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Multiple Large Inputs to Principal Cells in the Mouse Medial Nucleus of the Trapezoid Body.

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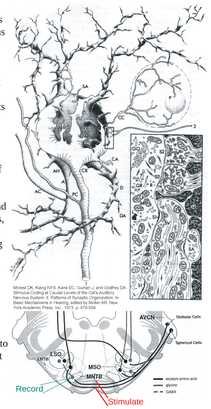
Abstract:

The calyx of Held is a giant nerve terminal that forms a synapse directly onto the principal cells of the medial nucleus of the trapezoid body (MNTB) in the mammalian auditory brainstem. This central synapse, which is involved in sound localization, has become widely used for studying synaptic transmission. Anatomical studies of this nucleus have indicated that each principal cell is innervated by only one calyx. Here we use previously established electrophysiological criteria of EPSC amplitude, kinetics, and transmitter type, as well as other characteristics commonly reported for this synapse, to examine the input properties of principal neurons. Our findings indicate that ~5% (5/94) of principal cells receive more than one strong, all-or-none, excitatory input. Each input had all the electrophysiological characteristics ascribed to the calyceal inputs to these cells, suggesting that some cells may receive multiple calyces.

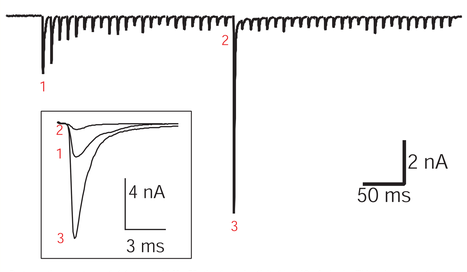
Introduction:

The architecture of the MNTB has been characterized anatomically at light and electron microscope levels. Axons from the globular bushy cells in the ventral cochlear nucleus cross the midline in the trapezoid body and give rise to one or two calyces [1]. Anatomical studies in the young and mature rat [2-5], cat [1, 6], and other animals [e.g. 7] have reported that each postsynaptic principal cell receives input from only one calyx. In addition to the calyceal synapse, other, smaller, bouton contacts onto the principal cell and its dendrites have been observed [e.g. 7]. One study in the cat indicated that the small contacts may cover as much of the principal cell as the calyx [1] although most studies have reported a lower fraction. These contacts have been distinguished from calyceal contacts by the size and type of vesicles they contain, and the curvature and symmetry (or lack thereof) of the active zones. Most of these small contacts are thought to be GABAergic or glycinergic [8] and did not appear to arise from the axons giving rise to calyces, with only up to 5% attributable to so-called "calycine collaterals"-boutons extending from calyces on neighboring principal cells [1, 6, 9-11]. Without serial sectioning and extensive reconstruction it is difficult to determine whether such contacts might originate from a calyceal process and whether all calyceal contacts truly arise from a single structure. Accordingly, it has been hypothesized that occasional instances of high calyceal-type coverage of MNTB principal cells may result from innervation by two calyces [8]. Nevertheless, a single calyx has been assumed to be the dominant source of input to the principal cell in most anatomical and physiological studies. This assumption is important for the interpretation of many of the experiments performed with this system.



- Anatomy has indicated that each principal cell receives only one calyx
- It is difficult to rule out the possibility of multiple calyces and it has been hypothesized that this sometimes occurs [8]
- Electrophysiologists using afferent fiber stimulation have no direct information on the morphology of their inputs

1 A Curious Observation (Cell A)



Postsynaptic current recorded during a 100 Hz, 50 stimulus train elicited by 110 µA shocks. This train produced the well-described presynaptic depression of the EPSC amplitude [e.g 12]. However, after the depression reached a steady state, one EPSC was much larger than the steady state amplitude (response marked "3"). In fact it was much larger than even the initial, non-depressed EPSC (response "1").

Responses marked 1-3 are expanded in inset and are aligned to the stimulus. Stimulus artifacts were blanked for clarity.

