Cholinergic Responses of Acoustically-Characterized Cochlear Nucleus Neurons: an *in vivo* iontophoretic study in guinea pig.

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ABSTRACT

The responses of guinea pig cochlear nucleus neurons to *in vivo* iontophoretic application of various neurotransmitter agonists were recorded with extracellular multi-barrelled electrodes. Where possible, neurons were physiologically identified using strict criteria. Emphasis was placed on the action of cholinergic agonists in relation to the possible action of olivocochlear collateral innervation. Excitatory responses (increase in action potential firing) to glutamate were confirmed in a number of neuronal response types. Application of acetylcholine (ACh) or the broad spectrum cholinergic agonist carbachol produced reliable excitatory responses in about 47% of neurons (n= 29 out of 61 neurons). The remaining neurons were unresponsive to cholinergic agonists and no inhibitory responses were observed. Cholinergic responses were more common in dorsal cochlear nucleus (DCN) (73% of 30 neurons tested) than in ventral cochlear nucleus (VCN) (23% of 31 neurons). Of the total neuron sample in which cholinergic responses were investigated, 41 neurons were able to be categorized according to established acoustic response features. Excitatory responses to cholinergic agonists were seen in "Pauser-buildup" (Pb) and "Transient chopper" (Ct) response types. Primary-like neurons (PL and Pn) as well as "Onset chopper" (Oc) neurons (n=6) were unresponsive to either ACh or carbachol. Oc neurons also did not show any effect on their acoustic responses. Robust cholinergic responses were also seen in several VCN and DCN neurons that were either unresponsive to sound, or had acoustic response properties that did not fit standard classification. The results suggest a relatively more robust cholinergic innervation of DCN compared to VCN. The excitatory cholinergic responses of some Ct neurons and the lack of effect on Oc neurons are consistent with previous results in mouse brain slice studies, but are in conflict with reports of medial olivocochlear collateral excitatory responses in onset-type neurons *in vivo*. The results also indicate that a number of neurons of unknown identity may also receive cholinergic input.

ABBREVIATIONS

ACh	acetylcholine
CAP	compound action potential
CF	characteristic frequency
CN	cochlear nucleus
Cs	sustained chopper
Ct	transient chopper
DCN	dorsal cochlear nucleus
MOC	medial olivocochlear
Oc	onset chopper
Pb	pauser-buildup
PL	primary-like
Pn	primary-like with notch
PSTH	peristimulus time histogram
VCN	ventral cochlear nucleus

1. INTRODUCTION

The mammalian cochlear nuclear complex receives descending cholinergic innervation from several sources. Superior olivary complex inputs comprise the collateral projection of medial olivocochlear (MOC) efferent neurons (Benson et al., 1990; Brown et al., 1992; Brown et al., 1991; Brown et al., 1988), and a separate direct projection from the ventral nuclei of the trapezoid body (Sherriff et al., 1994). Tegmental areas also are known to supply cholinergic input to both the dorsal (DCN) and ventral cochlear nucleus (VCN) (Mellott et al., 2011). The importance of these descending systems in the modulation of ascending auditory information is still a matter of conjecture and the specific neuronal targets that receive cholinergic input within the diverse populations of cell types in the cochlear nuclear complex, is not firmly established. Anatomical studies show that MOC collaterals make excitatory synaptic contacts with dendrites of multipolar neurons in VCN (Benson et al., 1990; Benson et al., 1996). Two major populations of multipolar cells in VCN are the D-stellate, thought to correspond to Onset Chopper (Oc) neurons and the T-stellate, thought to correspond to the transient chopper (Ct) and sustained chopper (Cs) neurons (Oertel et al., 2001; Oertel et al., 1990; Smith et al., 1989; Smith et al., 2005). *In vivo* electrophysiological studies using both intra and extracellular recordings, indicate that onset type and Oc neurons are excited by activation of olivocochlear collaterals (Mulders et al., 2002; Mulders et al., 2007; Mulders et al., 2003; Mulders et al., 2009).

However, in mouse brain slices, excitatory responses to application of cholinergic agonists have only been reported in T-stellate cells and bushy cells (Fujino et al., 2001; Oertel et al., 2001). The latter authors specifically report a lack of cholinergic responses in D-stellate neurons, which evidence suggests correspond to Oc neurons (Oertel et al., 1990; Smith et al., 1989). The present study attempts to resolve some of these conflicts by combining *in vivo*

recording of cholinergic responses of cochlear nucleus (CN) neurons with detailed classification of their acoustic response types.

