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T-TYPE CALCIUM CHANNELS MEDIATE REBOUND FIRING IN INTACT DEEP CEREBELLAR NEURONS

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Abstract—Neurons of the deep cerebellar nuclei (DCN) form the main output of the cerebellar circuitry and thus play an important role in cerebellar motor coordination. A prominent biophysical property observed in rat DCN neurons is rebound firing; a brief but strong hyperpolarizing input transiently increases their firing rate to much higher levels compared with that prior to the inhibitory input. Low-threshold T-type voltage-gated calcium channels have been suspected for a long time to be responsible for this phenomenon, but direct pharmacological evidence in support of this proposition is lacking. Even though a multitude of functional roles has been assigned to rebound firing in DCN neurons, their prevalence under physiological conditions is in question. Studies aimed at delineating the physiological role of rebound firing are hampered by the lack of a good pharmacological blocker. Here we show that mibefradil, a compound that blocks T-type calcium channels, potently blocks rebound firing in DCN neurons. In whole-cell experiments both mibefradil and NNC 55-0396 [(1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride]. a more selective T-type calcium channel blocker, effectively blocked rebound firing produced by direct current injection. Thus, mibefradil and other T-type channel modulators may prove to be invaluable tools for elucidating the functional importance of DCN rebound firing in cerebellar computation. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cerebellum, calcium channels, rebound, inhibition, rat.

Neurons of the deep cerebellar nuclei (DCN) form the major output of the computational circuitry of the cerebellum (Ito, 1984). Their rate and pattern of activity encode the information necessary for motor coordination. An interesting biophysical feature of DCN neurons is rebound depolarization (Jahnsen, 1986a; Llinas and Muhlethaler, 1988; Aizenman et al., 1998; Aizenman and Linden, 1999; Molineux et al., 2006; Pugh and Raman, 2006; Wetmore et al., 2007). When the membrane potential of a DCN neuron recovers from hyperpolarization induced by a strong inhibitory input, it transiently rises to a more depolarized level than that preceding the inhibitory input. This rebound depolarization significantly increases the firing rate of the DCN neuron after the stimulus-evoked pause. Given the inhibitory GABAergic nature of Purkinje cell synapses onto DCN neurons, rebound firing has been extensively incorporated into recent theories of cerebellar function (Medina et al., 2000; Kistler and De Zeeuw, 2003; Wetmore et al., 2007) and several functional roles, from timing to encoding information and mediating plasticity have been assigned to it (Aizenman et al., 1998; Kistler and De Zeeuw, 2003; Pugh and Raman, 2006; Wetmore et al., 2007).

However, despite the biophysical robustness of rebound firing in DCN neurons, there is a great deal of debate as to its prevalence and function in response to physiological stimuli (Telgkamp and Raman, 2002; Aksenov et al., 2004; Rowland and Jaeger, 2005, 2008; Holdefer et al., 2005; Alvina et al., 2008). Full characterization of rebound firing and scrutiny of its physiological role *in vivo* require pharmacological tools to manipulate it. These tools are presently lacking.

Low threshold voltage-gated calcium channels are thought to contribute to rebound firing in DCN neurons (Llinas and Muhlethaler, 1988; Aizenman and Linden, 1999; Molineux et al., 2006; Pugh and Raman, 2006). These cells express both high- and low-threshold calcium channels (Muri and Knopfel, 1994; Volsen et al., 1995; Gauck et al., 2001). These channels play an essential role in rebound depolarization in different cells because they activate and inactivate with relatively little depolarization. In fact it has recently been demonstrated that based on expression of different T-type calcium channel subtypes DCN neurons can be classified as strong or weak rebound bursters (Molineux et al., 2006). Here we report that mibefradil, a blocker of T-type calcium channels (McDonough and Bean, 1998; Martin et al., 2000), may prove to be an invaluable tool in resolving the function of rebound firing in the DCN because it effectively blocks rebound firing in these neurons without affecting their pacemaking.