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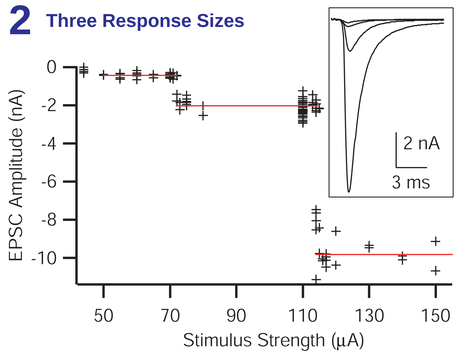
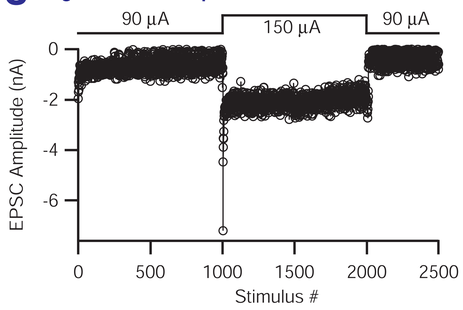
2 Three Response Sizes



EPSC amplitude as a function of the strength of single stimuli. Systematic variation of the stimulus strength applied to the bipolar electrode revealed three different EPSC size ranges in this cell. Below 50 µA little response was seen. Between 50 µA and 70 µA, the response averaged 0.44 nA. Stimulation between 72 and 113 µA elicited EPSCs with amplitudes of approximately 2 nA. Any stimulation over 115 µA elicited EPSCs with an average amplitude of 9.8 nA. Thus this cell appeared to have at least 3 inputs. Presumably the larger EPSC amplitudes included the EPSCs elicited with lower stimulation strengths, so by subtraction the three amplitudes were roughly 0.44, 1.6, and 7.8 nA (cell "A" in Table).

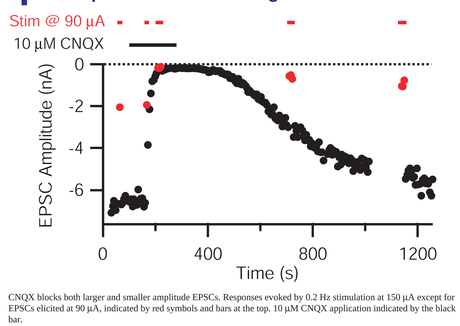
Red lines indicate average response levels for the stimulus ranges depicted. Inset depicts averages of 4-6 EPSCs at 44, 60, 75, and 117 µA.

3 High Threshold Input in Isolation



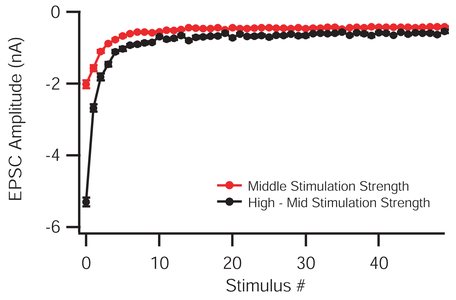
To confirm that the response to the higher stimulation strength was the sum of the lower and higher threshold inputs we utilized a protocol that allowed us to measure the response to the high threshold input in relative isolation. We first stimulated at 30 Hz at 90 µA-the middle of the medium threshold range. After ~1000 stimuli the EPSCs elicited by the medium and low threshold inputs were depressed to a low level (0.56 nA, 28% of their initial value). During continued 30 Hz stimulation the stimulus strength was increased to 150 µA, significantly above the high threshold level. The first EPSC following the increase in stimulus strength was 7.2 nA and presumably represented an undepressed high threshold input in addition to the 0.56 nA depressed response of the low and middle inputs. The EPSCs containing the high threshold input also quickly depressed to a steady state. In three such trials (one at 100 Hz) in this cell the first large EPSC averaged 7.5 ± 1.0 nA larger than the depressed responses preceding it, in accord with the previous estimate for the size of the high threshold input. Many failures occurred at 90 µA-this is common with extended high frequency stimulation near the threshold.

4 All Inputs are Glutamatergic



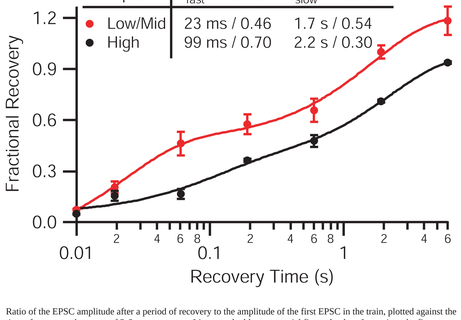
CNQX blocks both larger and smaller amplitude EPSCs. Responses evoked by 0.2 Hz stimulation at 150 µA except for EPSCs elicited at 90 µA, indicated by red symbols and bars at the top. 10 µM CNQX application indicated by the black bar.

