Rebound excitation triggered by synaptic inhibition in cerebellar nuclear neurons is suppressed by selective T-type calcium channel block

Rebecca Boehme,¹,²,³ Victor N. Uebele,⁴ John J. Renger,⁴ and Christine Pedroarena¹,²
¹Department of Cognitive Neurology, Hertie Institute for Clinical Brain Research, ²Systems Neurophysiology Group, Werner Reichardt Centre for Integrative Neuroscience, and ³Graduate School for Neural and Behavioral Sciences, University of Tübingen, Tübingen, Germany; and ⁴Merck Research Laboratories, West Point, Pennsylvania

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Boehme R, Uebele VN, Renger JJ, Pedroarena C. Rebound excitation triggered by synaptic inhibition in cerebellar nuclear neurons is suppressed by selective T-type calcium channel block. J Neurophysiol 106: 2653–2661, 2011. First published August 17, 2011; doi:10.1152/jn.00612.2011.—Following hyperpolarizing inputs, many neurons respond with an increase in firing rate, a phenomenon known as rebound excitation. Rebound excitation has been proposed as a mechanism to encode and process inhibitory signals and transfer them to target structures. Activation of low-voltage-activated T-type calcium channels and the ensuing low-threshold calcium spikes is one of the mechanisms proposed to support rebound excitation. However, there is still not enough evidence that the hyperpolarization provided by inhibitory inputs, particularly those dependent on chloride ions, is adequate to deinactivate a sufficient number of T-type calcium channels to drive rebound excitation on return to baseline. Here, this issue was investigated in the deep cerebellar nuclear neurons (DCNs), which receive the output of the cerebellar cortex conveyed exclusively by the inhibitory Purkinje cells and are also known to display rebound excitation. Using cerebellar slices and whole cell recordings of large DCNs, we show that a novel piperidine-based compound that selectively antagonizes T-type calcium channel activity, 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydropyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2), suppressed rebound excitation elicited by current injection as well as by synaptic inhibition, whereas other electrophysiological properties of large DCNs were unaltered. Furthermore, TTA-P2 suppressed transient high-frequency rebounds found in DCNs with low-threshold spikes as well as the slow rebounds present in DCNs without low-threshold spikes. These findings demonstrate that chloride-dependent synaptic inhibition effectively triggers T-type calcium channel-mediated rebounds and that the latter channels may support slow rebound excitation in neurons without low-threshold spikes.

Cav3.² channels; cerebellum; chloride inhibition; GABA; postinhibitory rebound

PIONEERING STUDIES showed a period of increased excitability in many neurons following hyperpolarizing potentials that was often associated with the discharge of calcium-dependent low-threshold spikes (LTSSs) and high-frequency bursts of sodium spikes (Andersen et al. 1964; Jahnsen and Llinás 1984; Kandel and Spencer 1961; Kuffler and Eyzaguirre 1955; Llinás and Yarom 1981). This property, known as rebound excitation (RE), provides a means to convert an inhibitory signal into an excitatory one that can be transferred to other neurons. RE has been found in many different neuronal types and is postulated to participate in many functions and brain processes under normal and pathological conditions (Perez-Reyes 2003). Notable examples include the prominent RE of thalamic neurons thought to contribute to sleep and epilepsy (Andersen et al. 1964; Kim et al. 2001; Steriade and Llinás 1988), the RE found in neurons of circuits generating rhythmic motor outputs (Bertrand and Cazalets 1998; Miller and Selverston 1982; Syed et al. 1990), the RE found in neurons of the olfactory and visual pathways (Balu and Strowbridge 2007; Liu and Shipley 2008; Lo et al. 1998; Margolis et al. 2010), the RE found in the GABAergic periaqueductal gray neurons involved in analgesia (Park et al. 2010), and the RE described in neurons of the inferior olive and deep cerebellar nuclei (Aizenman and Linden 1999; Alvina et al. 2008; Czubayko et al. 2001; Jahnsen 1986a; Llinás and Mühlethaler 1988; Llinás and Yarom 1981; Mol ineux et al. 2006; Pedroarena 2010; Pugh and Raman 2006; Tadayonnejad et al. 2009; Uusisaari et al. 2007) thought important for motor control and other aspects of cerebellar function.

In particular, the deep cerebellar nuclear neurons (DCNs) are the main target of the GABAergic Purkinje cells and carry the output of the cerebellum to other brain structures. Thus, since its early description, RE has been hypothesized to play an important role in cerebellar function (Llinás and Mühlethaler 1988). Purkinje cell synapses can be activated in cerebellar slices containing the cerebellar nuclei, and DCNs fire spontaneously in vitro with frequencies similar to those found in vivo recordings. Therefore, Purkinje cell-evoked RE can be investigated in vitro in DCNs at membrane potentials likely similar to those found in behaving animals.

The activation of T-type calcium channels, a group of calcium channels that display lower threshold than sodium channels, remain active only transiently, and deinactivate on hyperpolarization (Carbone and Lux 1984; Fox et al. 1987), has been previously proposed as the basis for RE and LTSSs in many neurons, including the DCNs (Llinás and Mühlethaler 1988). Indeed, several groups confirmed the expression of T-type channels and showed calcium potentials, calcium currents, or calcium concentration changes with voltage dependence, kinetics, and pharmacology consistent with T-type channel activation in DCNs (Aizenman and Linden 1999; Alvina et al. 2009; Czubayko et al. 2001; Llinás and Mühlethaler 1988; Molineux et al. 2006; Muri and Knöpfel 1994; Zheng and Raman 2009).

