

1 HCN channel-mediated neuromodulation can control action 2 potential velocity and fidelity in central axons

3
4 Niklas Byczkowicz¹, Abdelmoneim Eshra¹, Jacqueline Montanaro², Andrea Trevisiol³,
5 Johannes Hirrlinger^{1,3}, Maarten H. P. Kole^{4,5}, Ryuichi Shigemoto², and Stefan
6 Hallermann^{1,6}

7
8 ¹Carl-Ludwig-Institute for Physiology, Medical Faculty, University of Leipzig, Liebigstr. 27, 04103
9 Leipzig, Germany

10 ²Institute of Science and Technology Austria (IST Austria), Am Campus 1, 3400 Klosterneuburg, Austria

11 ³Department of Neurogenetics, Max-Planck-Institute for Experimental Medicine, Hermann-Rein-Strasse
12 3, 37075 Göttingen, Germany

13 ⁴Department of Axonal Signaling, Netherlands Institute for Neuroscience, Royal Netherlands Academy
14 of Arts and Sciences, Meibergdreef 47, 1105 BA, Amsterdam, Netherlands.

15 ⁵Cell Biology, Faculty of Science, University of Utrecht, Padualaan 8, 3584 CH, The Netherlands.

16 [#]Correspondence: hallermann@medizin.uni-leipzig.de

18 Abstract

19 Hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels control electrical
20 rhythmicity and excitability in the heart and brain, but the function of HCN channels at
21 subcellular level in axons remains poorly understood. Here, we show that the action
22 potential conduction velocity in both myelinated and unmyelinated central axons can
23 bidirectionally be modulated by HCN channel blockers, cyclic adenosine
24 monophosphate (cAMP), and neuromodulators. Recordings from mice cerebellar mossy
25 fiber boutons show that HCN channels ensure reliable high-frequency firing and are
26 strongly modulated by cAMP (EC_{50} 40 μ M; estimated endogenous cAMP concentration
27 13 μ M). In accord, immunogold-electron microscopy revealed HCN2 as the dominating
28 subunit in cerebellar mossy fibers. Computational modeling indicated that HCN2
29 channels control conduction velocity primarily via altering the resting membrane
30 potential and was associated with significant metabolic costs. These results suggest that
31 the cAMP-HCN pathway provides neuromodulators an opportunity to finely tune
32 energy consumption and temporal delays across axons in the brain.

34 Introduction

35 HCN channels are expressed in the heart and nervous system and comprise four
36 members (HCN1–HCN4) differing in their kinetics, voltage-dependence and degree of
37 sensitivity to cyclic nucleotides such as cAMP (Biel et al., 2009; Robinson and

38 Siegelbaum, 2003). Membrane hyperpolarization activates HCN channels and causes a
39 depolarizing mixed sodium/potassium (Na^+/K^+) current. In the heart, the current through
40 HCN channels (I_f) mediates the acceleratory effect of adrenaline on heart rate by direct
41 binding of cAMP (DiFrancesco, 2006). In neurons, the current through HCN channels
42 (I_h) controls a wide array of functions, such as rhythmic activity (Pape and McCormick,
43 1989) and excitability (Tang and Trussell, 2015). In addition to the somatic impact,
44 HCN channels are expressed throughout various subcellular compartments of neurons
45 (Nusser, 2012). For example, patch-clamp recordings from dendrites in pyramidal
46 neurons have revealed particularly high densities of HCN channels which acts to control
47 the local resting potential and leak conductance, thereby playing important roles in
48 regulating synaptic integration (George et al., 2009; Harnett et al., 2015; Kole et al.,
49 2006; Magee, 1999; Williams and Stuart, 2000).

50

51 In contrast, the expression and role of I_h in the axon is less studied and may vary with
52 species. I_h seems to critically control the strength of synaptic transmission in crayfish
53 and *Drosophila* neuromuscular junction (Beaumont and Zucker, 2000; Cheung et al.,
54 2006). However, presynaptic recordings from the vertebrate calyx of Held in the
55 auditory brainstem found I_h to marginally affect neurotransmitter release (Cuttle et al.,
56 2001), but to exert a strong influence on the resting membrane potential (Cuttle et al.,
57 2001; Kim and von Gersdorff, 2012) and vesicular neurotransmitter uptake (Huang and
58 Trussell, 2014). At synaptic terminals of pyramidal neurons in the cortex of mice, HCN
59 channels inhibit glutamate release by suppressing the activity of T-type Ca^{2+} channels
60 (Huang et al., 2011).

61

62 Besides a potential impact on neurotransmitter release, axonal I_h could play a role in the
63 propagation of action potentials. Indeed, in axons of the stomatogastric nervous system
64 of lobsters (Marder and Bucher, 2001) it was shown that the action potential conduction
65 was affected by dopamine via axonal HCN channels (Ballo et al., 2010; Ballo et al.,
66 2012). In vertebrates, studies on action potential propagation by Waxman and
67 coworkers indicated that I_h counteracts the hyperpolarization of the membrane potential
68 during periods of high-frequency firing (Baker et al., 1987; Birch et al., 1991; Waxman
69 et al., 1995) and that it participates in ionic homeostasis at the node of Ranvier

70 (Waxman and Ritchie, 1993). More recent investigations found I_h to be crucial for the
71 emergence of persistent action potential firing in axons of parvalbumin-positive
72 interneurons (Elgueta et al., 2015), but I_h seems to have an opposing effect on the
73 excitability at the axon initial segment, where its activation reduces the probability of
74 action potential initiation (Ko et al., 2016). Finally, there is evidence from extracellular
75 recordings that blocking I_h decreases the action potential conduction velocity in
76 unmyelinated central (Baginskas et al., 2009; Soleng et al., 2003) and peripheral axons
77 of vertebrates (Grafe et al., 1997). However, neuromodulation of conduction velocity
78 and the underlying cellular membrane mechanisms are not known in vertebrate axons.

79

80 Here, we demonstrate a decrease or increase in conduction velocity in central axons
81 through the application of HCN blockers or neuromodulators. To gain mechanistic
82 insights into the modulation of conduction velocity by HCN channels, we performed
83 recordings from *en passant* cerebellar mossy fiber boutons (cMFB; Ritzau-Jost et al.,
84 2014; Delvendahl et al., 2015). We found that HCN channels in cMFBs mainly consist
85 of the HCN2 subunit, are ~7% activated at resting membrane potential, ensure high-
86 frequency firing, and control the passive membrane properties. Whole-cell and
87 perforated patch clamp recordings from cMFBs demonstrated a strong dependence of
88 HCN channels on intracellular cAMP concentration with an EC₅₀ of 40 μ M and a high
89 endogenous cAMP concentration of 13 μ M. Computational modelling indicated that the
90 resting membrane potential controls conduction velocity and that the activity of HCN
91 channel is metabolically expensive. These data reveal a mechanism shared among
92 different types of axons to bidirectionally modulate conduction velocity in the central
93 nervous system.

94

95 **Results**

96 *Bidirectional modulation of conduction velocity*

97 To investigate whether HCNs affect conduction velocity, we recorded compound action
98 potentials in three different types of axons (Fig. 1): Application of the specific HCN
99 channel blocker ZD7288 (30 μ M) decreased the conduction velocity by $8.0 \pm 2.8\%$ in
100 myelinated cerebellar mossy fibers ($n = 14$), by $9.2 \pm 0.9\%$ in non-myelinated cerebellar
101 parallel fibers ($n = 15$), and by $4.0 \pm 0.8\%$ in optical nerves ($n = 4$; see Fig. 1 and legend

102 for statistical testing). Since some studies implied that ZD7288 might have some
103 unspecific side effects, such as blocking voltage-dependent Na^+ channels (Chevaleyre
104 and Castillo, 2002; Wu et al., 2012), we recorded Na^+ currents from 53 cMFBs and
105 found no change in amplitude or kinetics of voltage-dependent Na^+ currents after
106 ZD7288 application (Supplemental Fig. 1), suggesting that under our conditions and at
107 a concentration of 30 μM , ZD7288 did not affect the Na^+ currents. Because of the
108 modulation of HCN channels by intracellular cAMP, we measured conduction velocity
109 during application of 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP;
110 500 μM), a membrane-permeable cAMP-analogue. The conduction velocity increased
111 by $5.9 \pm 2.8\%$ in cerebellar mossy fibers ($n = 17$), by $3.7 \pm 1.4\%$ in parallel fibers ($n =$
112 10), and by $4.6 \pm 0.6\%$ in optic nerves ($n = 5$; see Fig. 1 and legend for statistical
113 testing). These results indicate that HCN channels control the conduction velocity both
114 in myelinated and non-myelinated central axons.

115

116 *Neuromodulators differentially regulate conduction velocity*

117 To investigate a modulation of conduction velocity by physiological neuromodulators,
118 we focused on the cerebellar parallel fibers, where the velocity could be most accurately
119 measured, and then applied several modulators known to act via cAMP-dependent
120 pathways (Fig. 2). Wash-in of 200 μM norepinephrine (NE) resulted in a relatively fast
121 increase in conduction velocity ($1.9 \pm 0.8\%$; $n = 6$; see Fig. 2B and legend for statistical
122 testing), consistent with the existence of β -adrenergic receptors in the cerebellar cortex
123 (Nicholas et al., 1993), which increase the cAMP concentration via G_s -proteins. On the
124 other hand, the application of either 200 μM serotonin ($-3.5 \pm 0.5\%$; $n = 11$), 200 μM
125 dopamine ($-5.0 \pm 0.7\%$; $n = 13$) or 200 μM adenosine ($-7.2 \pm 0.6\%$; $n = 5$) resulted in a
126 continuous decrease of the conduction velocity (Fig. 2B and C; see legend for statistical
127 testing), consistent with the existence of G_i -coupled receptors for serotonin, dopamine,
128 and adenosine in the molecular layer of the cerebellum (Geurts et al., 2002;
129 Schweighofer et al., 2004), which decrease the cAMP concentration. Although we used
130 rather high concentrations of the agonists and off-target effects cannot be excluded (e.g.,
131 NE activating dopamine receptors; Sánchez-Soto et al., 2016), these data nevertheless
132 indicate that physiological neuromodulators can both increase or decrease action
133 potential conduction velocity, depending on the type of neuromodulator and receptor.

