Subthreshold sodium current underlies essential functional specializations at primary auditory afferents

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ABSTRACT

Primary auditory afferents are generally perceived as passive, timing-preserving, lines of communication. Contrasting this view, identifiable auditory afferents to the goldfish Mauthner cell undergo potentiation of their mixed, electrical and chemical, synapses in response to high frequency bursts of activity. This property likely represents a mechanism of input sensitization as they provide the Mauthner cell with essential information for the initiation of an escape response. Consistent with this synaptic specialization, we show here that these afferents exhibit an intrinsic ability to respond with bursts of 200-600 Hz and that property critically relies on the activation of a persistent sodium current, which is counterbalanced by the delayed activation of an A-type potassium current. Furthermore, the interaction between these conductances with the membrane passive properties supports the presence of electrical resonance, whose frequency preference is consistent with both the effective range of hearing in goldfish and the firing frequencies required for synaptic facilitation, an obligatory requisite for the induction of activity-dependent changes. Thus, our data shows that the presence of a persistent sodium current is functionally essential and allows these afferents to translate behaviorally relevant auditory signals into patterns of activity that match the requirements of their fast and highly modifiable synapses. The functional specializations of these neurons suggest that auditory afferents might be capable of more sophisticated contributions to auditory processing than has been generally recognized.

Keywords: auditory afferent; Mauthner; persistent sodium current; electrical resonance; repetitive firing; electrical synapses; gap junctions; synaptic plasticity; LTP.
INTRODUCTION

Primary afferents are generally perceived as canonical passive lines of communication between peripheral receptors and second order sensory neurons located in the central nervous system. A special class of auditory afferents terminating as, single, “Large Myelinated Club Endings” on the goldfish Mauthner (M-) cells (Bartelmez 1915), a pair of large reticulospinal neurons that mediate tail-flip escape responses in fish (Eaton et al. 2001), challenge this perception as their synapses undergo activity-dependent potentiation (Yang et al. 1990; for review see Pereda et al. 2004). That is, discontinuous, “burst-like”, stimulation of these afferents with high frequency trains evokes a long-term potentiation of both components of their mixed, electrical (gap-junction mediated) and chemical, synaptic response (Yang et al. 1990). The plastic properties of these synapses likely represent a mechanism for input sensitization (Yang et al. 1990) and therefore an unusual specialization for a primary auditory afferent which, in contrast to most auditory afferents that faithfully rely critical timing information for its processing along the auditory pathway, provide a decision-making neuron (Eaton et al. 2001) with relevant sensory information which could be directly translated into a behavioral response that is essential for the survival of the fish.

While the properties and mechanisms of plasticity of their single synapses have been the subject of detailed analysis (see Pereda et al. 2004 for review), little is known regarding the electrophysiological characteristics of these identifiable auditory afferents, in particular their ability to undergo high frequency repetitive firing, which constitutes an essential requirement for induction of synaptic plastic changes (Yang et al. 1990; Pereda and Faber 1996; Smith and Pereda 2003). The electrophysiological properties of primary auditory afferents have been generally investigated in several species (Santos-Sacchi 1993), including goldfish saccular afferents (Davis 1996), which constitutes the auditory
component of the VIII\textsuperscript{th} cranial nerve organ in fish (Furukawa and Ishii 1967; Furukawa 1978). Detailed biophysical analysis revealed the presence of a variety of sodium (Na\textsuperscript{+}) and potassium (K\textsuperscript{+}) conductances at both, goldfish saccular afferents (Davis 1996) and mammalian spiral ganglion cells (Santos-Sacchi 1993; Adamson et al. 2002; Jagger and Housley 2002; Mo et al. 2002; Davis 2003; Hossain et al. 2005; Dulon et al. 2006). Yet, is still unclear how these conductances interact in these neurons to shape their firing pattern under more physiological conditions and how they relate to their function. Repetitive firing is an essential property of auditory afferents, as changes in their firing rate are known to encode variations in stimulus intensity (Sachs and Abbas 1974; Pickles 1982). Interestingly, it has been suggested that auditory afferents might not constitute a homogeneous cellular population but rather, like some inner-ear hair cells (Fettiplace and Fuchs 1999), could be electrophysiologically tailored to their functional roles (Adamson et al. 2002; Davis 2003).

Due to unfavorable anatomical characteristics (they span from peripheral receptors to their target in the central nervous system) the membrane and synaptic properties of primary auditory afferents have been generally investigated \textit{in-vitro} at either their central (Zhang and Trussell 1994) or peripheral ends (Santos-Sacchi 1993; Davos, 1996; Glowatzki and Fuchs 2002). Because of their advantageous experimental \textit{in vivo} accessibility (where anatomical integrity and synaptic connectivity are preserved) and critical role in the initiation of escape response, identifiable auditory afferents terminating as Large Myelinated Club endings on the M-cells provide with an ideal opportunity to link cellular biophysical analysis with system-level analysis of information processing. Here we show that these afferents are endowed with electrophysiological properties that allow them to translate their broad auditory frequency sensitivity into patterns of activity that are adapted to the requirements of their highly modifiable synapses. Consistent with
their ability to generate bursts of action potentials, these afferents are capable of sustaining high frequency repetitive firing and exhibit a strong frequency adaptation in response to depolarizing pulses. Our results also indicate that while their ability to sustain repetitive firing critically relies on the presence of a persistent Na\(^+\) current, its frequency adaptation results from the delayed activation of an A-type K\(^+\) current. Furthermore, the interplay of these conductances with passive membrane properties endows these specialized afferents with electrical resonance, whose band of frequency preference is consistent with both the goldfish’s hearing range and the firing frequencies required for facilitation of their chemical synapses, a requisite for the induction of long-term plastic changes.

**METHODS**

**Electrophysiological procedures.** The experiments were performed in adult goldfish (*Carassius auratus*) 3 to 5 inches long. The surgical and *in vivo* recording techniques were similar to those previously described (Lin and Faber 1988a; Pereda et al. 1995; Curti and Pereda 2004). Briefly, auditory afferents establishing mixed synapses on the M-cell known as “Club endings” (n=90) were intracellularly recorded outside the brain at the posterior branch of the VIIIth nerve, which contains these saccular afferents. Club ending afferents were identified by the presence of electrotonic coupling potentials after M-cell antidromic activation by stimulating the spinal cord (see Results). For most recordings, glass microelectrodes (30-45 M\(\Omega\)) were filled with 2.5 M KCl. Only afferents presenting resting potentials negative than -67 mV and action potentials larger than 70 mV in amplitude were used for this study. Due to the fast membrane time constant of the afferent fibers (estimated in \(\sim 200 \mu s\) in the present study) the bridge was balanced using the “spike-height method” (Frank and Fourtes 1956). For intracellular recordings of the
M-cell, a second electrode (5 M KAc or 2.5M KCl, 4-12 MΩ) was inserted either 350-400 µm lateral to this cell’s axon cap into the lateral dendrite or into its axon, placing the electrode more caudally between the vagal lobes. To activate the saccular afferents, a bipolar stimulating electrode was positioned on the posterior VIIIth nerve, distal to the recording site. In the case of acoustic stimulation, sound stimuli consisted of a 500 µs broadband noise square pulses delivered by a 2.5 inches speaker (Quam; frequency response 0.2–8 kHz) located about 4 inches from the animal’s head, and connected to a Grass AM8 Audio monitor. Experimental data was acquired and recorded using software developed in the laboratory and analyzed using Kaleida Graph (Synergy Software), Igor Pro (Wave Metrics) and Superscope II (GW Instruments) software. Student’s t test was used to assess statistical significance of the data. Group data were reported as mean ± s.e.m., unless otherwise stated.

**Drug application.** The fish’s brain was continuously superfused (1.5 ml/minute) with ACSF (124 mM NaCl; 5.1 mM KCl; 3.0 mM NaH2PO4-H2O; 0.9 mM MgSO4; 5.6 mM Dextrose; 1.6 mM CaCl2-H2O and 20 mM HEPES; pH 7.2-7.4). Experimental drugs were added either to the intracellular recording solution (50 mM QX-314; 0.5-1M TEA-Cl, 0.075 / 0.15 M 4-aminopyridine; 1-2 M CsCl), applied topically to the surface of the posterior VIIIth nerve (1-10 µM tetrodotoxin, TTX; 1-5 µM αDendrotoxin), or included in the superfusing ACSF (5 mM 4-aminopyridine, 4-AP). Because of limitations to diffusion in the intact brain, the effective concentration of extracellularly applied drugs is expected to be significantly lower (up to an order of magnitude in some cases; Pereda et al. 1992) to those of the superfusate.

**Computer simulations.** A first approximation to the characterization of Club endings electrical resonance was obtained modeling a combination of low-pass and high-pass

RESULTS
Identifiable saccular afferents terminate as single mixed (electrical and chemical) “Large Myelinated Club endings”, henceforth referred to as Club ending afferents, on the distal portion of the lateral dendrite of the M-cell (Fig. 1A). The afferents were intracellularly recorded in vivo in the posterior root of the VIIIth nerve and identified by the presence of the antidromic coupling potential (AD coupling), the electrotonic coupling potential due to the passive dendritic depolarization produced by the antidromically-evoked M-cell action potential (Fig. 1B, inset), and characteristic lack of spontaneous activity and high threshold for acoustic stimulation. Previous studies showed that intracellular labeling of fibers exhibiting these properties invariably resulted in fibers that because of their size, prominent myelinization, dendritic distribution and saccular origin unambiguously corresponded to Club endings afferents (Lin and Faber 1988a; Smith and Pereda 2003).

