amine, 10,000 MW, D-1817 Invitrogen, Carlsbad, CA, USA; 10% in saline) was injected into the left temporal cortex in each animal. Injections were made with a 10 μl Hamilton microsyringe, angled approximately perpendicular to the cortical surface. The syringe was inserted approximately 1 mm into cortex, 0.1–0.2 μl was injected as a single bolus, and then the syringe was removed. Injections were made at 5–18 sites in order to label projections from a wide area of auditory cortex. In all cases, the array of injections was centered on primary auditory cortex (A1), localized approximately according to surface landmarks (Bregma; pseudosylvian sulcus; cf. Wallace et al., 2000, 2002).

Stereotaxic coordinates were used to guide injections of Fast Blue (EMS-Chemie GmbH, Gross Umstadt, Germany; 5% in water) into one IC. Fast Blue was injected with a 1 μl Hamilton microsyringe. The tracer was injected at two to four sites within the IC (total volume=0.3–0.8 μl).

Table 1. Summary of injection sites

Experiment	Tracer in left AC	Tracer in left IC	Tracer in right IC
GP553	FR		FB
GP556	FR	FB	
GP557	FR	FB	
GP558	FR		FB
GP566	FR		FB
GP571	FR	FB	

List of tracers injected into the auditory cortex (AC) and the inferior colliculus (IC) in the experiments used for this study.

FB, Fast Blue; FR, FluoroRuby.

Perfusion and sectioning

After 7–14 days, the animal was perfused 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 at 4 °C in fixative with 25% sucrose. The following day the cerebellum was removed and the brain was frozen and cut in the transverse plane on a sliding microtome into 50 μm thick sections, which were collected serially in six sets. For identification of cytoarchitectural borders and landmarks, one series was stained with thionin. The remaining series were used for immunohistochemistry.

Immunohistochemistry

Putative cholinergic cells were stained with immunohistochemistry for choline acetyltransferase (ChAT). Details of the procedure 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 AlexaFluor 488 (green; Invitrogen, Carlsbad, CA, USA). 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, as used in the present experiments (Motts et al., 2008). Control studies included Western blot analysis of guinea-pig brain tissue as well as staining in tissue sections that was eliminated by pre-adsorption of the primary antibody with ChAT or by omission of primary or secondary antibody from the solutions.

Data analysis

Injection sites were plotted with a Neurolucida reconstruction system (MBF Bioscience, Williston, VT, USA) attached to a Zeiss Axioplan II microscope. 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. Cholinergic cell groups were identified as described previously (Motts et al., 2008).

Photomicrographs were obtained with a Zeiss Axioskop fluorescence microscope and Magnafire camera (Optronics Inc., Muskogee, OK, USA) or a Zeiss Imager Z1 fluorescence microscope and AxioCam HRm camera (Carl Zeiss, Inc., Thornwood, NY, USA). Adobe Photoshop CS3 (Adobe Systems, Inc., San Jose, CA, USA) was used to add scale bars, to adjust the size and cropping of images, to erase background around tissue sections, and to adjust brightness and contrast. Adobe Illustrator CS3 was used to add lines to the images in Fig. 1.

RESULTS

We combined anterograde and retrograde tracer injections with immunolabeling for ChAT to identify auditory cortical inputs to brainstem cholinergic cells that project to the IC. Details of the cortical injections and the distribution of labeled axons were described previously (Schofield and Motts, 2009). Briefly, the injections were centered in the A1 and, in some cases, extended into surrounding areas (e.g. the dorsocaudal field, ventrorostral and dorsorostral belt areas and most likely area S; Wallace et al., 2000). Fig. 1 shows several FluoroRuby (FR) deposit sites in the AC (Fig. 1A) and labeled axons in the ipsilateral PPT (Fig. 1B).

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