Voltage-Dependent Currents in Isolated Vestibular Afferent Calyx Terminals

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Rennie, Katherine J. and Michele A. Streeter. Voltage-dependent currents in isolated vestibular afferent calyx terminals. J Neurophysiol 95: 26–32, 2006; doi:10.1152/jn.00641.2005. Na+ currents were studied by whole cell patch clamp of calyx-shaped afferent terminals attached to type I hair cells isolated from the gerbil semicircular canal and utricle. Outward K+ currents were blocked with intracellular Cs+ or with extracellularly applied 20 μM linopirdine and 2.5 mM 4-aminopyridine (4-AP). With K+ currents blocked, inward currents activated and inactivated rapidly, had a maximum mean peak amplitude of 0.92 ± 0.60 (SD) nA (n = 24), and activated positive to ~60 mV from holding potentials of ~70 mV and more negative. The transient inward currents were blocked almost completely by 100 nM TTX, confirming their identity as Na+ currents. Half-inactivation of Na+ currents occurred at −82.6 ± 0.9 mV, with a slope factor of 9.2 ± 0.8 (n = 7) at room temperature. In current clamp, large overshooting action potential-like events were observed only after prior hyperpolarizing current injections. However, spontaneous currents consistent with quantal release from the hair cell were observed at holding potentials close to the zero-current potential. This is the first report of intrinsic conductances in calyx terminals postsynaptic to type I hair cells in the mammalian vestibular system.

INTRODUCTION

Two types of hair cell and three classes of primary afferent fibers have been identified in the vestibular sensory organs of mammals, birds, and reptiles. The cup-shape terminals of calyx afferent fibers encase the basolateral surface of one or more type I hair cells (Wersäll 1956). Bouton afferents contact type II hair cells and dimorphic afferents make synaptic contact with both type I and type II hair cells (Fernández et al. 1988). The three populations of afferents differ in several respects including their electrophysiological response properties. For instance calyx fibers, which comprise 13% of the total number of primary afferents in gerbil semicircular canals (Desai et al. 2005a), are irregularly firing and have a comparatively low gain in response to rotational stimuli (Baird et al. 1988; Lysakowski et al. 1995). In addition, different complements of basolateral K+ channel are found in type I and type II vestibular hair cell populations. These genetically distinct K+ channel populations are thought to differentially filter mechanically gated transduction currents and may shape synaptic and primary afferent responses (Brichta et al. 2002; Correia and Lang 1990; Lennan et al. 1999; Rennie and Correia 1994; Rüsch et al. 1998). A low-voltage activated K+ current in type I hair cells confers a lower input resistance, which has been suggested to set the low gain of calyx afferents (Goldberg and Brichta 2002; Rennie and Ashmore 1991). However, relatively little is known about the transfer of information across the synapse between the type I hair cell and the calyx terminal. The presence of ribbon synapses and vesicles within the type I hair cell is consistent with conventional chemical transmission at this synapse (Gulley and Bagger-Sjöbäck 1979; Lysakowski and Goldberg 1997). Furthermore, intracellular recordings from calyx afferents of the lizard semicircular canal revealed spontaneous postsynaptic potentials, which in some instances gave rise to action potentials (Schessel and Highstein 1981). However, mathematical modeling studies have suggested that nonquantal transmission may also take place, as a result of K+ accumulation in the narrow intercellular cleft that exists between the hair cell and calyx (Goldberg 1996). This would require the presence of K+ channels on the inner face of the calyx membrane, adjacent to the type I hair cell (Goldberg 1996). In a study of Ca2+ currents in type I and type II hair cells that were papain-dissociated from the anterior crista of the rat, currents in both cell types had similar kinetic and pharmacological properties and were consistent with L-type Ca2+ currents. The half-maximum Ca2+ current occurred at ~46 mV and only ~1% of the Ca2+ current was estimated to be available at rest (~70 mV) in type I hair cells, suggesting that there would be little quantal release of transmitter from the hair cell at this potential (Bao et al. 2003).