134

135 *Neuromodulation of conduction velocity is mediated by HCN channels*

136 In addition to HCN channels, some voltage gated Na^+ , K^+ , and Ca^{2+} channels can be
137 modulated via the intracellular cAMP-pathway (Burke et al., 2018; Yang et al., 2013;
138 Yin et al., 2017). To address the contribution of other channels on the neuromodulation
139 of the conduction velocity, we performed a set of experiments, in which HCN channels
140 were first blocked by 30 μM ZD7288 and subsequently three modulatory substances
141 that significantly increased or decreased conduction velocity in prior experiments were
142 applied. With ZD7288 continuously being present in the recording solution, conduction
143 velocity of parallel fibers slightly decreased over the course of 20 minutes (Fig. 3A).
144 Adding 8Br-cAMP (500 μM), Adenosine (200 μM) or NE (100 μM) at $t = 5$ min (i.e.
145 25 minutes after application of ZD7288) did not change the conduction velocity
146 significantly compared with control (only ZD7288). The average conduction velocity
147 between $t = 15$ and 20 min was decreased by $-3.3 \pm 2.4\%$ for cAMP ($n = 9$), $-4.6 \pm$
148 1.6% for Adenosine ($n = 9$) and $-3.7 \pm 1.2\%$ for NE ($n = 7$), compared to the average
149 velocity between $t = 0$ and 5 min baseline recording. This was not significantly different
150 from the decrease in the sole presence of ZD7288 ($-3.3 \pm 1.4\%$; $n = 7$, see Fig. 3B and
151 legend for statistical testing), indicating that the previously shown effects of cAMP and
152 neuromodulators on conduction velocity are mainly mediated by HCN channels.

153

154 *Cerebellar mossy fiber terminals have a prominent voltage sag*

155 To investigate the membrane and signaling mechanisms underlying the bidirectional
156 control of conduction velocity, we focused on cerebellar mossy fibers, which allow the
157 whole-cell recording configuration and a direct access to the cytoplasmic compartment
158 (Fig. 4A). Recordings from *en passant* cMFBs are well suited to investigate the ionic
159 basis of conduction velocity in the adjacent axonal compartments, because of a long
160 membrane length constant and slow HCN channel gating. Injection of depolarizing
161 currents during current-clamp recordings evoked a single action potential and injection
162 of hyperpolarizing currents generated a substantial ‘sag’ at cMFBs (Fig. 4B; Rancz et
163 al., 2007; Ritzau-Jost et al., 2014), i.e. a delayed depolarization towards the resting
164 potential, which is a hallmark of the presence of I_h (Biel et al., 2009; Robinson and
165 Siegelbaum, 2003). At a potential of on average -150 mV, the sag ratio, calculated from

166 the peak and steady state amplitude as indicated in Fig. 4C (George et al., 2009), was
167 0.497 ± 0.030 ($n = 12$).

168

169 *HCN channels support high frequency action potential firing*

170 Using direct presynaptic recordings from cMFBs, we first aimed to investigate the
171 impact of HCN channels on action potential firing. To this end, we analyzed action
172 potentials elicited by current injections into the cMFBs (data not shown) as well as
173 traveling action potentials elicited by axonal stimulation with a second pipette (Fig.
174 5A). In both cases, the amplitude and half-duration of action potentials elicited at 1 Hz
175 were not significantly affected by application of 30 μ M ZD7288 (data not shown and
176 Fig. 5B and C, respectively), indicating that HCN channels do not alter the active
177 membrane properties profoundly.

178

179 However, cerebellar mossy fibers can conduct trains of action potentials at frequencies
180 exceeding 1 kHz (Ritzau-Jost et al., 2014), making them an ideal target to study the
181 impact of axonal HCNs on the propagation of high-frequency action potentials as well.
182 Blocking HCN channels significantly impaired the ability of mossy fibers to fire at high
183 frequencies (20 action potentials at 200 – 1666 Hz). In the examples illustrated in Fig.
184 5D, the failure-free trains of action potentials could be elicited at up to 1.1 kHz under
185 control conditions and up to 500 Hz, when ZD7288 was present in the extracellular
186 solution. The average failure-free frequency reduced from 854 ± 60 in control to $426 \pm$
187 63 Hz in the presence of ZD7288 ($n = 20$ and 10, respectively; $P_{T-Test} = 0.0002$; Fig.
188 5E). Action potential broadening and amplitude reduction was more pronounced in the
189 presence of ZD7288. For example, during trains of action potentials at 200 Hz, the half-
190 duration of the 20th action potential was 109.6 ± 1.5 and $141.7 \pm 7.0\%$ of the half-
191 duration of the 1st action potential for control and ZD7288, respectively ($n = 20$ and 10;
192 $P_{T-Test} = 0.02$; Fig. 5E). The amplitude of the 20th action potential was 96.5 ± 0.8 and
193 $82.9 \pm 1.6\%$ of the 1st action potential for control and ZD7288, respectively ($n = 20$ and
194 10; $P_{T-Test} = 0.02$; Fig. 5E). Furthermore, the delay during trains of action potentials at
195 200 Hz increased by ~20% in the presence of ZD7288 but decreased by ~5% in control
196 recordings (Fig. 5F), indicating an acceleration and a slowing of conduction velocity
197 during high-frequency firing for control and ZD7288, respectively. The difference in

198 delay of the 20th action potential was maximal at intermediate frequencies (200 and 333
199 Hz; Fig. 5G). These experiments show, that HCNs, despite their slow kinetics, ensure
200 reliable high-frequency firing.

201

202 *The passive membrane properties of cMFBs are HCN- and cAMP-dependent*

203 To better understand how I_h impacts action potential firing, we next investigated the
204 passive membrane properties of cMFBs by recording the voltage response elicited by
205 small hyperpolarizing current injections (-10 pA for 300 ms) in the absence and
206 presence of 30 μ M ZD7288 (Fig. 6A and B). ZD7288 caused a hyperpolarization of the
207 resting membrane potential by, on average, 5.4 mV (-80.0 ± 0.6 mV and -85.4 ± 1.4
208 mV for control and ZD7288, $n = 94$ and 35, respectively), a doubling of the apparent
209 input resistance calculated from the steady state voltage at the end of the current step
210 (794 ± 48 M Ω and 1681 ± 185 M Ω), as well as a doubling of the apparent membrane
211 time constant, as determined by a mono-exponential fit to the initial decay of the
212 membrane potential (14.4 ± 0.8 ms and 35.0 ± 2.5 ms, respectively; see legend of Fig.
213 6B for statistical testing). To analyze the cAMP-dependence of the conduction velocity
214 (cf. Fig. 1), we determined the cAMP-dependence of the passive membrane properties
215 of cMFBs. Adding cAMP in various concentrations to the intracellular solution
216 depolarized the membrane potential and decreased both the input resistance and the
217 apparent membrane time constant in a concentration-dependent manner, which shows
218 an opposite effect compared to the application of ZD7288 (Fig. 6B). These data suggest
219 that HCN channels in cerebellar mossy fibers determine the passive membrane
220 properties as a function of the intracellular cAMP concentration.

221

222 *HCN2 is uniformly distributed in mossy fiber axons and boutons*

223 Of the four HCN subunits (HCN1–HCN4), the subunits HCN1 and HCN2 are
224 predominantly expressed in the cerebellar cortex (Notomi and Shigemoto, 2004;
225 Santoro et al., 2000). Previous studies in the cortex, hippocampus, and auditory
226 brainstem primarily detected HCN1 in axons (Elgueta et al., 2015; Huang et al., 2011;
227 Ko et al., 2016), but HCN2 was found to be more sensitive to cAMP in comparison to
228 HCN1 (Wang et al., 2001; Zagotta et al., 2003). To understand the pronounced cAMP
229 dependence of conduction velocity (cf. Fig. 1) and passive membrane properties (cf.

230 Fig. 6) at the molecular level, we investigated the identity and distribution of HCN
231 channels using pre-embedding immunogold labeling for HCN1 and 2 in cMFBs and
232 adjacent axons. At the electron microscopic level, we found only background
233 immunoreactivity for HCN1 (data not shown) but significant labeling for HCN2 (Fig.
234 7A). HCN2 immunogold particles were diffusely distributed along the plasma
235 membrane of cMFBs, with similar labeling density in the adjacent mossy fiber axon
236 (Fig. 7B), which could be traced back up to 3.5 μm from cMFBs. In addition, we
237 created a 3D reconstruction of a cMFB (Fig. 7C and Supplemental Video), including
238 gold particles for HCN2 and identified synaptic connections. While synapses onto
239 granule cell dendrites were observed within invaginated parts of the bouton, HCN2 was
240 uniformly distributed without apparent spatial relations within those synapses. The
241 density of immunogold particles for HCN2 in this reconstructed bouton was 17.1
242 particles/ μm^2 (in total 1260 particles per $73.65 \mu\text{m}^2$). The mean density of immunogold
243 particles for HCN2 was 22.7 ± 2.4 per μm^2 ($n = 6$ cMFBs from 2 mice). These data
244 indicate that HCN2 is the dominant subunit mediating I_h in cMFBs, consistent with its
245 pronounced cAMP-dependence.

246

247 *HCN channels in cMFB are strongly modulated by cAMP*

248 To better understand the function of axonal HCN2 channels and their modulation by
249 intracellular cAMP, we performed voltage-clamp recordings from cMFBs combined
250 with different cAMP concentrations within the intracellular patch solution.
251 Hyperpolarizing voltage steps evoked a slowly activating, non-inactivating inward
252 current, which was inhibited by ZD7288 (Fig. 8A). Using tail currents of ZD7288-
253 sensitive currents evoked by voltage steps between -80 mV and -150 mV from a
254 holding potential of -70 mV, we calculated the activation curve of I_h with a mean $V_{1/2}$ of
255 -103.3 ± 0.8 mV (Fig. 8B; $n = 36$ $V_{1/2}$ -values, each from a different cMFB). Based on
256 the average resting membrane potential of cMFBs, this means about 7% of the overall
257 HCN2-mediated current is active at rest.