However, several issues raise the question of whether T-type calcium currents are involved in all cases of RE. First, although RE can be evoked by inhibitory postsynaptic potentials (IPSPs) in DCNs in vitro (Llinás and Mühlethaler 1988; Aizenman et
al. 1998; Aizenman and Linden 1999; Pedroarena 2010; Sangrey and Jaeger 2010; Tadayonnejad et al. 2009; Zheng and Raman 2009) and in vivo preparations (Hoebeek et al. 2010), the limited hyperpolarization attained by chloride-dependent IPSPs has been noted as an obstacle for the deactivation of T-type channels in DCNs (Alvina et al. 2008; Zheng and Raman 2009). Indeed, this issue applies to all neurons where the deactivation of T-type calcium channels is expected to occur as a result of chloride-dependent inhibition. Second, LTSs are considered a hallmark of T-type channel activation and thought to provide the depolarization underlying RE. However, only some DCNs display LTSs (Molineux et al. 2008; Pedroarena 2010), calling into question the role of T-type calcium channels in the RE of neurons without LTSs.

Third, other ionic mechanisms could support DCN RE, in particular, hyperpolarization-activated current ($I_h$) activation (Aizenman and Linden 1999; Czubayko et al. 2001), increased availability of sodium channels (Aman and Raman 2007), activation of other calcium currents (Zheng and Raman 2009), and activation of a persistent sodium current ($I_{NaP}$) (Jahnsen 1986b; Sangrey and Jaeger 2010).

The understanding of the ionic basis of RE has been hindered by the lack of specific and potent T-type calcium channel blockers (reviewed in Isope et al., in press). Here, using whole cell recordings of large DCNs in cerebellar slices from rats and mice and the application of 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydropryan-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2), a new compound that selectively blocked T-type calcium channels in recombinant (Shipe et al. 2008) and native systems (Dreyfus et al. 2010), it is demonstrated that T-type calcium channel activation is involved in RE evoked by current injection as well as IPSPs. Moreover, TTA-P2 blocked the slow RE typical of DCNs without LTSs, indicating that T-type calcium currents also contributed to RE in these neurons.

MATERIALS AND METHODS

Cerebellar slice preparation. The animal protocol was reviewed and approved by an independent local committee and the Regional Council of Tübingen and conducted according to the standards of German law and the Society for Neuroscience (SFN). Experiments were performed as previously described (Pedroarena and Schwarz 2003). Briefly, cerebellar slices from C57BL/6 mice or Sprague-Dawley rats (postnatal days 14–17) were prepared using a vibratome (Leica, Bensheim, Germany) and ice-cold artificial cerebrospinal fluid (containing in mM: 125 NaCl, 2.5 KCl, 1.3 NaH2PO4, 1.5 MgCl2, 26 NaHCO3, 20 glucose, 2.5 CaCl2, bubbled with 95% O2-5% CO2).

Patch-clamp recordings and extracellular stimulation. Recordings were performed using a submersed type of recording chamber. Whole cell current-clamp recordings were made from large (>18–µm) DCNs in the lateral or interpositus nuclei using an AxoClamp-2B amplifier (Axon Instruments). The electrode solution contained (in mM): 134 K-glucosinate, 6 KCl, 10 K-HEPES, 0.1 EGTA, 0.3 NaGTP, 2 KATP, 10 phosphocreatine, 2 MgCl2. The calculated junction potential (between the bath and the pipette solution) was 15.1 mV, and, as usual, it was nulled by adjusting the direct current (DC) offset before patch recording. In many studies, under the assumption that the intracellular composition of neurons recorded under whole cell patch recording is identical to the pipette solution, the readings of the membrane potential are manually or automatically corrected for the calculated junction potential. The assumption that the intracellular composition is identical to the intracellular solution is probably correct for small cells, but deviations may occur in large neurons and neurons with active membrane ion transporters (Neher 1992). Therefore, and to facilitate comparisons with other studies, we provide both the noncorrected and corrected membrane values that may indicate the lower and upper bounds of the actual membrane potential. According to the extra- and intracellular concentration of chloride ions used in the present study, the calculated equilibrium potential for chloride ions was −68.6 mV at 32°C and −66.8 mV at 24°C. To stimulate Purkinje cell axons, a pair of tungsten microelectrodes (Frederick Haer) was located in the white matter surrounding the cerebellar nuclei. Single or repetitive current pulses (100 µA, 1–100 µA) were delivered using a constant-current unit (stimulus isolator; WPI) every 10–50 s. Recordings were digitized (25 kHz), stored, and analyzed using programmable software ( Spike2; CED). IOR (WaveMetrics) and SigmaStat (SPSS) were used for further analysis. Data are presented as means ± SE.

Drugs. The following drugs were bath applied: kynurenic acid (3–5 mM; Tocris), a broad-spectrum antagonist of ionotropic glutamate receptors, to isolate pharmacologically Purkinje cell axon activation; gabazine (3 µM; Tocris), a GABA_A antagonist in selected experiments; and TTA-P2 (Merck), a specific T-type calcium channel blocker. TTA-P2 was diluted in DMSO to prepare stock solutions. The final concentration of TTA-P2 chosen for this study was 1 µM TTA-P2 (SISO, 0.01%), since concentrations ≥2 µM did not result in further attenuation of RE, and lower doses (0.1–0.5 µM) induced submaximal effects. SNX-482 (300 nM; BioTrend), a blocker of R-type calcium channels, was applied after the recording chamber perfusion was stopped. In control recordings (without SNX-482 added), stopped inflow resulted in hyperpolarization of all recorded neurons.