As a first step to investigating the mechanisms of transmission between type I hair cell and calyx, as well as the initiation and propagation of action potentials in vestibular afferents contacting type I hair cells, we recorded from calyx terminals nonenzymatically dissociated from gerbil vestibular end organs. The calyx fiber transforms vestibular signals from type I hair cells into a frequency code of action potentials, but the ionic currents underlying this modulation have not previously been described. We report here that calyx terminals express a voltage-dependent inward current with rapid activation and inactivation kinetics, which was sensitive to TTX and abolished in Na+-free extracellular solution. These properties are consistent with a Na+ current. Outward K+ currents were also identified, based on their sensitivity to the K+ channel blockers linopirdine and 4-aminopyridine (4-AP). Furthermore, spontaneous currents consistent with transmitter release occurred at calyx holding potentials of approximately ~70 mV and action potential–like events with large overshoot could be elicited in current clamp from potentials more hyperpolarized than rest.

Preliminary accounts of this work have been published (Rennie 2003; Rennie et al. 2005).

METHODS

Cell preparation

Type I hair cells and their associated calyces were dissociated without the use of exogenous enzymes from the vestibular system of
Mongolian gerbils as described previously (Rennie and Correia 2000). Animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee and were within guidelines established by the American Physiological Society. Gerbils (3–12 wk old) were injected with pentobarbital sodium (50 mg/kg, ip) and ketamine (40 mg/kg, im), and the ampullae of the semicircular canals and utricles were removed from both ears. Animals were decapitated under deep anesthesia immediately after the removal of end organs. The end organs were transferred to saline containing (in mM) 135 NaCl, 5 KCl, 10 MgCl₂, 0.02 CaCl₂, 2 NaH₂PO₄, 8 Na₂HPO₄, and 3 d-glucose (pH 7.4 with NaOH and osmolality 305 mmol/kg). Epithelia were incubated for 32 min at 37°C (this improved the cell yield compared with keeping them at room temperature) and washed in a solution of Leibovitz’s L-15 medium (Life Technologies) containing bovine albumin (0.5–1 mg/ml) for ≥50 min at room temperature (18–22°C). Individual end organs were placed in standard L-15 medium (osmolality adjusted to 300–305 mOsm with distilled H₂O) in the recording chamber, and the epithelial surface was stroked with a probe to dislodge cells. Cells were allowed to settle for 10–15 min and were viewed on an Olympus upright microscope (BX50WI) equipped with DIC optics. Type I hair cells were recognized by their characteristic morphology (Ricci et al. 1997). In some cases, one or two type I hair cells remained attached to the chalice-shaped terminal portion of their calyx, which enclosed the basolateral regions of the hair cell. Occasionally, a short afferent stalk was attached to the calyx; however, most recordings were from calyces that lacked an obvious stalk (Fig. 1A).

**Electrophysiological recording and solutions**

Patch pipettes were pulled on a Sutter Instruments micropipette puller (P-87) from capillary tubing (PG165T, Warner Instrument Corp.) and coated with silicone elastomer (Sylgard, Dow Corning) after fire-polishing of the tips on a microforge (MF 83, Narishige). The electrode solution was “high K⁺” (in mM): 110 KF, 15 KCl, 27 KOH, 1 NaCl, 10 HEPES, 3 d-glucose, 1.8 MgCl₂, and 10 EGTA (pH 7.4) or “high Cs⁺,” where K⁺ in the solution was replaced with Cs⁺ to block outward K⁺ currents.

Conventional whole cell tight-seal patch-clamp experiments were carried out at room temperature (18–22°C) with an Axopatch-1D patch amplifier connected to a PC through an AD converter (Digidata 1320A, Axon Instruments). The patch electrode (initial resistance in the bath ranged from 1.0 to 3.5 MΩ) was sealed onto the outer face of the calyx, usually close to the base of the hair cell. After calyx recordings, it was often possible to remove the nerve terminal by gentle mechanical tugging with the patch electrode and make patch-clamp recordings from the associated type I hair cell with a second patch electrode. In whole cell voltage clamp, gerbil type I hair cells showed a large resting K⁺ conductance that activated at potentials above approximately −90 mV, i.e., 30–40 mV more hyperpolarized than outward currents in calyx terminals. This current, termed $I_{K,W}$, has been characterized previously in gerbil type I hair cells (Rennie and Correia 1994) and did not resemble K⁺ conductances in calyces. Furthermore, input resistance values for calyx fibers in our recordings were consistently >1.2 GΩ, whereas the mean input resistance in rodent type I hair cells is reported to be <50 MΩ (Chen and Eatock 2000; Rennie et al. 1996).

