Corticotropin Releasing Factor Increases Purkinje Neuron Excitability by Modulating Sodium, Potassium and Ih currents.
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Running Head: CRF increases the excitability of Purkinje neurons

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Abstract

Corticotropin releasing factor (CRF) is a neuromodulator closely associated with stress responses. It is synthesized and released in the central nervous system by various neurons, including neurons of the inferior olive. The targets of inferior olivary neurons, the cerebellar Purkinje neurons, are endowed with CRF receptors. CRF increases the excitability of Purkinje neurons in vivo but the biophysical mechanism is not clear. Here we examine the effect of CRF on the firing properties of Purkinje neurons using acute rat cerebellar slices. CRF increased the Purkinje neuron firing rate, regardless of whether they were firing tonically or switching between firing and quiescent periods. Current- and voltage-clamp experiments showed that the increase in firing rate was associated with a voltage shift of the activation curve of the persistent sodium current and hyperpolarizing activated current (Ih) as well as activation of voltage-dependent potassium current. The multiple effects on various ionic currents, which are in agreement with the possibility that activation of CRF receptors triggers several intracellular pathways, are manifested as an increase excitability of PN.

Keywords: CRF; cerebellum; Purkinje neurons

Introduction
Corticotropin releasing factor (CRF), popularly called the ‘stress hormone’, is the major neuromodulator orchestrating the stress response. Its main site of production lies within the hypothalamic region activating the hypothalamic-pituitary-adrenal axis. Additional CRF-producing neurons are found in a variety of brain regions regulating stress responses, including the amygdala, thalamus, hippocampus, and various brainstem nuclei (Bale and Vale 2004; Palkovits et al. 1985). Cerebellar involvement in the stress response is also well documented (Baldacara et al. 2011; Liu et al. 2010; Strick et al. 2009). Neurons of the inferior olive targeting the cerebellar Purkinje neurons produce CRF (Palkovits et al. 1987), suggesting that CRF plays a role in the information processing within the cerebellum. Its release from climbing fibers can be induced by electrical or chemical stimulation of the inferior olive, as well as by specific sensory stimulation (Barmack et al. 1993; Barmack and Young 1990; Beitz and Saxon 2004; Tian and Bishop 2003).

Although it is commonly agreed that cerebellar Purkinje neurons (PNs) express CRF receptors, it is still arguable which subtype and how many are operating. According to King and Bishop (Bishop and King 2013), CRF-R1 receptors are mostly located on the PN primary dendrites and somata, whereas Tao et al (2009) show that cerebellar tissue is devoid of CRF-R1 receptors and express CRF-R2α receptors. Furthermore, in transgenic mice, where cells expressing CRF-R1 receptors are GFP labeled, no labeling was observed in PNs (Justice et al. 2008). Other reports demonstrate that CRF-R2α-tr receptors are found in the axon initial segment, while expression of CRF-R2α receptors
is restricted to a subpopulation of PNs and Bergmann glial cells (Bishop and King 2013; Bishop et al. 2006; Lee et al. 2004; Swinny et al. 2003).

Despite the presence of CRF in the cerebellar cortex and its receptors in the PNs, only a few studies have addressed the physiological effects of CRF in the cerebellum. Early work by Bishop and colleagues demonstrated that CRF increases PN firing rate either directly or in response to neurotransmitter application (Bishop 2002; 1990; Bishop and King 1992b; Bishop et al. 2006; Tao et al. 2009). Other studies examined the effects of CRF on the synaptic potentials and its role in synaptic plasticity (Miyata et al. 1999; Schmolesky et al. 2007). A reduction in P-type calcium current in isolated PNs has been reported in the only study examining the biophysical mechanism of PN response to CRF application (Tao et al. 2009).

Here we examine the effects of CRF on PNs in the acute cerebellar slice preparation. Using current- and voltage-clamp methodologies we demonstrate that CRF application increases PN excitability by modulating three different ionic currents: CRF shifts the activation of persistent sodium current (NaP) toward more negative values while shifting the hyperpolarizing activated cationic current (Ih) activation curve to more positive values. In addition CRF activates non-inactivated voltage-dependent potassium current (K_nia).

**Materials and Methods**

Housing and experimental procedures conformed to the guidelines of the Hebrew University Institutional Animal Care and Use Committee.
Slice preparation: Sprague-Dawley rats (18–25 days old) were anesthetized with pentobarbitone (60 mg/kg) and decapitated. The cerebellum was rapidly removed and placed in ice-cold physiological solution containing (in mM): 124 NaCl, 2.4 KCl, 1 MgCl₂, 1.3 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 2 CaCl₂, saturated with 95% O₂-5% CO₂, pH 7.4 at room temperature. 250-300 µm parasagittal slices of the vermal area were cut on a microslicer (7000 SMZ, Campden Instruments, UK). The slices were incubated with oxygenated physiological solution and maintained at 34°C. After 1 h incubation the slices were transferred to a recording chamber and maintained at room temperature under continuous superfusion with the oxygenated physiological solution containing CNQX (15µM) and picrotoxin (100µM) to block synaptic activity. Neurons were visualized using differential interference contrast infrared microscopy (BX61WI, Olympus, Tokyo, Japan). PNs were identified by the location of their somata between the granular layer and molecular layer, by soma size and by the presence of a clear primary dendrite. All recordings were performed at room temperature.