This effect is probably due to the decrease in the bath temperature associated with the stopped inflow, since we used an in-line temperature controller. Therefore, recordings during application of SNX-482 were obtained during DC injection to match the level of the membrane potential found in control conditions.

Experimental protocols and analysis. Measurements were performed, unless specified, without injecting DC. All DCNs investigated were spontaneously active. Several parameters were analyzed. First, changes in the membrane potential of spontaneously firing DCNs were assessed. These were estimated as changes in the average interspike trough. Second, we investigated the presence of LTSs, here defined as a transient, slow depolarizing event (lasting tens of milliseconds), with lower threshold than fast spontaneous spikes. For this purpose, 1-s-long current steps were injected while the neuron was hyperpolarized by DC injection up to −100 mV [membrane potential corrected for the junction potential (Vj): −115 mV]. LTSs were observed only in part of the recorded DCNs in variable proportions according to the animal species and room temperature: in recordings from mouse slices at room temperature, 38% (n = 34), in mouse slices at 32°C, 53% (n = 13), in rat slices at 32°C, 94% (n = 35). Although the methods of slicing and recording were identical for both species, it cannot be ruled out that the recordings were inadvertently obtained more often from particular subnuclei in one species than in another.

Third, the presence of RE in response to hyperpolarizing stimulus was investigated in spontaneously firing DCNs without injecting DC and evoked either by 1-s-long hyperpolarizing steps of increasing magnitude or by trains of IPSPs (0.2–1 s long, 30–100 Hz). The magnitude of current pulses was increased until the hyperpolarization peaked close to −100 mV (Vj: −115 mV). RE was defined as an increase in spontaneous firing above baseline level (Pedroarena 2010). The magnitude of RE was evaluated over the 1st s after the end of the hyperpolarizing stimulus using three parameters: 1) absolute maximal rebound frequency (MRF), calculated as the peak of absolute instantaneous frequency during this period; 2) the relative MRF (RMRF), calculated as the difference between the MRF and the basal frequency; and 3) the averaged rebound frequency (ARF), which was calculated as the difference between the averaged frequency over 1 s starting 200 ms after the end of the hyperpolarizing stimulus (to exclude the initial transient period of fast rebound) and the basal frequency.
Two types of RE were distinguished according to their time course: transient (T) and slow (S) rebounds (see RESULTS). The following heuristic criterion was used to classify rebounds: whenever the instantaneous frequency decayed within at least 40 ms from the time of the maximum to a value equal to 60% of the RMRF, the rebound was classified as transient. This criterion was successful in predicting which neurons would display LTSs on hyperpolarization. In contrast, using MRF was not 100% predictive. Particularly, low MRF could be associated with the presence of LTS on hyperpolarization. Different types of DCN rebounds were previously reported in the literature using similar but not identical classifying criteria. Thus it is important to clarify the similarities and differences for comparison purposes. In particular, transient and weak rebounds have been previously distinguished (Molineux et al. 2006). Specifically, in the study by Molineux et al. (2006) and subsequent ones from the same group, the MRF was used as the main classifying criteria, whereas in our classification, the time course of the frequency change was used. Because high-frequency rebounds usually show a transient time course, this group of rebounds is most likely coincident in both classifications. However, within the weak rebound DCNs, some of the cells were reported to display LTSs. The latter instances most likely would have been classified as T-rebounds using the criteria employed in the present study. In studies from a different laboratory, a different type of rebound was found in recordings from GABAergic DCNs (Uusisaari et al. 2007), which typically showed a burst with a long plateau of sustained high-frequency firing in recordings from more hyperpolarized potentials. Although the recording conditions were not identical, evidence for this type of rebound was not found in the present study, suggesting that most of the recorded neurons in the present study were non-GABAergic DCNs.

RESULTS

LTSs and their associated high-frequency burst of spikes are considered a hallmark of T-type channel activation (Fig. 1A, left, control trace). However, because only a subset of DCNs displayed LTSs, even when explored from hyperpolarized membrane levels (see MATERIALS AND METHODS), the effect of TTA-P2 was separately analyzed in DCNs that did or did not display LTSs. RE, a posthyperpolarization increase in firing frequency above the baseline level, was investigated using hyperpolarizing stimuli in spontaneously firing DCNs. It differed between the two subsets of DCNs (Pedroarena 2010). DCNs displaying LTSs showed T-rebounds, different from DCNs without LTSs, which showed S-rebounds. Briefly, T-rebounds showed a transient increase in instantaneous frequency that lasted few intervals (1–4 typically) and decreased sharply afterward (Fig. 1; see MATERIALS AND METHODS for detailed criteria). S-rebounds showed a smooth decay in firing fre-