258

259 To analyze the cAMP concentration-dependence of I_h , we added different
260 concentrations of cAMP (30 μM to 10 mM) to the intracellular patch solution. With 1
261 mM cAMP $V_{1/2}$ shifted by 17 mV to on average -86.6 ± 1.2 mV ($n = 16$ cMFBs; $P_{\text{T-Test}} <$

262 10^{-10} ; Fig. 8B). The resulting average shifts of $V_{1/2}$ revealed an EC_{50} of $40.4 \mu M$ intracellular cAMP (Fig. 8D). In order to estimate the endogenous presynaptic cAMP concentration, we performed presynaptic perforated-patch recordings on cMFBs. Under perforated patch conditions, $V_{1/2}$ of I_h was -96.4 ± 1.2 mV ($n = 10$), significantly more depolarized compared to the corresponding whole-cell recordings after rupturing of the perforated patch (-101.3 ± 1.0 mV; $n = 15$; $P_{T-Test} = 0.0076$; Fig. 8C; see methods for comparison with addition control groups). This voltage shift indicates an endogenous cAMP concentration of $12.6 \mu M$ in cMFBs, with a 68%-confidence interval of 1.8 to $60.7 \mu M$ cAMP (Fig. 8D). These data reveal a high endogenous resting cAMP concentration.

272

273 *Hodgkin-Huxley model describing HCN2 channel gating*

274 For our ultimate aim, to obtain a mechanistic and quantitative understanding of axonal
275 HCN2 function in cerebellar mossy-fiber axons, we developed a computational
276 Hodgkin-Huxley (HH) model. The model was constrained to the experimentally
277 recorded I_h kinetics derived from the activation and deactivation time constants of I_h
278 (Fig. 9A) measured at potentials between -70 and -150 mV. The activation curve (cf.
279 Fig. 9B), as well as the averaged time constants for both activation ($n = 20$) and
280 deactivation ($n = 15$; Fig. 9B) were well described by a HH-model with one activation
281 gate. In addition, we generated an alternative HH-model to describe the HCN2 current
282 in the presence of 1 mM intracellular cAMP (for a more detailed implementation of the
283 cAMP-dependence of HCN2 gating see Hummert et al., 2018). Furthermore, we
284 estimated the reversal potential of I_h with short voltage ramps as described previously
285 (Cuttle et al., 2001) and found a value of -23.4 ± 1.4 mV ($n = 7$; Fig. 9C), similar to
286 previous estimates (Aponte et al., 2006; Cuttle et al., 2001). These data provide a
287 quantitative description of axonal I_h at cMFBs.

288

289 *Mechanism of conduction velocity-control and metabolic costs of HCN channels*

290 What are the mechanisms by which axonal HCN2 channels control conduction
291 velocity? In principle, the depolarization caused by HCN2 channels will bring the
292 resting membrane potential closer to the threshold of voltage-gated Na^+ channel
293 activation, which could accelerate the initiation of the action potential (see discussion).

294 Alternatively, the increased membrane conductance caused by HCN2 channels will
295 decrease the membrane time constant, which could accelerate the voltage responses, as
296 has been shown, e.g., for dendritic signals in auditory pathways (Golding and Oertel,
297 2012; Mathews et al., 2010). To distinguish between these two possibilities, we
298 generated a conductance-based NEURON model consisting of cylindrical compartments
299 representing cMFBs connected by myelinated axons (Fig. 10A; Ritzau-Jost et al., 2014).
300 The model contained voltage-dependent axonal Na^+ and K^+ channels, passive Na^+ and
301 K^+ leak channels, and the established HH model of I_h (cf. Fig. 9). After adjustments of
302 the peak conductance densities the model captured the current clamp responses to -10
303 pA current injections (Fig. 10B), the resting membrane potential (Fig. 10C) as well as
304 the apparent input resistance (Fig. 10D). Removing the HH model of I_h or replacing it
305 with the 1-mM-cAMP-HH-model of I_h , reproduced the corresponding voltage
306 responses, the shift in the resting membrane potential, and the change in the apparent
307 input resistance obtained in the presence of ZD7288 or 1 mM intracellular cAMP (Fig.
308 10B-D). Interestingly, the models predicted a decrease of the conduction velocity when
309 the control HH model was removed and, conversely, an increase with the 1-mM-cAMP-
310 HH model (Fig. 10E), to a similar extent as experimentally measured with ZD7288 and
311 8-Br-cAMP (cf. Fig. 1). These findings support our conclusion that HCN2 channel
312 modulation suffices to bi-directionally tune conduction velocity.

313

314 Next, we generated two additional models, in which either only the depolarizing effect
315 of HCN2 channels (V_m -model) or the decreased input resistance, i.e. the decreased
316 membrane resistance (R_m -model) was implemented (by modifying the K^+ or the I_h
317 reversal potential, respectively, see methods). The results showed that the V_m -model but
318 not the R_m -model caused an increase in conduction velocity, indicating that the
319 depolarizing effect of axonal HCN2 channels determines conduction velocity (Fig.
320 10E). Interestingly, increasing the resting membrane potential from -90 to -65 ,
321 decreased the availability of voltage dependent Na^+ (Na_V) channels but increased the
322 conduction velocity (Fig. S2). Only at resting membrane potentials above -65 mV the
323 conduction velocity decreased in our model. Together, these data indicate that the
324 depolarization mediated by HCN2 channels accelerates the conduction velocity by
325 bringing the membrane potential closer to the firing threshold.

326

327 The non-inactivating nature of HCN channels and the accompanying shunt at the resting
328 membrane potentials suggest that I_h is metabolically expensive. Therefore, we
329 calculated the Na^+ influx in each model and converted it into the required ATP
330 consumption to restore the Na^+ gradient (Hallermann et al., 2012). Computational
331 modeling showed that it is $\sim 100\%$ more expensive to maintain the resting membrane
332 potential with I_h than without or by depolarization alone (V_m -model; Fig. 10F).
333 Furthermore, the metabolic costs to maintain the resting membrane potential with I_h for
334 one second was ~ 3 -fold higher than the costs to generate one action potential (Fig. 10F).
335 Assuming an average frequency of cerebellar mossy fibers of 4 Hz *in vivo* (Chadderton
336 et al.; Rancz et al., 2007), the HCN2 channels increased the required energy of
337 cerebellar mossy fibers by $\sim 30\%$. With increasing firing frequency, the metabolic costs
338 of action potential firing will become dominating compared with the HCN2-mediated
339 costs for resting membrane potentials (e.g., $\sim 3\%$ at 40 Hz). These data indicate that
340 HCN2 channels are a major contribution for the energetic demands of axons.

341

342 **Discussion**

343 Here, we demonstrate that the presence of HCN channels in axons accelerates
344 conduction velocity in various types of central axons and that neuromodulators change
345 axonal conduction velocity. By combining advanced electrophysiological, electron-
346 microscopic, and computational techniques, we reveal the mechanism and the metabolic
347 costs of the dynamic control of axonal conduction velocity by HCN channels in the
348 vertebrate central nervous system.

349

350 *Dynamic control of conduction velocity*

351 We describe both an increase and decrease of the baseline axonal conduction velocity in
352 the range of $\sim 5\%$ mediated by HCN channels (Figs. 1 – 3). Furthermore, HCN channel
353 accelerate action potential propagation by $\sim 25\%$ and increase the maximal failure-free
354 firing frequency by a factor of 2. Although the changes in baseline conduction velocity
355 are relatively small, considering the long distances that axons traverse in the brain, HCN
356 channels can be expected to change the arrival time of the action potential by for
357 example 0.5 ms in the case of cerebellar parallel fibers (assuming 3 mm length and 0.3

358 m/s velocity; see also Swadlow and Waxman, 2012). Such temporal delays will
359 influence information processing in the central nervous system, because spike-timing
360 dependent plasticity (Caporale and Dan, 2008), coincidence detection (Softky, 1994),
361 and neuronal rhythms of cell ensembles (Buzsáki et al., 2013) precisely tune the arrival
362 times of action potentials. There are several examples for specific tuning of conduction
363 velocity in the sub-millisecond domain: the diameter and the degree of myelination of
364 cerebellar climbing fibers (Sugihara et al., 1993; Lang and Rosenbluth, 2003; but see
365 Baker and Edgley, 2006), the degree of myelination of thalamocortical axons (Salami et
366 al., 2003), and the internode distance of auditory axons (Ford et al., 2015) are tuned to
367 exactly offset different arrival times of action potentials with a temporal precision of
368 ~100 μ s.

369

370 The cerebellum is involved in the accurate control of muscle contraction with a
371 temporal precision of 1-100 ms (Hore et al., 1991). Submillisecond correlations in spike
372 timing occurring between neighboring Purkinje cells have been noted previously
373 (reviewed in Isope et al., 2002; Person and Raman, 2012). Furthermore, submillisecond
374 precision of the mossy/parallel fiber input are critical for information processing in the
375 cerebellar circuits (Braitenberg et al., 1997; Heck et al., 2001). Together, the here-
376 described changes in action potential conduction velocity in mossy and parallel fibers
377 (Figs. 1-3) may thus play an important role in cerebellar computation. Furthermore, the
378 observed impairment in high-frequency firing without HCN channels (Fig. 5) is
379 expected to negatively impact such functions (Delvendahl and Hallermann, 2016).

380

381 Our findings that the cAMP-HCN pathway and neuromodulators can finely tune
382 conduction velocity in the vertebrate central nervous system adds to the emerging idea
383 that axons directly contribute to computation in neuronal circuits. Indeed, the view of
384 the axon as a simple and cable-like compartment in which conduction velocity is static
385 has substantially changed over the recent years in favor for a model that allows
386 flexibility and complex forms of axonal computation (Debanne et al., 2011). Recent
387 findings showed that axon diameters change during the time scales of LTP induction
388 (Chéreau et al., 2017) and changes in myelination in the motor cortex were resolved
389 during learning of complex motor skills (McKenzie et al., 2014; for environmental

390 effects on myelination see also Forbes and Gallo, 2017). One caveat of our study, is the
391 rather high concentrations of the used neuromodulators and the lack of *in vivo* evidence
392 for neuromodulation of conduction velocity. However, our data demonstrate that under
393 certain conditions an active control of conduction velocity could occur in the vertebrate
394 CNS via the cAMP-HCN pathway.