2. METHODS

2.1. Animals

Eighteen pigmented guinea pigs were used in this study. All anaesthetic and surgical methods conformed to the Code of Practice of the National Health and Medical Research Council of Australia and were approved by the Animal Ethics Committee of the University of Western Australia. All procedures, anaesthetic and surgical methods have been reported in detail previously (Vogler et al., 2011; Vogler et al., 2014). Briefly, animals received a subcutaneous injection of 0.1 ml atropine followed by an intraperitoneal injection of pentobarbitone sodium, 30 mg/kg and a 0.15 ml intramuscular injection of Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone). Anaesthesia was maintained with full Hypnorm doses every hour and half doses of Nembutal every 2 h. A tracheostomy was performed and animals were artificially ventilated using a mixture of 95% O_2 and 5% CO_2 (carbogen). Animals were mounted in hollow ear bars, and acoustic stimuli were delivered to the left ear using a closed calibrated sound system (½" condenser microphone Bruel and Kjaer, type 4134 driven in reverse). Body temperature was maintained between 37.5 and 38 ºC by a thermostatically-controlled heating pad. After exposure of the left cochlea a silver wire electrode was placed on the round window and a compound action potential (CAP) audiogram was routinely determined for each animal for the frequency range 4-24 kHz in order to establish normal hearing (Johnstone et al., 1979). Pure tone stimuli and the broad band noise search stimulus were synthesized by a computer equipped with DIGI 96 soundcard connected to an analog/digital interface (ADI-9 DS, RME Intelligent Audio Solution). This interface was also used to collect single neuron spikes for response classification.

2.2. Recording and iontophoresis

The left CN was exposed by a posterior craniotomy and aspiration of part of the cerebellum overlying the DCN. A recording and iontophoresis microelectrode assembly (Carbostar-4, Kation Scientific) was introduced into the CN at an angle of approximately 45degrees from the vertical plane. 4% Agar in 0.9% saline was used to fill the craniotomy and reduce respiratory pulsations of the brainstem. The electrode was advanced using a stepping motor microdrive and broad band noise was used as an acoustic search stimulus. For all neurons the depth from the DCN surface was recorded and the characteristic frequency (CF) was determined. Where single-to-noise ratio and stability of recording permitted, the neuron response type was classified as follows. Spontaneous firing rate was measured using a 10 second sample. Peri-stimulus time histograms (PSTHs) were then acquired using up to 250 repetitions (4/s) of a 50ms CF tone 20dB above threshold. For onset chopper neurons (Oc) neurons, a comparison of the response to a CF tone and broad band noise was also made. Response type classification was based on previously published criteria (Winter et al., 1995; Young et al., 1988). Primary-like (PL), transient (Ct) and sustained (Cs) chopper neurons were distinguished from each other using regularity analysis of the discharge during the tone burst as described previously (Winter et al., 1995; Young et al., 1988). Neurons were classified as Oc if they showed no spontaneous firing rate, two clear onset peaks with only weak sustained firing to tones 20-40dB above CF threshold, and a strong sustained firing in response to noise stimuli that was more robust than the response to CF tones of matched intensity. Onset "L" (OL) neurons were distinguished according to previously described criteria (Winter et al., 1995)by their absence of spontaneous firing, broad tuning, single narrow onset peak and a ratio of peak-to-sustained firing in their PSTH of greater than 10. Figure 1A-E shows examples of typical PSTHs for 4 of the classical response categories.

A number of neurons could not be classified with certainty because of a less than optimal signal-to-noise ratio, especially at the onset of CF tones, where multiunit activity could

contaminate the recording and prevent accurate triggering. These neurons were dubbed "unclassified". In addition to unclassified neurons, there were also neurons that did not fit the standard classification criteria, despite an adequate signal-to-noise ratio in the recording. An example of these "unidentified" neurons is shown in figure 1F. This neuron, recorded in DCN, showed a rather sluggish onset response, followed by a marked inhibition below the pre-stimulus spontaneous firing rate during the remainder of the tone burst and no evidence of a buildup in firing above spontaneous firing rate in contrast to typical pauser buildup (Pb) neurons (Fig. 1C). Other neurons in the "unidentified" category, were unresponsive to acoustic stimulation and were only detected by a non-zero spontaneous firing rate.