Fig. 1. 3.5-Dichloro-N-[1-(2,2-dimethyl-tetrahydropyran-4-ylmethyl)-4-fluoropiperidin-4-ylmethyl]-benzamide (TTA-P2) blocks low-threshold spikes (LTSs) and transient (T) rebounds in deep cerebellar nuclear neurons (DCNs). A: typical traces from a mouse DCN recording illustrating block of mouse LTSs and the associated burst of spikes by TTA-P2 application (left). The membrane potential was hyperpolarized by direct current (DC) injection (−300 pA). Right: only tonic spiking could be evoked from the same level using stronger depolarizing steps. Bars below the recordings indicate the time of the depolarizing current pulses. The depolarizing current pulses were 100, 200, and 300 pA. Vm indicates in this and all following figures the membrane potential (Vm) corrected for the junction potential. Because of the uncertainty about the actual value, both values are reported (see text for further discussion). B: traces illustrate recordings from a rat DCN displaying a T-rebound (top) blocked by the application of TTA-P2 application (bottom). Traces show the posthyperpolarization response to current steps that hyperpolarized the DCN close to −70 mV. Black circles, here and in the next panels, indicate the instantaneous frequency corresponding to DCNs firing (note that the left scale applies to both Vm and frequency. The right scale applies to Vc). The arrow indicates the maximal rebound frequency (MRF) before the application of TTA-P2. The dashed lines indicate the basal firing frequency. C: summary plots of relative MRF (RMRF)-current relationships from recordings obtained before and during the application of TTA-P2 (top; n = 8, rat DCNs). Bottom: the curve during TTA-P2 is plotted at magnified scale. These curves were constructed using the RMRF of rebounds evoked by 1-s hyperpolarizing current pulses. The magnitude of the current was increased until the Vm peaked at approximately −100 mV (see MATERIALS AND METHODS for further details). D: typical voltage responses to hyperpolarizing steps before and during TTA-P2 are shown semioverlapped. D’: summary bar plot of the depolarizing sag, calculated as Vm/VSS (peak voltage/steady-state voltage; further details in text). E and E’: typical example showing no effect of TTA-P2 application on action potentials (E) or its derivative values (E’). F: summary graph of derivative values of action potentials (details in text). G: summary plot showing no effect of TTA-P2 on the frequency-current curves constructed using responses to depolarizing pulses (n = 5, rat DCNs).
frequency (Fig. 2). In addition, the rebound frequency-current relationship of T- and S-rebounds differed: T-rebounds displayed a step-like curve (Fig. 1), but S-rebounds showed a linear function (Fig. 2). To quantify changes in rebound frequency, we calculated the absolute MRF, the difference between basal frequency and MRF (RMRF), and the rebound frequency averaged over the 1st s of rebound (ARF) as detailed in MATERIALS AND METHODS.

TTA-P2 effect on LTSs and T-rebounds. In agreement with the idea that LTSs result from T-type channel activation, TTA-P2 (1 μM) blocked LTS in all neurons investigated (mice, n = 10; rats, n = 9; Fig. 1A). After the maximal TTA-P2 effect was achieved (with ~10 min of bath application), spiking from the same holding level could only be evoked using higher depolarizing currents and only in tonic spiking mode (Fig. 1A, right).

If T-rebounds were based on the same ionic mechanism as LTSs, TTA-P2 would also be expected to block these events. Consistent with this idea, TTA-P2 blocked T-rebounds in all neurons investigated (n = 17). The example in Fig. 1B is characterized in control conditions by a spike “doublet” (top). In this case, an LTS is not obvious under these conditions of recording. The doublet was blocked by the application of TTA-P2, and the instantaneous frequency (black circles) was markedly reduced, peaked later, and decayed slowly (bottom). In addition, TTA-P2 application changed the MRF- and RMRF-current relationships of T-rebounds from step-like functions into linear ones (Fig. 1C summary plot illustrates the changes in RMRF induced by TTA-P2 in rat DCNs, top). The rebound frequency continued to increase with increasing current intensity, but values were several times lower than the control (Fig. 1C, bottom). Similar results were observed in mouse DCNs (data not shown). On average, pooling together data from mice and rat DCNs, the application of TTA-P2 reduced MRF to 15% of the control values (control, 238 ± 23 Hz; TTA-P2, 32 ± 4 Hz; n = 13; P < 0.001) and RMRF to 10.5 ± 2.25% of control (control, 227 ± 23 Hz; TTA-P2, 21 ± 3 Hz; n = 13; P < 0.001). The TTA-P2 effect was not limited to the transient high-frequency period: it also reduced the slow increase in firing frequency that followed it, as estimated by the reduction in the ARF to 41 ± 6.5% (control, 12.5 ± 1.5 Hz; TTA-P2, 5.3 ± 1.3 Hz; n = 11; P < 0.001).

Next, to assess the selectivity of TTA-P2, the effects of this drug on other electrophysiological properties were analyzed. In particular, we examined whether responses dependent on other membrane channels potentially contributing to RE in DCNs were affected by TTA-P2. First, I_h has been proposed to be involved in RE (Aizenman and Linden 1999; Czubayko et al. 2001). However, during TTA-P2 application, no significant changes were observed in the depolarizing sag evoked by hyperpolarizing pulses, and thus it is unlikely that the effect of TTA-P2 was due to I_h suppression (control, 0.54 ± 0.02; TTA-P2, 0.55 ± 0.02; n = 13; P = 0.625; Fig. 1, D and D’), consistent with previous findings (Huang et al. 2011). As illustrated in Fig. 1, B and C, RE was not completely abolished by TTA-P2, and thus it is possible that I_h activation may be one of the mechanisms explaining the remaining RE (Aizenman and Linden 1999). Next, the increased availability of high-voltage-activated (HVA) calcium channels has also been considered a mechanism supporting RE (Zheng and Raman 2009).