Signals were low-pass filtered on-line at 2 or 5 kHz, and the sampling rate was set between 10 and 20 kHz depending on the protocol used. Residual series resistance ($R_s$; mean, 3.3 ± 2.2 MΩ; $n = 44$) is stated for individual cells in the figure legends. Maximum voltage errors are estimated to be <6.4 mV and were not corrected; however, voltages have been corrected for liquid junction potentials. The mean input capacitance ($C_m$) for calyces was 3.6 ± 1.9 pF ($n = 44$). No leak subtraction was performed.

The extracellular superfusion solution was L-15. For Na⁺ replacement experiments, an extracellular solution containing (in mM) 140 choline chloride, 5 KCl, 1.8 MgCl₂, 1.3 CaCl₂, 10 HEPES, and 3 d-glucose (pH 7.4 with KOH) was used and replaced a similar HEPES-buffered solution containing 140 mM NaCl. Chemicals were obtained from Sigma (St. Louis, MO), and solutions containing drugs were applied locally to cells using gravity-fed flow pipes, with the exception of TTX, which was applied directly to the bath solution from a 1 μM stock solution made up in normal saline.

The stock solution of linopirdine was made up in DMSO before final dilution.

**Data analysis**

Analyses were performed and figures were generated using SigmaPlot (version 8, Jandel Scientific). Values presented are means ± SD unless stated otherwise. Spontaneous excitatory postsynaptic currents (EPSCs) in Fig. 5 were detected and analyzed using Mini Analysis software (version 6.0.3, Synaptosoft, Decatur, GA) with manual confirmation of events detected above a threshold approximately three times root mean square noise.

**RESULTS**

**Voltage-dependent ionic currents in calyx terminals**

Figure 1A shows a single type I hair cell with a cup-shaped calyx afferent surrounding the base and neck of the hair cell. In whole cell voltage clamp from holding potentials of −70 mV and more negative, most calyx endings showed rapid inward currents, followed by more slowly activating outward currents to applied steps when the electrode solution contained high K⁺ (Fig. 1B). In Fig. 1B, a slowly activating outward current was

![Figure 1](http://jn.physiology.org/)
Inward current is TTX sensitive

The Na⁺ channel blocker TTX (100 nM) was an effective blocker of inward currents as shown in Fig. 2. Figure 2A shows typical control currents for three different voltage steps after a prepulse potential to −120 mV. The transient inward current activated at membrane potentials positive to −60 mV. The maximum inward current was attained at approximately −30 mV, and the current magnitude then decreased with further depolarizations (Fig. 2B, ○). TTX reduced peak inward currents by a mean value of 81.1 ± 9.4% in four cells studied, and the block by TTX was partially reversible in two cells after washout (data not shown). These results indicate that a major component of the transient inward component is carried by TTX-sensitive Na⁺ channels.

In addition, the transient inward currents were completely abolished when the Na⁺ in the extracellular solution was replaced by choline in two cells and returned when Na⁺-containing extracellular solution was reintroduced (data not shown).

Block of outward K⁺ currents in calyx terminals

To allow a more detailed examination of the transient inward current, the outward current component was also minimized with externally applied K⁺ channel blockers. The transient inward current and sustained outward current, after a hyperpolarizing prepulse to maximally activate currents, are shown in control conditions and in the presence of 2.5 mM 4-AP in Fig. 3A. 4-AP reversibly blocked a large portion of the outward current and had no effect on the peak transient inward Na⁺ current. Outward currents were reduced at all potentials above −50 mV in 4-AP as shown in Fig. 3B. At positive potentials, 4-AP produced a block of peak current of 65.1 ± 7.6% in five cells tested.

Previously, immunoreactivity to the K⁺ channel KCNQ4 has been shown in calyx terminals in the vestibular periphery (Kharkovets et al. 2000; Lysakowski and Price 2002). The KCNQ channel blocker linopirdine (Schnee and Brown 1998; Wang et al. 1998) was therefore tested and was also found to inhibit a large portion of the outward current in calyx terminals (Fig. 3C). At 20 μM, linopirdine blocked outward currents by 76.9 ± 9.5% (n = 4), whereas a combination of 20 μM linopirdine and 4-AP (1 mM; Fig. 3C) produced a block of

![Image](http://jn.physiology.org/DownloadedFrom)
steady-state currents >90% at depolarized potentials, as shown in the I-V plot of Fig. 3C.