Cell-attached recordings: Extracellular recordings were obtained in normal bath solution (see above) using borosilicate pipettes (4-6 MΩ) pulled on a Narishige pc-10 puller (Narishige, Tokyo, Japan) and filled with physiological solution. The glass pipette was positioned in close contact with cell membrane without forming a seal and signals were recorded in current-clamp mode using either MultiClamp 700B (Molecular Devices, CA, USA), or AxoClamp 2B (Molecular Devices) and further amplified (DP-301 Warner Instruments, CT, USA).
Whole-cell recordings: Whole-cell recordings were obtained using borosilicate pipettes (4-6 MΩ) pulled on a Narishige pp-83 puller. For current clamp experiments, signals were recorded at 10-20 kHz and low-pass filtered at 3 kHz. Electrode access resistance was routinely checked and recordings with values larger than 20 MΩ were not included in the analysis. For voltage-clamp experiments, data were sampled at 5 KHz, and low-pass filtered at 1 kHz. Voltage-clamp recordings were commenced 5-10 minutes after seal breaking. Experiments were discarded if more than 20% change in holding current was observed. Homemade software based on the LabView platform (National Instruments, TX, USA,) was used for monitoring and data acquisition.

Current double-ramp protocol: Bath solution was the same as the cutting solution. Borosilicate pipettes were filled with internal solution containing (in mM): K-gluconate 145, KCl 2.8, NaCl 2, CaCl₂ 0.1, Mg-ATP 2.4, GTP 0.4, and Hepes 10, EGTA 1 (pH adjusted to 7.3 with KOH). PNs were held at ~-70 mV and double-ramp current was given for a total duration of 2 seconds every 45 seconds. CRF was applied in every alternating repeat, from 5.5 seconds before the ramp to 1-2 seconds after the ramp.

NaP voltage ramp protocol: Bath solution was composed of (in mM): NaCl 120, KCl 5, MgCl₂ 1, KH₂PO₄ 1.3, CaCl₂ 1.6, NaHCO₃ 26, glucose 20, CdCl₂ 0.2, ZD-7288 0.01 saturated with 95% O₂-5% CO₂, pH 7.4. Borosilicate pipettes were filled with the following internal solution (designed to reduce the potassium conductance) CsCl 120, TEA-Cl 20, NaCl 4, CaCl₂ 0.5, EGTA 5, MgATP 3, NaGTP 0.4 Hepes 10 (pH adjusted to 7.3 with CsOH). The PNs were held at -98 mV and a 6 sec ramp was applied, to a value of -45
mV. CRF was applied during alternating repeats. CRF (3 µM) was applied 5 sec before ramp start.

**K\textsubscript{nia} voltage ramp protocol:** Bath solution contained (in mM) NaCl 120, KCl 2.4, MgCl\textsubscript{2} 1, KH\textsubscript{2}PO\textsubscript{4} 1.3, CaCl\textsubscript{2} 1.6, NaHCO\textsubscript{3} 26, glucose 20, NiCl\textsubscript{2} 1.5, CdCl\textsubscript{2} 0.2, TTX 0.0005 saturated with 95% O\textsubscript{2}-5% CO\textsubscript{2}, pH 7.4. In some experiments calcium was omitted (replaced by MgCl\textsubscript{2}) and EGTA 0.2 mM was added. Borosilicate pipettes were filled with the same internal solution as in the double-ramp protocol. PNs were held at -60 mV and a 6 sec ramp was applied to a value of 0 mV. CRF (4 µM) was applied as described above.

**Ih voltage pulses protocol:** Bath solution contained (in mM): NaCl 115, KCl 5, CaCl\textsubscript{2} 2, NaH\textsubscript{2}PO\textsubscript{4} 1.2, MgCl\textsubscript{2} 1, glucose 20, NaHCO\textsubscript{3} 25, NiCl\textsubscript{2} 1, CdCl\textsubscript{2} 0.2, BaCl\textsubscript{2} 1, TEACl 5, TTX 0.0005, 4-AP 1 (Nolan et al. 2003; Rinaldi et al. 2013). Borosilicate pipettes were filled with the same internal solution as in the double-ramp protocol. PNs were held at -50 mV and 2 sec voltage pulses were given, every 50 seconds, from -55 to -120 mV in 5 mV steps or from -60 to -120 mV in 10 mV steps. Leak and capacitance currents were estimated by giving 250 msec test pulses of ±2 mV from -50 mV holding potential, and offline subtracted from the recorded currents.

**CRF application:** borosilicate pipettes (tip size 2-5µm) were filled with either CRF or physiological solution and positioned near the recorded PN. An electronic pressure valve controlled the CRF or the physiological solution discharged from the pipette. Pressure pulses of 5-20 kPa and various durations were used. Care was taken to minimize
movement of the tissue during the application. To that end, the pipette was located 20-
30 µm above the slice surface and the selected PN somata was located at maximal
depth of 50-70 µm. Furthermore, application of the physiological solution was used to
assess possible artifacts due to pressure. CRF concentrations used were at the range of
2-20 µM. Although this range of concentration is well below the reported values used in
this type of local application (Bishop 2002; Bishop and King 1992a; Cahusac et al. 1998;
Jedema and Grace 2004), it is higher than those used in other type of applications (such
as bath applications, which tend to range from 100 nM to 1.5 µm i.e. (Blank et al. 2003;
Schmolesky et al. 2007; Miyata et al. 1999). However, we estimated that the final
concentration, to which the receptors located on the PNs are actually exposed, might be
an order of magnitude smaller. This first approximation is based on diffusion from a
constant concentration source. Finally, in few experiments we positioned the puff
pipette into the slice close (5 µm) to the recorded cell somata, while reducing the CRF
concentration to 1µM. We have obtained similar responses, but under those conditions
the recording quality rapidly deteriorated. Thus, we are confident that our experimental
results represent physiological responses to physiological concentrations of CRF.