In addition to be phase-locked to higher frequencies, these afferents characteristically respond with multiple action potentials to brief (Fig. 1B) or low frequency (Furukawa and Ishii 1967) acoustic stimulation. In order to investigate the mechanisms underlying this firing property we examined the responses of Club ending afferents to depolarizing current pulses. We found that depolarizing pulses of 1.5 their threshold typically evoked an initial high frequency burst of action potentials, which was immediately followed by a complete absence of activity (Fig. 1C). In contrast, depolarization of the M-cell axon with a depolarization of equivalent (1.5 threshold; Fig. 1D) or stronger magnitude invariably resulted in a single action potential. These
results suggest that: 1) Club ending afferents are endowed with electrophysiological properties that favor the generation of high frequency bursts in response to strong depolarizations, and 2) because the M-cells are not capable of repetitive firing (an otherwise inconvenient feature for a cell in which a single action potential initiates an escape response that lasts several hundred milliseconds; Eaton et al. 1988; Korn and Faber 2005), we hypothesize that their ability to generate high frequency bursts must represent a functional specialization of these afferents with the goal of providing the M-cell with adequate patterns of synaptic activation.

**Characterization of repetitive responses**

A characterization of the firing properties of Club ending afferents using pulses of increasing magnitude revealed the presence of a bi-linear relationship between firing frequency and the injected current (Fig. 2A). Whereas a pulse of current at its “threshold” or “rheobasic” intensity (minimum stimulus strength of infinite duration that evokes a response) evokes a single action potential, a current step 1.5 times the threshold intensity evokes a repetitive response that averaged 6-7 spikes (6.8 ± 0.83, n=11; Fig. 2A, middle panel). Finally, a current pulse twice its threshold intensity elicited a repetitive response that lasted for the duration of the pulse (Fig. 2A, lower panel). In order to characterize the dependence of repetitive firing on the magnitude of the injected current, we plotted instantaneous frequency versus current injection for the first, second, and third interspike intervals (ISI; Fig. 2B). The slope of the primary range for the first, second and third ISI averaged 516.5 ± 62.4, 596.6 ± 61.5 and 571.5 ± 64.6 Hz/nA respectively, whereas the slope of the secondary range averaged 165.6 ± 12.8, 232.2 ± 20.5 and 269.8 ± 27.2 Hz/nA (n=11) for the first, second and third ISI respectively. In all the examined examples, the slope of the primary range was significantly higher than that of the secondary range (p<0.0005).
Repetitive responses at Club ending afferents showed the presence of a strong frequency adaptation, a property that allows these afferents to respond with an initial brief burst of action potentials to depolarizing stimuli (Fig. 2). The time course of this progressive decrease in firing rate in response to a constant current step (Fig. 2A, lower panel) can be better appreciated during stronger stimulation (Fig. 2C), in which the response lasted for the duration of the pulse. The time course of this phenomenon could be described by a single exponential function ($\tau = 18 \pm 1.5$ ms, $n=12$) plus a constant. Depending on the strength of the current pulse, the repetitive discharge decayed from an initial frequency ($F_i$) of $448.4 \pm 45$ Hz to a final value ($F_f$) of $153.5 \pm 19$ Hz ($n=11$); the initial and final frequency values ($F_i$ and $F_f$) represent the frequency at $t=0$ and the value of the constant obtained from the fit, respectively. The degree of adaptation $[F_{adapt}=(F_i - F_f)/F_i]$ (Liu and Wang 2001) was quantified and expressed as percentage of the initial rate, averaging $64 \pm 4 \%$ ($n=11$). Thus, Club ending afferents are endowed with mechanisms that allow them to respond with brief bursts of 200-600 Hz to stimulus of increasing intensity. These electrophysiological properties likely represents those of the ending of the afferent contacted by the hair cell, as brief acoustic stimulation was also able to trigger repetitive discharges of characteristics similar to those observed by depolarizing the axon in our recording site (Fig. 1B). This observation is consistent with recent data, based on the ubiquitous distribution of Nav1.6 Na\(^+\) channels in primary auditory afferents, which indicates that these neurons generate (and regenerate) action potentials at multiple sites along their anatomy, including the afferent endings, ganglionic initial segments and nodes of Ranvier (Hossain et al. 2005).

A subthreshold Na\(^+\) conductance is essential for repetitive firing
Subthreshold Na\(^+\) conductances are known to underlie repetitive firing in many neuronal types (Crill 1996; Enomoto 2006). Furthermore, we have previously described the presence in Club ending afferents of a subthreshold conductance with properties similar to those of a persistent Na\(^+\) current (Curti and Pereda 2004). To investigate the possible involvement of a persistent Na\(^+\) current in repetitive firing at Club ending afferents we tested the effect of intracellularly injected QX-314 (that blocks Na\(^+\) channels) on repetitive firing responses evoked by depolarizing current pulses (Fig. 3A). Subthreshold Na\(^+\) currents are generally affected earlier than transient Na\(^+\) currents to application of blockers (Stauftrum et al. 1985; Hu 1991; Brumberg et al. 2000). QX-314 dramatically abolished repetitive firing (Fig. 3A, center, n=10). These changes occurred within a time window in which the action potentials of the afferents remained largely unaffected (Fig. 3A, center and Fig. 3B; amplitude averaged 92.5 ± 4.63 and 89.1 ± 3.82 mV for control and QX-314 respectively, n=7, p=0.13) and was accompanied by a drastic reduction of the retrograde coupling potential amplitude obtained at depolarized membrane potentials (Fig. 3A, right and Fig. 3C), a phenomenon that results from the amplification of this retrogradely transmitted coupling by the subthreshold Na\(^+\) current (Curti and Pereda 2004). Because QX-314 has been reported to have non-specific actions on other than Na\(^+\) channels we tested the effect of extracellular application of TTX (1-10 µM), which specifically blocks Na\(^+\) channels, on the repetitive responses evoked by depolarizing current pulses. As illustrated in Fig. 3D, application of TTX also led to a dramatic suppression of repetitive responses (n=6). Like in the case of QX-314, the observed suppression of repetitive firing took place within a time window in which the action potentials of the afferents remained largely unaffected; amplitude averaged 97.5 ± 4.3 and 95.1 ± 3.8 mV for control and TTX respectively (n=6, p=0.7) (Fig.3E,F). Both QX-314 and TTX ultimately led, as previously reported (Curti and Pereda 2004), to the complete blockade of the first action potential (not shown). Finally, consistent with the
elimination of a subthreshold amplifying mechanism, the depolarizing prepotentials that usually lead to initiation of the next action potential in repetitive responses (Lanthorn et al. 1984; MacVicar 1985; Amir et al. 2002a,b), were no longer observed after TTX application (Fig.3G).

To directly demonstrate the presence of a subthreshold voltage-sensitive Na⁺ conductance, the membrane properties of the afferents were examined by measuring their current-voltage relations (Fig.4A). For a given depolarizing current pulse, we found a non-linear voltage response that was maximal within ~2-5 ms of the pulse onset and then rapidly decayed to a steady-state value. The magnitude of this initial response was not linearly correlated to the injected current and was more obvious for “near-threshold” depolarizations, defined as the membrane potential in which a given pulse elicits an action potential in about 50% of the trials (Fig. 4A,B; only the responses lacking action potentials were averaged). We found that this initial non-linear component was abolished by TTX. Voltage responses to near-threshold depolarizing current pulses averaged 62.5 ± 3.5 % (range: 56.5 – 68.7 %, n=3) of its control amplitude (Fig.4A) following TTX application, and the V-I relationship became linear (Fig. 4B). The non-linear nature and time course of the TTX-sensitive subthreshold component was also revealed by subtracting the V-I relationship obtained in TTX from that obtained in control conditions (Fig.4C). The membrane responses to current steps were similar in all studied fibers (see also Curti and Pereda 2004). To investigate if this slowly decaying TTX-sensitive subthreshold membrane response was the underlying mechanism responsible for the adapting firing pattern observed in Club ending afferents, we compared the time courses of both phenomena. As illustrated in Fig. 4D, the decay of the membrane response to a near-threshold depolarizing pulse closely followed the time course of the instantaneous firing frequency obtained for a suprathreshold current pulse in the same fiber (in which
the response lasted for the duration of the pulse; see above), suggesting a primary role of subthreshold mechanisms in shaping the firing patterns at Club ending afferents.

**Subthreshold K⁺ conductance underlines spike frequency adaptation**

The results so far indicate that subthreshold mechanisms are largely responsible for the firing pattern of Club ending afferents. However, because subthreshold Na⁺ currents have a slow process of inactivation with time constants in the order of hundred to thousands of milliseconds (French et al. 1990; Crill 1996; Ogata and Ohishi 2002), the relatively faster decay of the TTX-sensitive subthreshold responses to depolarizing current pulses (Fig. 4A-C) suggests the involvement of an opposing repolarizing conductance. The existence and contribution of more than one active membrane mechanism to near-threshold responses was revealed by subtracting the response of a 0.2 nA pulse (mostly determined by resistive and capacitive properties of the membrane) multiplied by a factor of 10, from a near-threshold response evoked by a 2 nA depolarizing pulse (Fig. 5A). This subtraction revealed the presence of an initial depolarization (likely corresponding to the activation of a subthreshold Na⁺ current), which was followed by a sustained hyperpolarization (Fig. 5A, lower panel). Because subthreshold Na⁺ currents are generally opposed by repolarizing K⁺ conductances (Crill 1996) we tested the effects of intracellular applications of a combination of K⁺ channel blockers (see Methods), covering a wide spectrum of these channels, on the membrane responses to depolarizing current steps. We found that blockade of K⁺ channels prolonged the initial voltage response to a depolarizing pulse (Fig. 5B), suggesting the presence of a persistent Na⁺ current which was able to reveal, unopposed, its time course. Accordingly, membrane responses, which lacked under these conditions their typical decay, were greatly attenuated by extracellular application of TTX (Fig. 5B)
confirming the presence of a none, or very slowly, inactivating component with the characteristics of a persistent Na⁺ current (Crill, 1996).

