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 Pedroarena 1,2

1Department of Cognitive Neurology, Hertie Institute for Clinical Brain Research, 2Systems Neurophysiology Group, Werner Reichardt Centre for Integrative Neuroscience, 3Graduate School for Neural and Behavioral Sciences, University of Tübingen, Otfrid MüllerStr 27, 72076 Tübingen, Germany. 4Merck Research Labs, 770 Sumneytown Pike, West Point, PA 19486, US

Running title: Selective T-type Ca^{2+} channel block in the cerebellar nuclei.

Correspondence: Christine M. Pedroarena
Department of Cognitive Neurology
Hertie Institute, University of Tübingen
Otfrid Müller Str. 27
72076 Tübingen, Germany
Phone: +49 7071 298 0437
Fax: +49 7071 295724
Email: christine.pedroarena@uni-tuebingen.de

*Present address: Humboldt University Berlin, Cognitive Neurobiology, Dorotheenstrasse 94, 10117 Berlin, Germany
ABSTRACT

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 de-inactivate a sufficient number of T-type calcium channels to drive rebound excitation upon 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 TTA-P2, \(\text{3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydropyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide}\), 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.
INTRODUCTION

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 (LTS) and high frequency bursts of sodium spikes (Andersen et al. 1964; Kandel and Spencer 1961; Kuffler and Eyzaguirre 1955; Llinás and Yarom 1981; Jahnsen and Llinás 1984). 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; Steriade and Llinás 1988; Kim et al. 2001), the RE found in neurons of circuits generating rhythmic motor outputs (Miller and Selverston 1982; Syed et al. 1990; Bertrand and Cazalets 1998), the RE found in neurons of the olfactory and visual pathways (Lo et al. 1998; Balu and Strowbridge 2007; Liu and Shipley 2008; 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 (Llinás and Yarom 1981; Jahnsen 1986a; Llinás and Mühlethaler 1988; Aizenman and Linden 1999; Czubayko et al. 2001; Molineux et al. 2006; Uusisaari et al. 2007; Pedroarena 2010; Tadayonnejad et al. 2009; Alvina et al. 2008; Pugh and Raman 2006) 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 played 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 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, remains active only transiently and de-inactivate upon hyperpolarization (Carbone and Lux 1984; Fox et al. 1987), has been previously proposed as the basis for RE and LTSs 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 (Llinás and Mühlethaler 1988; Muri and Knöpfel 1994; Aizenman and Linden 1999; Czubayko et al. 2001; Molineux et al. 2006; Alvina et al. 2009; Zheng and Raman 2009).

However, several issues raise the question of whether T-type calcium currents are involved in all cases of rebound excitation. First, although rebound excitation can be evoked by inhibitory synaptic potentials (IPSPs) in DCNs in vitro (Llinás and Mühlethaler 1988; Aizenman and Linden 1999; Aizenman et al. 1998; Zheng and Raman 2009; Pedroarena 2010; Tadayonnejad et al. 2009; Sangrey and Jaeger 2010) and in vivo
preparations (Hoebeek et al. 2010), the limited hyperpolarization attained by chloride-dependent IPSPs has been noted as an obstacle for the de-inactivation of T-type channels in DCNs (Alvina et al. 2008; Zheng and Raman 2009). Indeed, this issue applies to all neurons where the de-inactivation 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 rebound excitation. 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 rebound excitation, in particular $I_h$ 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 (Jahnsen 1986b; Sangrey and Jaeger 2010).

The understanding of the ionic basis of RE has been hindered by the lack of a specific and potent T-type calcium channel blockers (reviewed in (Isope et al. 2010). Here, using whole cell recordings of large DCNs in cerebellar slices from rats and mice and the application of TTA-P2 (3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydropyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide ), 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.
MATERIAL AND METHODS

Cerebellar slices preparation: Experiments were performed as previously described and according to the SFN rules and German law (Pedroarena and Schwarz 2003). Briefly, cerebellar slices from C57 BL/6 mice, or Sprague-Dawley rats, P-14-17 were prepared using a vibrotome (Leica, Bensheim, Germany) and ice-cold ACSF (containing in mM: 125 NaCl, 2.5 KCl, 1.3 NaH$_2$PO$_4$, 1.5 MgCl$_2$, 26 NaHCO$_3$, 20 Glucose, 2.5 CaCl$_2$) bubbled with 95% O$_2$ and 5% CO$_2$).