Drugs: CRF (Bachem, Switzerland) was solubilized and prepared as a stock solution of
100µM, stored at -80°C and diluted in physiological solution to a final concentration of 1
-20 µM. A stock solution of 100µM astressin (Bachem), a non-specific CRF receptor
antagonist, was stored at -80°C. Astressin was added to the external bath solution (1
µM). A stock solution of 5 mM NBI-35965 hydrochloride, a CRFR1-specific antagonist,
(Tocris, UK) was prepared in DDW. NBI-35965 was added to the external solution (5µM) when we sought to block CRF-R1. 100µM picrotoxin (Tocris, UK), and 15µM CNQX (Tocris) were always added to the bath to block ionotropic synaptic transmission. 1.5-2mM NiCl₂ (Sigma, Israel) and 0.3mM CdCl₂ (Sigma) were used as broad-spectrum calcium current blockers. 10-15 µM ZD-7288 (Sigma) and 400nM-1 µM TTX (Alomone Labs, Israel) were used to block Iₜh current and transient sodium current, respectively. KT-5720 0.2µM (Sigma) was added to the bath to block PKA activity. GF109203x hydrochloride (Tocris), a potent PKC antagonist, was dissolved in DMSO and used externally in the bath with 5µM concentration. Chelerythrine 10µM (Alomone Labs) was added externally bath to block the PKC pathway. In all experiments in which an intracellular pathway was blocked, the slices were submerged in the slice chamber for 50 minutes before recordings started. When KT-5720 or GF109203x were used, they were also added to the pipette solution, with the same concentration.

**Data Analysis and statistics:** A Non-linear least square algorithm was used to fit the experimental results (Matlab; Mathworks MA, USA). Paired t-tests were performed on experimental results collected from the same cell and Welch t-tests were used, when comparing two non-dependent populations. When appropriate, F-tests were used to test for variance equivalence following a Shapiro-Wilk test for normality. Matlab 2009 (Mathworks, MA, USA) was used for data analysis and statistics. Errors are presented as standard error of the mean, unless differently indicated.

**Firing Probability:** At least 20 repetitions of the response to CRF application were used for calculating firing probability. Following detection of the spikes, binary vectors were
created by dividing each trace into 10 msec bins (maximal firing rate was lower than 100 Hz) and a bin was assigned a value of 1 if it contained a spike. Firing probability was calculated as the average of the vectors. All firing probability curves were smoothed using a moving average (window length 60 bins).

**Delay of increase in firing rate in tonically firing PNs:** Following the calculation of the PN firing probability dynamics, we calculated the maximum change in probability of firing rate ($\Delta P_{\text{max}}$), due to CRF application. The delay to the increase in the PN firing rate was determined from the time point, following CRF application, in which the change in probability for firing was 10% of $\Delta P_{\text{max}}$ value.

**Burst duration:** A burst was defined as a group of at least 4 spikes whose maximum inter-spike-interval (ISI) was < 300 msec. Burst duration was the interval from the first to the last spike peak.

**Instantaneous firing frequency and histograms:** Instantaneous firing frequency at the beginning and end of CRF application was calculated as the inverse of the mean ISIs of 4 consecutive spikes. Bin size for firing rate histograms are given in the text.

**Detection and probability of up- and down-states in whole-cell recordings:** After filtering out the action potentials using a moving average filter with window length of 4000-5000 samples, each voltage point was labeled as "up" or "down" by using either the k-means clustering algorithm or the Expectation Maximization (EM) algorithm for a Gaussian mixture of two normal distributions. A cell was rejected from the analysis if the distance between the two centers (k-means) or means (EM) was smaller than 8 mV. The
probability of being in the up-state was calculated as described above for firing probability but with 50 msec bins.

Calculation of double-ramp max frequency, upstroke slope and voltage integral:

Spikes were detected and the instantaneous frequency of the current spike, occurring at $t_{\text{current spike}}$, was calculated as $\frac{1}{t_{\text{current spike}} - t_{\text{previous spike}}}$. Upstroke slope was calculated as the slope of the linear regression of the instant frequency as a function of current. The initial 2-4 spikes usually appear at high frequency. This initial response was removed from the curve if the instantaneous frequency was higher than 80% of the maximal frequency. Traces, in which CRF caused spiking before the beginning of the ramp, were discarded. Maximum frequency was calculated as the max frequency of the fitted linear regression. The voltage integral was calculated over the period of 5 seconds before the ramp start, where zero is the voltage value at the puff start.

Calculation of outward and inward currents (voltage ramps): The maximal outward current was calculated as the difference between, the average current during the last 10 msec of the voltage ramp and the holding current during 50 msec preceding the voltage ramp. Persistent inward current was calculated as the current during the 10 msec average at the minimum point of the TTX sensitive current. Leak current was estimated, offline, from current recorded during the ramp between -90 to -80 mV and subtracted.

Calculation of Sodium conductance: The conductance curve, $G_{NaP}$, was calculated as: $G_{NaP} = \frac{I}{V - V_{Na}}$, where $I$ was the current obtained during voltage ramp protocol and $V_{Na}$ was set to 85 mV. The conductance curve was fitted using the Boltzmann equation
\[
\frac{G_{NaP}}{\text{max}(G_{NaP})} = \frac{1}{1 + \exp\left(-\frac{V - V_{\text{half}}}{k}\right)}
\]

Calculation of \(I_h\): Steady state current \((I_{\text{steady state}})\) was measured as the current during the average of the last 100ms of the voltage pulse. \(I_{\text{steady state}}\) was fitted using the equation:

\[
I_{\text{steady state}} = \text{max}(G_{th}) \left( \frac{V - E_{th}}{1 + e^{\left(-\frac{V - V_{\text{half}}}{k}\right)}} \right)
\]

The fitted conductance is:

\[
G_{th}(V) = \frac{I_{\text{steady state}}}{V - E_{th}} = \frac{\text{max}(G_{th})}{1 + e^{\left(-\frac{V - V_{\text{half}}}{k}\right)}}
\]