By opposing the action of a persistent Na⁺ current, the delayed activation of a subthreshold K⁺ conductance is responsible for the decay observed at near-threshold membrane responses and therefore responsible for the frequency adaptation observed during repetitive responses at Club ending afferents. To investigate the identity of the involved K⁺ conductance we tested the effects of extracellularly applied 4-AP (5 mM, bath applied; see Materials and Methods), known to affect some subthreshold K⁺ conductances (Rudy 1988; Faber and Sah 2003; Jerng et al. 2004), on the membrane responses to depolarizing current steps. As with the intracellular application of K⁺ channel blockers, extracellular application of 4-AP prolonged the initial voltage response to a depolarizing pulse and lacked its characteristic sag, as illustrated by the modification of the ratio between the late and early amplitudes of pulse responses which became ~1 (see Fig. 5C). The off response to depolarizing pulses was followed by a small "tail" which was greatly reduced by TTX (arrows in Figs. 4A and 5B) suggesting that the decay corresponds to the action of some persistent Na⁺ channels, which deactivate slowly relative to the rapid variations of the membrane potential produced by the injected current step. Consistent with this observation, this tail was enhanced by 4-AP, which by removing IA leaves persistent Na⁺ channels acting unopposed (arrow in Fig. 5C). Thus, near-threshold depolarizing responses at Club ending afferents are dominated by the interplay of two independent active mechanisms that are sequentially activated. Initially, the response is dominated by the rapid activation of an amplifying persistent Na⁺ current, which is counterbalanced by the relative slow activation of a subthreshold K⁺ current, whose activation opposes membrane depolarizations (Fig. 5D).
The sensitivity to 4-AP of subthreshold membrane responses suggested the involvement of an A-type K⁺ current (IA), which is known to operate within this voltage range (Rudy 1988; Storm 1990). We confirmed this possibility by using a standard protocol to reveal the presence of this current (Storm 1988). More specifically, we measured the delay to the first spike when bringing the cell to threshold from hyperpolarized potentials, which removes IA steady state inactivation (Fig. 6A). As IA became more activated, it characteristically introduced a delay in the generation of the spike that was inversely proportional to the prepulse membrane potential (Fig. 6B). Finally, and consistent with its pharmacological effects on subthreshold membrane responses, this delay was greatly reduced by extracellular application of 4-AP (Fig. 6C, D). To further characterize the properties of this subthreshold conductance, we estimated its recovery from inactivation following a similar protocol. For this purpose, a hyperpolarizing prepulse of variable duration and fixed amplitude (adjusted to drive the membrane potential to about -100 mV) was followed by a suprathreshold depolarizing current pulse; the availability of IA channels was inferred from the delay to the first action potential (Fig. 6E). As illustrated in Figure 6F, this delay increased with the prepulse duration, indicating that the recovery from inactivation followed an exponential time course with a time constant of 56.2 ± 4.73 ms (range: 46.8 – 61.8 ms, n=3), a value that is consistent with those found for similar A-type conductances in other cell types (Petersen and Nerbonne 1999; Jerng et al. 2004; Koyama and Appel 2006; Wang and Schreurs 2006), including in the auditory system (Rothman and Manis 2003). In an attempt to determine the identity of the involved K⁺ channels we tested the effects of α-dendrotoxin (αDTX, 1-5 µM), which blocks channels of the Kv1 family known to be responsible for low threshold K⁺ conductances in the auditory system (Mo et al. 2002; Rathouz and Trusell, 1998; Trussell 1999; Klug and Trussell 2006), on subthreshold
responses to depolarizing current pulses. In contrast to the effects of 4-AP, we did not detect changes in the decay of near-threshold responses as a result of the application of this toxin (see Fig. 5C), suggesting that channels other than those of the Kv1 family are responsible for this hyperpolarizing conductance. Consistent with a primary role of this subthreshold mechanism and confirming an early report (Davis 1996), we did not observe evidence for the involvement of a Ca\(^{++}\)-dependent K\(^+\) current (IK\(_{ca}\)) (see supplementary Fig. 1A and legend). In addition, our analysis revealed that the observed firing pattern couldn’t be explained by a progressive decrease in the availability of transient Na\(^+\) channels (see supplementary Figure 1B-F, and legend).

Our data is consistent with the notion that a balance between a persistent Na\(^+\) and a IA currents dominate near-threshold responses to depolarizing current pulses and are likely responsible for determining the observed firing pattern at Club ending afferents (Fig. 4D). To confirm the primary functional role of this interaction, we investigated how the relative availability of these channels affects the firing pattern of Club ending afferents by modifying the balance between these two conductances (Fig. 7). For this purpose, we increased the contribution of persistent Na\(^+\) current by bringing the cell to threshold from more hyperpolarized membrane potentials, which is expected to increase the availability of these channels by removing their inactivation (they also present inactivation but of slower time course; Fleidervish et al. 1996). As illustrated in Figure 7A, depolarization of the afferent to about -55 mV from resting potential (-75 mV in this case) elicited a single action potential; in contrast, depolarization to the same membrane potential from about -120 mV (held at this potential by DC current injection during at least 10 seconds) elicited instead a high frequency repetitive response. Furthermore, the frequency adaptation of repetitive responses obtained by depolarizing from hyperpolarized potentials was significantly attenuated (Fig. 7B). These effects were
prevented by of QX-314 (Fig. 7C), confirming that an increased availability of persistent Na⁺ channels was responsible for the observed increased firing during repetitive responses overcoming the actions of IA, whose increased availability initially causes the delay to the first spike but later begins to inactivate (such delay is consistent with the time constant of inactivation of IA currents in other cell types; Rothman and Manis 2003). To independently confirm this conclusion, we tested the effects of pharmacological blockade of IA on responses to suprathreshold depolarizing pulses. As illustrated in Figure 7D (left), while depolarization to -65 mV from resting potential (-75 mV) evoked a single action potential, the same depolarization led to a high frequency response following extracellular application of 4-AP (middle panel). Thus, blockade of IA led to the unopposed action of persistent Na⁺ channels, resulting in an enhancement of the repetitive responses. Supporting this conclusion, both 4-AP and a combination of K⁺ channel blockers, ultimately led to the development of a pronounced plateau potential (Fig. 6C and Fig. 7D, right panel) potential that was greatly suppressed by extracellular application of TTX (Fig. 7D, right panel). Consistent with its lack of effect on subthreshold responses, application of αDTX did not enhance firing responses at Club ending afferents, confirming that αDTX-insensitive channels are responsible for spike frequency adaptation at Club ending afferents (Fig. 7E, upper panel). In contrast, as previously reported (Nakayama and Oda 2004), αDTX induced repetitive firing in the M-cell during parallel control experiments (Fig. 7E, lower panel).

**Club ending afferents exhibit electrical resonance.**

Membrane oscillations were occasionally observed following the initial burst of action potentials, suggesting that underlying oscillatory membrane mechanisms could be responsible for repetitive firing at Club ending afferents (Fig. 8A). Subthreshold oscillations have been shown to be responsible for repetitive firing in primary sensory
neurons (Pedroarena et al. 1999; Wu et al. 2001; Liu at al. 2002; Amir et al. 2002a) and are generally caused by the existence of membrane electrical resonance (Hutcheon and Yarom 2000). Electrical resonance characterizes the frequency at which neurons respond best to depolarization and therefore describes its frequency-dependent properties, in particular how neurons process oscillatory inputs at subthreshold potentials (Hutcheon and Yarom 2000). Furthermore, interplays between subthreshold K$^+$ conductances such as that found at Club ending afferents are responsible for the generation of electrical resonant behavior in various neuronal types (Hutcheon et al. 1996; Hutcheon and Yarom 2000), and produce a characteristic “sag” in the subthreshold membrane response to depolarizing pulses (Fig. 5D). The presence of both subthreshold “sags” and membrane oscillations, which are considered time-domain signatures of electrical resonance (Hutcheon and Yarom 2000), suggest that these auditory afferents are endowed with similar membrane properties.