Inactivation of the Na⁺ current

The voltage-dependent inactivation of the Na⁺ current was examined using the protocol shown in Fig. 4A (bottom). The cell membrane was stepped to a range of conditioning potentials for 40 ms before stepping to the test potential at −20 mV. The largest inward currents were seen following steps to −110 mV and more hyperpolarized. The voltage-dependent inactivation plot for the transient inward current ($I_{\text{hmax}}$) for seven cells using the voltage protocol shown in Fig. 4A is shown in Fig. 4B and was fit with a Boltzmann function of the form

$$I_{\text{hmax}} = I_0 + \frac{1}{1 + \exp(-x)}$$

where $V$ is the conditioning potential, $V_{1/2}$ is the half-maximum inactivation potential, and $S$ determines the slope factor for inactivation. The mean value for $V_{1/2}$ was −82.6 ± 0.9 mV and for $S$ was 9.2 ± 0.8 (n = 7) at room temperature (18–22°C).

For neurons that fire at high-frequency rates, the speed of recovery from Na⁺ channel inactivation is important. We therefore studied the effect of altering the duration of the conditioning pulse in the inactivation protocol on Na⁺ current size (Fig. 4C). Conditioning pulses of duration 8, 40, 80, and 196 ms were used. As shown in the bar chart, where peak current values were normalized to values after the longest prepulse, the Na⁺ current was statistically significantly smaller after a prepulse of 8 ms compared with the longer pulses. However, currents were not significantly different in amplitude after prepulses of 40, 80, or 196 ms in duration. For this reason, a prepulse duration of 40 ms was routinely used for inactivation protocols.

Responses in current clamp

To study the role of Na⁺ currents in action potential firing, membrane responses to current injection were studied in current clamp with high K⁺ in the electrode solution. The mean zero-current potential for 14 cells was −53.6 ± 8.5 mV, and no spontaneous action potentials were recorded at rest in the neurons studied in current clamp. However, action potential-like responses with large overshoots were observed if the membrane was first hyperpolarized (Fig. 4D). In the example shown, the cell was held in current clamp and given a 100-ms hyperpolarizing current injection of 300 pA. After the termination of the current pulse, a biphasic membrane depolarization, subsequent rapid depolarization, and repolarization were observed (Fig. 4D, top trace). The large spike seen after hyperpolarizing pulses was consistent with recruitment of Na⁺ channels after hyperpolarization to remove inactivation of the channels. Although cells would repeatedly fire a single action potential after consecutive hyperpolarizations, no more than one action potential could be elicited after a single hyperpolarization.

As shown in Fig. 4E, the time-dependent inactivation kinetics of the current were well fitted with a double exponential function of the form

$$I(t) = I_{\text{hmax}}[\exp(-t/\tau_1) + \alpha_2\exp(-t/\tau_2)]$$

where $\tau_1$ and $\tau_2$ are the fast and slow time constants. For a voltage step from −109 to −31 mV, the values for $\tau_1$ and $\tau_2$ were 0.53 ± 0.14 (n = 7 cells) and 3.3 ± 0.86 ms (n = 7 cells), respectively.

Spontaneous activity

In voltage-clamp mode, spontaneous inward currents could frequently be recorded from isolated calyx afferents as shown in Fig. 5A. These currents are thought to be EPSCs resulting from the binding of transmitter, presumably glutamate, quantally released from the hair cell to the postsynaptic inner face of the calyx membrane. Similar results were seen previously in...
This may reflect a differential distribution of different types of Na$^+$ channel between the calyx terminal and the rest of the ganglion cell. Indeed the half-maximum inactivation value for calyx terminals is closer to values reported for Na$^+$ currents in vestibular hair cells of $-80$ mV or more negative (Chabbert et al. 2003; Géléc et al. 2004; Masetto et al. 2003; Mechaly et al. 2005; Rüsch and Eatock 1997). As described previously for hippocampal CA1 neurons (Bruehl and Witte 2003), mouse vestibular ganglion neurons (Chabbert et al. 1997), and cells transfected with the Na$^+$ channel α3-subunit (Moran et al. 2000; Ukomadu et al. 1992), the exponential inactivation kinetics of the Na$^+$ current were best described by a fast and slow component.