EXPERIMENTAL PROCEDURES

Cerebellar slices

All procedures were in accordance with the policies established by the Animal Institute Committee of the Albert Einstein College of Medicine and in agreement with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Measures to minimize the amount of animals used and their suffering were taken. Wistar rats (P12-20) were anesthetized with halothane and

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Abbreviation: DCN, deep cerebellar nuclei; NNC55-0396, [(1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride].

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decapitated. The brain was quickly removed and placed on cold extracellular solution containing (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, and 10 glucose, pH=7.4 when gassed with 95% O_2:5% CO_2. The cerebellum was dissected and mounted on a modified Oxford vibratome (and 300-μm thick sagittal slices were made. The slices were kept in oxygenated extracellular solution at 34 °C for 1 h, and then at room temperature until use.

Electrophysiological recording

Slices were placed in a recording chamber on the stage of a Zeiss Axioskop microscope. DCN neurons were visually identified using a 40× water-immersion objective with infrared optics. The slices were superfused with the recording solution at a rate of 1.5–2 ml/min and the temperature adjusted to 35±1 °C. 5 mM kynurenic acid (Spectrum Chemical MFG Corporation, Gardena, CA, USA), a broad-spectrum ionotropic glutamate receptor antagonist (Stone, 1993) was added to the recording solution to isolate inhibitory inputs.

Extracellular recordings were obtained from single DCN neurons from all three cerebellar nuclei using a homemade differential amplifier and glass pipette electrodes filled with extracellular solution. Data were sampled at 10 kHz using an analog-to-digital converter (PCI-MIO-16XE-10; National Instruments, Austin, TX, USA), and acquired and analyzed using custom software written in LabView (National Instruments).

Whole-cell recordings were performed using an Optopatch amplifier (Cairn Research, Kent, UK) with electrodes pulled from borosilicate glass (1–3 MΩ resistance when filled with intracellular solution). The internal solution used to measure GABA-induced currents consisted of (in mM): 70 Cs-gluconate, 10 CsF, 20 CsCl, 10 EGTA, 10 HEPES, and 3 Na_2ATP, pH=7.4 (CsOH). To measure rebound firing in current-clamp mode, the internal solution used contained the following (in mM): 125 K methylsulfate, 10 NaCl, 0.01 EGTA, 9 Hepes, 14 creatine phosphate, 4 MgATP, and 0.3 Tris-GTP, pH=7.2 (KOH).

Mibefradil was a generous gift from Hoffmann-La Roche (Basel, Switzerland). [(1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride] (NNC 55-0396) was purchased from Tocris (Ellisville, MO, USA).

Photorelease of caged GABA

4-Carboxy-7-nitroindolinyl-GABA (caged GABA) was synthesized in the laboratory. Caged GABA was dissolved in the extracellular solution at a final concentration of 325 μM. A 1 ms pulse of UV light from a flash lamp (Cairn Research, Kent, UK) was delivered to the epifluorescence port of the microscope using a liquid light guide and was set up as Köhler illumination to uniformly illuminate the field of view. The energy of the UV pulse was adjusted to photorelease enough GABA to produce a well-defined pause of 200–700 ms duration in the spontaneous activity of the cell. Extracellular recording was performed as described above.

Electrical stimulation

Monopolar and bipolar stimulation electrodes were used for electrical stimulation of inhibitory inputs. The monopolar electrode consisted of a glass pipette with a tip diameter of ≈80 μm filled with the extracellular solution. The bipolar electrode was made by twisting a pair of 50 μm nickel wires with a final tip separation of ≈200 μm. The stimulation electrode was positioned between the DCN and the cerebellar cortex to stimulate the axons of Purkinje cells and to avoid either a direct effect on the DCN neuron under study or the direct stimulation of intra-DCN axons. The electrodes were driven by constant current stimulator (Digitimer Ltd, Hertfordshire, England) which was set to deliver 100–200 μs long current pulses. The stimulation paradigm consisted of either a single pulse, or a train of 10 pulses at 100 Hz, with intensities ranging from typically 50 μA to maximally 500 μA.