5 Depression of Low/Middle and High Threshold Inputs



EPSC amplitudes for a 100 Hz train of 50 stimuli. The average responses to the middle stimulus strength (78-110 µA) are shown with red circles (N=31). These values were subtracted from the average responses to high stimulation strength (150 µA, N=18) and the differences are shown with black circles. Although the high threshold input started off almost three times as large as the low/middle threshold inputs combined, it reached a similar steady state response level after 8-10 stimuli. Some middle stimulation strength trials contained occasional large responses and were discarded.

6 Recovery from Depression



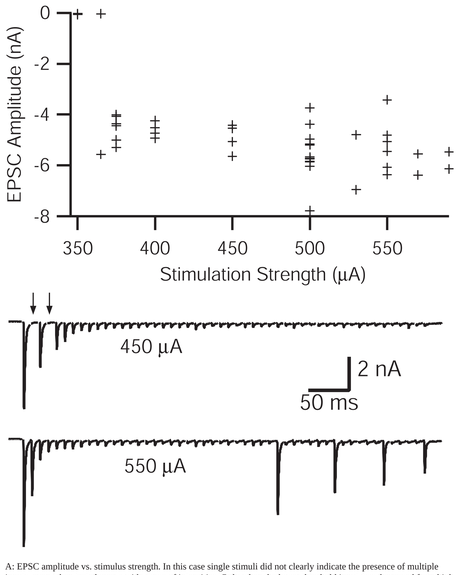
Ratio of the EPSC amplitude after a period of recovery to the amplitude of the first EPSC in the train, plotted against the time of recovery. Averages of 3-6 measurements. Lines are double exponential fits to the data. Inset gives the fit parameters. Trains were given every 40 s and only one recovery point was measured for each train.

7 Characteristics of Cells with Multiple Large Inputs

Cell	Age (days)	Strain	Stimulation Threshold (µA)	EPSC Amplitude[a] (nA)	Maximum Frequency[b] (Hz)	Latency[c] (ms)
A	10	C57BL/6	50	- 0.44 ±0.12	Not Tested	2.1 ±0.1
			72	- 1.6±0.4	300[d]	2.2 ±0.1
			115	- 7.8±0.6	300	2.2 ±0.05
B	7	C57BL/6 X 129SV	365	- 5.1±0.5	100	2.6 ±0.08
			500	- 3.5±0.6*	Unreliable	3.0 ±0.2
C	10	C57BL/6 X 129SV	240	- 2.3±0.4	≤ 200	2.0 ±0.07
			410	- 3.7±0.5	≤ 100	2.5 ±0.08
D	11	C57BL/6 X 129SV	105	- 0.73 ±0.23	Not Tested	1.9 ±0.07
			130	- 4.3 ±2.2	Not Tested	2.2 ±0.05
E	12	C57BL/6	215	- 1.1 ±0.2	Not Tested	2.2 ±0.09
			225	- 3.7 ±0.4	≤ 100	1.7 ±0.04
F	9	C57BL/6 X 129SV	365	- 2.5 ±0.5	Not Tested	2.2 ±0.1
			565	- 3.9 ±0.4	Not Tested	2.1 ±0.05

a Value for high threshold inputs determined by subtracting the value of lower threshold inputs except as indicated (*, tested after depression).
b Highest stimulation frequency at which no or very few failures were seen in 50 stimuli. Some cells were not tested to their limit.
c Time from onset of the stimulus artifact to the peak of the EPSC; higher threshold response latencies were measured after depression of the lower threshold response(s).
d Includes lower threshold response.

8 Properties of MNTB Principal Cell B



A: EPSC amplitude vs. stimulus strength. In this case single stimuli did not clearly indicate the presence of multiple inputs, even when tested over a wide range of intensities. Only when the lower threshold input was depressed from high frequency stimulation (see B) was the presence of a second input clear.

B: Postsynaptic currents during 100 Hz, 50 stimulus trains. For example, a train elicited by 450 µA (upper trace) stimulation resulted in typical depression, with two failures, to the second and fourth stimuli (arrows). An identical train with a stimulation strength of 550 µA (lower trace) resulted in a similar depression, with no failures and occasional large, undepressed EPSCs superimposed on the depressed lower threshold EPSCs. Stimulation artifacts were blanked for clarity.