A rapid Na$^+$ current with similar properties has also been identified in type I spiral ganglion neurons of the mammalian cochlea (Moore et al. 1996; Santos-Sacchi 1993; Sun and Salvi 2001). The Na$^+$ current in type I neurons activated positive to $-70$ mV and like the Na$^+$ current described here was almost completely inactivated at $-60$ mV (Moore et al. 1996; Santos-Sacchi 1993). Only single spikes were observed in current clamp in response to a short depolarizing step from rest in spiral ganglion neurons (Lin 1997; Sun and Salvi 2001; Szabo et al. 2002).

The molecular identity of Na$^+$ channels on vestibular primary afferents is unknown. In mammals, there are at least nine sodium channel isoforms described to date (Goldin et al. 2000). In a preparation of the neonatal rat utricle, which would contain hair cells, supporting cells, and nerve terminals, the TTX-sensitive Na$^+$ channel α3 subunits 1.1, 1.2, 1.3, 1.6, and 1.7 were detected using RT-PCR (Mechaly et al. 2005). The same subunits were also detected in neonatal utricular hair cells using single cell PCR, with Na$^+$ 1.2 and 1.6 showing the most abundance (Mechaly et al. 2005). In another preliminary study, the TTX-insensitive subunit Na$^+$ 1.5 was reported to be present in calyx afferents in the rat utricle and crista using immunocytochemistry (Gaboyard et al. 2005). Both TTX-sensitive and TTX-resistant subunits may therefore contribute to Na$^+$ currents in calyx fibers and could explain why Na$^+$ currents were not completely blocked by 100 nM TTX in this study.

K$^+$ currents

The large outward K$^+$ current in calyx terminals was blocked $>90\%$ by a combination of 0.02 mM linopirdine and 1 mM 4-AP. Linopirdine at low concentrations (10 $\mu$M) is thought to be selective for K$^+$ channels of the KCNQ type (Wang et al. 1998) and has been shown to be an effective blocker of currents mediated by KCNQ4 in outer hair cells (Marconi and Kros 1999). Linopirdine at 20 $\mu$M does not block the type I-specific current $I_{K1}$ in type I hair cells, but does block a component of the $K^+$ current in type II vestibular hair cells (Rennie et al. 2001; Wong et al. 2004). The linopirdine-sensitive component described here suggests the presence of KCNQ channels in calyx endings. A likely candidate is the KCNQ4 channel, which has been detected on the inner face of calyx terminals using immunocytochemistry (Kharkovets et al. 2000; Lysakowski and Price 2002). Three types of outward K$^+$ currents, two of which were blocked by 4-AP, have previously been identified in enzymatically dissociated mouse vestibular ganglion neurons (Chabbert et al. 2001). One of the 4-AP-sensitive currents was blocked by dextrotoxin and margatoxin;
the other was selectively blocked by blood-depressing sub-
stance (250 nM). However, because neurons were isolated
from the superior branch of the vestibular nerve, the type of
hair cell originally contacted by the afferent fiber was not
known in these previous studies (Chabbert et al. 1997, 2001).
A third slowly activating K\(^+\) current, sensitive to tetraethyl-
ammonium but insensitive to 4-AP, was also described in
ganglion cells (Chabbert et al. 2001). It has been suggested that
calcium-activated K\(^+\) channels may underlie the afterhyperpo-
larization current and irregular firing pattern typical of calyx
afferents (Desai et al. 2005b; Goldberg 2000). Further exper-
iments are needed to determine the molecular identity of K\(^+\)
channels and to study possible differences between irregularly
and regularly firing vestibular afferents.

Vestibular hair cells and transmitter release

The electrophysiological properties of vestibular type I and
type II hair cells differ greatly, owing to the presence of
different populations of K\(^+\) channels in these cells (Brichta
et al. 2002; Correia and Lang 1990; Rennie and Correia 1994;
Rüsch et al. 1998). One notable observation is the presence of
a delayed rectifier active at unusually negative potentials in
type I hair cells, which in mammalian and turtle cells produces
an input resistance typically <50 MΩ (Chen and Eaton 2000;
Goldberg and Brichta 2002; Rennie et al. 1996). The relation-
ship between hair cell currents and afferent firing patterns has
not been elucidated, but the low input resistance of type I hair
cells may contribute to the low gain of calyx afferents (Gold-
berg and Brichta 2002; Rennie and Correia 1994; Rüsch and
Eaton 1996).