Fig. 2. TTA-P2 application inhibits slow (S) rebounds. A: typical S-rebound (top) suppressed by TTA-P2 (bottom). Traces correspond to the intracellular recordings from a mouse DCN depicting the posthyperpolarization response. Black circles illustrate the corresponding instantaneous frequency (note that the left scale applies to both V_m and frequency. The right scale applies to V_m). Note the slow decay of frequency. Dashed lines indicate baseline frequency in A and A’. A’: the effect of TTA-P2 is more obvious in the plots of instantaneous firing frequency for the same neuron depicted in Fig. 1A at a condensed time scale. Plots correspond to representative spontaneous firing (left) and the rebound excitation (RE) evoked by 2 increasing levels of hyperpolarization (middle and right) before (top) and during (bottom) TTA-P2 application. Arrowheads indicate the end the hyperpolarizing stimuli. B: summary plot illustrates the RMRF-current relationship for neurons with S-rebounds before and during the application of TTA-P2 (n = 5, mouse DCNs). C and D: typical example of spikes from neurons displaying S-rebounds before and during TTA-P2 application (C) and the corresponding derivative traces (D). E: summary plot showing no significant effect of TTA-P2 application on the derivative values for up- and downstroke phases of the spontaneous action potentials of DCNs with S-rebounds (details in text).
Previous results showed that DCN spontaneous firing rate is tightly regulated by HVA calcium channels (Alvina and Khodakhah 2008; Zheng and Raman 2009). However, TTA-P2 did not induce consistent changes in the membrane potential of spontaneously firing DCNs (control, $-52 \pm 2.7$ mV; TTA-P2, $-52 \pm 2.7$ mV; $n = 10$; $P = 0.49$; $V_c$: control, $-67 \pm 2.7$ mV; TTA-P2, $-67 \pm 2.7$ mV) nor in the spontaneous firing rate (control, $12 \pm 2.2$ Hz; TTA-P2, $11.5 \pm 2.0$ Hz; $n = 11$; $P = 0.31$), strongly suggesting that TTA-P2 did not modify DCN HVA calcium currents. These results are in agreement with previous studies showing no effect of TTA-P2 on HVA calcium channels (Dreyfus et al. 2010; Huang et al. 2011). The lack of effect of TTA-P2 on firing rate also indicates that TTA-P2 did not modify the calcium-dependent potassium channels that are known to regulate DCNs firing rate (Aizenman and Linden 1999; Czubayko et al. 2001; Raman et al. 2000). Thus it is unlikely that TTA-P2 suppressed RE by modifying HVA calcium channels or calcium-dependent potassium currents. Another mechanism for DCN RE discussed in the literature is the hyperpolarization-induced increase in the availability of sodium channels (Aman and Raman 2007). Thus it was determined next whether TTA-P2 modified the transmembrane currents associated with spontaneous spikes. These were estimated by the derivative of spontaneous action potentials. As illustrated in Fig. 1, $E$, $E'$, and $F$, no changes were detected, suggesting that TTA-P2 application did not modify the sodium conductance involved in the upstroke or the potassium current responsible for the downstroke phases of action potentials (control, $265 \pm 23$ V/s; TTA-P2, $266 \pm 23$ V/s; $n = 10$; $P = 0.92$; control, $140 \pm 18$ V/s; TTA-P2, $-140 \pm 18$ V/s; $n = 10$; $P = 0.91$; for up- and downstroke phases, respectively).

Moreover, no changes were detected in the spontaneous firing frequency (see above) or in the frequency-current relationships constructed using the mean firing rate of responses evoked by 1-s depolarizing steps (gain: control, $251 \pm 0.03$ Hz/nA; TTA-P2, $253 \pm 0.04$ Hz/nA; $n = 9$; $P = 0.96$; Fig. 1G). These results argue against the idea that TTA-P2 suppressed rebounds by modifying sodium channels. Finally, TTA-P2 had no effect on the input resistance measured with small hyperpolarizing pulses applied at potentials below $-70$ mV (−85 mV $V_c$: control, $149 \pm 26$ MΩ; TTA-P2, $145 \pm 27$ MΩ; $n = 12$; $P = 0.42$). The latter results indicated that the TTA-P2 block of rebounds could not be explained by a reduction in input resistance and a subsequent decrease in the effectiveness of the hyperpolarizing current pulses. However, it was found that in 9 of the 12 DCNs investigated, TTA-P2 application increased the apparent input resistance measured using small hyperpolarizing pulses from the spontaneous membrane potential. As noted above, TTA-P2 did not result in changes in the membrane potential or spontaneous firing rate as would have been expected in the case where TTA-P2 blocked a tonic T-current (Dreyfus et al. 2010). However, the increased apparent input resistance measured at relatively depolarized potentials could be a hint that a tonic T-inward current is present in some neurons but that its voltage effect is balanced by an associated outward current. Considering all of these data together, the efficiency and specificity of TTA-P2 action are in agreement with previous data (Dreyfus et al. 2010; Huang et al. 2011; Shipe et al. 2008) and show that TTA-P2 selectively suppressed LTSs and T-rebounds in DCNs.

**TTA-P2 effect on S-rebounds.** Although the involvement of T channels in the generation of T-rebounds seemed a highly plausible notion because the same cells displayed typical LTSs, the opposite could be postulated for S-rebounds. Therefore, we investigated the effect of TTA-P2 on the S-rebounds shown by neurons without LTSs. As illustrated in the example depicted in Fig. 2, $A$ and $A'$, the application of TTA-P2 (1 μM) clearly reduced the S-rebound frequency as well. The effect of TTA-P2 is also evident in the RMRF-current relationship (Fig. 2B). On average, pooling together data from mice and rat DCNs, the RMRF was reduced to 48 ± 8.6% of control values by TTA-P2 (control, $45 \pm 4.5$ Hz; TTA-P2, $22 \pm 5.0$ Hz; $n = 6$; $P = 0.003$). In addition, similar to the results on T-rebounds, the late phase of the rebound was also reduced during TTA-P2 application as estimated by the change in ARF to 42 ± 9.4% (control, $16 \pm 2.3$ Hz; TTA-P2, $7 \pm 1.5$ Hz; $n = 6$; $P = 0.008$).