Conclusions:

We have shown that a small fraction of principal cells in the mouse MNTB exhibit multiple discrete EPSC amplitudes in response to afferent fiber stimulation, indicating that these cells received multiple large excitatory inputs. These inputs met commonly used electrophysiological criteria for classification as originating from the calyx of Held (Barnes-Davies and Forsythe 1995; Forsythe and Barnes-Davies 1993a; Futai et al. 2001). Thus the results were consistent with innervation by multiple calyces of Held.

Without retrospective anatomical studies we could not be sure that the inputs were calyces, however their large amplitudes implied that whatever their form they must have covered a significant amount of the cell surface. Most MNTB principal cells have small dendritic arbors (Sommer et al. 1993), arguing against the possibility of a large collection of small dendritic contacts arising from a single axon or from a group of axons with extremely similar activation thresholds and other properties.

We found that careful orientation of the tissue during sectioning resulted in a relatively high fraction of MNTB cells on the surface of the slice retaining enough of their axons to be stimulated by the electrode placed at the midline of the slice (see Methods). It was possible that this may have increased the number of cells in which we were able to stimulate more than one input compared to other studies with a lower fraction of connected cells. Nevertheless, assuming all principal cells received calyceal input, we were rarely able to stimulate even half of the calyces near the surface of the slice. This implied that the fraction of cells which received multiple inputs may have been higher than our data indicated. If axons heading for the same cell ran together through the trapezoid body they may have had similar outcomes from the cutting procedure and the underestimate may be small, but if there was variation in the axons' paths the estimate could be low by a factor of two or more.

- Approximately 5% (or more) of MNTB principal cells receive multiple large inputs
- These inputs meet published criteria used to identify calyces of Held by electrophysiology
- Without detailed anatomical studies it is unknown whether any input is a calyx in this experimental paradigm
- The estimate of 5% may be low depending on axon severing during slicing

Implications:

If indeed some MNTB principal cells do receive multiple calyces it will be important to determine whether this is a transitory phenomenon during development like that seen in the other instances of strong single inputs or whether it persists in adulthood. If this is a transitory phenomenon it may prove to be a useful system for studying competition between inputs during development; conversely if it is a persistent phenomenon it will be important to determine its impact on the processing of auditory information.

The potential presence of multiple large inputs should be taken into consideration when performing experiments at this synapse which utilize afferent fiber stimulation. Our findings suggest two procedural safeguards in experiments of this type. First, in order to avoid unknowingly recording from cells receiving multiple inputs, the experimenter must test that the response size is constant with changing stimulation strength over a fairly broad range covering the stimulation frequencies used in the experiments, as stimulus threshold depends on the frequency of stimulation. As shown in Fig. 8, even this is not a perfect test. Choosing stimulation rates that produce a high fraction of failures, even if such frequencies will not subsequently be used in the experimental paradigm, may be more likely to reveal an instance of multiple inputs by revealing one input when the other fails. In addition to these strategies for avoiding instances of multiple inputs, a second safeguard may be to avoid recordings with smaller EPSC amplitudes. The combination of the fact that several of the inputs seen in the cases of multiple inputs were small, and the lack of anatomical evidence for multiple calyces, begs the question whether these small inputs are simply one end of a spectrum of calyx sizes, or whether they have a different underlying structure, which would likely imply other functional differences. Since limiting recordings to those with amplitudes greater than, for example, 2.5 nA, is not very restricting, such a criterion seems a reasonable precaution. Experiments utilizing simultaneous pre- and postsynaptic recording avoid most of the problems of multiple inputs, although it must be considered when monitoring spontaneous minature EPSCs that they may not arise from the input being studied.

- Possible developmental model for synapse elimination
- May affect processing of auditory information
- Must test over a range of stimulation strengths and at high frequency
- May be prudent to restrict experiments to inputs over 2.5 nA

Materials and Methods:

Slice Preparation. P7 to P12 C57BL/6 or C57BL/6X129SV mice were decapitated in accord with Yale's IACUC policies and the brainstems rapidly transferred to ice-cold low-Ca^{2+} artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl (125), KCl (2.5), $NaHCO_3$ (25), NaH_2PO_4 (1.25), dextrose (25), $CaCl_2$ (0.1), $MgCl_2$ (3), myo-inositol (3), sodium pyruvate (2), and ascorbic acid (0.4), bubbled with 95% O2/5% CO_2. Transverse brainstem slices (200-210 µm) were cut rostral to the seventh nerve. Careful orientation of the plane of sectioning was important to maximize the number of connected cells. Slices were cut at a slight (~5°) angle relative to the medial-lateral axis such that the axons giving rise to the calyces of Held ran up through the slice to the MNTB on one side, and recordings were only made from this side (see figure below). The dorsal-ventral axis was also controlled such that the rostral extent of the fourth ventricle was sliced with the ventro-rostral extent of the seventh nerve in the brainstem. Slices were immediately transferred to normal Ca^{2+} ACSF (as above with 2 mM $CaCl_2$ and 1 mM $MgCl_2$) and held at 35°C for 40-50 minutes and at room temperature thereafter until they were used.