The frequency preference of Club ending afferents was estimated by determining its underlying resonant mechanisms from near-threshold membrane responses (“sag”). Although indirect, this approach has the advantage of accurately estimating the impact of this voltage-dependent behavior at the membrane potential where is primarily expressed, the threshold of the cell. Electrical membrane resonance is known to result from the interaction of two mechanisms with specific frequency-dependent properties: a low-pass filter determined by the membrane passive properties (membrane time constant) that attenuates responses to inputs with high frequency content, and a high-pass filter, which is set by slowly activating voltage-dependent K$^+$ currents that by opposing to membrane depolarization attenuates voltage responses to inputs with low frequency content (“resonant currents”; Hutcheon and Yarom 2000; Izhikevich 2007). Thus, the approximate resonant properties can be estimated if the values of the
activation time constant of the “resonant K⁺ current” and the membrane time constant are determined experimentally. The membrane time constant (low pass filter) was measured by fitting a single exponential function to the decay that followed the cessation of current pulses of different polarities and amplitudes and averaged 0.2 ± 0.01 ms (n=12; Fig. 8B, upper panel). The time constant representing the activation of the A-type current (high pass filter) was estimated from the decaying portion of the near-threshold membrane response (Fig. 8B). The decay was best fitted with a double-exponential function (p<0.001) and the time constants of the first and second exponential averaged 2.9 ± 0.26 ms and 30.1 ± 2.01 ms (n=12). The fastest and more prominent time constant dominating the decay of the pulse (10 times faster and 30 times the magnitude of the second one) was used for the estimates of resonance (Fig. 8B, upper panel). This measurement proved to be a good approximation to the activation kinetics of IA, as the obtained values were consistent with the activation kinetics of A-type currents obtained elsewhere (Rudy 1988; Storm 1990; Huguenard et al. 1991; Huguenard and McCormick 1992; Rathouz and Trusell 1998; Rothman and Manis 2003; Jerng et al. 2004). The second slower time constant might represent a minor contribution of an unidentified, less prominent, outward current to the decay and it was not included in the estimates. The filtering properties determined by the obtained IA and membrane times constants are illustrated in the Bode plots of Fig. 8B, determining cutoff frequencies for the high-pass (middle panel) and low-pass filters (lower panel) of 61 ± 6 and 803 ± 45 Hz (n=12), respectively. When combined (Fig. 8C), these values determined a band-pass filter with a bandwidth (defined as the range contained by the cutoff frequencies) of 742 Hz and a peak value at 220 Hz, suggesting the existence of resonant properties at Club ending afferents.
Independently supporting these measurements, the estimated bandwidth of the electrical resonance matched the frequency range of repetitive firing in Club ending afferents (200 to 600 Hz; see Fig. 2). Furthermore, the estimated peak resonant frequency was also in agreement with that calculated for the “minimum frequency”, the instantaneous frequency of a minimal repetitive response consisting of only two spikes triggered by a depolarizing pulse, indicating a propensity of Club ending afferents to generate repetitive responses within this frequency range (Fig. 8E). A second consequence of electrical resonance is that, in addition to allow neurons to undergo high frequency repetitive firing within a given frequency range, it also make them more susceptible to respond to inputs with this particular frequency content. Accordingly, the bandwidth of this resonance matched the effective range of hearing of this specie, estimated to be about 100 to 1000 Hz (Fay 1995). The tuning curves of two fibers, representative of the “high” and “low” frequency types of broadly-tuned afferents identified in goldfish (Fay 1978, 1995), are illustrated superimposed to the Bode plot (Fig. 8C; examples taken from Fay 1995).

The estimates of electrical resonance predict that Club ending afferents would be more easily depolarized in response to intracellularly injected currents with a frequency content close to their resonant frequency. Unfortunately, this direct approach proved to be inapplicable to our in vivo recording conditions. Because the band of resonant frequencies found in Club ending afferents is 20-40 fold higher (10-15 Hz vs. 200 Hz) than those found in previous studies where resonant behaviors were explored with this method (Puil et al. 1986; Huguenard et al. 1991; Hutcheon et al. 1996; Wu et al. 2005), the filtering properties of our high resistance electrode (~40 MΩ) made it impossible to reliably induce and monitor changes in voltage at the required high frequencies (up to more of 1000 Hz, as the band of resonance is 61-803 Hz).
Given these experimental limitations, we sought additional independent support for the resonant properties of IA and the potential participation of the persistent Na\(^+\) current by using computer simulations. For this purpose, a Club ending afferent was modeled, following available anatomical and physiological data (Furukawa and Ishii 1967; Sento and Furukawa 1987; Rosenbluth and Palay 1961; see Supplementary Methods for details). Because of the impossibility to obtain direct measurements, we ran computer simulations using parameters of A-type currents described in auditory neurons that are similar to those estimated for these afferents. We found that addition of an A-type current with the kinetics properties estimated experimentally for the ventral cochlear nucleus (Rothman and Manis 2003), a clear resonant behavior appeared with a peak at 220 Hz (Fig. 8D). This approach also allowed us to evaluate the role of the persistent Na\(^+\) current in this resonant behavior. As previously shown (Hutcheon and Yarom 2000), the addition of a persistent Na\(^+\) current produced a strong amplification (~35%) of the predicted membrane resonance without modifying the resonant frequency suggesting that this conductance is likely to play a relevant functional role by allowing the expression of this resonance (Fig. 8D). Thus, despite the low stringency of this model, computer simulations adequately reproduced the frequency range of the predicted membrane resonance, suggesting that IA has robust resonant properties in these neurons. In addition, it indicated an essential role for the persistent Na\(^+\) current in amplifying these resonant properties.

**Properties of synaptic facilitation at synapses between Club endings and Mauthner cells**

Stimulation of the posterior VIII\(^{th}\) nerve, where Club ending afferents run, evokes a mixed (electrical and chemical) synaptic potential in the lateral dendrite of the M-cell
(Fig. 9A; Furshpan 1964). In contrast to most primary auditory afferent synapses that undergo depression (Zhang and Trusell 1994), high frequency stimulation of these afferents with trains of 2 to 6 pulses (Fig. 9A) was shown to evoke a strong facilitation of the glutamate-mediated chemical component (Lin and Faber 1998b; Pereda and Faber 1996; Wolszon et al. 1997). Synaptic facilitation is essential for the induction of long-term potentiation of both components of the mixed synaptic response, as it optimizes the required activation of NMDA receptors (Yang et al. 1990; Pereda and Faber 1996).

Given the firing properties of Club ending afferents, we explored the impact of various frequencies on the time course of paired pulse facilitation of the chemical component of the mixed synaptic potential (Fig. 9B). Paired pulses were used instead of trains to minimize the postsynaptic contribution of NMDA receptors to the facilitated responses, which was shown to be prominent during the last pulses of a short train (Pereda and Faber 1996; Wolszon et al. 1997; the changes in the facilitated synaptic potential were measured by subtracting the response to a single pulse, see Wolszon et al. 1997). We found that paired pulse facilitation decayed following an exponential time course, with a time constant of $4.5 \pm 0.81$ ms ($n=8$). This facilitation took place within a narrow temporal window (Fig. 9C, upper panel) and, remarkably, it did not extend beyond the frequency range determined by the firing properties of Club ending afferents, indicating that bursts of 200-600 Hz are optimal in producing facilitation of the chemical component of synaptic response. Further, the time course of the synaptic facilitation was extremely fast when compared with that of other well-characterized contacts (Fig. 9C, lower panel), suggesting that firing characteristics of these auditory afferents are adapted to the requirements of synaptic transmission at Club endings. We found that repetitive firing within the 200-600 Hz range was also essential to obtain temporal summation of successive synaptic responses (Fig. 9A). Because of the brief time
constant of the M-cell (~400 µs; Fukami et al. 1965), temporal summation can only take place during the chemical component of the mixed synaptic response. The duration of the chemical component was found in ~5 ms, an integrating interval which is consistent with the band of frequency preference determined by the electrical resonance of the membrane.

DISCUSSION

In contrast to the M-cells, Club ending afferents undergo repetitive firing as a result of intracellular depolarization. While the lack of repetitive firing on M-cells is thought to be due to the presence of DTX-sensitive K⁺ channels of the Kv1 family (Nakayama and Oda 2004), our results show that the ability of Club ending afferents to respond with high frequency bursts of action potentials results from the interaction of a subthreshold Na⁺ current, which is required for repetitive responses, with an A-type K⁺ conductance. This subthreshold Na⁺ conductance has all the characteristics of a persistent Na⁺ current. That is, the voltage sensitivity and membrane potential activation range (10-15 mV above resting potential), susceptibility to TTX and QX-314 and prolonged time course in the presence of K⁺ channel blockers, are typical of persistent Na⁺ currents described in fish (Watanabe et al. 2000; Berman et al. 2001; Doiron et al. 2003) and mammalian neurons (Crill 1996). The A-type K⁺ conductance was, in contrast to that of the M-cell, insensitive to DTX and therefore unlikely to represent one of the typical “low threshold” K⁺ currents, which act to prevent repetitive firing in the auditory system (Trusell 1999; Davis 2003; Klug and Trussell 2006). Rather, this conductance presented values of kinetics of activation and recovery from inactivation and a pharmacological profile (lack of sensitivity to DTX and sensitivity to 4-AP in the mM range) that were comparable to those of another A-type current also found in the auditory system (Rothman and Manis 2003), which possibly involves channels of the Kv4 family (Jerng et al. 2004; Patel and
Unfortunately, we did not observe changes as a result of bath application of rHeteropodatoxin-2 (which blocks Kv4 channels; not shown) suggesting that either this toxin, unlike DTX, has limited experimental access in our in vivo conditions or that the involved fish isoforms are less sensitive to its actions. Thus, while the presence of Kv1 channels is probably beneficial for the M-cell, where a single action potential is sufficient to initiate an escape response that lasts several hundreds of milliseconds, the lack of these channels in Club ending afferents allows these afferents to undergo high frequency repetitive firing, a pattern that is likely to play an important physiological role by producing a strong dendritic depolarization (see below).