In this set of neurons, the specificity of the effect of TTA-P2 was also estimated by analyzing other electrophysiological properties before and during TTA-P2 application. No significant changes were detected in the depolarizing sag observed in the voltage response elicited by hyperpolarizing current pulses (control, $0.46 \pm 0.015$; TTA-P2, $0.47 \pm 0.021$; $n = 6$; $P = 0.65$), the spontaneous firing rate (control, $28 \pm 7.2$ Hz; TTA-P2, $28 \pm 6.9$ Hz; $n = 6$; $P = 0.165$), the membrane potential trough between spontaneous spikes (control, $-48 \pm 1.9$ mV; TTA-P2, $-48 \pm 1.8$ mV; $n = 5$; $P = 0.9$; $V_c$: control, $-63 \pm 1.9$ mV; TTA-P2, $-63 \pm 1.8$ mV), the derivative of the spontaneous action potentials (control, $199 \pm 38$ V/s; TTA-P2, $196 \pm 38$ V/s; $n = 6$; $P = 0.66$; and control, $-130 \pm 22$; TTA-P2, $-129 \pm 20.5$ V/s; $n = 6$; $P = 0.79$; for the upstroke and downstroke phases, respectively; Fig. 2, $C$–$E$), and the input resistance measured below $-70$ mV ($V_c$: $-85$ mV; control, $126 \pm 23$ MΩ; TTA-P2, $121 \pm 2.6$ MΩ; $n = 6$; $P = 0.51$). These results indicate that S-rebounds were also selectively suppressed by TTA-P2, supporting the idea that S-rebounds are dependent on T-type calcium channel activation as well.

Given the selective effect of TTA-P2 on rebounds elicited by current pulses, the effect of TTA-P2 on RE elicited by IPSPs was investigated next.

**TTA-P2 effect on IPSPs and synaptic-induced rebounds.** First, a series of control experiments was conducted to determine whether TTA-P2 affected synaptic transmission itself. The action of TTA-P2 on inhibitory synaptic responses induced by single or trains of stimuli delivered to Purkinje cell axons was tested (Fig. 3A). Only IPSPs that did not reach the reversal potential (see MATERIALS AND METHODS) were analyzed to be able to detect increases and decreases in IPSP amplitude. Application of TTA-P2 at concentrations that fully block LTSs (1 μM) did not attenuate the peak amplitude of single or trains of IPSPs, rather the peak amplitude of single IPSPs was slightly increased (single IPSP: control, $17.4 \pm 3$ mV; TTA-P2, $18.2 \pm 3.2$ mV; $n = 6$; $P = 0.025$; trains: control, $19.3 \pm 1.9$ mV; TTA-P2, $19.5 \pm 1.9$ mV; $n = 7$; $P = 1$). These results showed that TTA-P2 did not attenuate inhibitory responses and could therefore be used to investigate synaptic-induced rebounds.

Thus the effect of TTA-P2 on rebounds elicited by trains of IPSPs was explored. Trains of IPSPs elicited RE of the same type as current pulses (i.e., DCNs displaying T-rebounds with...
current pulses also showed T-rebounds with trains of IPSPs). TTA-P2 suppressed synaptic-triggered rebounds in all cells investigated ($n = 11$; Fig. 3B). In the example illustrated in Fig. 3B, a train of stimulus triggered a typical T-rebound with a transient high-frequency firing period (top). The application of TTA-P2 to the slice blocked the transient period of high-frequency firing (bottom) and consequently reduced the rebound frequency. On average, TTA-P2 reduced RMRF to 16 ± 3.4% of control (Fig. 3C; control, 142 ± 20 Hz; TTA-P2, 20 ± 6 Hz; $n = 11$; $P < 0.001$). The ARF was also decreased (control, 13 ± 1.6 Hz; TTA-P2, 8 ± 1.3 Hz; $n = 10$; $P < 0.001$). Moreover, the application of TTA-P2 also blocked S-rebounds elicited by trains of IPSPs ($n = 3$, data not shown).

There is no evidence that the effect of TTA-P2 could be due to nonspecific R-type channel block (Dreyfus et al. 2010; Uebele et al. 2009); however, because R-type channels were postulated to mediate synaptic rebounds in DCNs (Zheng and Raman 2009), the effect of SNX-482, a compound that at low concentration is relatively specific for R-type calcium channels (Isope et al., in press), was tested. In three experiments, application of SNX-482 (300 nM) did not block synaptically elicited rebounds, which were instead blocked by further application of TTA-P2 (Fig. 3D). These results indicated first that R-channels (at least those sensitive to low doses of SNX-482) are not necessary for T-rebounds in DCNs, which are instead dependent on channels sensitive to TTA-P2. Second, nonspecific block of R-type channels was not mediating the effect of TTA-P2 on DCNs. There was no attempt to analyze quantitatively other rebound properties because the recordings obtained during SNX-482 application were performed by necessity under conditions different from the control recordings (similar to Zheng and Raman 2009; see MATERIALS AND METHODS for details).

In summary, these results together show that synaptically elicited RE is sensitive to TTA-P2, indicating that T-type channels mediated these responses.