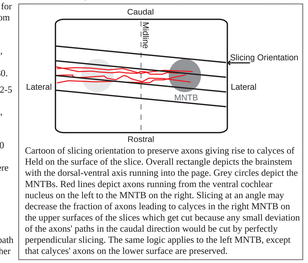
Electrophysiology. Slices were placed in a perfusion chamber (RC-26G Warner Instruments) on a Zeiss Axioskop 2 microscope and visualized with a 40X water-immersion lens and video (Hamamatsu C400-79H camera and C2400 controller) infrared differential interference microscopy. Slices were continuously perfused with ACSF warmed to 26°C (Warner SH-27A) at ~1 mL/min. ACSF contained 25 µM DL-2-amino-5-phosphonovaleric acid, 10 µM (-)-bicuculline methiodide 1S9R, and 500 nM strychnine HCl to block NMDA, GABA, and glycine receptors respectively.

Patch pipettes were pulled (P97, Sutter Instruments) from leaded glass capillaries (PG10165 World Precision Instruments (WPI)) and had a tip resistance of 1.5-2.5 MΩ when filled with an internal solution containing (in mM) CsF (110), CsCl (30), EGTA (5), HEPES (10), and QX-314 (2), adjusted to pH 7.3 with CsOH and to 295 mOsm with H_2O. After obtaining the whole cell configuration the series resistance was usually less than 7 MΩ and experiments were discarded if the series resistance was over 10 MΩ or changed significantly during the experiment. Experiments were also discarded if the leak conductance was greater than 5 nS. Series resistance was compensated by 80% with a lag of 10 µs using the built-in functions of the Axopatch 200B amplifier (Axon Instruments). The holding potential was -80 mV except for reversal potential experiments. No correction was made for the liquid junction potential which was estimated to be ~7 mV. Axons giving rise to the calyces of Held were stimulated via a bipolar electrode (15-30 KΩ typical impedance, FHC) positioned just ipsilateral to the midline of the trapezoid body by delivering brief (0.15 ms) constant current pulses. Stimulation was triggered by Clampex 8.2 (Axon Instruments) protocols through a Master 8 timer (AMPI) connected to a stimulus isolator (A360, WPI).

Cell selection. A glass micropipette was used as an extracellular electrode to search for cells on or near the surface of the slice that received input evoked by stimulation [12]. We began our search for cells on the lateral side of the MNTB, which might have biased our recordings for cells on this side, which receive input from globular bushy cells with a lower characteristic frequency due to the tonotopic organization of this nucleus [8, 13]. Slices varied in their fraction of connected cells, from ~ 1 in 2 to ~ 1 in 30.

Data Analysis. Currents were filtered at 2-5 KHz and digitally sampled (Digidata 1322A Axon Instruments) at 10-25 KHz, and recorded on a PC running Clampex. Data were analyzed offline in Igor Pro 4 (Wavemetrics) and Microsoft Excel 2000 (Microsoft) and expressed as mean ± standard deviation. EPSC amplitudes were computed as the peak current during the EPSC after subtraction of the baseline current immediately preceding the stimulation.

Chemicals. QX-314 was from Alomone Labs, bicuculline was from RBI, major bath constituents were from JT Baker, and other chemicals were from Sigma.



Cartoon of slicing orientation to preserve axons giving rise to calyces of Held on the surface of the slice. Overall rectangle depicts the brainstem with the dorsal-ventral axis running into the page. Grey circles depict the MNTBs. Red lines depict axons running from the ventral cochlear nucleus on the left to the MNTB on the right. Slicing at an angle may decrease the fraction of axons leading to calyces in the right MNTB on the upper surfaces of the slices which get cut because any small deviation of the axons' paths in the caudal direction would be cut by perfectly perpendicular slicing. The same logic applies to the left MNTB, except that calyces' axons on the lower surface are preserved.

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