The central glass insulated carbon fiber of the electrode assembly was used for recording and three separate iontophoresis barrels were used connected to a microiontophoresis pump (Union-200, Kation Scientific). One barrel contained the drug of interest, a second barrel contained 200mM NaCl for delivering control injections and the third barrel containing 200mM NaCl was used as the current return path. In a small number of early experiments, glutamate and glycine, known excitatory and inhibitory transmitters in the CN, were used to test the overall feasibility of the iontophoretic methods. In the case of glutamate and glycine, the drug barrel was connected to the negative current output of the current source during injections of varying amplitudes of current. In the case of ACh and carbachol applications, the drug barrel was positive. Injection current amplitudes between 10 and 60nA were tested, depending on the occurrence of artifacts (see section on iontophoretic criteria below). A holding current ranging from 3-5nA of opposite polarity to the injection current was continuously applied when drugs were not being tested. Drug solutions consisted of acetylcholine chloride 0.2M pH 4.0, carbachol (carbamylcholine chloride) 0.2M pH 4.0, glutamate 0.5-1.0M pH 10, glycine 0.5M pH 4.0).

Single neuron responses to drug and control NaCl application were recorded using LabChart software (AD Instruments). In general, excitation or inhibition in neurons in both DCN and VCN was considered present when there was a clear increase or decrease in the spontaneous firing rate. For neurons with little or no spontaneous discharge (notably onset type neurons), the effect of drug application on the responses to sound was also investigated. The raw data were inspected offline and time-histograms of spikes before during and after iontophoresis were constructed using the Spike Histogram module in LabChart 7 (AD Instruments). In cases where multi-neuron activity was present, the spike sorting facility in LabChart was used to separate single neuron spikes on the basis of amplitude, duration and polarity. The same software tool was used to eliminate switching artifacts at the onset and offset of current injection, when constructing time histograms.

2.3. Iontophoresis criteria

Several difficulties were encountered while attempting to achieve successful iontophoretic application of drugs. Frequently, the iontophoresis barrels did not fill down to their tips, possibly due to fine particles trapped in the fine taper of the barrel, and their resistance remained high (greater than 12MΩ). This occurred despite stringent filtering of the filling solutions and the use of electrodes soon after manufacture. Reliable effects could not be elicited with such electrodes. On other occasions, current application caused marked and rapid changes in single neuron spike height, suggestive of a direct effect of the current on the cell membrane. Several criteria therefore had to be satisfied before the effect of a drug application was regarded as real. First, changes in neuron firing rate had to occur in the absence of spike height changes. Second, the onset and cessation of any changes in spike rate had to be delayed relative to the onset and offset of the injection current (this criterion made it less likely that

direct current effects were involved, and was consistent with a finite time taken for the drug to be ejected from the pipette tip and reach the target cell). Third, an absence of response was only accepted as genuine if the same iontophoresis electrode could be demonstrated to produce unambiguous responses in at least one other neuron in that experiment. In the case of cholinergic drug application, this latter criterion was most easily met by first recording in DCN, where it was found that strong excitatory responses were relatively common compared to VCN. Failure to elicit strong cholinergic responses in DCN required either a change of electrode, or termination of the experiment. Finally, wherever possible, a control application of the same current using the 200mM NaCl barrel was used to check for any effects that were not drug-related. Because of the difficulty in obtaining reliable filling of all iontophoresis barrels, this was not able to be performed in every experiment or in every neuron.

Figures 2 and 3 show examples of responses to iontophoretic drug application which satisfied the above criteria. In Fig.2B the applied drug was glutamate (positive holding current, negative injection current), and the neuron was an unclassified VCN neuron (CF=13.9 kHz). Figure 2A shows the absence of any effect in the same neuron when current was applied via the NaCl barrel. Figure 3B shows another neuron with an excitatory response to ACh (negative holding current and positive injection current). This neuron was unidentifiable on the basis of its PSTH. This neuron had a CF of 16 kHz and was located (based on the CF progression and depth within the electrode track,) close to the boundary between DCN and VCN. Again, when the same magnitude of current was applied via the NaCl barrel (Fig. 3A) no effect was observed. In both cases, the delayed effect of the iontophoretic current and the absence of any effect when current was applied via the NaCl barrel, are consistent with a specific drug action.