**Interaction of membrane and synaptic properties.**

Our results indicate that the Club ending afferents have the propensity to respond with high frequency (200 to 600 Hz) bursts of action potentials to strong depolarizing inputs, and that this frequency range is ideally suited to produce facilitation of the chemical component of their mixed synaptic response. Bursts are thought to represent reliable neural codes during information processing (Lisman 1997; Izhikevich et al. 2003; Krahe and Gabbiani 2004), and play special roles for the induction of synaptic plasticity (Lisman 1997). The generation of bursts in response to strong acoustic stimuli seems a particularly advantageous strategy for the M-cell system, and it could constitute an efficient and desirable code of information. That is, a burst of action potentials would generate, as opposed to a single action potential, a prolonged synaptic response that can efficiently depolarize the large and unusually low input resistance M-cell (Faber and Korn 1978). Due to the longer duration of the chemical component, high frequency bursts also allow temporal summation of the mixed synaptic responses, an otherwise unlikely possibility given the short duration of the electrical component and the brief membrane time constant of the M-cell. Because the firing frequency of the burst was
found to be proportional to the strength of the depolarization (see Fig. 2B), the results suggest that louder acoustic stimuli (likely required to trigger the escape response) would produce stronger facilitation of synaptic responses. Thus, by producing synaptic facilitation and allowing temporal summation of successive synaptic responses, high frequency bursts of action potentials can lead to stronger and longer-lasting synaptic activation of the M-cell lateral dendrite.

Equally relevant, brief high frequency bursts of action potentials are required for the induction of activity-dependent long-term potentiation of the mixed synaptic response (Yang et al. 1990), as they optimize the activation of NMDA receptors by providing enhanced glutamate release at more depolarized potentials, whose involvement is essential for the induction of the plastic changes (Pereda and Faber 1996; Wolszon et al. 1997; Pereda and Faber 1996; Smith and Pereda 2003). Thus, both membrane and synaptic properties of Club ending afferents act in a concerted fashion and seem to be adapted to the M-cell system, where the increased synaptic gain of these auditory nerve synapses will sensitze a vital escape response, lowering its threshold to acoustic stimuli (Korn and Faber 2005).

Resonant membrane properties

Bursts of action potentials are often generated by the interaction of synaptic inputs with intrinsic membrane properties (Lisman 1997). Our results also suggest that the propensity to respond with high frequency (200 to 600 Hz) bursts of action potentials to strong depolarizing inputs is supported by the existence of electrical resonant properties of the Club ending afferent membrane. Furthermore, the optimal intervals required for the synaptic facilitation of the glutamatergic component of the mixed synaptic potential also matched the estimated frequency preference of this resonance, suggesting that this
membrane behavior is essential for the interaction between afferent firing and synaptic properties.

Unfortunately, the estimated band of membrane frequency preference was significantly higher that those found in other preparations (5-10 Hz vs. 100-800 Hz) and thus the filtering properties of our sharp recording microelectrode prevented us from obtaining direct evidence of this membrane behavior while depolarizing the membrane with a sinusoidal current of varying frequency. In contrast to those previous examples in which resonance could be explored directly in in vitro conditions (Puil et al. 1986; Huguenard et al. 1991; Hutcheon et al. 1996; Wu et al. 2005), these properties were estimated in Club ending afferents by obtaining measurements of the filtering characteristics of the membrane passive properties and the activation of the subthreshold, A-type, K⁺ conductance at near-threshold responses in an in vivo (more physiological) situation where anatomical integrity is preserved. Because electrical resonance is a voltage-dependent property, while indirect, this method has the advantage of accurately estimating the impact of this behavior at the membrane potential where is primarily expressed, namely about this cell’s threshold. As a result of activation kinetics slower than the membrane time constant, subthreshold K⁺ conductances of this type determine “resonant” behavior in many cells types by opposing to membrane depolarizations (Hutcheon and Yarom 2000; Izhikevich 2007). These estimates of resonant behavior were independently supported by: 1) the presence of subthreshold oscillations underlying repetitive responses, 2) the propensity of these afferents to respond within this frequency range, and finally 3) by computer simulations generated using the kinetic values obtained for the A-type current found in auditory neurons (Rothman and Manis 2003) of similar physiological and pharmacological characteristics to that found in Club ending afferents, given the impossibility to obtain
direct measurements under our experimental conditions. Despite their low stringency, computer simulations adequately reproduced the frequency range of the predicted membrane resonance, suggesting that IA has robust resonant properties in Club ending afferents.

While “resonant” currents can by themselves generate membrane resonance, they are often enhanced by “amplifying” currents (such as persistent Na\(^+\) currents) that allow them to manifest their functional impact (Izhikevich 2007). That is, because of their depolarized reversal potential and quick activation properties these currents actively enhance, rather than oppose, voltage depolarizing changes amplifying otherwise weak membrane resonant behaviors (Hutcheon and Yarom 2000). Numerical computer simulations also suggested an essential contribution of the persistent Na\(^+\) current to this property in Club ending afferents by providing with strong amplification of membrane responses at resonant frequencies (~35%). If the interaction between “resonant” and “amplifying” currents is sufficiently strong, it can destabilize the membrane potential allowing the generation of spontaneous oscillatory pacemaker-like activity (Hutcheon and Yarom 2000). Most commonly, this interaction is not very strong so the frequency preference of a given cell is “latent” and oscillatory activity is only revealed in the presence of its inputs. Such “weak” resonance makes a neuron a “good listener” within a specialized frequency band (Hutcheon and Yarom 2000). This second possibility seems to be the case of Club ending afferents, where oscillatory activity was observed only in response to depolarization and the estimated band of electrical resonance matched that of the effective frequency range of hearing of the goldfish (~100-1000 Hz; Fay 1995), making these afferents more sensitive to a broad range of behaviorally relevant frequencies.
Neurons are excitable because they are near transitions from resting to spiking. From the dynamical systems point of view this transition corresponds to a bifurcation (Izhikevich, 2007). This approach provides an alternative framework in which the excitable properties of neurons can be understood. Regardless of the cellular type and ionic conductances involved, they are only four different types of bifurcation of equilibrium that a system can undergo (Izhikevich 2007). In this context neurons are divided into integrators and resonators with bistable or monostable activity. The presence of resonant properties indicates that the behavior of this neuron corresponds to an Andronov-Hopf type of bifurcation. The lack of a bistability of this neuron's electrical behavior suggests that it more specifically corresponds to a supercritcal Andronov-Hopf bifurcation. Nevertheless, this categorization should be more appropriately explored and confirmed in the future with more adequate stimuli such as current ramps (Izhikevich 2007). Consistent with our results, Andronov-Hopf bifurcations can be reproduced in reduced models that combine a persistent Na$^+$ current with a delayed K$^+$ current ("persistent sodium plus potassium model"; Izhikevich 2007) indicating that the electrophysiological properties of Club ending afferents can be adequately explained by the interaction between these two currents.

**Frequency tuning in goldfish primary afferents.**

Unlike their mammalian counterparts, fish auditory afferents are broadly tuned and exhibit limited frequency selectivity (Fay 1995; Popper and Fay 1998). The anatomical characteristics of the Club ending afferents (large diameter and characteristic myelinization) meet those of S1 type saccular fibers originated from the rostral part of the sacculus (the auditory organ in fish), following the initial characterization of goldfish auditory afferents by Furukawa and Ishii (Furukawa and Ishii 1967). In contrast with S2, a second type of smaller fibers, S1 afferents were shown to respond to higher
frequencies (>500 Hz; Furukawa and Ishii 1967). More rigorous characterization of
goldfish afferent responses confirmed the presence of both high frequency and low
frequency afferent types (Fay 1978, 1995). Although in these studies physiological
responses were not correlated with anatomical identification of the afferent fibers, both
low and high tuning were observed in afferents lacking spontaneous activity (Fay 1978),
a notable characteristic and identification criteria for Club ending afferents, suggesting
that these larger afferents can respond to a broader range of frequencies.

Club endings constitute a relatively homogenous population of ~100 afferents
terminating in the lateral dendrite of the M-cell (Bartelmez 1915). Our results suggest
that they are also relatively physiologically homogeneous, as all of the studied fibers
exhibited similar biophysical properties. Mechanisms of frequency selectivity in lower
vertebrates, including goldfish (Sugihara and Furukawa 1989), are known to involve the
contribution of resonant electrical membrane properties, which allow inner-ear hair cells
to be tuned to stimuli of specific frequency content (Fettiplace and Fuchs 1999). The
reported tuning curves and characteristic frequencies of both types of afferent response
(Fay 1978, 1995) fell within the band of electrical resonance estimated for Club ending
afferents (see Fig. 8C; Fay 1978, 1995), indicating that this mechanism is unlikely to
underlie their individual frequency tuning. Rather, because most saccular fibers will
respond to any frequency within the goldfish’s effective range of hearing at sound levels
40 dB or more above best threshold (see Fig. 8C for the Q₁₀dB [range of frequency
response at 10dB above threshold] of each afferent; Fay 1995), this resonant
mechanism would act to synchronize this population of large afferents to loud acoustic
stimuli of behavioral relevance by making them, regardless their characteristic
frequency, electrically tuned to the whole hearing range. Accordingly, strong acoustic
stimuli are necessary for triggering an escape response in the M-cell system, which has a characteristic high threshold (Fay 1995).