Pre- and post-pause firing rates were obtained by averaging five interspike intervals prior to, and after the stimulus-evoked pause in the spontaneous activity of the target neuron. Statistical analysis was performed by one-way ANOVA and data were considered not to be statistically different if $P>0.05$. All data are presented as mean±S.E.M.

RESULTS

Contribution of T-type calcium channels to rebound firing in intact DCN neurons

To mimic inhibitory GABAergic inputs and avoid the complications that GABAergic inputs in slices might have been damaged by the slicing procedure, GABA was photoreleased on the soma and dendrites of DCN neurons from caged GABA pre-equilibrated with the slice. We first examined the kinetics of such GABA-evoked currents by whole cell voltage-clamping DCN neurons. As shown in Fig. 1, photorelease of GABA using a 1 ms pulse of UV light resulted in a current that typically had a rise time of a few milliseconds and decayed monotonically with an average time constant of 78.7±9.0 ms (n=8 cells).

The fast rise time and decay time constant of GABA-induced currents suggests that photorelease of GABA on intact DCN neurons is likely to hyperpolarize the cells for a well-defined period of time. We thus examined the consequences of photorelease of GABA on the spontaneous activity of DCN neurons. We avoided whole-cell recordings because it is well-established that they alter the extent of contribution of calcium channels to spontaneous firing of these cells, presumably as a consequence of dialysis of the intracellular milieu (Alvina and Khodakhah, 2008). Therefore we monitored the activity of individual DCN neurons by single cell extracellular recordings in the presence of blockers of excitatory synaptic transmission (kynurenic acid). The spontaneous activity of the neuron was recorded for a minimum of 10 min to obtain a baseline and subsequently GABA was photoreleased onto the target cell as it was done in the whole-cell experiments described above. The energy of the pulse of photolysis UV light was

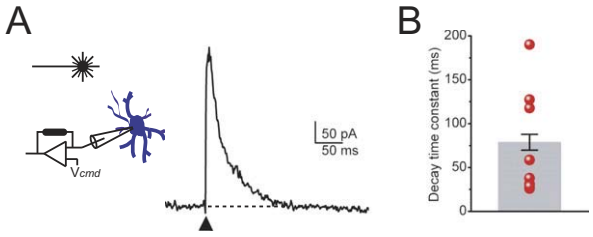

Fig. 1. Photorelease of GABA induces fast inhibitory currents in DCN neurons. (A) Photorelease of GABA above the soma and dendrites of voltage-clamped DCN neuron (see schematic on the left) by a 1 ms pulse of UV light delivered at the time indicated by the black triangle produced a fast rising outward current (V_{cmd}=+30 mV). (B) Average and individual (red circles) values of decay time constant of the GABA-induced outward currents measured at+30 mV in eight cells. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