3. RESULTS

Table 1 summarizes the results of iontophoretic application of glutamate, glycine, carbachol and acetylcholine. The responses to iontophoretic applications which satisfied the criteria described above (see Methods), were recorded in 82 neurons. Of these, glutamate and glycine responses were tested in 19 and 2 neurons respectively. In the remaining 61 neurons, carbachol responses were tested in 17 and ACh responses in 44 neurons.

With respect to cholinergic responses, no inhibitory effects were recorded in any neurons in either VCN or DCN. A notable feature of the data overall was that excitatory responses to ACh were seen quite commonly in DCN (>70%, or 22 out of a total of 30 neurons). Of the 22 DCN neurons that showed excitatory responses, 5 were classified as Pb (CF range 0.9-15.9kHz) and of the remainder, 12 were unclassified (CF range 0.8-8.1kHz) because of technical limitations and 5 were unidentifiable despite a PSTH to sound being collected (see for example Figure 1F, CF= 3.5 kHz). Not all DCN Pb neurons were responsive to ACh, a lack of response being found in 4 (CF range 1.1-8.3kHz) out of the total of 9 Pb neurons included in the analysis. Three unclassified and 1 unidentifiable DCN neurons were also unresponsive to cholinergic drug application (CF range 7.3-27kHz). It was unlikely that this lack of responsiveness in some neurons was due to technical problems with the iontophoresis because of the requirement that the same electrode assembly produced successful responses in other neurons in the same animals

In VCN, excitatory responses to ACh or carbachol were seen much less frequently than in DCN. Of a total of 31 VCN neurons that met the criteria for accepting results of iontophoretic application, only 7 (~23%) showed excitatory responses as manifested by an increase in background firing rate. No neurons classified as PL (n=8, CF range 1.1-24.5kHz), Pn (n=2,

CF 6 and 4.7kHz), Oc (n=6, CF range 2.7-19.3kHz), OL (n=3, CFs 2.9, 8.5 and 14.7kHz) or Cs (n=1, CF=18.5kHz) showed any change in background action potential firing rate in response to applied ACh or carbachol. The low incidence of excitatory responses in VCN, which is deeper in the electrode track than DCN could be a result of blocking of the iontophoretic barrel with cellular debris such as myelin. We believe this is unlikely, because in many of the experiments, excitatory responses could be detected in DCN neurons and neuron clusters in multiple penetrations. In addition, several good examples of excitatory responses in VCN were obtained towards the end of the experiments, further suggesting that pipette blockage was not an issue when our criteria for successful iontophoresis were applied.

Of the total of 7 VCN neurons that showed clear excitatory responses to either ACh or carbachol, 3 were classified as Ct (CFs 2.7, 26.6 and 7.4kHz), one was unclassified for technical reasons and 3 were unidentifiable on the basis of their PSTHs in response to tones (two responded to 2.6 and 16kHz). The remaining unidentified VCN neuron was unresponsive to acoustic stimulation but showed robust increases in firing in response to application of ACh. Figure 4A shows, as a raw spike recording, an example of the excitatory effect of ACh on an identified Ct neuron. Figure 4B shows the same data presented as a spike rate-versus-time histogram and Figure 4C shows the lack of response of the same neuron to an identical current injection via the NaCl barrel.

The absence of effect of cholinergic agonists on Oc neurons was of particular interest because this response type has been suggested as a potential target of olivocochlear collaterals. Oc neurons have no background firing rate and in all 6 cases studied, there was no appearance of any action potential discharge in response to cholinergic agonists using a wide range of ejection current and durations. In order to investigate the possibility of a neuromodulatory effect on acoustically-driven responses, in 3 of the 6 Oc neurons, the effect of cholinergic drugs was further tested on the response to fixed intensity tones at the neuron's CF and in 1 Oc neuron

the response to broad band noise was also examined. In no case was there any detectable change in the strength of the acoustic response as a result of application of cholinergic agonists. A typical result is shown in figure 5, in which the CF tone intensity was set just above threshold so that the neuron responded to the tone approximately 50% of the time. There was no obvious change in this firing pattern during iontophoretic application of ACh. In this example the average number of spikes per tone burst was 0.33, 0.33 and 0.38 before, during and after Ach application, respectively (average of 19 tone bursts per epoch). A qualitatively similar result was found in the other two Oc neurons tested. It is worth noting that an additional two Oc neurons were tested for their responses to glutamate and in both cases, no action potential discharge could be elicited.