395

396 *Mechanism and metabolic costs of HCN channel mediated control of conduction*
397 *velocity*

398 Our analysis revealed that the control of conduction velocity is solely mediated by
399 changes in resting membrane potential. Isolated changes of membrane conductance and
400 thus of the membrane time and length constant had no effect on conduction velocity
401 (Fig. 10E). The speeding of conduction upon depolarization is consistent with a
402 previously observed correlation between conduction velocity and the depolarization
403 from the resting potential required to reach the firing threshold in motoneurons (Carp et
404 al., 2003). On the other hand, Na^+ channels have a steep steady-state inactivation and
405 are partially inactivated at the resting membrane potential in axons (Battefeld et al.,
406 2014; Engel and Jonas, 2005; Rama et al., 2015). Depolarization could thus be expected
407 to further inactivate Na^+ channels and decrease conduction velocity. However, our
408 modelling results showed that increasing the membrane potential from -90 to -60 mV
409 increased the conduction velocity despite significantly decreasing Na^+ channels
410 availability (Fig. S2). Interestingly, these findings are in agreement with the nonlinear
411 cable theory predicting that the difference between the resting membrane potential and
412 the firing threshold is a critical parameter for action potential conduction velocity (see,
413 e.g., Fig. 12.25 in Jack et al., 1983, for increasing velocity with increasing safety factor,
414 i.e. decreasing excitation threshold V_B). Intuitively, the HCN channel mediated
415 acceleration of conduction velocity can be understood as follows; in a more depolarized
416 axon, Na_v mediated current influx in one axonal location will depolarize neighboring
417 locations faster above the threshold. In our model, this effect outweighs the
418 disadvantage of the increased steady-state inactivation of Na^+ channels up to a
419 membrane potential of about -65 mV and a Na_v availability of 50%. The exact values,
420 above which Na_v availability limits conduction velocity, critically depend on
421 assumptions of the model, such as the voltage-dependence of inactivation and the

422 density of Na_v channel. Interestingly, Ca^{2+} entering through axonal voltage-gated Ca^{2+}
423 channels (Bender et al., 2010) could interact with the cAMP pathway by activating or
424 inhibiting different subtypes of adenylyl cyclase and phosphodiesterase (Bruce et al.,
425 2003). Thereby, HCN could also serve a homeostatic role, by bringing the resting
426 membrane potential closer to threshold and offsetting the inactivation of Na_v channels
427 under conditions of high-frequency action potential firing.

428

429 Our findings indicate that the evolutionary design of HCN channels as a continuously
430 open shunt for Na^+ influx causes significant metabolic costs. The high costs might
431 appear surprising, because a metabolically cheaper way to depolarize the membrane
432 would be the expression of less Na^+ - K^+ -ATPases resulting in a depolarized K^+ reversal
433 potential (cf. V_m -model in Fig. 10). However, as discussed in the following paragraph,
434 our finding that conduction velocity can be rapidly regulated via the cAMP-HCN
435 pathway might provide a justification for the metabolic costs of axonal HCN channels.

436

437 *Modulation of conduction velocity via the intracellular cAMP concentration*

438 Using direct whole-cell recordings and immunogold EM from *en passant* boutons in
439 cerebellar axons, we identified near exclusive HCN2 isoforms expression and a half-
440 maximal shift of the activation of HCN2 channels at a cAMP concentration of 40 μM
441 (Fig. 7D). Furthermore, our perforated patch-recordings from axonal compartments
442 provide, to our knowledge, the first direct estimate of endogenous cAMP concentration
443 in vertebrate central axons of 13 μM (Fig. 7D). This is higher compared to previous
444 estimates of 50 nM in Aplysia sensory neurons (Bacskaï et al., 1993; but see Greenberg
445 et al., 1987) and 1 μM in cardiomyocytes (Börner et al., 2011). A recently reported low
446 cAMP-sensitivity of protein kinase A (Koschinski and Zaccolo, 2017), a prototypical
447 cAMP-regulated protein, also argues for high intracellular cAMP concentrations. On the
448 other hand, our data do not rule out that such high cAMP concentration occur only in
449 spatially restricted domains. The possibility for local cAMP signaling-compartments
450 was recently observed in *Drosophila* axons (Maiellaro et al., 2016).

451

452 A high endogenous cAMP concentration and expression of the HCN2 isoform
453 facilitates neuromodulators to bidirectionally and dynamically control conduction

454 velocity. Only norepinephrine increased the conduction velocity in cerebellar parallel
455 fibers whereas the other neuromodulators reduced the velocity (Fig. 2), consistent with
456 the expression of both G_i - and G_s -coupled receptors, respectively. Indeed, G_s -coupled
457 receptors for serotonin, dopamine, and adenosine are expressed in the molecular layer of
458 the cerebellum (see e.g. Geurts et al., 2002; Schweighofer et al., 2004). Interestingly,
459 adenosine, which decreased the conduction velocity (Fig. 2), has been shown to be an
460 endogenous sleep factor (Basheer et al., 2004; Porkka-Heiskanen et al., 1997).
461 Moreover, serotonin, dopamine, and norepinephrine play important regulatory functions
462 during sleep in, e.g., the cerebellum (Canto et al., 2017). Therefore, it is tempting to
463 speculate that the cAMP-HCN pathway allows not only the increase in conduction
464 velocity during arousal but also the decrease in velocity and saving of metabolic costs
465 during periods of rest or sleep. The cAMP-HCN pathway in axons could thus contribute
466 to the reduced energy consumption of the brain during sleep (Boyle et al., 1994;
467 Townsend et al., 1973). It should be noted that the observed modulation of conduction
468 velocity by neurotransmitters (Fig. 2) is consistent with a modulation via the cAMP-
469 HCN pathway but other mechanisms, such as direct influences on voltage-dependent
470 Na^+ (Yin et al., 2017), K^+ (Yang et al., 2013), and Ca^{2+} channels (Burke et al., 2018)
471 could contribute to the modulation of conduction velocity.

472

473 *Clinical relevance of axonal HCN channels*

474 The function of HCN channels has been studied in human peripheral nerves using non-
475 invasive threshold tracking techniques (Howells et al., 2016; Howells et al., 2012;
476 Lorenz and Jones, 2014). Significant alterations of HCN channel expression and/or
477 function have been described in pathologies such as stroke (Jankelowitz et al., 2007),
478 porphyria (Lin et al., 2008), diabetic neuropathy (Horn et al., 1996), neuropathic pain
479 (Chaplan et al., 2003), and inflammation (Momin and McNaughton, 2009) as well as a
480 vertebrate model of demyelination (Fledrich et al., 2014). In some of these cases, the
481 alterations are consistent with an activity-dependent modulation of HCN channels
482 (Jankelowitz et al., 2007). Furthermore, HCN channel seem to be causally related to
483 pain symptoms (Chaplan et al., 2003; Momin and McNaughton, 2009) and therapeutic
484 blockade of HCN channels are also considered (Wickenden et al., 2009). Based on our

485 findings, HCN could also play a compensatory role in some diseases to restore
486 conduction velocity.

487 **Methods**

488 *Cerebellar slice preparation*

489 Cerebellar slices were prepared from P21-P46 C57BL/6 mice of either sex as reported
490 previously (Delvendahl et al., 2015; Ritzau-Jost et al., 2014). In short, after
491 anesthetization with isoflurane, mice were killed by rapid decapitation; the cerebellar
492 vermis was quickly removed and placed in a slicing chamber filled with ice-cold
493 extracellular solution (ACSF) containing (in mM): NaCl 125, KCl 2.5, NaHCO₃ 26,
494 NaH₂PO₄ 1.25, glucose 20, CaCl₂ 2, MgCl₂ 1 (pH adjusted to 7.3–7.4 with HCl).
495 Parasagittal or horizontal slices were cut from the vermis of the cerebellum using a
496 microtome with a vibrating blade (VT1200, Leica Biosystems, Nussloch, Germany),
497 incubated at 35°C for approximately 30 minutes and subsequently stored at room
498 temperature until use. For electrophysiological recordings, a slice was transferred into
499 the recording chamber mounted on the stage of an upright Nikon microscope. The
500 recording chamber was perfused with ACSF and the temperature in the center of the
501 recording chamber was set to 35°C using a TC-324B perfusion heat controller (Warner
502 Instruments, Hamden CT, USA).

503

504 *Measuring conduction velocity in cerebellar parallel and mossy fibers*

505 Compound action potentials were evoked by electrical stimulation using a bipolar
506 platinum/iridium electrode (from Microprobes for Life Science, Gaithersburg MD,
507 USA) placed either in the white matter or in the molecular layer (Fig. 1) of the
508 cerebellum. For the extracellular recording of compound action potentials, two pipettes
509 were filled with a 1M NaCl solution (tip resistance of 1–3 MΩ) and placed within the
510 respective fiber bundle, and the voltage was measured in current clamp mode with an
511 EPC10 amplifier (CC gain 10x). Compound action potentials were evoked at 0.5 Hz in
512 parallel fibers and 1 Hz in the white matter. All recordings were performed in the
513 presence of 10 μM NBQX to block synaptic potentials. The conduction velocity of PFs
514 was measured at 35°C. Due to the higher conduction velocity in myelinated mossy
515 fibers, action potentials evoked by white matter stimulation had to be recorded at room
516 temperature to allow separation of the compound action potential from the stimulation
517 artifact. To calculate the conduction velocity, we determined the delays of the peaks of
518 the compound action potential component recorded with the proximal and distal

519 electrode. Compound action potentials from mossy fibers were analyzed offline using
520 the smoothing spline interpolation operation of Igor Pro to increase the signal to noise
521 ratio. Control recordings were performed interleaved with application of different drugs.
522 The conduction velocity experienced a small rundown over 20 minutes under control
523 conditions (Fig. 1 – 3).

524

525 *Measuring conduction velocity in the optic nerve*

526 Male wildtype mice of the C57BL6/N strain (P63 ± 4) were euthanized by decapitation.
527 After the brain was exposed, the optic nerves (ON) were separated from the retina at the
528 ocular cavity and both ONs were detached by cutting posterior to the optic chiasm. The
529 preparation was gently placed into an interface brain/tissue slice (BTS) perfusion
530 chamber (Harvard Apparatus) and continuously superfused with ACSF, bubbled with
531 carbogen (95% O₂, 5% CO₂) at 36.5 °C during the experiment (Trevisiol et al., 2017).
532 In case both nerves were used for experiments, the non-recorded ON was transferred in
533 a different incubation chamber (Leica HI 1210) that provided similar incubation
534 conditions to the recorded nerve while preventing exposure to ZD7288 and 8-Br-cAMP.
535 The temperature was maintained constant using a feedback-driven temperature
536 controller (model TC-10, NPI electronic) connected to a temperature probe (TS-100-S;
537 NPI electronic) inserted in the BTS incubation chamber near the nerve. Each ON was
538 detached from the optic chiasm and individually placed into the suction electrodes for
539 stimulation/recording. The stimulation's direction of the ON was maintained constant
540 (orthodromic) throughout the experiments by inserting the proximal (retinal) end of the
541 nerve into the stimulation electrode as illustrated in Fig. 1I. The stimulating electrode
542 was connected to a battery (Stimulus Isolator A385; WPI) that delivered a
543 supramaximal stimulus to the nerve. The voltage was pre-amplified 500 times and fed
544 to the AD ports of the EPC9 or acquired directly via the EPC9 headstage (HEKA
545 Elektronik, Lambrecht/Pfalz). The reference channel was obtained from an ACSF-filled
546 glass capillary next to the recording suction electrode, in contact with the bathing
547 ACSF. Initial equilibration of the ONs was performed at 0.1 Hz stimulation, until the
548 recorded compound action potentials showed a steady shape (typically around 45-60
549 min from preparation). 5 nerves from 4 animals and 4 nerves from 4 animals were used
550 for ZD7288 and 8-Br-cAMP treatment, respectively. Compound action potentials were

551 analyzed as described above using the smoothing spline interpolation operation of Igor
552 Pro to increase the signal to noise ratio.