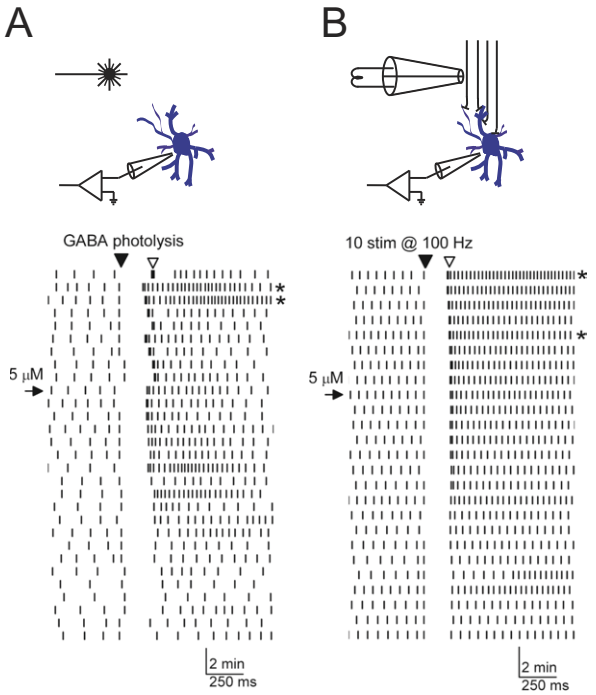

Fig. 2. The rebound firing post-inhibition is abolished by the T-type calcium channel blocker mibefradil in DCN neurons. The raster plots show spontaneous activity of individual DCN neurons recorded extracellularly. (A) Photorelease of GABA, at the time indicated by the black triangle, induced a pause followed by rebound firing in the form of a short high frequency burst (marked with a white triangle above the first raster). Bath application of mibefradil (added at the time marked on the left) blocked rebound firing. (B) Ten electrical stimuli at 100 Hz were delivered at the time indicated by the black triangle to activate inhibitory synaptic inputs to a DCN neuron. After the stimulus-induced pause, prominent rebound firing was observed as a high-frequency burst of action potentials (white triangle). * Indicates traces in which a clear long lasting increase in firing rate was seen. Bath application of mibefradil (at the indicated time on the left) blocked rebound firing, including the long responses.

adjusted such that photorelease of GABA produced a pause of 200–700 ms in the spontaneous activity (Fig. 2A). In accord with our earlier observation (Alvina et al., 2008), in a small fraction of DCN neurons (8/58 cells) clear rebound firing was evident after the pause. This manifested in the form of high-frequency bursts of action potentials after the pause. The 58 DCN neurons sampled and the eight cells that responded with rebound firing were distributed in all three nuclei. Recently it has been shown that the DCN are made of three different types of neurons, which can be partly distinguished based on their baseline firing rate (Uusisaari et al., 2007). On the basis of an analysis of the baseline firing rate of our cells, we have no evidence to believe that the cells that showed rebound firing belonged to any specific type of DCN neurons.

Fig. 2A shows consecutive raster plots in time obtained every 45 s. GABA was photoreleased at the time indicated by the black triangle. The approximately 250 ms long pause produced by GABA in this cell resulted in prominent rebound firing as evidenced by the highly reproducible burst of action potentials (white triangle on the top). In all cells that showed rebound firing the bursts were robust, and were present trial after trial with repeated photorelease of GABA (Fig. 2A). In five of these eight cells we examined the consequences of blocking T-type calcium channels with mibefradil (5–10 μM). In all five cells examined, mibefradil completely abolished rebound firing (Figs. 2A and 3).

We also examined whether mibefradil could equally prevent rebound firing when it was evoked synaptically, because it is possible that under these conditions the GABAergic conductance may differentially hyperpolarize electrical compartments of the cell (e.g. soma vs. dendrites). Inhibitory synaptic inputs were activated by a train of 10 constant current electrical pulses delivered at 100 Hz, or in a small number of cells with a single pulse (nine cells). Similarly to that seen when rebound firing was evoked by GABA photorelease, only 8 out of 39 cells showed rebound firing after electrical stimulation. One of these eight cells was stimulated with a single pulse; the rest was stimulated with a train of 10 pulses. In five of these eight cells (including the one cell stimulated with a single pulse), 5 μM mibefradil was applied to block T-type calcium channels. Fig. 2B shows an example of one such