4. DISCUSSION

The present study has a number of limitations. The strict criteria we chose to apply before accepting drug-related responses as genuine, and the need to fully characterize acoustic response properties of each neuron, means that the final sample size is rather small. In addition, single cell iontophoresis combined with extracellular recording of spike activity suffers from problems of interpretation. For example, a lack of response in terms of spike rate changes does not exclude the possibility of more subtle neuro-modulatory changes that might be revealed by intracellular recording, either with sharp electrodes or patch clamping. A lack of response could be due to the drug not reaching the appropriate receptors that may be located on parts of the neuronal structure spatially too distant from the iontophoretic barrels. Alternatively, the response of some receptors may be critically dependent on the time course of arrival of the drug and iontophoretic application cannot readily mimic the rapid, pulsatile delivery normally associated with vesicular transmitter release from synaptic terminals. Hence a lack of effect must be interpreted with caution. Conversely, a positive response could be an indirect effect of the drug acting on neurons other than the one from which spike recordings are being made. By deliberately confining our observations to the short term effects of brief iontophoretic pulses we have attempted to reduce this possibility, but it cannot be definitively excluded. In addition, the fact that, except in rare instances (as in the case of Oc neurons), we only studied the effects of drug application on spontaneous firing rate, means that inhibitory effects could only be observed in neurons that exhibited a significant spontaneous firing rate.

Bearing these limitations in mind, the key findings of the present study in relation to cholinergic responses are as follows. Cholinergic excitatory responses were significantly more common and robust in DCN neurons compared to VCN. Our finding of exclusively excitatory responses in VCN is in broad agreement with an earlier study (Caspary et al., 1983) but in contrast these authors reported frequent inhibitory effects in DCN. There could be several reasons for this

latter discrepancy. Our technique of looking only at effects on background firing rate could not reveal inhibitory effects in neurons with low or zero spontaneous firing. Perhaps the most significant difference between the study by Caspary et al. (1983) and the present study is the duration of iontophoretic current employed. Caspary and co-workers (1983) mainly monitored effects as changes in PSTHs derived from acoustic presentations and to do this, they routinely used 25s of continuous drug applications. In some cases in DCN they report effects that required up to 180s to become evident as a reduced spike count. Such long drug applications may be necessary to activate more complex intracellular process than our brief current applications. However, by presumably creating greater spatial spread of the drug, the risk of activating remote elements of the DCN (and VCN) circuitry is increased, potentially generating indirect effects on the neurons from which recordings are being made. Finally, gathering summed firing rate data over a very long drug application time, could obscure initial rapid effects, as processes such as receptor desensitization and spike adaptation could become prominent. We therefore feel that our technique of inspecting rapid initial firing rate changes may more faithfully reflect the immediate and local physiological action of the applied drug.

Another potential complication when considering the relative paucity of cholinergic effects in VCN, is that cholinergic innervation might vary according to cell CF. For example, it is well known that MOC efferent innervation of the cochlea is maximal approximately in the 8-14kHz region of the tonotopically-organized receptor and conversely, is sparse in the extreme low and high frequency regions (Brown, 2016). If MOC collateral innervation of the VCN were similarly tonotopically organized, then low and high CF neurons might be less likely to exhibit cholinergic responses. However, there was no obvious relationship in the present data between cell CF and cholinergic responsiveness. Indeed, numerous examples of responsive and unresponsive neurons were found for neurons of low, medium and high CF across most categories, including unidentifiable neurons. We did not systematically characterize the