Hair cells continuously release neurotransmitter and vestib-
ular afferents in intact preparations are tonically active (re-
viewed in Goldberg 2000). Spontaneously firing action poten-
tials were not recorded in this study; instead, neurons required
prior hyperpolarization to evoke action potential-like events in
current clamp. The negative inactivation range of the Na\(^+\)
current reported here and paucity of open Ca\(^{2+}\) channels in
type I hair cells at −70 mV (Bao et al. 2003) would tend to
prevent spontaneous firing.

A calyx terminal would require a hyperpolarization of sev-
eral millivolts from the measured zero-current potential to
remove Na\(^+\) channel inactivation and allow action potential
generation. One source of this hyperpolarization could be
through the efferent system. Efferent fibers are known to make
synaptic contact with the outer face of calyces and stimulation
of the efferent fibers excites calyx afferents (Goldberg
1996). The efferent effect could result from a hyperpolarization
mediated through the α9/α10 nicotinic acetylcholine receptor,
coupled to the activation of small conductance calcium-acti-
vated K\(^+\) channels (Holt et al. 2003). Through this mechanism,
activation of efferent fibers could hyperpolarize calyx termin-
als, remove Na\(^+\) channel inactivation, and increase the like-
ilhood of action potential firing. In addition, the K\(^+\) concen-
tration in the intercellular cleft between the type I hair cell and
its calyx is unknown. In vivo K\(^+\) levels may be lower than in
the cut nerve preparation described here, resulting in a more
hyperpolarized resting potential than reported in isolated cells.

Isolated type I hair cells have a very low input resistance and
a resting membrane potential much more negative than the
activation threshold for their Ca\(^{2+}\) channels (Bao et al. 2003).

Transmitter release in hair cells is linked to Ca\(^{2+}\) influx and
release increases as a function of depolarization. However, the
detection of EPSCs indicates that spontaneous release of trans-
mitter from the type I hair cell occurs readily in this preparation
and supports chemical transmission across the synapse. Results
from a theoretical study by Goldberg (1996) suggested that K\(^+
\) could accumulate in the intercellular cleft during vestibular
transduction. A K\(^+\)-induced depolarization of both type I hair
cells and calyx could boost chemical transmission across the
synapse. Although our results do not rule out such an effect,
they indicate that transmitter release can occur from type I hair
cells at rest and in normal extracellular levels of K\(^+\). The
postsynaptic events are similar to those described in afferents
contacting inner hair cells and the decay time constant we
found for calyx EPSCs of ~1 ms is comparable with that
described in auditory afferents (Glowatzki and Fuchs 2002).
Similar to other cells that release neurotransmitter in a graded
fashion, hair cells have ribbon synapses consisting of a dense
core surrounded by a halo of vesicles (Gulley and Bagger-
Sjöbäck 1979). In the chinchilla crista, each type I hair cell was
found to have, on average, 15–20 ribbon synapses (Lysakovski
and Goldberg 1997). In auditory hair cells, individual ribbons
can release several vesicles simultaneously (multivesicular
release) and as described here for type I hair cells, release can
occur spontaneously in the absence of depolarization. In audi-
tory fibers, amplitude distributions remained skewed even at
depolarized membrane potentials and the mean number of
vesicles for each EPSC did not increase, indicating a lack of
Ca\(^{2+}\) dependence (Glowatzki and Fuchs 2002). Further exper-
iments in isolated calyx fibers at depolarized potentials and in
the presence of raised extracellular K\(^+\) should determine
whether vestibular calyces behave similarly and will also shed
light on the K\(^+\) accumulation hypothesis proposed by Gold-
berg (1996).

Primary vestibular afferents often integrate information from
several hair cells; in the case of type I hair cells, a single calyx
afferent may contact one or more type I hair cells and can also
receive input from type II hair cells (Goldberg 2000; Lysa-
kowski et al. 1995). This newly described isolated preparation
comprising a single type I hair cell and its associated calyx
terminal should prove valuable in elucidating the synaptic
properties of this unusual synapse.

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