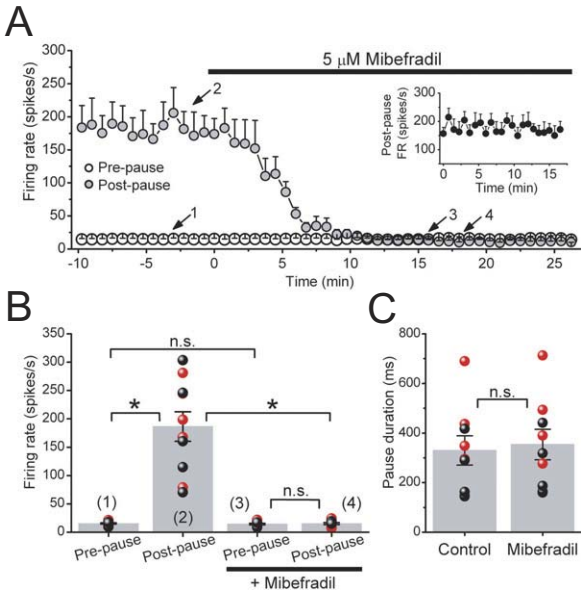

Fig. 3. Mibefradil inhibits rebound firing without affecting baseline spontaneous activity or inhibition. (A) Average time course of the effect of mibefradil on rebound firing induced by GABA photorelease and electrical stimulation (pooled data from the 10 cells examined). The inset shows the average rebound firing rate of six cells in the absence of mibefradil. At the times indicated on the graph, representative pre-pause and post-pause firing rates were measured by averaging the values obtained for 10 trials for each condition. (B) Average and individual firing rate of the cells shown in A, before and after the stimulus-induced pause, in the presence and absence of mibefradil respectively. Red symbols and black symbols represent the experiments done with GABA photorelease and electrical stimulation respectively. * $P<0.001$, n.s. denotes "not significant." (C) The average and individual pause durations for the 10 cells shown in (B) in the presence and absence of mibefradil. n.s. denotes "not significant." For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

experiment. In this cell the stimulation paradigm resulted in a pause of approximately 200 ms duration and caused clear rebound firing as evident by the robust high-frequency bursts seen in the raster plots (white triangle on the top). In some cases, again as seen with GABA photorelease, rebound firing was longer lasting than just a few initial high frequency bursts (note trials denoted by * in Fig. 2). Such long responses have been previously reported and may be mediated by a persistent sodium current (Llinas and Muhlethaler, 1988). As can be seen in the example shown in Fig. 2B and in all five cells examined, application of 5 μM mibefradil effectively blocked rebound firing. Mibefradil also blocked the long rebound responses. This finding is not inconsistent with the notion that these long responses are mediated by a persistent sodium current because the T-type calcium channel–mediated depolarization is likely to be required to activate the persistent sodium current.

To examine the time course of the effect of mibefradil on rebound firing, we measured the firing rate previous to the stimulus-evoked pause (pre-pause), and immediately after the pause (post-pause) in every trial for each cell examined (Fig. 3A). The full effect of mibefradil on the post-pause firing rate took several minutes to complete, perhaps as a consequence of the tortuous diffusion path in the slices. The decline in the post-pause firing rate was clearly not caused by rundown because in the absence of mibefradil, the rebound firing was stable for as long as examined ($\approx$15 min) (inset in Fig. 3A).

The average firing rate prior to photorelease of GABA was 15.3$\pm$1.1 spikes per second and it increased to 194.0$\pm$34.7 during the bursts associated with rebound firing (Figs. 2A and 3B). Application of mibefradil reduced the high-frequency bursts such that on average the post-pause firing rate was comparable to that prior to the GABA-induced pause (15.5$\pm$1.8 spikes per second before mibefradil vs. 13.7$\pm$2.9 spikes per second after, Figs. 2A and 3B, P=0.62). Similarly, the average post-pause firing rate in the electrically-induced rebound firing was 179.0$\pm$42.5 spikes per second and after application of 5 μM mibefradil it decreased to 16.8$\pm$0.8 spikes per second (Figs. 2B and 3B), which was comparable to the firing rate preceding the stimulus-induced pause (13.5$\pm$1.6 spikes per second, Fig. 3B, P=0.15).

In agreement with prior observations (Alvina and Khodakhah, 2008) mibefradil did not alter the rate of spontaneous activity in DCN neurons. The average pre-pause firing rate for the cells tested with GABA photolysis was 15.3$\pm$1.1 spikes per second vs. 15.5$\pm$1.8 spikes per second pre-pause after adding mibefradil (Figs. 2A and 3B, P=0.71). For the cells tested with electrical stimulation in turn, the average pre-pause spontaneous firing rate was 14.8$\pm$1.5 spikes per second before mibefradil vs. 13.5$\pm$1.6 spikes per second after (Figs. 2B and 3B, P=0.58). This suggests not only that T-type calcium channels do not contribute to pacemaking in these cells, but also that at the concentrations used the potential non-specific actions of mibefradil on other channels required for pacemaking in DCN neurons (such as voltage-gated sodium, potassium and N-type calcium channels) is insignificant.