**The essential role of a persistent Na\(^+\) current**

Our data indicates that the properties of the fast and modifiable Club ending mixed synapses are supported by biophysical specializations that allow these afferents to translate the goldfish’s effective range of hearing into adequate patterns of afferent activity. Amongst these properties, a persistent Na\(^+\) current plays an essential functional role as is required for the generation of repetitive responses and likely to enhance an otherwise weak electrical resonance. Although persistent Na\(^+\) currents represent a small (~1%) non-inactivating fraction of the total Na\(^+\) current they have a significant functional impact because they are activated about 10 mV negative to the transient Na\(^+\) current, where few voltage-gated channels are activated and neuron input resistance is high (Crill 1996). As a result of this property, subthreshold Na\(^+\) currents play essential cellular roles amplifying dendritic synaptic potentials, regulating repetitive firing, and producing depolarizing responses (Crill 1996; Ogata and Ohishi 2002). Persistent Na\(^+\) currents have been reported to be present in primary afferents (Kocsis and Waxman 1983; Bowe et al. 1985; Honmou et al. 1994; Wu et al. 2005; Rush et al. 2007) where they are thought to mediate important functional and pathological roles (Stys et al. 1992, 1993). Consistent with these observations, Nav1.6 channels, thought to underlie persistent Na\(^+\) currents (Ogata and Ohishi 2002; Rush et al. 2007), were reported to be expressed in primary auditory afferents (Hossain et al. 2005). Thus, the presence of a relatively small number of Na\(^+\) channels lacking fast inactivation is essential for this neuron’s function and define its electrophysiological phenotype, allowing these “anatomically simple” afferents to link behaviorally relevant auditory signals into patterns of activity that match the requirements of their fast and highly modifiable synapses (Fig. 9D). In addition, this...
subthreshold Na⁺ current was shown to play an essential role in amplifying retrograde synaptic communication via electrical synapses that, by providing a mechanism of lateral excitation, contributes to the synchronization of Club ending afferents (Pereda et al. 1995; Curti and Pereda 2004).

Thus, the specialized functional properties of these neurons suggest that primary afferents can be endowed with complex membrane and synaptic properties and be capable of more sophisticated contributions to auditory processing than has been generally recognized.

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FIGURE LEGENDS

FIGURE 1. Club endings undergo high frequency repetitive firing. A, Experimental arrangement. Large identifiable auditory afferents innervate the rostral portion of the saccular macula (Sacculus), the auditory component of the goldfish ear, and terminate as mixed (electrical and chemical) synapses known as Large Myelinated Club endings (“Club endings”) on the lateral dendrite of the M-cell. Intracellular recordings were obtained in the posterior branch of the VIIIth nerve, where Club ending afferents run. In some occasions, intradendritic recordings were also obtained to monitor changes produced by the activation of these afferents. B, Traces illustrate electrophysiological responses obtained sequentially from the M-cell lateral dendrite and a Club ending in response to brief acoustic stimulation (500 µs duration sound click, lower trace). Brief acoustic stimulation evokes a high frequency burst of action potentials in the recorded Club ending (upper trace) that temporally correlates with the sound-evoked synaptic potential (PSP) recorded in the M-cell lateral dendrite (middle trace), produced by the activity of this and an undetermined number of these afferents. Inset: Club ending afferents were routinely identified by the presence of the coupling potential of the M-cell antidromic spike (AD coupling) evoked by stimulation of the spinal cord. C, Direct intracellular activation of Club ending afferents with depolarizing current pulses (50 ms duration, lower trace) at 1.5 its rheobasic strength or threshold (1.5 T) evokes a repetitive discharge consisting in a train of 7 action potentials that exhibits marked frequency adaptation. D, In contrast, direct intracellular activation of the M-cell axon with a depolarizing current pulse of equivalent magnitude (1.5 T) evokes a single action potential.
FIGURE 2. Characterization of repetitive firing at Club ending afferents. A, Responses of a Club ending to intracellular depolarizing current pulses of increasing magnitude. A 1.7 nA current step (rheobasic intensity) evokes a single action potential at the beginning of the pulse (upper trace). In contrast, a 2.3 nA depolarizing pulse (1.5 its rheobasic intensity) evokes an initial burst of 7 action potentials (middle trace). Finally, a current step almost twice the rheobasic intensity (3.0 nA, lower trace) evokes repetitive firing throughout the duration of the current pulse (note the strong frequency adaptation).

B, Frequency (ordinate) – current (abscissa) plot obtained in the same afferent shown in A for the first (1st ISI, open circles), second (2nd ISI, filled circles) and third (3rd ISI, open triangles) interspike intervals, indicating the presence of primary and secondary firing ranges. The experimental data was fitted with straight lines to the primary and secondary ranges (solid and dashed lines respectively). The slopes of the two ranges for the 1st ISI are indicated.

C, Plot of instantaneous firing frequency versus time for a repetitive discharge evoked by a depolarizing current pulse of 3 nA and 50 ms duration illustrating the time course of the spike frequency adaptation (same example shown in panel A, lower trace; each point represents the average of 15 individual single responses). The data was fitted to a single exponential function with a time constant (τ) of 15 ms and the estimated initial (Fi) and final (Ff) frequencies are indicated.

FIGURE 3. Repetitive firing is abolished by application of QX-314 and TTX. A, Repetitive discharges evoked by an intracellular depolarizing current pulse (1.8 nA, 50 ms duration) 2 minutes (Control, left panel) and 11 minutes after the penetration of the cell with an electrode containing 50 mM QX-314 (QX-314, middle panel). Right: intracellular application of QX-314 simultaneously produced a marked reduction of the amplitude AD coupling potential at depolarized potentials (AD coupling). Superimposed traces of AD coupling recorded 2 minutes (control) and 11 minutes after penetration of
the cell with the QX-314 (QX-314) containing electrode (each trace represents the average of 10-12 single recordings). B, The effects of QX-314 were observed within the time window in which the action potential of the afferents remained largely unaffected by the drug, suggesting that only persistent sodium channels were affected. Graph plots both, the number of action potentials per train (left ordinate, filled circles) and the amplitude of the first action potential (right ordinate, open circles), as a function of time after QX-314 application (abscissa). C, Correlation between the decrease in spike number and the amplification of the AD coupling after QX-314 application. Error bars represent standard deviation. D, Repetitive firing evoked by a depolarizing current pulse (3.3 nA, 50 ms duration) before (Control, left) and 30 minutes after extracellular application of 1 µM TTX (TTX, right). E, As with QX-314, the effects of TTX were observed within the time window in which the action potential of the afferents remained largely unaffected by the drug, suggesting that only persistent sodium channels were affected. Graph plots both, the number of action potentials per train (left ordinate, filled circles) and the amplitude of the first action potential (right ordinate, open circles), as a function of time after TTX application (abscissa). While repetitive firing was quickly abolished after TTX application (action potentials per train were reduced from 11 to 1 within a few minutes), the amplitude of the first action potential remained largely unchanged. F, Summary of the effects of TTX on the number of action potentials per train and action potential amplitude in 6 fish. The mean amplitude of the first action potential (First spike; ordinates) is plotted against the mean number of action potentials per train (Spikes number; abscissa), before (control, filled square) and after TTX application (TTX, open circle). Error bars represent standard deviation. G, Traces shown in D (Control and TTX) are illustrated superimposed and aligned by the first action potential. Note the marked reduction of the depolarizing prepotential that generally precedes repetitive firing (arrow after TTX application).
FIGURE 4. Membrane responses to current steps reveal the presence of a subthreshold Na⁺ current. A, Superimposed voltage responses to current pulses of both polarities (50 ms duration, +2.0 and -0.5 nA, lower panel) before (Control) and 10 minutes after extracellular application of 10 µM TTX (TTX, thicker trace). Note the larger response at the beginning of depolarizing current pulses obtained in control conditions. Each trace represents the average of at least 10 single responses. B, Voltage (ΔV, ordinate) - Current (abscissa) relation obtained in another fiber before (Control, filled circles) and after TTX application (TTX, open circles). The voltage responses were measured 5 ms after the onset of the current step. Membrane response characteristically exhibited a non-linear response to larger depolarizing current pulses in the form of an apparent increase in the slope resistance (Control), which was abolished by application of TTX (TTX; voltage - current relation after TTX was fit with a straight-line function). C, Plot illustrates the difference between the voltage - current relations obtained before (Control) and after TTX application for the experiment shown in panel B, revealing the active component blocked by TTX. Note that the TTX-sensitive component was activated only by current pulses larger than 0.8 nA. Inset, subtraction of the voltage responses to depolarizing current pulses illustrated in panel A (obtained before and after TTX application) revealed the time course of the TTX-sensitive membrane component. This component reached its maximum within 5 ms of the onset of the current pulse and slowly decays towards the end of the pulse. D, Subthreshold membrane mechanisms are the major determinant of the firing behavior at Club ending afferents. The time course of the spike frequency adaptation followed that of membrane responses to near-threshold depolarizing current pulses. To illustrate this property, the scaled decay of the membrane response to a near-threshold current pulse is illustrated superimposed to the
plot of instantaneous frequency versus time of a repetitive discharge induced in the same fiber by a suprathreshold depolarizing current pulse (filled circles, same data shown in Fig. 2C).