553

554 *Recordings from cMFBs*

555 cMFBs were visualized as previously described (Delvendahl et al., 2015; Ritzau-Jost et
556 al., 2014) with infrared differential interference contrast (DIC) optics using a FN-1
557 microscope from Nikon with a 100x objective (NA 1.1) or infrared oblique illumination
558 optics using a Femto-2D two-photon microscope (Femtonics, Budapest) with a 60x
559 Olympus (NA 1.0) objective. The passive properties of the cMFB were determined as
560 previously described (Hallermann et al., 2003) and revealed similar values for a two-
561 compartment model (data not shown) as previously described for cMFBs (Ritzau-Jost et
562 al., 2014), indicating that we indeed recorded from cMFBs. Furthermore, the access
563 resistance was on average $16.9 \pm 0.9 \text{ M}\Omega$ ($n = 53$ cMFBs), indicating optimal voltage
564 clamp conditions.

565

566 To elicit traveling action potentials by axonal stimulation with a second pipette (Fig.
567 5A), whole-cell recordings from cMFBs were performed with 50 μM green fluorescent
568 dye Atto488 in the intracellular solution to visualize single mossy fiber axons. The
569 additional stimulation pipettes filled with ACSF and 50 μM of the red-fluorescent dye
570 Atto594 had the same opening diameter as patch pipettes and were positioned close to
571 the axon and approximately 100 μm apart from the patched terminal. Stimulation pulses
572 with durations of 100 μs were delivered by a voltage-stimulator (ISO-Pulser ISOP1,
573 AD-Elektronik, Buchenbach, Germany). The stimulation intensity (1-30 V) was
574 adjusted to ensure failure-free initiation of action potentials at 1 Hz (~ 1.5 time the firing
575 threshold). High-frequency trains of action potentials were evoked at 100, 200 333, 500,
576 750, 1000, 1111 and 1666 Hz. Amplitudes were measured from peak to baseline. The
577 duration was determined at half-maximal amplitude and is referred to as half-width.
578 Action potentials were treated as failures if the peak did not exceed -40 mV.

579

580 Recordings were performed with an EPC10/2 patch-clamp amplifier, operated by the
581 corresponding software PatchMaster (HEKA Elektronik), running on a personal
582 computer. Recording electrodes were pulled from borosilicate glass capillaries (inner

583 diameter 1.16 mm, outer diameter 2 mm) by a microelectrode puller (DMZ-Universal
584 Puller, Zeitz Instruments, Augsburg). Pipettes used for patch-clamp recordings had
585 open-tip resistances of 5–12 MΩ. The intracellular presynaptic patch pipette contained
586 (in mM): K-gluconate 150, MgATP 3, NaGTP 0.3, NaCl 10, HEPES 10 and EGTA
587 0.05. The apparent input resistance of cMFBs was estimated by linear regression of the
588 steady-state voltage in response to 300 ms hyperpolarizing current pulses of increasing
589 amplitude (−5 to −20 pA), while the apparent membrane time constant was determined
590 by fitting the voltage response to a -10 pA hyperpolarizing pulse with a mono-
591 exponential function.

592

593 I_h activation curves determined from analysis of normalized tail current were fitted with
594 a Boltzmann function:

$$595 \frac{I}{I_{max}} = \frac{1}{1 + e^{\frac{V - V_{1/2}}{k}}}$$

596 where V is the holding potential, $V_{1/2}$ is the voltage of half-maximal activation and k the
597 slope factor. The reversal potential of I_h was calculated from leak-subtracted currents
598 evoked by 10 ms long voltage ramps extending across the activation range of I_h (Cuttle
599 et al., 2001). Three I-V relationships recorded at holding potentials of −80, −110 and −
600 140 mV were linearly extrapolated and the reversal potential was measured from the
601 point of intersection of the three linear fits.

602

603 *Perforated patch recordings*

604 For perforated-patch recordings from cMFBs, a nystatin stock solution was prepared by
605 dissolving the pore-forming antimycotic in DMSO (25 mg/ml). Immediately before the
606 experiments, the nystatin-stock was added to the intracellular solution at a final
607 concentration of 50 µg/ml. In order to monitor the integrity of the perforated membrane
608 patch, the green-fluorescent dye Atto 488 (from Atto-Tec, Siegen,
609 Germany) was added at a concentration of 50 µM. Since nystatin is known to impair the
610 formation of the GΩ seal, the initial ~500 µm of the pipette tip was filled with a
611 perforating agent-free internal solution before back-filling the pipette shaft with the
612 perforating agent-containing solution. After establishing a GΩ seal, the holding
613 potential was set to −70 mV and the access resistance (R_a) was continuously monitored

614 by applying 10 ms long depolarizing pulses to -60 mV at 1 Hz. Recording the voltage-
615 dependent activation of I_h was started after R_a dropped below 150 M Ω . Because the
616 perforated membrane patch ruptured spontaneously with $R_a < 50$ M Ω , the access
617 resistance was not comparable to standard whole-cell recordings. To exclude the
618 possibility that the right-shift of the I_h activation curve in the perforated configuration
619 (Fig. 8C) was caused by the comparatively higher R_a , the voltage-dependent activation
620 of I_h was measured under normal whole-cell patch-clamp conditions, using pipettes with
621 small openings resulting in high access resistances ($R_a = 119 \pm 12$ M Ω). However, in
622 these recordings, the midpoint of I_h activation (-105.5 ± 1.4 mV; $n = 8$) had a tendency
623 to be left-shifted compared with regular whole-cell recordings with standard patch
624 pipettes ($R_a \approx 30$ - 60 M Ω ; $V_{1/2} = -103.3 \pm 0.8$ mV; $n = 36$; $P_{T-Test} = 0.13$). The left-shift of
625 the I_h activation curve measured with high access resistances indicates that the right-
626 shift measured with perforated patch recordings might be underestimated due to the
627 higher R_a , which would result in an even higher estimate of the endogenous cAMP
628 concentration (Fig. 7D).

629

630 *Analysis of ZD sensitivity of Na⁺ currents*

631 Sodium currents (Fig. S1) were isolated using a modified ACSF containing (in mM):
632 NaCl 105, KCl 2.5, NaHCO₃ 25, NaH₂PO₄ 1.25, glucose 20, CaCl₂ 2, MgCl₂ 1, TEA
633 20, 4-AP 5 and CdCl₂ 0.2. To avoid underestimating the true size of the presynaptic Na⁺
634 currents due to the voltage-drop through the access resistance, we blocked a portion of
635 the Na⁺ current with 30 nM TTX. Na⁺ currents were elicited from a holding potential of
636 -80 mV by a 3 ms long depolarization to 0 mV. Peak amplitudes and half-durations of
637 Na⁺ currents were measured from leak-subtracted traces.

638

639 *Immunoelectron microscopy*

640 Preembedding immunogold labeling was performed as described (Notomi and
641 Shigemoto, 2004). Briefly, adult C57Bl/6 mice were anesthetized with sodium
642 pentobarbital (50 mg/kg, i.p.) and perfused transcardially with a fixative containing 4%
643 formaldehyde, 0.05% glutaraldehyde and 15% of a saturated picric acid in 0.1 M
644 phosphate buffer (PB, pH 7.4). Parasagittal sections through the cerebellum were cut at
645 50 μ m, cryoprotected with 30% sucrose, flash frozen on liquid nitrogen and rapidly

646 thawed. Sections were blocked in 10% normal goat serum and 2% bovine serum
647 albumin (BSA) in Tris-buffered saline (TBS) for 2 h at room temperature, incubated in
648 TBS containing 2% BSA and either guinea pig anti-HCN1 or anti-HCN2 antibody
649 (1 µg/ml, Notomi and Shigemoto, 2004) for 48 h at 4°C, and finally reacted with
650 nanogold-conjugated secondary antibody (Nanoprobes, 1:100) for 24 h at 4°C.
651 Nanogold particles were amplified with HQ Silver Enhancement kit (Nanoprobes) for
652 8 min. Sections were then treated in 0.5% osmium tetroxide in PB for 40 min, 1%
653 aqueous uranyl acetate for 30 min at room temperature, dehydrated, and flat embedded
654 in Durcopan resin (Sigma-Aldrich). Ultrathin sections were cut at 70 nm and observed
655 by a transmission electron microscope (Tecnai 12, FEI, Oregon). Sequential images
656 were recorded from the granule cell layer within a few microns from the surface of
657 ultrathin sections at X26,500 using a CCD camera (VELETA, Olympus). For the
658 reconstruction of a half mossy fiber bouton, 36 serial ultrathin sections were used.
659 Sequential images were aligned and stacked using TrakEM2 program (Cardona et al.,
660 2012). For the measurement of density of immunogold particles for HCN2 on this
661 reconstructed profile, 1260 immunogold particles were counted on the mossy fiber
662 bouton membrane area (73.7 µm²), giving a density of 17.1 particles/µm². Immunogold
663 particles within 30 nm from the bouton membranes were included in the analysis based
664 on the possible distance of the immunogold particle from the epitope (Matsubara et al.,
665 1996). The density of non-specific labeling was estimated using nuclear membrane of a
666 granule cell located adjacent to the reconstructed mossy fiber bouton. We found
667 40 immunogold particles on the nuclear membrane area of 60.5 µm² giving a density of
668 0.66 particles/µm², which was 3.9% of the HCN2 labeling density on the mossy fiber
669 bouton.