Application of mibefradil also did not change either the duration of the GABA-induced pauses (442.6$\pm$68.7 ms before mibefradil vs. 468.7$\pm$73.0 ms after, Figs. 2A and 3C, P=0.80), or the duration of the electrically-evoked pauses (200.5$\pm$29.9 ms in control vs. 219.9$\pm$31.1 ms in mibefradil, Figs. 2B and 3C, P=0.66). The fact that the synaptically-evoked pauses were not affected by mibefradil again suggests that at the concentrations used mibefradil did not affect synaptic transmission and thus any of the ions channels critical for this process (i.e. voltage-gated sodium and potassium channels and P/Q- and perhaps N- and R-type calcium channels).

Rebound firing induced by direct hyperpolarizing current injection is abolished by T-type calcium channel blockers

Using whole-cell current clamp recordings, we also examined whether blocking T-type calcium channels abolished rebound firing produced by direct current injection in DCN neurons. Fig. 4A shows an example of a cell that from a membrane potential of $\approx-$60 mV was hyperpolarized to $\approx-$85 mV for 500 ms. The hyperpolarization produced a strong rebound firing in the form of a high-frequency burst of two to three action potentials (Fig. 4A). Application of 5 μM mibefradil completely inhibited rebound firing in four out of five cells. In the 5th cell, 5 μM only reduced the firing rate and 10 μM mibefradil was needed to completely abolish it. On average, rebound firing consisted of 2.77$\pm$0.35 spikes with an average firing rate of 112.9$\pm$23.9 spikes per second (Fig. 4D and E). The number of spikes composing the rebound response decreased to 1.07$\pm$0.32 spikes in the presence of mibefradil (Fig. 4E, P=0.009). It was not possible to quantify the rebound firing rate in the presence of mibefradil because it was either absent or consisted of a single spike. It is noteworthy that the level of maximum hyperpolarization produced by the current injection was not affected by mibefradil ($-$80.6$\pm$3.19 mV in control vs. $-$83.45$\pm$3.67 mV in mibefradil, Fig. 4C, P=0.56).

We examined the efficacy of NNC 55-0396, a modified and much more specific mibefradil analog (Huang et al., 2004; Li et al., 2005) in blocking rebound firing in DCN neurons. We performed the same experiments in whole-cell configuration, and induced rebound firing by injecting hyperpolarizing currents as described. An example of such experiment is shown in Fig. 4B. The rebound high-frequency burst of action potentials induced under control conditions was completely abolished after application of 15 μM NNC 055-0396 (a very low concentration at which NNC 055-0396 is exquisitely selective for T-type calcium channels). On average, in control conditions the maximum firing rate after the hyperpolarizing pulse was 157.07$\pm$35.70 spikes per second (n=4 cells), after adding NNC 55-0396 there was on average less than one spike and therefore the firing rate was considered to be zero (Fig. 4D, $P<$0.001 vs. control). Thus, NNC 55-0396 reduced the number of spikes from 2.68$\pm$0.32 to 0.59$\pm$0.06 (Fig. 4E, $P<$0.001 vs. control), without altering the maximum level