RESULTS

CRF increased the excitability of Purkinje neurons

The effects of CRF on Purkinje neuron (PN) firing were examined in cell-attached and whole-cell patch recordings. Both methods revealed that PNs in the anterior regions of the sagittal slice showed a bimodal firing pattern, firing in bursts (up-state) separated by quiescent periods (down-state. Fig. 1A upper panel). In contrast, PNs in the posterior regions showed mostly continuous firing behavior. Regardless of PN firing mode or recording method, local application of CRF increased the firing rate (Fig. 1&2).
CRF response measured in cell attached recording: Local application of CRF (2µM; red bars) to PNs with a bimodal firing distribution reliably elicited a burst of firing resembling spontaneous shifts to an up-state (Fig. 1A lower trace). The consistency of the response is demonstrated in a raster plot (Fig. 1B). CRF application increased the probability of burst firing from 0.03 to almost 100% after a delay of 0.68 sec. On average CRF increased the probability of being in firing mode to 80% with an average delay of 1.44±0.3 sec (SD, n=19).

While resembling the bursts during spontaneous activity, the evoked firing differed in the intra-burst frequency. In the representative cell shown in Figure 1C the mean instantaneous firing frequency at the end of CRF application was 25.24±3.1 Hz (SD) compared to 18.5±2.62 (SD) of spontaneous burst. The mean firing frequency, which during spontaneous up-state was 20.17±2.33 Hz, increased to 27.76±2.17 Hz in CRF induced up-state (SE, n=19).

In order to quantify the CRF effect and extract the dynamics of the response, we calculated the spike probability in a time window of 10msec before, during and after CRF application (see methods). This calculation revealed a fast increase in firing probability for the duration of CRF application (Fig. 1D), reaching a peak value of 0.27+0.09 (SD, n=27), followed by a slow, monotonic decay to baseline.

Tonically firing PNs also increased their firing rate from an average of 30.19 ±1.6 Hz to 40.63 ± 1.9 Hz (SE, n=35). The dynamics of the population response is summarized in figure 1E. The firing rate increased with an average delay of 0.53±0.25 sec (SD, n=35, see methods), reaching a peak value and slowly decaying monotonically to control level.
The increase in probability of firing in both PN types depended on CRF concentration. The average response of 65 cells for 4 different CRF concentrations is shown in figure 1F. The response increased with concentration up to 4 µM CRF while maintaining similar dynamics. At higher concentrations a reduction in firing probability was, sometimes, observed toward the end of CRF application. Within these limitations the dose-response curve displays a typical logarithmic relationship (Fig. 1G).

**CRF response measured in whole-cell recordings:** The effect of CRF application on PN somata was examined in whole-cell recordings in 9 bimodal neurons (Fig. 2A). All showed a robust and consistent shift to an up-state during CRF application (Fig. 2B&C). CRF applied during the up-state caused an increase in firing frequency (Fig. 2B, bottom trace). The up-state probability before CRF application was about 30%; this increased rapidly to almost 100% after 2.53 sec from the start of CRF application, a consistent response seen in the raster plot (Fig 2C). On the population level, the average maximal probability during CRF application was 95± 0.04% (SD, n=9). Thus, like the cell-attached recording, whole-cell recording revealed that CRF elicited an all-or-none type of response, resembling the periodic shifts to an up-state that characterize PN activity both *in vivo* and *in vitro* (Engbers et al. 2012; Loewenstein et al. 2005; Williams et al. 2002).

Like the extracellular recordings, the whole-cell recordings showed that the frequency during CRF-induced firing was higher than during spontaneous activity (Fig. 2D). The CRF effect was quantified by calculating the spike probability within a time window of 10 msec before, during and after CRF application (Fig. 2E). The spiking probability started to
increase with an average delay of $1.29 \pm 0.39$ sec, reaching a peak of $0.30 \pm 0.07$ (SD, $n=9$), demonstrating a similar dynamic to that measured in extracellular recordings (Fig. 1E).

**Measurement of Excitability changes:** The above description suggests that CRF increases the excitability of PN, driving them to firing mode. The excitability changes were examined by analyzing the responses of PN to double-ramp current injections while holding the neuron at -70 mV (see methods). This mode of stimulation induced typical PN firing that was limited to the time of the current injection (Fig 3A black trace).

In the presence of CRF, firing onset occurred earlier and lasted longer (Fig 3A red trace), suggesting a lower threshold for spike initiation. Indeed, careful examinations of the threshold level (inset in figure 3A) reveal a decrease of 2.49 mV. Furthermore, analyzing the instantaneous firing frequency as a function of the current (Figure 3B) showed that in the presence of CRF the firing rate increased during the up-stroke (B upper panel) and the down-stroke (B lower panel). The slope of the frequency-current relationship increased during the up-stroke from 87.9 to 195.8 (Hz/nA) whereas the slope of the frequency-current relationship during the down-stroke increased from 205 to 288 (Hz/nA). However, on the population level the change in the down stroke was insignificant (paired t-test, $p > 0.1$), suggesting that it is almost independent of the presence of CRF. The latter, suggests that the firing during this phase is governed by intrinsic mechanisms. These changes are summarized in figure 3C (see also Table1) where the increase in maximal firing rate (Figure 3C, left panel) and the change in the slope of the current frequency relations (Figure 3C, right panel) are plotted against three
different concentrations of CRF. A significant increase in these two parameters as a function of concentration dependence is clearly evident.

Close examination of the recorded responses (Fig 3A arrow) revealed a small depolarizing shift in the membrane potential induced by the application of CRF. This depolarization is shown at larger gain in figure 3D (left) and the integral of these voltage traces are summarized in the right panel for three different CRF concentrations (see also Table 1). A significant, concentration-dependent increase in the integral is evident.