**DISCUSSION**

We report here that TTA-P2 selectively suppressed RE evoked by inhibitory synaptic inputs as well as current injections, providing crucial evidence that T-type calcium channels can be activated by physiological hyperpolarizing stimuli. Moreover, only part of DCNs displayed LTS, yet TTA-P2 attenuated RE in all DCNs, indicating that T-type channel activation was involved in all cases of RE. As a corollary, this outcome also implies that the expression and activation of T-type calcium channels does not necessarily result in the ability to discharge LTSs.

T-type calcium channels and RE elicited by IPSPs. RE was a ubiquitous finding: it was present in all recordings from mouse and rat DCNs, as previously reported (Pedroarena 2010; Fig. 3. TTA-P2 application blocks rebounds evoked by inhibitory postsynaptic potentials (IPSPs) but not synaptic transmission. A, top: averaged traces of 15 successive IPSPs obtained before and during TTA application from a mouse DCN. B, control: averaged traces of single (left; $n = 6$) and trains of (right; $n = 7$) IPSPs. See details in the main text. B, control: averaged traces of single (left; $n = 6$) and trains of (right; $n = 7$) IPSPs. See details in the main text. C, control: averaged traces of single (left; $n = 6$) and trains of (right; $n = 7$) IPSPs. See details in the main text.
Moreover, in the present study, it was found that the hyperpolarization elicited by trains of IPSPs could evoke RE in all neurons investigated. These findings agree with previous studies of DCNs in vitro (Aizenman et al. 1998; Aizenman and Linden 1999; Llinás and Mühlethaler 1988; Pedroarena 2010; Sangrey and Jaeger 2010; Tadayonnejad et al. 2009; Zheng and Raman 2009) as well as in vivo (Hoebeek et al. 2010; Rowland and Jaeger 2008). We previously showed that IPSPs elicited using the same paradigm of Purkinje cell axon stimulation could be completely blocked by application of selective GABA_A antagonists (Pedroarena and Schwarz 2003), indicating that they were mediated by chloride-dependent GABA_A receptors. DCNs are known to receive other chloride-dependent inhibitory inputs originating in glycinergic DCNs (Pedroarena and Kamphausen 2008), which could generate as well RE in DCNs.

Regarding the ionic mechanisms of rebounds, our results showed that RE was blocked by TTA-P2, whether elicited by current injection or synaptic inhibition, demonstrating that T-type channels were involved in the RE elicited by the GABA_A IPSPs. Suggestive evidence in this direction was previously provided by earlier studies using less-selective channel blockers (Aizenman et al. 1998; Aizenman and Linden 1999; Llinás and Mühlethaler 1988; Tadayonnejad et al. 2009). Two additional considerations further support our conclusion. First, previous studies showed that TTA-P2 selectively and potently blocked different T-type calcium channel isoforms (Cav3.1, -3.2, and -3.3) without unspecific suppression of sodium or calcium currents or modifications of electrophysiological properties not related to T-type calcium channel activation (Dreyfus et al. 2010; Huang et al. 2011; Shipe et al. 2008). In the present study, TTA-P2 suppressed RE and LTSs in all DCNs investigated without modifying the transmembrane currents associated with spontaneous spikes, the depolarizing sag observed in responses to hyperpolarizing current pulses, the spontaneous firing rate, the firing response to depolarizing current steps, nor the input resistance, in agreement with the idea that TTA-P2 also selectively blocked T-type calcium channels in DCNs. Second, according to the chloride concentration of the intra- and extracellular solutions used in the present study, the reversal potential of IPSPs was approximately −69 mV at 32°C and −67 mV at −24°C. These values are similar or slightly depolarized relative to the GABA_A IPSP reversal potential found using non- or minimally invasive methods in DCNs (Zheng and Raman 2009) and other neurons (Canepari et al. 2010; Glickfeld et al. 2009). Thus the RE elicited by synaptic inputs in the present study cannot be the result of an artificial hyperpolarizing shift of the IPSP reversal potentials due to experimentally imposed low intracellular chloride concentration. The hyperpolarization attained during trains of IPSPs in this study was ~20 mV, corresponding to a membrane potential of approximately −70 mV (V_c − 85 mV). The latter value, calculated after correcting for the junction potential, is discrepant with the calculated reversal potential for chloride ions mentioned before. At least two possible reasons may explain the discrepancy. First, as discussed in MATERIALS AND METHODS, the correction for the junction potential may be incorrect for large neurons like large DCNs. Second, active ion membrane transporters may be able to maintain intracellular chloride concentration lower than the one in the recording pipette even under whole cell recording conditions (Canepari et al. 2010). Indeed, values of equilibrium potential for chloride ions (E_Cl) close to −80 mV or more hyperpolarized levels have been reported for other neurons (Canepari et al. 2010; Chavas and Marty 2003). DCN hyperpolarization reaching ~85 mV or more hyperpolarized levels is expected to result in substantial levels of T-current deinactivation (Zheng and Raman 2009). Therefore, if the second possibility discussed above is true, trains of IPSPs may lead to significant levels of T-type calcium channel deinactivation under our recording conditions and under physiological conditions. If the latter is not the case, as discussed elsewhere, several other mechanisms could explain how T-type channel-mediated rebounds can be elicited by IPSPs despite the apparent disparity between the deinactivation voltage of T-type calcium channels and the hyperpolarization attained during IPSP. These include more efficient hyperpolarization in distal dendrites where T-type channels are also expressed and a redundancy of T-type channels (Tadayonnejad et al. 2010). Finally, because T-type calcium channels are also located in distal dendrites, there is a degree of uncertainty about their voltage dependence estimated using somatic recordings. Indeed, the reported values in the literature for deinactivation/activation voltages for T-type channels are ~10 mV more hyperpolarized in slices than in recordings from isolated cells or dendrites (reviewed in Perez-Reyes 2003), a finding attributed to the difficulty in controlling distal membrane potentials from somatic recordings. In summary, and regarding our original question, from our results, it can be firmly concluded that the hyperpolarization reached during the GABAergic IPSPs can deinactivate a sufficient number of T-type calcium channels to generate postinhibitory rebounds.