670

671 *Hodgkin-Huxley model of axonal HCN channels*

672 Because we did not intend to implement the cAMP dependence of HCN channels
673 explicitly (Hummert et al., 2018), we created two separate models for 0 and 1 mM
674 intracellular cAMP, which were based on a previously described Hodgkin-Huxley
675 model (Kole et al., 2006) with one activation gate and no inactivation (Hodgkin and
676 Huxley, 1952). In short, the activation gate was described by

677
$$\frac{dm}{dt} = \alpha_m(1 - m) - \beta_m m$$

678 with

$$\alpha_m(V) = A e^{\frac{-(V_m - V_{1/2})}{V_\alpha}}$$

680 and

$$\beta_m(V) = A e^{\frac{(V_m - V_{1/2})}{V_\beta}}$$

682

683 The four free parameters, A , $V_{1/2}$, V_α , and V_β were determined by simultaneously fitting
684 $\alpha_m / (\alpha_m + \beta_m)$ to the steady-state activation curve (see Fig. 7B) and $1 / (\alpha_m + \beta_m)$ to the
685 voltage dependence of the time constant of I_h activation and deactivation (Fig. 9B). The
686 sum of squared errors was minimized using the FindMinimum routine of Mathematica
687 (version 10; Wolfram Research, Champaign, IL), with the time constants of activation
688 and deactivation weighed with the inverse of the square of the maximum value in each
689 of the three datasets (time constant of activation, time constant of activation, steady-
690 state activation curve). The resulting parameters for 0 mM cAMP were $A = 6.907 \text{ ms}^{-1}$,
691 $V_{1/2} = -102.1 \text{ mV}$, $V_\alpha = 18.71 \text{ mV}$, and $V_\beta = 21.73 \text{ mV}$. To confirm that the global
692 minimum was reached, the best-fit parameters were shown to be independent of the
693 starting values within a plausible range. The 68% confidence interval was calculated as
694 the square roots of the diagonals of the inverse of the Hessian matrix (Press et al., 2002)
695 resulting in $\pm 2.71 \text{ ms}^{-1}$, $\pm 16.5 \text{ mV}$, $\pm 17.5 \text{ mV}$, and $\pm 24.3 \text{ mV}$ for A , $V_{1/2}$, V_α , and V_β ,
696 respectively. We also generated a model for the corresponding data obtained with 1 mM
697 cAMP in the intracellular solution (cf. Fig 5B), resulting in $A = 7.570 \text{ ms}^{-1}$, $V_{1/2} = -$
698 87.31 mV , $V_\alpha = 31.46 \text{ mV}$, and $V_\beta = 10.84 \text{ mV}$.

699

700 *NEURON model of cMFB*

701 The model of the cMFB consisted of connected cylindrical compartments representing
702 15 boutons (length and a diameter 8 μm) and 15 myelinated axonal compartments
703 (length 35 μm and a diameter 0.8 μm ; cf. Palay and Chan-Palay, 1974; Fig. 10A). In
704 addition, at one side of this chain a long cylinder was added presenting the axon in the
705 white matter (length 150 μm and a diameter 1.2 μm). The specific membrane resistance
706 was $0.9 \mu\text{F/cm}^2$ (Gentet et al., 2000) and the cytoplasmatic resistivity was $120 \Omega/\text{cm}$
707 (Hallermann et al., 2003). The specific membrane resistance of the axonal
708 compartments was reduced by a factor of 10 representing myelination.

709

710 The active membrane conductances were similar to Ritzau-Jost et al. (2014) and were
711 adjusted to reproduce the action potential duration and maximal firing frequency as well
712 as the data shown in Figs. 10B-D. Namely, an axonal Na^+ channel (Schmidt-Hieber and
713 Bischofberger, 2010) and K^+ channel NMODL model (Hallermann et al., 2012) was
714 added with a density of 2000 and 1000 $\text{pS}/\mu\text{m}^2$ in the boutons and 0 and 0 $\text{pS}/\mu\text{m}^2$ in the
715 axonal compartments, respectively. The Na^+ and K^+ reversal potentials were 55 and -97
716 mV, respectively. To investigate ATP consumption, separate Na^+ and K^+ leak channel
717 models were added, with a conductance of 0.0138 and 0.18 $\text{pS}/\mu\text{m}^2$, respectively, in the
718 bouton compartments. In the axonal compartments, both conductances were reduced by
719 a factor of 10. The above described Hodgkin-Huxley model of axonal HCN channels for
720 either 0 or 1 mM intracellular cAMP was added with a density of $g_{\text{HCN}} = 0.3$ and 0.03
721 $\text{pS}/\mu\text{m}^2$ for the bouton and axonal compartments, respectively, to reproduce the data
722 shown in Fig. 10B-D. To investigate ATP consumption, the conductance was separated
723 in a Na^+ and a K^+ conductance according to $g_{\text{HCN}(\text{Na})} = (1 - \text{ratio}_{\text{K}/\text{Na}}) g_{\text{HCN}}$ and $g_{\text{HCN}(\text{K})} =$
724 $\text{ratio}_{\text{K}/\text{Na}} g_{\text{HCN}}$, where $\text{ratio}_{\text{K}/\text{Na}} = (e_{\text{Na}} + e_{\text{HCN}})/(e_{\text{Na}} - e_{\text{K}})$, where e_{Na} and e_{K} are the Na^+
725 and K^+ reversal potential as described above and e_{HCN} is the reversal potential of I_h
726 measured as -23.3 mV (cf. Fig. 9C). Assuming a single channel conductance of 1.7 pS
727 for HCN2 channels (Thon et al.), this conductance corresponds to a density of 0.18
728 HCN channels/ μm^2 , which is much lower than the estimate from preembedding
729 immunogold labeling (22 particles/ μm^2 ; Fig. 8). However, the optimal density of the
730 model critically depends on the geometry of the structure, which was not obtained from
731 the recorded boutons. To obtain the required structural information including the
732 fenestration of the cMFB (cf. Fig. 8) and the level of myelination, electron microscopic
733 reconstructions of large volumes of the recorded cMFB and the entire axon would be
734 needed. When we used a g_{HCN} , as determined with preembedding immunogold labeling
735 in our model, the model also predicted that I_h critically effects conduction velocity and
736 that the depolarization is the main reason for the velocity to change. In general, these
737 two conclusions of the model were very insensitive to the specific parameters of the
738 model and were, e.g., also obtained with additional interleaved cylindrical
739 compartments with high Na^+ and K^+ channel density representing nodes of Ranvier or
740 with a long cylindrical compartment with homogenous channel densities representing

741 an unmyelinated axon. This further supports our finding that HCN channels accelerate
742 conduction velocity independent of the exact parameters of the axon and the degree of
743 myelination (cf. Fig. 1).

744

745 Starting from the model that reproduced the control data, the following four additional
746 models were generated: (1) To simulate ZD application, the HCN HH model was
747 removed. (2) To simulate 8-br-cAMP application, parameters of the HCN HH model
748 were exchanged with the parameters obtained from the experiments with 1 mM cAMP
749 as described in the section above. (3) To simulate only the depolarization by HCN
750 channels (V_m -model), the HCN HH channel model was removed and the K^+ reversal
751 potential was increased from -97 mV to -90 mV. (4) To simulate only the increase in
752 membrane conductance by HCN channels (R_m -model), the reversal potential of the HCN
753 HH model was decreased from -23.3 mV to -85.5 mV and the density was increased
754 from 0.3 pS/ μ m² to 1 pS/ μ m².

755

756 All simulations were run with a simulation time interval (dt) of 0.2 ms, preceded by a
757 simulation of 1 s with a dt of 5 ms to allow equilibration of all conductances.
758 Conduction velocity was calculated from the peak of the action potentials in different
759 boutons of the model. The apparent input resistance was calculated identical to the
760 experimental recordings, i.e. from the voltage after 300 ms of a -10 pA current
761 injection. Mathematica was used to execute the NEURON simulations and to visualize
762 and analyze the automatically imported NEURON results.

763

764 *Statistics*

765 Statistical analysis was performed using built-in functions of Igor Pro (Wavemetrics,
766 Lake Oswego, OR). The suffix of the P values provided in the legends and the main test
767 indicate the used statistical test. Results were considered significant with P < 0.05.

768

769 *Code*

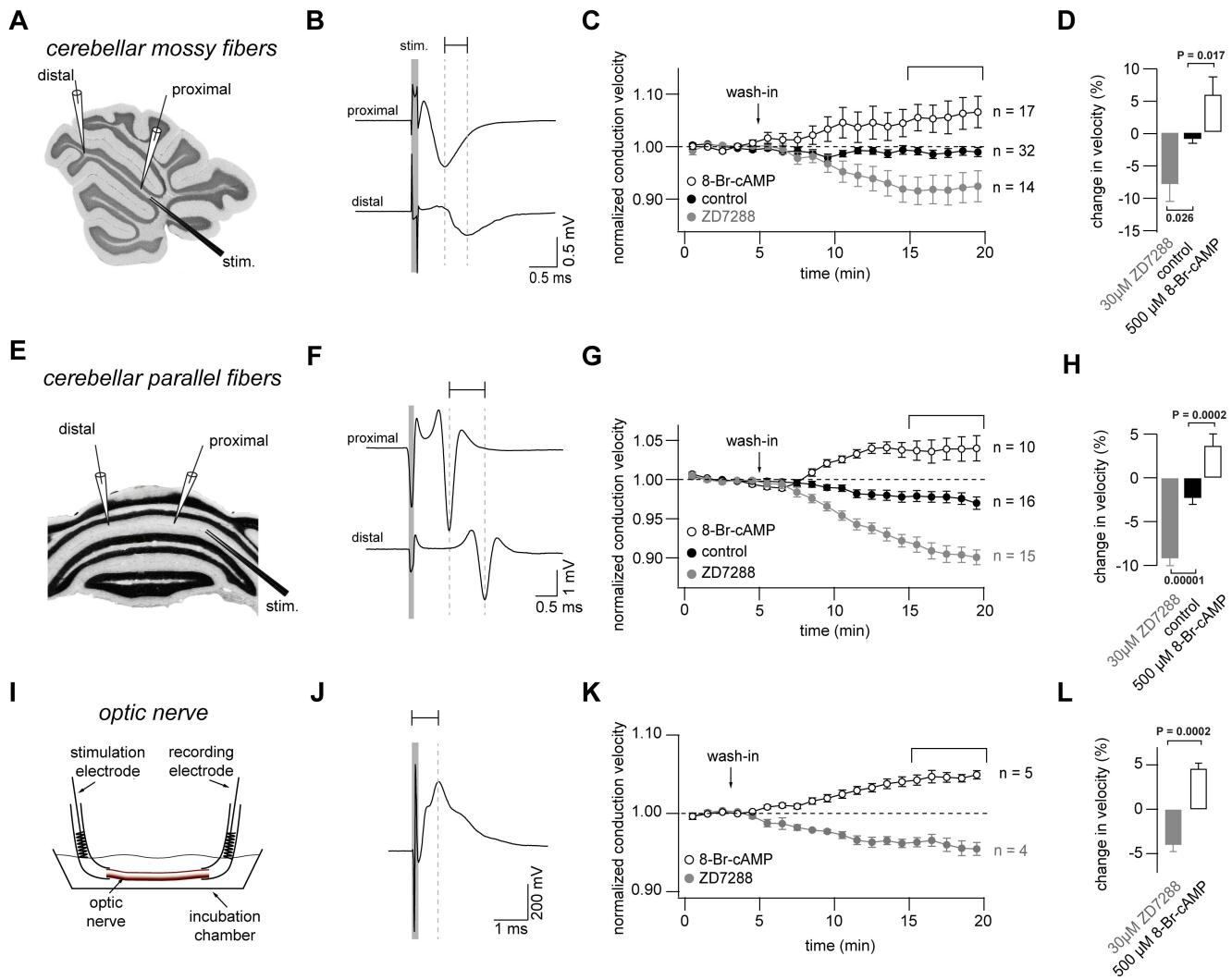
770 The NEURON and Mathematica scripts to reproduce the model results will be available
771 at: https://github.com/HallermannLab/2018_eLife_HCN.