**FIGURE 5.** Time course of membrane responses to depolarizing current steps is determined by the interplay between a persistent Na\(^+\) current and a subthreshold K\(^+\) current. A, Kinetics of membrane responses to near-threshold depolarizing current pulses (50 ms) suggests the interplay between depolarizing and hyperpolarizing active conductances. *Upper panel:* average voltage responses to +2.0 nA and +0.2 nA (thicker trace) current pulses are represented superimposed (n=15). Also superimposed, is the response to the +0.2 nA current pulse multiplied by a factor of 10 ["(+0.2 nA)*10", gray thick trace]. Note the difference between the waveforms of the scaled response, mostly determined by resistive and capacitive properties of the membrane, and that obtained in response to a current pulse 10 times stronger, indicating the contribution of active membrane mechanisms to near-threshold responses. *Lower panel:* trace corresponds to the difference between the response to a +2.0 nA current pulse and the response to a +0.2 nA current pulse scaled by a factor of 10 ["(+2.0 nA)-[(+0.2 nA)*10]"; dashed line indicates the 0 mV level and the area above this level is represented in gray for clarity. Consistent with the presence of a TTX-sensitive subthreshold Na\(^+\) inward current, near-threshold responses are initially supralinear (grey area). This response gradually attenuates to become sublinear (arrow), suggesting the slower and delayed activation of a subthreshold outward current. B, Sustained depolarizing responses observed in the presence of K\(^+\) channel blockers are abolished by extracellular application of TTX, revealing the presence of a persistent Na\(^+\) current. Traces illustrate voltage responses to depolarizing current pulses (+1.0 nA, 50 ms duration) obtained after intracellular application of a combination of K\(^+\) channel blockers in the absence (K\(^+\) blockers) and
presence of 10 µM TTX (K⁺ blockers + TTX, thicker trace; traces illustrate the average of 15 individual responses). The grey area represents the magnitude and time course of the TTX-sensitive component evoked by membrane depolarization. C, Extracellular application of 4-AP (5 mM), known to block A-type K⁺ conductances, prolongs the duration of the depolarizing active responses. Upper panel: voltage responses to a +0.8 nA current pulse (50 ms duration) are illustrated superimposed before (control, thin trace) and after (4-AP, thick trace) extracellular application of 4-AP (traces illustrate the average of 10 individual responses). Lower panel: graph represents the mean values of the percentual changes in the ratio between the amplitudes of the voltage responses measured at 5 ms (open circle in upper panel) and 45 ms after the onset of the pulse (filled circle in upper panel), obtained after application of 4-AP and αDTX (error bars indicate standard error of the mean). D, Participation of active mechanisms in near-threshold membrane responses. The figure shows a representative response of a Club ending to a depolarizing current pulse. At the beginning of the pulse the response is dominated by the activation of a persistent Na⁺ current (Na⁺p). The amplifying action of this Na⁺ current is counterbalanced by the delayed activation of an A-type K⁺ current (K⁺).

FIGURE 6. Electrophysiological and pharmacological evidence confirm the presence of an A-type K⁺ current. A, Intracellular activation of Club ending afferents from hyperpolarized membrane potentials induces a marked increase on the time to the first action potential. Superimposed traces illustrate membrane responses to suprathreshold current pulses (50 ms duration) delivered at resting (-73 mV; pulse: +0.9 nA) and at a more hyperpolarized membrane potential (-113 mV; pulse: +2.5 nA; membrane potential held by DC current application; -1.7 nA). Note that regardless of
their different initial membrane potentials, both current pulses reach the same level of depolarization. B, Graph plots the time to the first action potential (Time to first spike; ordinate) as a function of the membrane potential (held by DC current application; abscissa) for the same fiber as shown in A. C, The time to the first action potential observed at hyperpolarized membrane potentials is reduced by extracellular application of 4-AP (5 mM). Superimposed traces illustrate membrane responses to depolarizing current pulses delivered at hyperpolarized potentials (-100 mV; pulse: +2.7 nA; DC current: -1.7 nA), before (Control) and after 4-AP application (thicker trace). 4-AP ultimately leads to the unmasking of a prominent depolarizing plateau potential (see Results and Supplementary Fig. 2). D, Graph summarizes the effect of 4-AP. Plot illustrates the mean time to the first action potential (Time to first spike) obtained for depolarizing current pulses delivered at resting potential (Control) and at a hyperpolarized potential, in the absence (Hyperpolarization) and presence of 4-AP (Hyperpol. + 4-AP). Errors bars represent s.e.m. (p<0.05). E, Estimate of the A-type current recovery from inactivation. The availability of IA channels was inferred from the delay to the first action potential. Superimposed traces illustrate membrane responses to suprathreshold depolarizing current pulses (-64 mV; +5.8 nA). The pulses were preceded by hyperpolarizing prepulses of variable duration (5 to 500 ms) but fixed magnitude (-3.6 nA) that bring the membrane potential to about -100 mV. F, Graph plots the time to the first action potential (Time to first spike; ordinate) as a function of the hyperpolarizing prepulse duration (abscissa) for the same fiber as shown in E. The data was fitted to a single exponential function with a time constant (τ) of 60 ms.

**FIGURE 7.** Balance between persistent Na⁺ and an A-type K⁺ currents determines the patterns of repetitive responses at Club ending afferents. A, Superimposed traces illustrate membrane responses to suprathreshold current pulses delivered at
resting (-75 mV; pulse: +0.5 nA) and at a more hyperpolarized membrane potential (-119 mV; pulse: +2.5 nA; DC current membrane potential held by DC current application; -2.0 nA). Both depolarizing current pulses reach the same final membrane potential. While the pulse applied at resting potential induces just a single spike, that applied from a hyperpolarized membrane level triggers a delayed and sustained repetitive discharge, likely due to an increased availability of both A-type (delay) and persistent sodium (repetitive firing) channels. B, Plot of instantaneous frequency (Frequency) versus time during repetitive discharge (Time) evoked by current pulses applied at these two membrane potentials (open circles: -75 mV; filled circles: -119 mV) in the same fiber. The intensity of the current pulse applied at resting potential was adjusted to give rise to a repetitive response, and its time course fitted to a single exponential function (solid line). Again, note the increased firing response of the Club ending when activated at a hyperpolarized membrane potential. C, Membrane responses to suprathreshold current pulses delivered at a hyperpolarized level (-100 mV; pulse: +2.9 nA, DC current: -1.5 nA) recorded 2 minutes (Control, left panel) and 13 minutes after penetrating the cell with an electrode containing QX-314 (QX-314; 50 mM; right panel). Intracellular QX-314 application induced a marked reduction in the number of spikes. D, Membrane responses to current pulses obtained before (control, left panel) and 5 min. after extracellular application of 5mM 4-AP (4-AP; middle panel). The current pulse that in control conditions was just sufficient to evoke a single action potential, was capable of inducing a vigorous repetitive discharge 5 minutes after 4-AP application. Right Panel: Intracellular application of a combination of K⁺ channel blockers ultimately leads to the development of a prominent plateau potential, which follows the initial fast action potential (triggered by a pulse of 5 ms duration, and 1.3 nA in control and 2.0 nA after TTX). Superimposed is the response obtained 7 minutes after extracellular application of TTX (10 µM). Both, the amplitude and duration of this plateau potential were greatly
suppressed by extracellular application of TTX. E, Top: Membrane responses to current pulses of the same amplitude obtained before (control, left panel) and 11 minutes after extracellular application of αDTX (αDTX; middle panel). No changes in the firing behavior of the afferent were observed after the application of this toxin. Bottom: responses to current pulses of 1.5 its rheobasic strength (threshold) obtained in the M-cell axon before (control, left panel) and 10 minutes after extracellular application of αDTX.

**FIGURE 8. Club endings exhibit resonant membrane properties.** A, Electrophysiological recordings revealed the presence of subthreshold membrane oscillations. Trace illustrates the membrane response to a depolarizing current pulse, consisting of four action potentials followed by a damped oscillation of the membrane potential (trace represents the average of 5 single responses centered in the oscillation; action potentials appear truncated). The frequency of this subthreshold oscillation was estimated fitting a function consisting in the sum of a sinusoidal and an exponential function (143 Hz, thicker trace). B, Combination of active and passive membrane properties determines electrical resonance. Indirect estimates of electrical resonance were obtained by measuring cutoff frequencies of the low-pass and high-pass filtering properties of the membrane from subthreshold membrane responses. **Top panel:** representative membrane response to a near-threshold depolarizing current pulse (50 ms duration). The time constant of a high-pass filter representing the activation of the A-type current (τh), was estimated by fitting a double-exponential function to the decaying portion of the response about 5 ms after the pulse onset. Only the faster and most prominent constant (30 fold the magnitude and 10 times faster than the second one), which likely represents the activation of the A-type current, is represented here and its
value used for the estimates of electrical resonance. The time constant of a low-pass filter, representing membrane passive properties ($\tau_m$), was accurately estimated by fitting a single exponential function to the decay that followed the cessation of current pulses of different polarities and amplitudes (only one of these pulses is illustrated in this example and indicated as $\tau_m$). The time constants were estimated in 2.6 ms and 0.2 ms respectively, for the illustrated example. Bode plots (magnitude versus frequency) were constructed with a model of linear filters (LTI system) using the mean values of $\tau_k$ and $\tau_m$ for the high- (middle panel) and low-pass (lower panel) filters respectively. Cutoff frequencies of the high-pass ($1/2\pi*\tau_k$) and low-pass ($1/2\pi*\tau_m$) filters averaged 61 ± 6 Hz and 803 ± 45 Hz respectively (vertical dashed lines, n=12). C, Bode plot (magnitude versus frequency) constructed using a model of linear filters (LTI system) combining high- and low-pass filter properties of the membrane. The combination of membrane mechanisms with high-pass and low-pass filtering properties determines a band-pass filter with a bandwidth of 742 Hz and a maximum at 220 Hz, suggesting the existence of resonant membrane properties. For comparison, the tuning curves of two representative saccular afferents are also illustrated in the same graph (gray traces; curves originally illustrated in Fay, 1995). Threshold in dB (right side ordinates) is plotted against sound frequency. Note that the characteristic frequencies and Q10dB responses (horizontal lines) of both tuning curves (corresponding to the responses of the two types of afferents found in goldfish) matched the estimated bandwidth of the electrical resonance at Club endings. D, Computer simulations with NEURON revealed the relative contributions of A-type (IA) and persistent Na⁺ (INap) currents to membrane resonance. Plot illustrates the computed input impedance (ordinates, normalized to the magnitude of the membrane response at DC in the absence of any active mechanism) versus frequency (abscissa). An ideal Club ending afferent fiber was modeled as a section consisting in a
cylindrical process with passive properties (see Supplementary Methods). When a delayed rectifier and an A-type current with the kinetics estimated experimentally for the ventral cochlear nucleus (Rothman and Manis, 2003) were added to the model, a clear resonant behavior appeared centered at 220 Hz (K + IA, gray trace). The addition of a persistent Na\(^+\) current produced a significant amplification of the membrane resonance (K + IA + INap, black trace), without modifying the resonant frequency. E, Summary plots of the estimated resonant frequency (left panel; estimated following the procedure illustrated in panel B), and minimum frequency (right panel; defined as the instantaneous frequency of a train consisting of only two spikes). Individual experiments (open circles) and mean (filled circles) and s.d. of the population are illustrated superimposed (n=11).