T-type calcium channels and different types of RE. In this study, two classes of rebounds were distinguished, T- and S-rebounds, based on their different time course and rebound frequency-current relationship. Are T- and S-rebounds two genuine classes or a continuum separated by the defining criteria? This question is important in light of their potential functional significance. In principle, different encoding and processing of inhibitory signals could be supported by the different rebounds. The transient increase in frequency and step-like rebound frequency-current relationship are compatible with the idea that T-rebounds could signal the timing of events. In contrast, the slower time course of S-rebounds and the linear relationship between frequency and stimulus intensity suggest that S-rebounds may carry information about the magnitude of the preceding inhibitory event to target structures. An argument in favor of the idea that two genuine classes exist is that only DCNs showing LTSs displayed T-rebounds. Given the properties of T-rebounds, short-duration, high-firing frequencies and step-like rebound frequency-current relationship, it is very likely that T-rebounds are supported by the deinactivation provided by an underlying somatic LTS, which is absent in DCNs with S-rebounds. Heterogeneous types of rebounds in rat DCNs were previously distinguished using a classification similar (but not identical) to the one used in the present study (see MATERIALS AND METHODS for detailed comparison of classifying criteria) (Molineux et al. 2006). Different type of rebounds found in GABAergic DCNs (Uusisaari et al. 2007) were not observed in the present study. The present study shows that both T- and S-rebounds were selectively suppressed by TTA-P2, providing conclusive evidence that both are dependent on T-type calcium channel activation. The

\[ E_{Cl} = -80 \text{ mV} \]
universal dependence on T-type calcium channel activation of the different type of rebounds found in large DCNs was previously presumed based on voltage dependence and susceptibility to partially selective T-type calcium channel blockers (Molineux et al. 2006, 2008). A recent study showed that rebounds in DCNs could be blocked by NNC 55-0396 (Alvina et al. 2009), a more selective version of the T-type channel blocker mibebradil (Huang et al. 2004; but see Zheng and Raman 2009). However, the type of rebound investigated was not reported, and the effect of NNC 55-0396 on synapticly elicited rebounds was not investigated. The results of the present study thus provide a first, necessary step toward the elucidation of the mechanisms supporting heterogeneous rebounds by confirming that T-type channels are involved in both types of rebounds. Several mechanisms have been proposed to explain the differences in RE, which could also explain the differential ability to fire LTSs. These explanations include the differential expression of Cav3 channel isoforms with different voltage dependency or kinetics (Molineux et al. 2006), the different amplitudes of the total T-type calcium current (Molineux et al. 2006; Steuber et al. 2011), and the modulation exerted by other inward or outward coactivated currents (Molineux et al. 2008; Steuber et al. 2011). Clarification of whether the latter mechanisms alone or cooperatively explain the differences in rebounds and ability to fire LTSs requires further specific studies.

Finally, TTA-P2 suppressed not only the early phase of rebounds, but also a later phase. A late, slower phase in DCNs rebounds has been described previously (Jahnsen 1986a; Llinäs and Mühlethaler 1988; Sangrey and Jaeger 2010). The ionic mechanism of the late phase is unclear. One mechanism that has been proposed (Sangrey and Jaeger 2010) is the activation of \( I_{\text{Nap}} \) (Jahnsen 1986b; Llinäs and Mühlethaler 1988). However, it is unlikely that TTA-P2 affected \( I_{\text{Nap}} \) directly given that TTA-P2 did not affect this current in other brain areas (Dreyfus et al. 2010). Instead, the effect of TTA-P2 could be explained if the current responsible for it, e.g., \( I_{\text{Nap}} \), was gated by an early and transient depolarization. Alternatively, activation of T-type calcium channel isoforms with slow kinetics (e.g., containing Cav3.3 subunits) and/or preferentially located in compartments with long-time constants could result in slow and lasting effects on firing rate. Indeed, it has been shown that Cav3.3 isoforms only attenuate slowly during bursts (Chemin et al. 2002). A third possibility is that a tonic T-type calcium inward current is temporarly facilitated by the hyperpolarization, resulting in a slower form of rebound. As mentioned in RESULTS, evidence suggests that a T-type tonic current is present in some DCNs. Finally, it must be noted that TTA-P2 did not completely block the late phase, and thus other currents, different and independent of T-type calcium currents, are likely to contribute to the late rebound.

Overall, the present study provides conclusive evidence that chloride-dependent IPSPs can elicit RE mediated by T-type calcium channels and, in particular for DCNs, suggests that T-type channels contribute to shape their spike output.

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Present address of R. Boehme: Cognitive Neurobiology, Humboldt Univ. Berlin, Dorotheenstrasse 94, 10117 Berlin, Germany.

GRANTS

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DISCLOSURES

V. N. Uebele and J. J. Renger are employees of Merck and Co., Inc. (USA) and potentially own stock and/or stock options in the company.

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