Patch clamp recordings and extracellular stimulation: Recordings were performed using a submerged type of recording chamber. Whole-cell current clamp recordings were made from large (>18µm) DCNs, in the lateral or interpositus nuclei using an Axoclamp2B-amplifier (Axon Instruments). The electrode solution contained (in mM): 134 Kgluconate, 6 KCl, 10 KHEPES; 0.1 EGTA, 0.3 NaGTP, 2 KATP, 10 Phosphocreatine, 2 MgCl$_2$. The calculated junction potential (between the bath and the pipette solution) was 15.1 mV and as usual, it was nulled by adjusting the 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
non-corrected and the corrected membrane values which 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 at 24 ºC. To stimulate PC axons, a pair of tungsten microelectrodes (Frederick Haer) was located in the white matter surrounding the cerebellar nuclei. Single or repetitive current pulses (100 µs, 1-100 µA) were delivered using a constant current unit (stimulus isolator, WPI) every 10 to 50 seconds. Recordings were digitized (25 kHz), stored and analyzed using programmable software (Spike 2, CED). Igor (Wavemetrics Inc.) and Sigma Stat (SPSS Inc.) were used for further analysis. Data are presented as means ± SE.

Drugs: The following drugs were bath applied: Kynurenate (3-5mM, Tocris), a broad spectrum antagonist of ionotropic glutamate receptors, to isolate pharmacologically PC axons activation; Gabazine, (3µM, 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 (DMSO, 0.01%), since concentrations ≥2µM did not result in further attenuation of RE and lower doses (0.1µM-0.5µM) induced submaximal effects. SNX-482 (BioTrend, 300 nM), 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 current 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 current. All DCNs investigated were spontaneously active. Several parameters were analyzed. First, changes in the membrane potential of spontaneously firing DCNs. These were estimated as changes in the average inter-spike trough. Second, we investigated the presence of LTSs, here defined as a transient, slow depolarizing event (lasting tens of ms), with lower threshold than fast spontaneous spikes. For this purpose one second long current steps were injected while the neuron was hyperpolarized by DC injection up to -100 mV ($V_c$: -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 sub-nuclei 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 current and evoked either by one second long hyperpolarizing steps of increasing magnitude or by trains of inhibitory synaptic potentials (IPSPs) (0.2 -1s long, 30 to 100 Hz). The magnitude of current pulses was increased until the hyperpolarization peaked close to -100 mV (membrane potential corrected for the junction potential ($V_c$): -115 mV). RE, was defined as an increase in spontaneous firing above baseline level (Pedroarena 2010). The magnitude of RE was evaluated over the first second 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 maximal-rebound frequency (RMRF), calculated as the difference between the
maximal rebound frequency and the basal frequency and 3) the averaged-rebound-
frequency (ARF), which was calculated as the difference between the averaged frequency
over one second 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: called transient (T)
and slow (S) rebounds (see the results section). 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 relative maximal rebound
frequency, the rebound was classified as “transient”. This criterion was successful in
predicting which neurons would display LTSs upon hyperpolarization. This criterion was
successful in predicting which neurons would display LTSs upon hyperpolarization. In
contrast, using MRF was not 100% predictive. Particularly, low MRF could be associated
with the presence of LTS upon hyperpolarization. Different types of DCN rebounds were
previously described. 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 of Molineux and following ones of the same group, the
maximum frequency of rebounds was used as the main classifying criteria while 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 probably 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 such 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 (Figure 1A, left, control trace). However, because only a subset of DCNs displayed LTSs, even when explored from hyperpolarized membrane levels (see methods), the effect of TTA-P2 was separately analyzed in DCNs that did or did not display LTSs. Rebound excitation, a post-hyperpolarization 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 transient type of rebounds (T-rebounds), different than DCNs without LTSs, which showed slow rebounds (S-rebounds). Briefly, T-rebounds showed a transient increase in instantaneous frequency that lasted few intervals (one to four typically) and decreased sharply afterward (Figure 1, see methods for detailed criteria). Slow rebounds (S), showed a smooth decay in firing frequency (Figure 2). In addition, the rebound frequency/current relationship of T and S rebounds differed: T-rebounds displayed a step-like curve (Figure 1), but S rebounds showed a linear function (Figure 2). To quantify changes in rebound frequency, we calculated the absolute maximal rebound frequency (MRF), the difference between basal frequency and MRF (RMRF) and the rebound frequency averaged over the first second of rebound (ARF) as detailed in the methods section.