**FIGURE 9.** Time course of synaptic facilitation at Club ending afferents. A, Synaptic responses evoked by a train of four VIII\(^{th}\) nerve stimuli at 5 ms interval (200 Hz). Stimulation of the posterior VIII\(^{th}\) nerve evokes a mixed synaptic response consisting of a fast electrical, gap junction-mediated, coupling potential (electrical; the fast time course reflects the duration of the presynaptic action potentials) followed by a delayed chemically-mediated EPSP (chemical). Note the progressive facilitation of the chemical synaptic response with the increasing number of pulses (illustrated traces represent, here and below, the average of 10 single responses). B, Characterization of the frequency-dependent facilitation of the chemical component with a two stimuli protocol (paired pulse facilitation). The stimuli were delivered at different intervals (2 ms, 5 ms and 10 ms are illustrated; the electrical components of the synaptic responses appear truncated). C, Upper panel: facilitation of the chemical component, estimated as (second EPSP amplitude – first EPSP amplitude / first EPSP amplitude)*100, is plotted as a function of the paired pulse protocol interval in a representative experiment. The data was fitted to a single exponential function (solid line) with a time constant in this
case of 6.9 ms. The vertical dashed line and the gray rectangular area approximately indicate the peak value and bandwidth of the estimated electrical resonance, respectively. Lower panel: For comparison, the time constant of paired pulse facilitation of chemical EPSP at Club endings is illustrated together with those obtained for the neuromuscular junction (NMJ; Magleby 1987) and Granule to Purkinje cell synapse (Granule cell; Atluri and Regehr 1996). D, Club ending afferents are endowed with electrophysiological properties that allow these afferents to translate behaviorally relevant acoustic signals into patterns of activity that match the requirements of their fast and highly modifiable synapses.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURE 1. Spike frequency adaptation is not due to neither the activation of a Ca^{++}-dependent K^{+} current nor cumulative Na^{+} channel inactivation. A, Lack of slow afterhyperpolarizing potentials (AHP) following a burst of action potentials, characteristic of Ca^{++}-activated potassium currents (Madison and Nicoll 1984; Faber and Sah 2003), suggesting the lack of involvement of these conductances in mediating spike frequency adaptation at Club ending afferents (action potentials appear truncated; see Fig. 1C for an example at a more depolarized membrane potential). Inset: boxed area marked by the asterisk is illustrated magnified for clarity. This interpretation is consistent with the fact that large afferents terminating in the rostral part of the saccular macula, from where Club endings originate, do not contain Ca^{++}-activated K^{+} channels (Davis 1996). B, Spike frequency adaptation is not due to a progressive decrease in Na^{+} channel availability as a result of slow channel inactivation (Fleidervish et al. 1996; Powers et al. 1999; Blair and Bean 2003; Miles et al. 2005). To investigate
this possibility, we quantified several action potential parameters during repetitive firing; these parameters included measurements of fast AHP ("Minimum level"), action potential overshoot ("Peak level") and maximum rate of depolarization ("Slope"; see supplementary Fig. 1B-C). Panel B illustrates a repetitive response of a Club ending to a current step of about twice its rheobasic intensity. Together with the progressive increase in duration of the successive interspike intervals (spike frequency adaptation) there is an increase in the post-spike hyperpolarizing potential ("Minimum level"). C, The 1st, 5th, 10th and 16th action potentials of the train shown in B, are illustrated superimposed. Note the progressive increase in “Minimum level”, and concomitant change in the slope of membrane potential during interspike intervals, which contrasts with the lack of change observed in the action potential “peak level” (measured as the amplitude above 0 mV) and “slope”, during the train (2nd to 16th). D, Evolution of these parameters along the repetitive discharge. Plots of post-spike hyperpolarization potential (“Minimum level”), action potential slope (“Slope”) and amplitude (“Peak level”), as a function of time during the train. Minimum level is plotted against time and was fitted to a single exponential function with a time constant (τ) of 20 ms, which is comparable to that of the spike frequency adaptation (see Fig. 2C). E, Graph plots the “Minimum level” (ordinate) versus instantaneous frequency (abscissa) for the same train. The data was fitted to a straight-line function (solid line). Neither the Slope nor the Peak level, parameters which are most sensitive to Na⁺ channel availability (Hodgkin and Huxley 1952; Miles et al 2005) showed substantial changes during repetitive responses (supplementary Fig. 1D, middle and right panels) suggesting that spike frequency adaptation at Club endings cannot be explained by inactivation of fast Na⁺ channels. F, The absence of cumulative Na⁺ channel inactivation, the sensitivity of “Peak level” and “Slope” parameters to Na⁺ channel availability was independently supported by computer simulations obtained using NEURON software, in which a progressive
decrease in the Na⁺ conductance (in 5% steps) was used to model the progressive inactivation of the Na⁺ channel population during a train of action potentials. The calculated action potential’s amplitude or “Peak level” (left ordinates, open circles) and “Slope” (right ordinates, filled circles) are plotted as a function of Na⁺ channel inactivation (Inactivation, abscissas). Both parameters exhibited a parallel decrease with increasing Na⁺ channel inactivation. Because the density of Na⁺ channels in Club ending afferents is unknown, default values of the Hodgkin and Huxley model were used for these estimates. This simulation confirmed that decreased Na⁺ channel availability is expected to significantly modify both the Peak level and the Slope of the action potentials during a repetitive response (supplementary Fig. 1F).

SUPPLEMENTARY METHODS.

Computer model (Figure 8D). Simulations were performed with the NEURON program (version 5.8; Hines and Carnevale 1997) using a fixed time step of 25 µs. The morphology of Club endings was based on previous studies including anatomical reconstructions following intracellular labeling (Furukawa and Ishii 1967; Sento and Furukawa 1987) and ultrastructural analysis with electronic microscopy (Rosenbluth and Palay 1961). The model consisted of one cylindrical section of 2 mm of longitude and 15 µm in diameter with 50 segments and a specific cytoplasmatic resistivity of 123 Ω-cm. The leak conductance and specific capacitance were set to 0.06 µF/cm² and 0.00015 S/cm² respectively, which matched those expected by experimentally measuring membrane time constant and input resistance (see Results). The reversal potential of the leakage conductance was set to -70 mV, consistent with the values of resting potential experimentally obtained at these neurons of -71 mV (Curti and Pereda 2004).
order to test their impact on membrane properties in the frequency domain, several voltage-dependent conductances were added to the model. The modeled active mechanisms included a Hodgkin-Huxley type K⁺ current (delayed rectifier, K= 0.036 S/cm²; Hodgkin and Huxley 1952), an A-type K⁺ current (IA; IA = 0.03 S/cm²; Rothman and Manis 2003; Traub et al. 2003) and a persistent Na⁺ current (INap = 0.00015 S/cm², ~ 0.1% of the standard values for the squid axon transient sodium current; Crill 1996; Traub et al. 2003). All channel mechanisms used in the present study were obtained form the web-accessible Model DB (Hines et al. 2004) and implemented without modifications. Simulations were performed at 18°C. For the assessment of the effects of Na⁺ channel availability on the amplitude and slope of the action potential, a second model was used. This model consisted of a single compartment containing the program's default properties (number of segments = 1, diameter = 500 µm, length = 100 µm, cytoplasmatic resistivity = 35.4 Ω·cm, specific capacitance = 1 µF/cm²) and default values of the Hodgkin-Huxley conductances (Na⁺, K⁺ and leakage), as the density of these channels in Club endings is unknown.
Figure 1
Figure 2
Figure 4
Figure 6
Figure 7
Figure 8
Figure 9