acoustic response properties of all DCN neurons encountered, but Pb neurons were commonly found to increase their firing as a result of either carbachol or ACh application. Previous studies have reported a range of both inhibitory and excitatory cholinergic effects in cartwheel, fusiform and granule cells of DCN (He et al., 2014; Koszeghy et al., 2012; Stefanescu et al., 2017). Fusiform cells probably correspond to the Pb response type (Godfrey et al., 1975; Rhode et al., 1983) that we found to frequently exhibit excitatory responses to cholinergic agonists. Cartwheel cells are large inhibitory DCN neurons with generally complex acoustic response properties (Portfors et al., 2007). We encountered a number of unidentifiable DCN neurons that were excited by cholinergic agonists and these may possibly have corresponded to cartwheel cells. Granule cells do not receive direct input from primary auditory nerve fibers, but instead receive a variety of descending auditory and non-auditory inputs (Weedman et al., 1996a; Weedman et al., 1996b). The granule cell domain is also known to receive input from MOC collaterals and it is therefore of interest that several unidentified and one acoustically-unresponsive neuron in our sample showed strong responses to cholinergic drug application. It has been reported that DCN hyperactivity after loud sound exposure can be reduced by application of carbachol to the surface of the DCN (Manzoor et al., 2013). Fusiform cells are generally agreed to be responsible for DCN hyperactivity and this finding is therefore in apparent conflict with our finding of excitatory cholinergic effects in Pb neurons. However, unlike our highly localized drug application to individual neurons, carbachol applied to the surface of DCN is likely to influence widespread and complex circuitry.

There are likely to be multiple sources of cholinergic input to DCN. Innervation of the CN by cholinergic collaterals of the MOC system is generally regarded as being mainly confined to VCN (Osen et al., 1969; Spangler et al., 1987), although recently, innervation of DCN by this system has been reported (Baashar et al., 2015). On the other hand, there is evidence of cholinergic innervation of DCN and VCN from non-olivocochlear neurons of the superior

olivary complex (Sherriff et al., 1994) and from non-auditory structures such as various tegmental nuclei (Mellott et al., 2011; Schofield et al., 2011).

As mentioned above, the primary focus of the present study was on the cholinergic responses of physiologically characterized cells of the VCN. In agreement with the findings of Caspary et al (1983) who found that 66% of VCN neurons did not change their activity after ACh application, many VCN neurons were not responsive to cholinergic drug application, suggesting a rather sparse cholinergic innervation of this structure. Although MOC collateral terminals are found within the body of VCN, most reports show that they tend to terminate in or close to granule cell and medial regions of the VCN (Brown et al., 1988). We did not find any cholinergic responses in PL and Pn neurons, known to correspond to the bushy cell category. Slow cholinergic modulation of properties of spherical bushy cells has been reported *in vitro* and *in vivo* (Goyer et al., 2016) and it is likely that our simple test of changes in spontaneous firing rate would not detect such more subtle, longer term changes.

In the present study, the only fully characterized VCN cells that did show an increase in background firing rate, were those that satisfied the specifications of Ct neurons. These excitatory cholinergic effects on Ct neurons are consistent with excitatory responses of T-stellate neurons to cholinomimetics described in mouse brain slices (Fujino et al., 2001). These authors also reported that D-stellate neurons were not responsive to cholinergic drugs, consistent with the present *in vivo* observation that Oc neurons did not show any effects of iontophoretic application of either ACh or carbachol. These findings however, are seemingly at variance with a number of electrophysiological studies *in vivo* reporting apparent excitatory effects of MOC stimulation in onset-like and identified Oc neurons and a lack of excitatory effects on Ct or Cs neurons (Mulders et al., 2002; Mulders et al., 2007; Mulders et al., 2003; Mulders et al., 2009). Moreover, one *in vivo* intracellular recording study reported inhibitory synaptic potentials in Ct or Cs neurons evoked by MOC stimulation (Mulders et al., 2003).

Anatomical studies report that MOC collaterals form synapses exhibiting excitatory structural characteristics on the proximal dendrites of multipolar neurons in VCN (Benson et al., 1990; Benson et al., 1996; Brown et al., 1988; Mulders et al., 2003) but it is unclear whether these correspond to D or T stellate neurons.

This lack of clear correspondence between various findings with regard to the targets of cholinergic MOC collaterals and the relationship to the present results, could be due to a number of issues. Responses to bath application of cholinomimetics may not relate in a simple manner to the effects of *in vivo* iontophoresis nor to the effects of electrical activation of cholinergic pathways. In addition, it is worth noting that we failed to evoke excitatory responses to applied glutamate in several Oc neurons, although they undoubtedly receive glutamatergic input from the primary cochlear afferents. This suggests that in Oc (and possibly other neuronal types) iontophoretic application from electrodes presumably positioned near to spike generating sites, may not always reach the appropriate neurotransmitter receptors that could be located on more distal cell structures. Hence it cannot be established with certainty that Oc neurons do not receive cholinergic input. Finally, we cannot exclude the possibility that the general anaesthetic used affected in some way the cholinergic response profile of particular cochlear nucleus neurons.