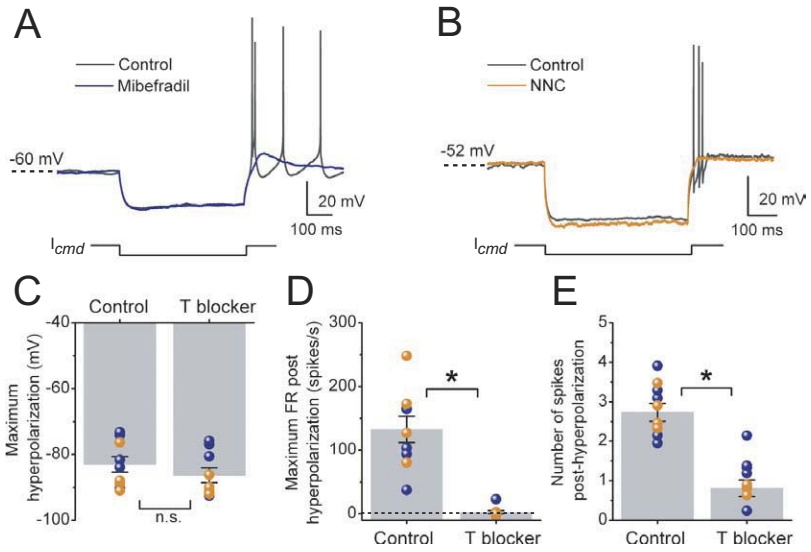

Fig. 4. Blockers of T-type calcium channels abolish rebound firing evoked by direct current injection. (A) Example of a whole-cell current-clamped DCN neuron showing robust rebound firing after a 500 ms long hyperpolarizing pulse to ≈−85 mV, in control conditions (dark gray trace) and after 5 μM mibefradil was added to the bath perfusion (blue trace). (B) Comparable experiment as in (A) but using 15 μM NNC 55-0396 (NNC, orange trace). (C) Average and individual values of maximum hyperpolarization during the current injection in control and after application of T-type channel blocker (T blocker). Blue symbols represent five cells treated with mibefradil and orange symbols represent four cells treated with NNC 55-0396. n.s. denotes "not significant." (D) Average and individual values of maximum rebound firing rate in control conditions, and after the addition of T-type channel blockers (T blocker). Blue symbols represent mibefradil and orange symbols represent NNC 55-0396, * $P<0.001$. (E) Average and individual values of the number of spikes after the hyperpolarizing pulse in control conditions and after adding the T-type channels blockers (T blocker). Blue symbols represent mibefradil and orange symbols represent NNC 55-0396, * $P<0.001$. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

of hyperpolarization (−86.03±3.29 mV in control vs. −89.84±1.28 mV, Fig. 4C $P=0.33$). In summary, these results support the idea that low-threshold T-type calcium channels mediate rebound firing in DCN neurons.

DISCUSSION

A stereotypic biophysical characteristic of DCN neurons is a pronounced rebound depolarization after a strong hyperpolarization (Jahnsen, 1986a; Llinas and Muhlethaler, 1988; Aizenman and Linden, 1999; Molineux et al., 2006). This rebound depolarization, which can result in rebound firing, depends on several factors such as the membrane potential prior to the hyperpolarizing pulse, and the amplitude and duration of the inhibitory stimulus (Jahnsen, 1986a; Aizenman and Linden, 1999). The voltage ranges required to induce rebound firing in DCN neurons are incompatible with high-threshold voltage-gated calcium channels and low-threshold calcium channels were implicated early on (Jahnsen, 1986b; Llinas and Muhlethaler, 1988; Aizenman and Linden, 1999). Whole-cell recordings have shown that rebound firing is obtained at membrane potentials consistent with activation and inactivation of low-threshold T-type calcium channels (Aizenman and Linden, 1999). Moreover, the expression of different T-type channel isoforms has been correlated with particular rebound behaviors in different DCN neurons (Molineux et al., 2006). However, it has been reported that blockade of T-type calcium channels with mibefradil does not abolish rebound firing (Aizenman and Linden, 1999).