Three parameters, the relative change in max firing rate, the relative change in slope of the current frequency relations, and the integral of the voltage change were used to examine the effects of various known blockers of CRF receptors (Table 2). Most of the changes were found during the slope of the upstroke of the current frequency, showing a significant effect of Astressin (CRF R1/2 antagonist), KT-5720 (PKA antagonist), GF109203x and Chelerythrine (PKC antagonists), whereas the effect of NBI-35965 (CRF-R1 antagonists) was insignificant (see discussion).

The biophysical mechanism underlying the CRF effect

Membrane potential changes: The initial depolarization evoked by CRF was further examined by exposing the slice to TTX and holding the membrane at -70 mV (Fig 4A, lower trace). Under these conditions CRF induce a depolarization that follows with slow onset and decay kinetics. Reducing the holding potential to -60 mV and -50 mV decreased the amplitude of the response while maintaining the slow decay kinetics.
Finally, we compared the depolarizing response evoked by CRF and ACSF under normal conditions, in the presence of TTX and in the presence of TTX and ZD-7288 (a known Ih current blocker; Fig 4B). The almost complete blockade of the response in the presence of ZD-7288 and TTX, strongly suggests that the depolarization occurring at -70 mV is due to activation of Ih current. The reversal of the response that occurred at -40 mV is rather surprising since the activation curve of Ih is known to approach zero at -50 mV (Kole et al. 2006), thus suggesting that a different ionic process generates the hyperpolarizing response.

**Conductance changes:** The hyperpolarized response measured at -40 mV was further investigated by recording the voltage response to a 3 sec local application of CRF in the presence of TTX (0.4 µM). A hyperpolarizing response to CRF was readily observed when the cell was held at -44 mV (Fig. 4C, top trace). The hyperpolarizing response was associated with increased membrane conductance, measured by applying a train of hyperpolarizing current pulses of constant amplitude before, during, and after CRF application (Fig. 4C, second trace). Subtracting the response with the pulses from the response to the CRF alone (Fig. 4C, third trace) revealed a reduction in input resistance $R_{in}$. Quantification of this result is shown in figure 4D where a significant reduction of $21±13$% (SD, n=5) in input resistance is shown.

The decrease in $R_{in}$ implied that the amplitude of the hyperpolarizing response should depend on the driving force. Indeed, a 10-fold increase in amplitude of the CRF response was obtained by shifting the membrane potential from -75 mV to -35 mV (Fig. 4E; traces are aligned by the voltage at the start of the response). Plotting the amplitude of the
hyperpolarizing response as a function of the membrane potential (Fig. 4F, red curve) revealed that the increase in the hyperpolarizing response occurred only at membrane potentials more positive than -65 mV and that it increased monotonically with depolarization. Whereas the latter suggested that an increase in potassium conductance was involved in the hyperpolarizing CRF response, the former indicated that this presumptive potassium conductance was voltage-dependent.

Similar results were obtained in another 5 cells (Fig. 4D, black curves) with response onsets occurring between -60 and -45 mV. The increase in amplitude of the hyperpolarizing response was non-linearly related to the membrane voltage, supporting the possibility that CRF activates a voltage-dependent conductance. An average amplitude of -5.43±0.73 mV was measured at an average holding potential of -33.76±1.34 mV (SE, n=6).

**Outward Current:** Outward currents were examined after blocking Calcium, Ih, and Sodium currents (CdCl₂, 0.3mM, ZD-7288 (15 μM) and TTX (1-2μM, respectively).

To activate the outward currents we used a voltage ramp that varied the membrane potential from -60 mV up to 0 mV (Fig. 5A; 10 mV/sec; see Methods). A voltage-dependent outward current was reliably recorded, reaching an amplitude of 2.94 nA at 0 mV membrane potential (Fig. 5A, blue trace; 3.09±0.1 nA, SE, n=24 cells). In the presence of CRF the outward current increased to 3.32 nA (Fig. 5A, green trace; 3.44±0.11nA, SE, n= 24 PNs). Subtracting the blue from the green curves (Fig. 5B) and plotting the result as a function of voltage, revealed that, as with the current-clamp
experiments, CRF increased an outward current in a voltage-dependent manner that reached 380 pA at 0 mV (348.3±41.2 pA, SE, n = 24 PNs).

The most likely explanation for these results is that the CRF-sensitive current was carried by potassium ions. We therefore tested the effects of TEA and 4AP on the CRF-sensitive outward current using the voltage ramp protocol as in figure 5D. While TEA caused only a small reduction (Fig. 5D; an average reduction of 14.94% was measured in 6 cells), 4AP reduced the CRF-sensitive current by 70.4% (Fig. 5E; n=3). These voltage-clamp results are in agreement with the current clamp results shown in figure 4F. Both suggest an increase in membrane conductance at membrane potentials less negative than -60 mV.

**Na dependent Current:** Sodium currents were examined in the presence of CdCl₂ (0.2 mM) and ZD-7288 (10 μM) in the external solution and CsCl in the pipette solution (see Methods). To activate the Na current we used a voltage ramp that shifted the membrane voltage from -98 mV up to -45 mV (Fig. 5A; 8.83 mV/sec; see Methods). Under these conditions, the measured inward current was most consistent with non-inactivating sodium current (NaP) that modulates PN firing (Kay et al. 1998). The membrane current-voltage relation was plotted under three conditions: (1) control (Fig. 6A, black curve), (2) during CRF application (Fig. 6A, red curve) and (3) after adding TTX to the external solution with and without the presence of CRF (Fig. 6A, green, blue curves respectively). Subtracting the current under condition 3 from 1 and 2 (Fig. 6B) showed that CRF alters the magnitude and the voltage dependence of the Na current.
The maximal NaP current increased from -312 pA to -497 pA (-390.43±34.59 to -529.72±56.2 pA, SE, n=15).