772

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775 Planck Institute of Experimental Medicine for scientific support. This work was
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777 **Figures and Legends**



779 **Figure 1. Bidirectional modulation of conduction velocity**

780 (A) Recording configuration of conduction velocity in mossy fibers using a bipolar
781 tungsten stimulation electrode (stim.) and two glass electrodes filled with 1M NaCl
782 solution.

783 (B) Example of compound action potentials recorded with two electrodes positioned
784 with different distance in relation to the stimulation electrode. Stimulation was 100
785 μ s as indicated by the grey bar. Each trace is an average of 50 individual compound
786 action potentials recorded at 1Hz.

787 (C) Average normalized mossy fiber conduction velocity, during bath application
788 ZD7288 (30 μ M) or 8-Br-cAMP (500 μ M) at t = 5 min.

789 (D) Average relative change in mossy fiber conduction velocity after application of
790 ZD7288 or 8-Br-cAMP measured 10 to 15 minutes after wash-in (bracket in C).
791 $P_{\text{ANOVA}} = 0.00015$. $P_{\text{Kruskal-Wallis}} = 0.00044$. The individual P values of the Dunnett
792 test for multiple comparisons with control are indicated.

793 (E) Schematic illustration of the experimental configuration used to record from
794 cerebellar parallel fibers.

795 (F) Examples of compound action potentials recorded from parallel fibers, as in panel
796 B.

797 (G) Normalized conduction velocity in parallel fibers, as in panel C.

798 (H) Average relative changes in parallel fiber, as in panel D. $P_{\text{ANOVA}} = 10^{-9}$. $P_{\text{Kruskal-Wallis}}$
799 $= 10^{-8}$. The individual P values of the Dunnett test for multiple comparisons with
800 control are indicated.

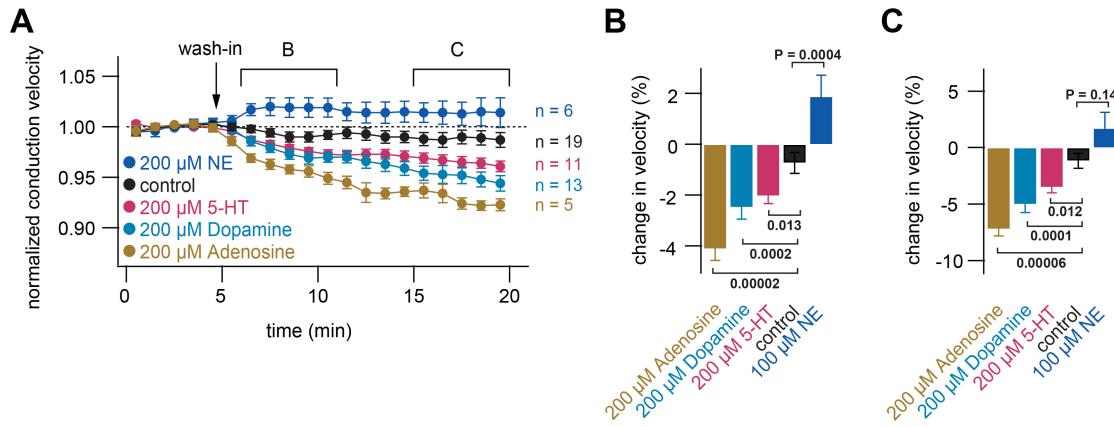
801 (I) Schematic of the experimental configuration used to record from optic nerve.

802 (J) Examples of compound action potentials recorded from optic nerve, as in panel B.

803 (K) Normalized conduction velocity in optic nerve, as in panel C.

804 (L) Average relative changes in optic nerve, as in panel D. $P_{\text{T-Test}} = 0.0002$. $P_{\text{Wilcoxon-}}$
805 $\text{Mann-Whitney-Test} = 0.004$.

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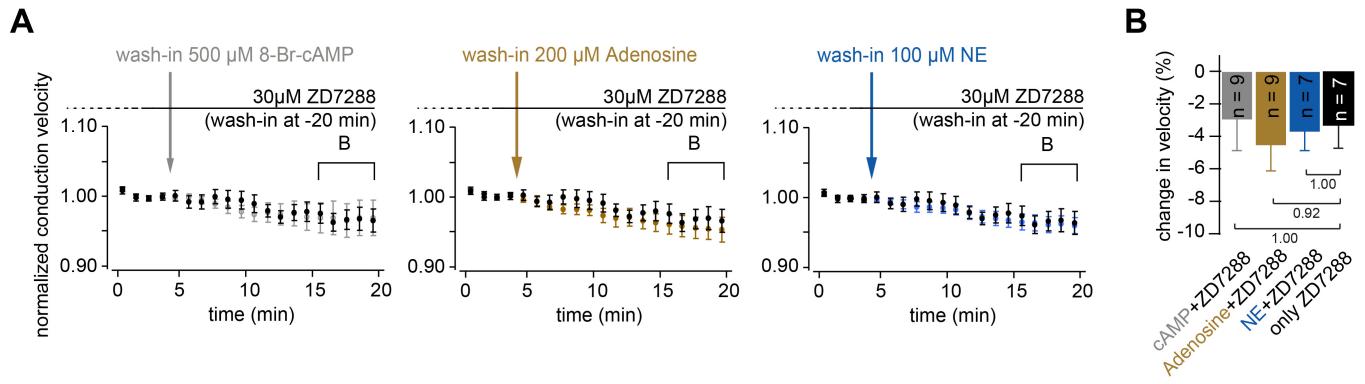
809 **Figure 2. Neuromodulators differentially regulate conduction velocity**

810 (A) Average normalized conduction velocity in cerebellar parallel fibers during wash-in
811 at $t = 5$ min of various neuromodulators known to act via cAMP-dependent
812 pathways.

813 (B) Average relative change in conduction velocity after application of the
814 neuromodulators measured from 1 to 6 minutes after wash-in (bracket marked B in
815 panel A). $P_{\text{ANOVA}} = 9 \times 10^{-10}$. $P_{\text{Kruskal-Wallis}} = 3 \times 10^{-8}$. The individual P values of the
816 Dunnett test for multiple comparisons with control are indicated.

817 (C) Average relative change in conduction velocity after application of the
818 neuromodulators measured 10 to 15 minutes after wash-in (bracket marked C in
819 panel A). $P_{\text{ANOVA}} = 3 \times 10^{-7}$. $P_{\text{Kruskal-Wallis}} = 3 \times 10^{-7}$. The individual P values of the
820 Dunnett test for multiple comparisons with control are indicated.

821



823

824 **Figure 3. Neuromodulation of conduction velocity is mediated by HCN channels**

825 (A) Average normalized conduction velocity in parallel fibers. Wash-in of 30 μ M
826 ZD7288 20 minutes before the start of the recording. The substance remained in
827 the solution during recording to ensure continuous block HCN channels. At $t = 5$
828 min, 8-Br-cAMP, Adenosine or NE was added to the solution.

829 (B) Average relative change in conduction velocity after application of the
830 neuromodulators measured 10 to 15 minutes after wash-in (bracket marked B in
831 panel A). $P_{ANOVA} = 0.91$. $P_{Kruskal-Wallis} = 0.77$. The individual P values of the
832 Dunnett test for multiple comparisons with control are indicated.