In an attempt to characterize the function of voltage-gated calcium channels in the regulation of rebound firing in DCN neurons and to find a tool to manipulate it, we revisited this issue under more physiological conditions. We monitored the activity of DCN neurons extracellularly and produced a prominent pause in their spontaneous firing by photoreleasing GABA onto their soma and dendrites. In a small fraction of DCN neurons (16 out of 97 cells) this procedure was effective in producing robust rebound firing. We found that in all cells examined mibefradil, a well-established T-type channel blocker (McDonough and Bean, 1998; Martin et al., 2000), completely abolished the increase in the firing rate observed after photoreleasing GABA. We similarly found that mibefradil blocked rebound firing in all cells in which inhibitory synaptic inputs were used as the stimuli to induce rebound firing. Our finding that mibefradil blocks rebound firing is in contrast to an earlier report (Aizenman and Linden, 1999). The difference is likely to be the consequence of the fact that in the earlier study a lower concentration of mibefradil was used and that we found that a relatively long time (several minutes) was required for mibefradil to equilibrate with the slice. Supporting our findings, a more recent publication reported that mibefradil decreases the maximum rebound firing rate after a very strong and prolonged hyperpolarization (Molineux et al., 2008). While in this study mibefradil did not completely block rebound firing it is noteworthy that the hyperpolarizations used were of much larger amplitude and greater duration compared with ours and may have recruited additional conductances. Given

that under physiological conditions it is unlikely that DCN neurons are hyperpolarized to such a great extent, we believe that blocking T-type calcium channels is an effective approach to block rebound firing.

An obvious concern would be non-specific actions of mibefradil on other calcium channels. In fact, mibefradil blocks some R-type calcium channels in addition to T-type calcium channels (Randall and Tsien, 1997; Viana et al., 1997; Jimenez et al., 2000). However, given that R-type calcium channels are high-threshold (Randall and Tsien, 1997; Tottene et al., 2000); our data suggest that low-threshold T -type calcium channels mediate rebound firing in DCN neurons. Moreover, the effect of the more specific T-type channel blocker NNC 55-0396 used at low concentration here (Huang et al., 2004), corroborates the fact the blocking T-type channels (as done so when using mibefradil) abolishes rebound firing. Depending on the subunit composition and expression of auxiliary subunits, mibefradil can also block other voltage-gated channels (Jimenez et al., 2000; Perchenet and Clement-Chomienne, 2000; Chouabe et al., 2000). However, our finding that at the concentrations used (mainly 5 μM and in some earlier experiments 10 μM) mibefradil affected neither spontaneous pacemaking nor synaptic transmission suggests that it is extremely unlikely that its efficacy in blocking rebound firing is the consequence of its non-specific actions on other channels. It is important to note that despite the very remote possibility that mibefradil may be blocking rebound firing in DCN neurons by a non-specific action on other channels, given its lack of an effect on intrinsic conductances and synaptic transmission, mibefradil remains an effective tool for examining the physiological function of rebound firing in the cerebellum. Taking into account the effect of NNC 55-0396, it is clear that T-type channels modulators can be effectively used to study the potential role of rebound firing in the cerebellum.

A multitude of functional roles has been assigned to DCN rebound firing (Aizenman et al., 1998; Pugh and Raman, 2006; Wetmore et al., 2007). There is, however, some debate as to how prevalent rebound firing is under physiological conditions (Telgkamp and Raman, 2002; Aksenov et al., 2004; Rowland and Jaeger, 2005, 2008; Holdefer et al., 2005; Alvina et al., 2008). After sensory stimulation, for example, the response of DCN neurons *in vivo* can consist of three components: a short-latency increase in spiking, an intermediate inhibition and a late increase in firing rate (Armstrong et al., 1975a,b; Rowland and Jaeger, 2005). The late increase, which could perhaps be the consequence of rebound depolarization, was the least frequent response component in all nuclei (Rowland and Jaeger, 2005). Moreover, these late increases are more likely to be the consequence of direct excitation of DCN neurons by mossy and olivary fibers rather than rebound firing because they are often not preceded by a pause in activity (Rowland and Jaeger, 2008). It has also recently been shown that direct and strong activation of Purkinje cell inputs of a DCN neuron *in vivo* does not result in rebound firing (Alvina et al., 2008).

It is plausible, but remains to be demonstrated, that even if rebound firing is uncommon it may be physiologically relevant and may play an important functional role in cerebellar function, perhaps in specialized motor tasks. It is hoped that the establishment of mibefradil (and other T-type calcium channels blockers) as an effective blocker of this phenomenon will aid future studies aimed at elucidating the functional relevance of DCN rebound firing in cerebellar computation.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2008.09.052.

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