To estimate the extent of the shift in the activation we took advantage of the fact that under ramp conditions the NaP (persistent sodium current) achieves a quasi-steady state (Carter et al. 2012). The conductance of the inward current was calculated as a function of the voltage (Fig. 6C) and both curves were well fitted by the Boltzmann equation (red curves in Fig. 6C; see Methods). The calculated fit showed a leftward shift of the Boltzmann V_half during CRF application and in 13 of 15 PNs it was also accompanied by an increase in the Gmax (see table 3). The goodness of fit by the Boltzmann equation supported our assumption that steady state conditions were achieved with the voltage ramp protocol (see Discussion).

**Hyperpolarizing activated current (Ih):** Ih was examined in the presence of a bath solution designed to minimize other voltage-activated currents (see methods). The membrane potential was held at -50 mV and voltage steps of either 5 or 10 mV were given to a maximum of -120 mV. The leak current was subtracted offline from the measured current (see methods). The resultant current traces are shown in figure 7A. The Ih current developed almost immediately on step onset with faster kinetics at more negative voltages, reaching values of 1.2 nA at -120 mV after two seconds. These currents show a consistent increase in the presence of CRF (red traces). These results are summarized in the voltage current plot (Fig 7B). We calculated the voltage dependence of the Ih conductance in 14 neurons and fitted the results with Boltzmann
function (fig. 7C). An increase in the maximal conductance as well as a rightward shift in the conductance curve is evident (See table 3).

These voltage-clamp results, which are in agreement with the current-clamp observations, support our suggestion that the activation of Ih current is involved in the response of PN to CRF.

Discussion

We have examined the effect of corticotrophin releasing factor (CRF) on the firing properties of Purkinje neurons (PN) in a rat cerebellar slice preparation. CRF increased the firing rate of PNs that fired tonically or bi-modally shifting between an active up-state and a quiescent down-state. The firing rate of tonically firing PNs increased during CRF application, after which it decayed monotonically over several seconds to baseline. CRF reliably triggered an up-state in the bimodal PNs. Current- and voltage-clamp recordings showed that the increase in firing rate was associated with a modulation of at least three voltage activated currents: (1) Slowly inactivated sodium current (NaP) (2) non-inactivated voltage-dependent potassium current (K_nia) (3) Ih current.

Voltage-clamp recordings of PNs using single electrodes in slice preparations should be treated cautiously, as their size prevents adequate space-clamp conditions. Although this problem is less severe when dealing with slow currents like the NaP current or K_nia, it definitely affected our measurements. Fortunately, the NaP channels, in PN, are mostly located within the somatic area and the axon hillock (Stuart and Hausser
1994) and therefore minimal distortion would be expected. The location of the potassium current is unknown, but the CRF receptors are found within the proximal dendritic tree and the cell body. The third current, Ih, has slow kinetics and thus should be affected to a lesser extent by the absence of space clamp conditions. We therefore presume that our results represented the ionic currents affected by CRF, although the precise kinetics changes may be affected by the inadequate space clamp conditions.

The CRF-sensitive outward current was: (1) a voltage-dependent current activated at a voltage range mostly above -55 mV; (2) not inactivated within the time frame used in our voltage ramp protocol and (3) was readily blocked by 4-AP, while showing low sensitivity to TEA. Several types of voltage-dependent potassium channels of the Kv1, Kv3 and Kv4 families have been described in PNs (Sacco and Tempia 2002; Sheng et al. 1994; Veys et al. 2013). Based on a detailed description of K channels (Coetzee et al. 1999), the CRF-sensitive outward current here was most likely mediated via Kv1.2 channels. Indeed, blocking the Kv1.2 has a profound effect on PN firing (Khavandgar et al. 2005; Williams et al. 2012).

The CRF-sensitive inward current was completely blocked by TTX and ZD-7288 and therefore is probably mediated via voltage-dependent sodium and Ih channels. In the presence of ZD-7288, an inward current persisted during our 6 sec voltage ramp, suggesting a rather slow inactivation process. That the voltage-dependence of the calculated conductance was well fitted by the Boltzmann equation further supports the absence or slow inactivation.
In most experiments the NaP shift in activation curve was associated with an increase in maximum conductance. It is less likely that this is due to a direct effect on the channel conductance, which has only been rarely documented (Aman et al. 2009; Grieco et al. 2002; Klinger et al. 2012). Also less likely is an increase in number of channels, as previous reports suggested that such a change occur over a rather prolonged time course (Nikitin et al. 2008). The possibility that the increased peak conductance is due to a change in the sodium ‘window current’ is also unlikely. In such a case, the shifted conductance curve could not be fitted by the Boltzmann equation, because it would be contaminated by the transient Na fast inactivation function. Furthermore adding riluzole, a known NaP current antagonist (Harvey et al. 2006; Kuo et al. 2006) abolished the increased excitability induced by CRF (data not shown). Finally, the division of labor between the transient and persistent sodium channels has been discussed in detail (Crill 1996). Whether the transient sodium channel differs from the persistent channel is still debated, but some reports claim that channels may shift from a transient to a persistent mode (Aman et al. 2009; Carter et al. 2012; Crill 1996; Grieco et al. 2002). Such a transition, which can occur within seconds, would best explain the measured increase in maximum conductance. The observed shift in the spike threshold in the presence of CRF may also support an effect on transient sodium current reflected in the modulation of persistent sodium current.