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

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 figure 1B is characterized in control conditions by a spike “doublet” (top panel). 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 panel). In addition, TTA-P2 application changed the MRF and RMRF/current relationships of T rebounds from step-like functions into linear ones (Figure 1C summary plot illustrates the changes in RMRF induced by TTA-P2 in rat DCNs, top panel). The rebound frequency continued to increase with increasing currents intensity, but values were several times lower than the control (Figure 1C bottom plot).

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, P<0.001, n= 13), and RMRF to 10.5 ± 2.25% of control.(control 227 ± 23 Hz, TTA_P2 21 ± 3 Hz, P<0.001, n=13). 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, 
P<0.001, n= 11).

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 rebound excitation in DCNs were affected by TTA-P2. First, $I_h$ has been proposed to be involved in rebound excitation (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; Figures 1D and D’), consistent with previous findings (Huang et al. 2011). As illustrated in Figures 1B 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). Previous results showed that DCNs 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 ± 2.7 mV , TTA-P2: -52 ± 2.7 mV, P=0.49, n= 10, (Vc: control: -67 ± 2.7 mV , TTA-P2: -67 ± 2.7 mV)), nor in the spontaneous firing rate (control, 12 ± 2.2 Hz, TTA-P2, 11.5 ± 2.0 Hz, P= 0.31, n= 11, strongly suggesting that TTA-P2 did not modify DCNs 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 indicate that TTA-P2 did not modify the calcium dependent potassium channels that are known to regulate DCNs firing rate (Aizenman and Linden 1999; Raman et al. 2000; Czubayko et al. 2001). 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 next determined whether TTA-P2 modified the trans-membrane currents associated to spontaneous spikes. These were estimated by the derivative of spontaneous action potentials. As illustrated in figures 1E, 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 ± 23 V/s, TTA-P2, 266 ± 23 V/s, n= 10 , P= 0.92; control-140 ± 18 V/s, TTA-P2 -140 ± 18 V/s, n= 10, P= 0.91, for up- and down-stroke 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 one second depolarizing steps (Gain, control 251± 0.03 Hz/nA, TTA-P2 253 ± 0.04 Hz/nA, n= 9, P= 0.96, Figure 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 Vc) (control, 149± 26 MΩ, TTA-P2, 145 ± 27 MΩ, P= 0.42, n= 12). 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 is in agreement with previous data (Dreyfus et al. 2010; Shipe et al. 2008; Huang et al. 2011), and shows 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 Figures 2A and 2A’, the application of TTA-P2 (1µM) clearly reduced the S-rebound frequency as well. The effect of TTA-P2 is also evident in the RMRF/ current relationship (Figure 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 ± 4.5 Hz, TTA-P2 22 ± 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 ± 2.3 Hz, TTA-P2 7 ± 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 ± 0.015, TTA-P2 0.47 ±
0.021, n=6, P= 0.65), the spontaneous firing rate (control, 28 ±7.2 Hz, TTA-P2 28 ± 6.9
Hz, n= 6, P= 0.165), the membrane potential trough between spontaneous spikes (control
-48 ± 1.9 mV, TTA-P2 -48 ± 1.8 mV, n= 5, P= 0.9, (Vc: control -63 ± 1.9 mV, TTA-P2 -
63 ± 1.8 mV), the derivative of the spontaneous action potentials (control 199± 38 V/s,
TTA-P2 196 ± 38 V/s, n=6, P= 0.66 and control -130 ± 22, TTA-P2 -129 ± 20.5 V/s,
6, P= 0.79 for the upstroke and downstroke phases respectively, Figures 2C,D,E) and
the input resistance measured below -70 mV (Vc: -85mV) (control 126 ± 23MΩ, TTA-P2
121 ± 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 inhibitory synaptic potentials and synaptic induced rebounds.**
First, a series of control experiments was conducted to determine if 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 (Figure, 3A). Only IPSPs that did not reach the reversal potential (see methods) were analyzed, to be able to detect increases and decreases in IPSP amplitude. Application of TTA-P2 at concentrations that fully block LTSs (1µM) 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 mV± 3, TTA-P2 18.2 ± 3.2 mV, n=6, $P= 0.025$; Trains, control 19.3 ± 1.9 mV, TTA-P2 19.5 ± 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, Figure 3B). In the example illustrated in Figure 3B a train of stimulus triggered a typical T-rebound with a transient high frequency firing period (top panel). The application of TTA-P2 to the slice blocked the transient period of high frequency firing (bottom panel) and consequently reduced the rebound frequency. On average, TTA-P2 reduced RMRF to 16 ± 3.4% of control (Figure 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 blocked as well 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 non-specific R-type channel block (Uebele et al. 2009; Dreyfus et al. 2010); however because R-type channels were postulated to mediate synaptic rebounds in DCNs (Zheng and Raman 2009), SNX-482, the effect of a compound that at low concentration is relatively specific for R-type calcium channels (Isoppe et al. 2010), was tested. In three experiments, application of SNX-482 (300nM) did not block synaptically elicited rebounds, which were instead blocked by further application of TTA-P2-(Figure 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, non-specific block of R type channels was not mediating the effect of TTA-P2 on DCNs. There was no attempt to quantitatively analyze other rebound properties because the recordings obtained during SNX-482 application were performed by necessity under different conditions than the control recordings (similar to (Zheng and Raman 2009), see methods for details).