An intriguing finding in the present study is that strong cholinergic responses were found in a small number of VCN neurons that were responsive to sound, but whose response characteristics did not fit traditional criteria, and also in one neuron that was acoustically unresponsive, despite robust acoustic responses from other neurons in the same animal. This suggests that neuronal classes other than those traditionally identified may receive cholinergic inputs, perhaps from MOC collaterals. The role of these unidentified neurons in auditory processing is unknown.

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FIGURE LEGENDS

Figure 1 Examples of PSTHs used as part of the basis for classification of response types of cochlear nucleus neurons. All stimuli 50ms CF tone burst or noise, 20dB above CF threshold. A,B primary-like (PL) and sustained chopper(Cs) VCN neurons. Note that regularity analysis was used to confirm identity of primary-like, Ct and Cs neurons. C, pauser buildup (Pb) neuron in DCN. D,E response of same Oc neuron to CF tone (D) and equivalent level broadband noise (E). Note two clear peaks in tone response and more vigorous sustained response to noise. F, unidentified neuron in DCN which did not fit standard classification of response type.

Figure 2 Raw trace example of neuron that met the criteria for successful iontophoretic responses in cochlear neurons. Unclassified VCN unit CF 13.9kHz. Response to negative current from NaCl pipette, B, response to negative current from glutamate pipette. Small arrows and large brief switching artifact denote start and end of application of iontophoresis current. Grey large arrows show duration of current application.

Figure 3 Raw trace example of response to ACh. A, response to positive current from NaCl pipette; B, response to positive current from ACh pipette. Unidentifiable VCN neuron CF 16.0 kHz. Start and end of iontophoretic current application as well as barrel used denoted as in Figure 2.

Figure 4. Example of the excitatory cholinergic effect in an identified Ct neuron A, raw trace showing spontaneous firing rate and delayed increase in rate in response to ACh application. B,C show histograms of spike counts obtained after off-line spike sorting, aligned with raw trace in A. B: shows response to ACh application, C: shows lack of response to NaCl. Start and end of iontophoretic current application as well as barrel used denoted as in Figures 2 and 3.

Figure 5. Lack of effect of the cholinergic agonist carbachol on activity of an identified Oc neuron. A, long sequence of responses to 4/s near-threshold 50 ms CF tone bursts, before, during and after drug application (Start and end of iontophoretic current application as well as barrel used denoted as in Figures 2, 3 and 4.). Note occasional single spikes at onset of each tone burst. B, expanded record (shown in figure A with grey bar) showing structure of responses shown in A. Each tone burst (small icons above trace) elicits low level "hash" activity from more distant neurons, and onset spike of Oc neuron occurs only in about 50% of tone burst presentations.

Table 1: Summary of data from all neurons for which responses to iontophoretic application of glutamate, glycine, ACh or carbachol were investigated and the criteria detailed in Methods section were satisfied. In each case, application of one drug and where possible the NaCl control were investigated. "Unclassified" refers to neurons in which no response classification was attempted. "Unidentifiable" refers to neurons in which PSTH data were collected that did not fit established criteria or the neuron was unresponsive to sound. + excitatory; - inhibitory; 0 no effect.

		glutamate			**glycine**			**carbachol**			**ACh**		
Acoustic response type	**n**	**+**	**-**	**0**	**+**	**-**	**0**	**+**	**-**	**0**	**+**	**-**	**0**
Pb	9							1		2	4		2
PL	10	2								3			5
Pn	2												2
Ct	8	2						1		1	2		2
Cs	1												1
Oc	8			2						3			3
OL	3												3
Unidentifiable VCN	4									1	3		
Unidentifiable DCN	6							3		1	2		
Unclassified VCN	7	6						1					
Unclassified DCN	24	7				2					12		3
total	**82**	**17**	**0**	**2**	**0**	**2**	**0**	**6**	**0**	**11**	**23**	**0**	**21**

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