Neuromodulators affecting ionic channel kinetics have been described previously. For example, a shift in the activation curve of I_h can be initiated by the activation of various metabotropic receptors that either increase the cAMP level or increase
phosphatidylinositol-4,5-bisphosphate (PIP2) concentration, or by activating tyrosine
phosphorylation (for review see Wahl-Schott and Biel, 2009 (Wahl-Schott and Biel 2009)). Shifts in the activation curve of sodium currents occur in cortical pyramidal cells
(Cheschetti et al. 2000) and spinal motor neurons (Dai et al. 2009). In both cases
these were triggered by activation of PKC and the shifts increased the neuron’s
excitability. A shift in the inactivation curve of T-type calcium channels induced by
activation of CRFR1 in HEK293 cells has also been reported (Tao et al. 2008). Thus,
activation of a G-protein-coupled cascade by CRF receptors could shift the activation
curves of ionic channels in PNs.

The fact that at least three currents were affected by CRF application is of no surprise. In
the literature several studies have suggested that CRF may activate several intracellular
pathways (Blank et al. 2003; Grammatopoulos et al. 2001; Grammatopoulos et al. 2000;
Hauger et al. 2009; Rossant et al. 1999). One might speculate that modulation of each
current, represents the activation of a different intracellular pathway, all of them
activated by CRF receptors.

Although the CRF effect is robust and highly significant, the specificity of the type of CRF
receptor is somewhat questionable. First, 80% blockade of increased excitability was
found using the nonspecific antagonist, Astressin, whereas the effect CRFR1 specific
blocker (NBI-35965) was insignificant. Second, neither of these drugs was effective on
the modulation of Ih by CRF. Third, targeting a single intracellular path by specific drugs
did not completely abolish all of the CRF effects. There are three possibilities that can
account for these observations: 1. the reliability of the CRF antagonists is questionable.
It varies from no effect to an agonistic effect (Jedema et al. 2004). 2. A non-specific activation of non-CRF receptor by CRF. 3. Another unknown type of CRF receptor, which is not sensitive to Astressin. Further studies are needed to resolve this point.

Our results fit those of in vivo experiments, in which CRF increased activity either directly or in the presence of excitatory transmission (Bishop 1990; Bishop and King 1992b). Yet, Fox and Gruol (Fox and Gruol 1993) reported that CRF decreased the excitability of cultured PNs, as measured by the I-f curve, and reduced the afterhyperpolarization. More recently, Tao et al. (Tao et al. 2009) found that activation of CRF-R2 by urocortin in enzymatically isolated PNs decreased P-type calcium current by shifting the inactivation curve to more negative values. However, they also report that CRF increased excitability as measured by the response to current injection. A reduction in afterhyperpolarization has also been reported (Fox and Gruol 1993; Miyata et al. 1999; Schmolesky et al. 2007) although we attributed such reduction to the increased Ih current (as seen in double-ramp protocol). These differences may be due to different experimental systems (cultured neurons or isolated neurons or slice preparation), different animals used (cats, rats, or mice) or different experimental protocols. Here we specifically studied NaP, K_nia and Ih currents that have direct impacts on PN excitability. The consensus emerging is that CRF increases excitability of cerebellar Purkinje cells.

The most straightforward interpretation of the functional role of CRF in increasing PN activity is that the increase in PN firing shuts down the deep cerebellar nuclei, preventing cerebellar output. However, PN firing also shuts down the nuclear inhibitory
neurons projecting to the inferior olive, thus increasing both the sensitivity of the inferior olive and its synchrony (Lefler et al. 2014). Increasing olivary sensitivity is thought to increase the speed of correcting motor output (Schweighofer et al. 2004) and a faster correction of motor output is most likely needed under stressful conditions. Finally, the increase in PN excitability bears direct relevance to interpretation of PN activity measured under awake conditions (Schonewille et al. 2006). It is difficult to assess the level of stress that the animal experience under this condition, but it is certainly there. Thus comparing between PN activity in awake and anesthetized conditions should be treated cautiously.

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References


**Figure Captions**

Figure 1: CRF shifts Purkinje Neuron to its firing mode – cell attached recordings. A. An extracellular recordings from PN displaying bimodal activity. Upper trace, spontaneous activity of PN exhibiting prolonged epochs of firing interspersed with quiescent periods. Lower trace, three applications of CRF (red bars) triggers three epochs of firing in the PN. B. Raster plot of 27 consecutive trials of the CRF responses of the same PN shown in A. (Red dashed line denote the time of CRF application) C. The firing frequency during CRF application is higher than the control. Distribution of firing frequency (bin size = 1 sec) during spontaneous activity (blue bars) and during CRF induced activity (red bars, CRF applications of 5 sec). D, E. the probability of spike
incidence in 10 msec time window during CRF application (red bars) for the cell presented in A and B (D) and in a population of 35 tonically firing neurons (E; gray area denotes the SD). Note the initial decrease in probability (prior to the CRF application) which is likely to be due to prolonged residual effect of CRF application. F. The average time course of the change in probability of spike incidence during CRF application (red bar) at 4 different concentrations in a population of 65 neurons (not all the neurons were exposed to all concentrations of CRF). G. The dose response curve of the CRF displayed as the maximum change of the probability of firing as a function of the concentration on logarithmic scale. (2 µM CRF was used in panels A to E)

Figure 2: CRF shifts Purkinje Neuron to its firing mode – whole cell recordings. A. Whole cell recordings from PN displaying bimodal activity. B. Upper trace – two applications of CRF (2µM, red bars), given during quiescent period (first 2 applications), trigger epochs of firing. Application of CRF during epoch of spontaneous activity (third application) increases the firing rate. The second and third applications are displayed (green rectangular) at extended time scale in the middle and lower traces. C. A raster plot of PN activity in 97 consecutive trials of CRF application denoted by vertical red lines. D. Distribution of firing frequency (bin size = 1 sec) during spontaneous activity (blue bars) and during CRF induced activity (red bars). E. The probability of spike incidence in 10 msec time window during CRF application in a population of 9 neurons (gray area denotes the SD). (2 µM CRF was used in panels B to E)