In summary, these result 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 of 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; Tadayonnejad et al. 2010). 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* (Llinás and Mühlethaler 1988; Aizenman and Linden 1999; Aizenman et al. 1998; Zheng and Raman 2009; Tadayonnejad et al. 2009; Pedroarena 2010; Sangrey and Jaeger 2010) as well as *in vivo* (Rowland and Jaeger 2008; Hoebeek et al. 2010). We previously showed that IPSPs elicited using the same paradigm of Purkinje cell axons stimulation could be completely blocked by application of selective GABA_\text{A} antagonists (Pedroarena and Schwarz 2003), indicating that they were mediated by chloride dependent GABA_\text{A} receptors. DCNs are known to receive other chloride dependent
inhibitory inputs originated in glycinergic DCNs (Pedroarena and Kamphausen 2008) which could as well generate 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 (Llinás and Mühlethaler 1988; Aizenman and Linden 1999; Aizenman et al. 1998; 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 (Shipe et al. 2008; Dreyfus et al. 2010; Huang et al. 2011). In the present study, TTA-P2 suppressed rebound excitation and LTSs in all DCNs investigated without modifying the trans-membrane 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 32ºC and -67 mV at -24 ºC. 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 ((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 IPSPs reversal potentials due to experimentally imposed low intracellular chloride concentration. The hyperpolarization attained during trains of IPSPs in this study was approximately 20 mV, corresponding to a membrane potential of approximately -70mV ($V_c$: -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 the methods section, 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 $E_{\text{Cl}}$ close -80 mV or more hyperpolarized have been reported for other neurons (Chavas and Marty 2003; Canepari et al. 2010). DCN hyperpolarization reaching -85 mV or more hyperpolarized levels is expected to result in substantial levels of T current de-inactivation (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 de-inactivation 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 in spite of the apparent disparity between the de-inactivation 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, or 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 de-inactivation/activation voltages for T-type channels are approximately 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 de-inactivate a sufficient number of T-type calcium channels to generate post-inhibitory rebounds.