Figure 3: CRF increases PN excitability. A. PN responses to double-ramp current injection (lower trace) with (red trace) and without (black trace) CRF application (red bar). Inset, an enlargement of the initial spiking period (marked by green dashed rectangle), where threshold is marked by horizontal lines. B. Instantaneous frequency as a function of current, of the cell shown in A. Upper and lower panels are, the frequency-current relationship curves of the up and down stroke respectively. Red curves were obtained in the presence of CRF. The solid lines are the linear regression of the data points. (8 µM CRF was used in panels A and B) C. Left panel – ratio of maximal firing rate with and without local application. The local application contained ACSF and CRF (4, 8, 16 µM). Right panel – The same as in the left panel for the upstroke slope of the current frequency curve. D. Left panel - Voltage trace of the initial small depolarization (marked
Figure 4: **CRF elicits voltage changes in PN.** 

**A.** The average of 4 responses of PN to local application of CRF (8 µM, red bar) at four different holding potentials in the presence of TTX. Note the reversal of the response between -40 mV and -50 mV. 

**B.** ZD-7288 blocks the TTX insensitive depolarizing response. The integral of the depolarizing response measured from CRF onset over a duration of 5.5 sec under control conditions (n=21), in the presence of TTX (n=13) and TTX + ZD-7288 (n=15). 

**C, top trace** - whole cell recording of the average response to CRF application in the presence of TTX. Second trace – 8 pulses of -40pA for 0.5 sec, delivered at 1Hz during CRF response. The third trace is the subtraction of the first trace from the second trace. Red bar denote the CRF application. Note the reduction in the response to current injections during CRF application. 

**D,** The average reduction in input resistance during CRF application in 5 PN. 

**E.** A representative example of seven superimposed traces of the hyperpolarizing response of PN to CRF applications obtained from different holding potentials (-75 to -35 mV) and aligned by the membrane voltage before the application. 

**F,** the result shown in E plotted as a function of the membrane potential (red curve) and similar curves measured from another 5 cells (black curves). (2 µM CRF was used in panels C to F)

Figure 5: **Voltage clamp measurement of CRF-Sensitive outward current.** 

**A.** Currents (upper traces) recorded during a voltage ramp protocol from -60 to 0 mV (lower trace). Blue and green traces are control and in the presence of CRF, respectively. 

**B.** The voltage-current relations of the difference between the two traces in A, represents the CRF sensitive outward current. 

**C, D.** CRF sensitive current, measured in the presence of TEA and 4-AP, respectively. The CRF sensitive currents in the presence of potassium current blockers are marked in red. (4 µM CRF was used in panels A to D which are an average of 2-3 responses)

Figure 6: **CRF increases the persistent sodium current and shifts its activation curve.** 

**A.** The average voltage-current relationship as measured from the current response to voltage ramp from -95 to -45 mV. Black and red curves are the control and in the presence of 3 µM CRF, respectively. Blue and green curves are the control and in the presence of CRF when TTX was
added. B. The voltage current relations of the TTX-sensitive current in control (black curve) and in the presence of CRF (red curve). C. The normalized conductance of TTX sensitive current as a function of membrane potential in the presence (left curve) and absence (right curve) of CRF. Red curves are the Boltzmann function fit.

Figure 7: **CRF increases the Ih current and shifts its activation curve.** A. A representative example of leak subtracted Ih current traces evoked by negative voltage steps (bottom traces) from a holding potential of -50 mV. Currents evoked in the presence of CRF are marked in red. B. A representative example of steady state currents as a function of the pulse voltage with (red) and without (black) CRF. C. The Boltzmann function fit of the average (n=14) Ih conductance curves, with (red) and without (black) CRF. Error bars are the SE of the calculated Ih conductance for each voltage point. (8 µM CRF was used in panels A to C)

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Table 1 – **Quantification of CRF effects in double-ramp current clamp experiments.** Three parameters (each column) were quantified: Upstroke slope, maximal firing rate and voltage integral (for the Ih effect). Four CRF concentrations were used: 0 (ACSF, where ACSF was locally applied), 4µM, 8µM and 16µM. Two values were measured for each concentration, with and without application. The upstroke slope and the maximal firing were further divided into the measured parameter and the percent change. The latter include the significances of the change calculated in paired t-test. All the groups for the voltage integral parameter had a statistical significant difference (1-Way Anova test, F<0.0001, for each couple p < 0.001). The other parameters were not tested using the 1-Way Anova test, as in both cases the distributions of the ACSF and CRF 4µM were not normally distributed as opposed to the CRF 8µM and CRF 16µM (Shapiro-wilk test). All errors are SE
Table 2 - Quantifying the effects of CRF related drugs. Three parameters (rows) were quantified: Upstroke slope, max fire and voltage integral. Each cell in the table contains the percentile change and the extent of blockade. In all conditions (columns) the same amount of CRF, 8µM, was applied. Effect of each antagonist was compared to the control condition (CRF alone) using welch t-test for un-paired samples (P-values shown for comparisons with statistical significance). In the case of the voltage integral, two additional conditions were added, TTX and ZD-7288. All errors are SE.
### Table 3 – Change in $G_{\text{max}}$ and $V_{\text{half}}$ of Ih and NaP currents.

The right column, $V_{\text{half}}$, describes the shift in the activation curve for both NaP and Ih. NaP shifts on average 5.02 mV to the left, while the activation curve of Ih shifts 3.43 mV to the right. In both cases, the maximal conductance, underlying the currents, is increased. All values shown are SE, and all cases are statistically significant with $p<0.01$ (paired t-test). NaP was tested with CRF 3µM and Ih with 8µM.

<table>
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