T-type calcium channels and different types of RE

In this study two classes of rebounds were distinguished, T or 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 depolarization 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 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 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; Molineux et al. 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 mibefradil (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 synaptically elicited rebounds was not investigated. The results of the present study provide thus 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. 2010) and the modulation exerted by other inward or outward co-activated currents (Molineux et al. 2008; Steuber et al. 2010). 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; Llinás 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 a persistent sodium current ($I_{Nap}$) (Jahnsen 1986b; Llinás and Mühlethaler 1988). However, it is unlikely that TTA-P2 affected $I_{Nap}$ 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. $I_{Nap}$, 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 temporarily facilitated by the hyperpolarization resulting in a slower form of rebound. As mentioned in the results section, 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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FIGURE LEGENDS

Figure 1 TTA-P2 blocks LTSs and T-rebounds in DCNs. A, Typical traces from a mouse DCN recording illustrating blocking of LTSs and the associated burst of spikes by TTA-P2 application (left panel). The membrane potential was hyperpolarized by DC current 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. \( V_c \) indicates in this and all following figure the Vm corrected for the junction potential. Due to uncertainty about the actual value, both values are reported (see methods and text for further discussion). B, Traces illustrate recordings from a rat DCN displaying a T-rebound (top panel) blocked by the application of TTA-P2 application (bottom panel). Traces show the post-hyperpolarization 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 V_c). The arrow indicates the maximum-rebound-frequency before the application of TTA-P2. The dashed lines indicate the basal firing frequency. 

C, Summary plots of 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 second hyperpolarizing current pulses. The magnitude of the current was increased until the Vm peaked approximately at -100 mV. (see methods for further details) D, Typical voltage responses to hyperpolarizing steps before and during TTA-P2 are shown semi-overlapped. D’, Summary bar plot of the depolarizing sag, calculated as V_P/V_SS (details in text). E, Typical example showing no effect of TTA-P2 application on action potentials or E’, its derivative values. 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).

Figure 2. TTA-P2 application inhibits S-rebounds. A, Typical S-Rebound (top), suppressed by TTA-P2 (bottom.). Traces correspond to the intracellular recordings from a mouse-DCN depicting the post-hyperpolarization response. Black circles illustrate the corresponding instantaneous frequency (Note that the left scale applies to both Vm and frequency. The right scale applies to V_c). 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 A at a condensed
time scale. Plots correspond to: representative spontaneous firing (left), and the RE
evoked by two increasing levels of hyperpolarization (middle and right) before (top) and
during TTA-P2 application (bottom). 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, typical
example of spikes from neurons displaying S-rebounds before and during TTA-P2
application and in D, the corresponding derivative traces. 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).

Figure3. TTA-P2 application blocks rebounds evoked by IPSPs but not synaptic
transmission. A, Top panel, averaged traces of 15 successive IPSPs obtained before and
during TTA application from a mouse DCN. Bottom, summary plot of TTA-P2 effect on
peak amplitude of single (left, n=6) and trains of IPSPs (right, n=7). See details in the
main text. B, TTA-P2 blocked a T-rebound evoked by a train of IPSPs (100 Hz) (rat
DCN). Top and Bottom traces correspond to control and TTA-P2 application conditions,
respectively. Black circles indicate the corresponding instantaneous frequency. Dashed
lines indicate baseline firing frequency. Insets show the early response at different scales.
C, Summary plot of TTA-P2 effect on the rebound-frequency induced by trains of IPSPs
(100 Hz 0.3-1s, see main text for details). D, The traces illustrate rebound responses in
control conditions (left), during the application of SNX-482 (300nM, middle), and TTA-
P2 (1µM, right), obtained from a rat DCN. The insets show the early responses (marked by *) at different scales.


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