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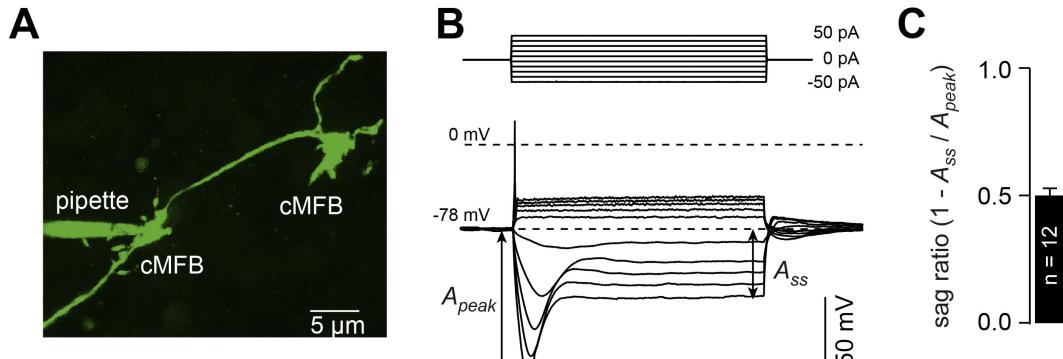
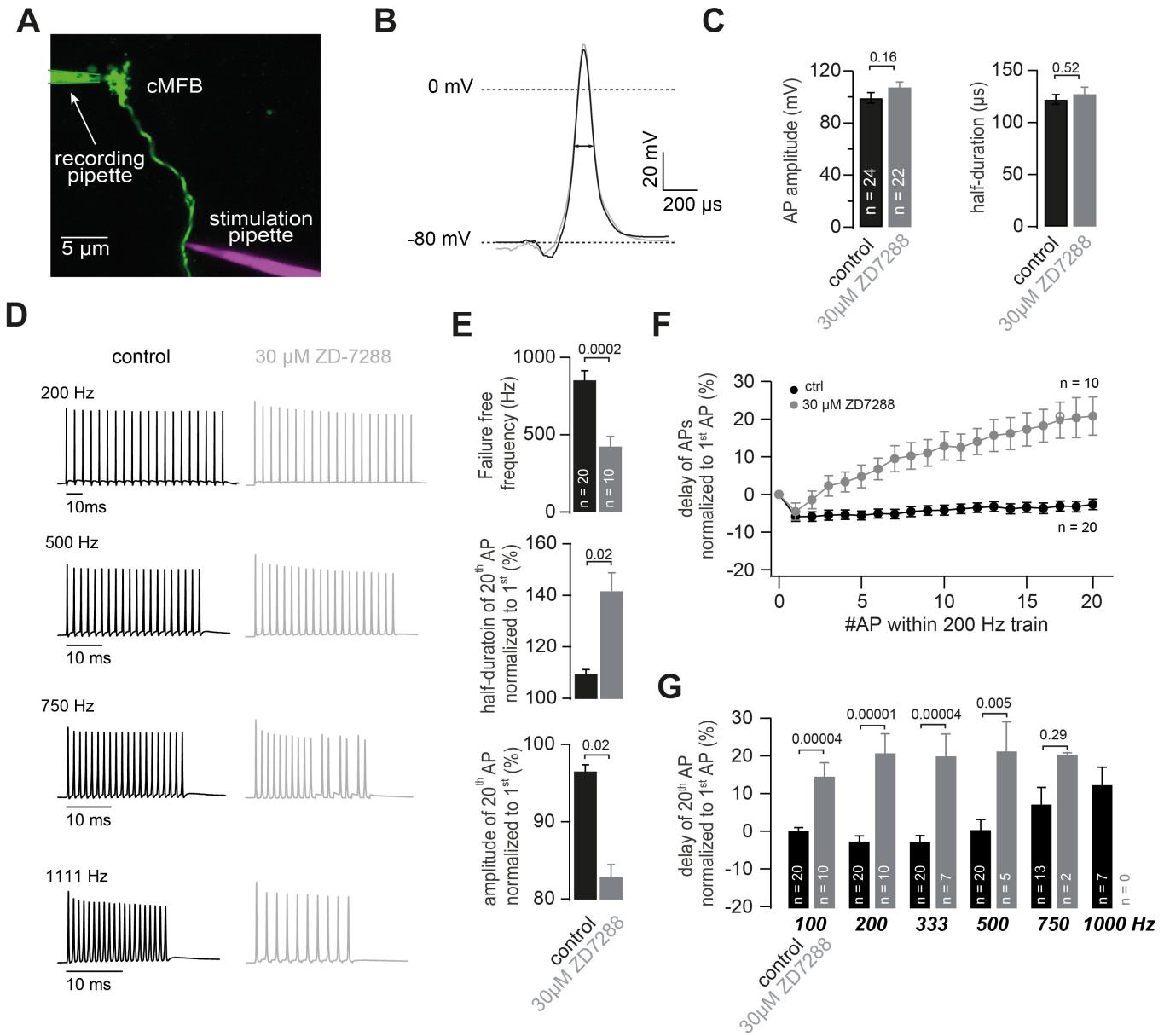


Figure 4. Cerebellar mossy fiber terminals have a prominent voltage sag

(A) Two-photon microscopic image of a whole-cell patch-clamp recording from a cMFB (green) filled with the fluorescence dye Atto 488 in an acute cerebellar brain slice of an adult 39-days old C57/Bl6 mouse (maximal projection of stack of images).

(B) Characteristic response of a cMFB to current injection: Depolarizing pulses evoked a single action potential while hyperpolarizing pulses evoked a strong hyperpolarization with a sag.

(C) Average sag ratio of 12 cMFB recordings.



847 **Figure 5. HCN channels support high frequency action potential firing**

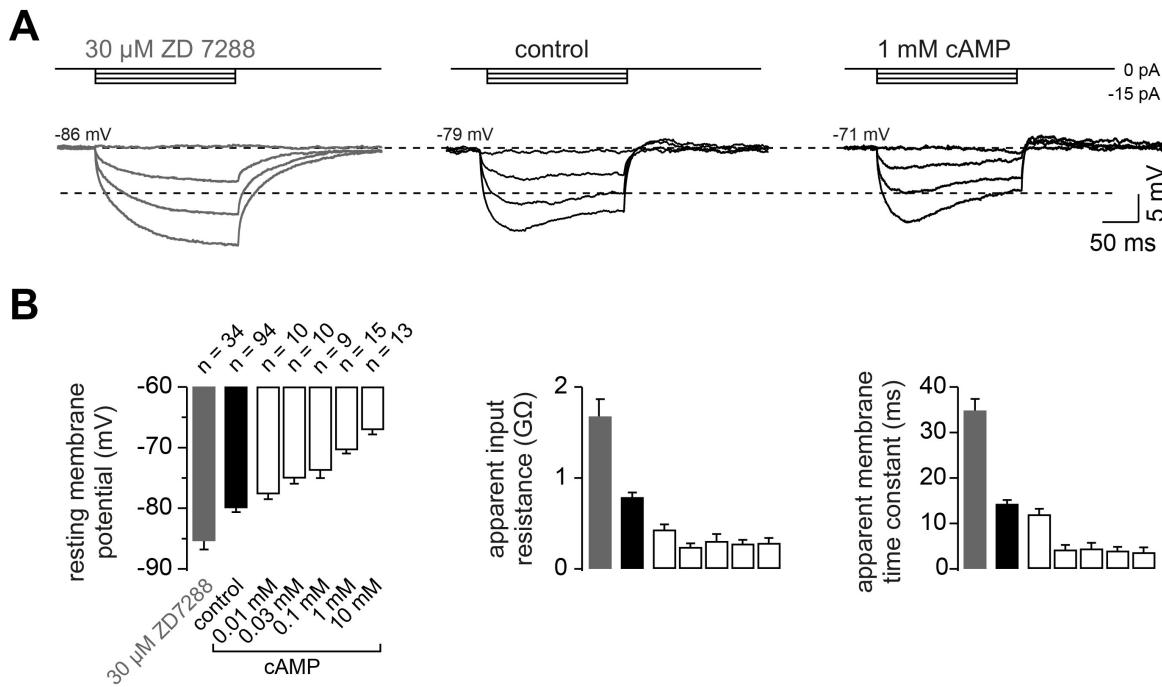
848 (A) Two-photon microscopic image of a whole-cell patch-clamp recording from a
849 cMFB (green) filled with the fluorescence dye Atto 488 in an acute cerebellar brain
850 slice of an adult 43-days old mouse (maximal projection of stack of images).
851 Targeted axonal stimulation was performed by adding a red dye Atto 594 to the
852 solution of the stimulation pipette.
853 (B) Grand average of action potentials evoked at 1 Hz under control conditions (black)
854 and in the presence of ZD7288 (grey).
855 (C) Average action potential amplitude (measured from resting to peak) and half-
856 duration $P_{T-Test} = 0.16$ and 0.51 , for amplitude and resting respectively.

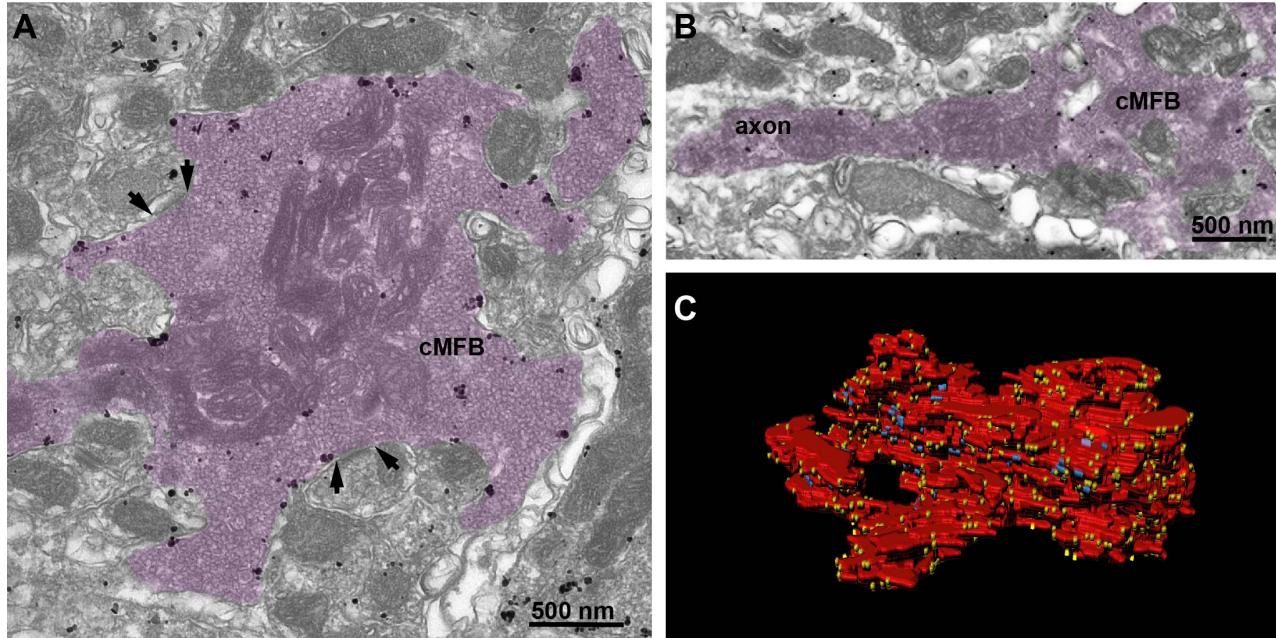
857 (D) ZD7288 effects the ability of cerebellar mossy fibers to fire high frequency action
858 potentials: Examples of two different cMFBs stimulated at frequencies between 200
859 Hz and 1111 Hz under control conditions (*left, black*) or in the presence of 30 μ M
860 ZD7288 (*right, grey*).
861 (E) cMFBs treated with ZD7288 showed an on average lower maximal failure-free
862 firing frequency. In addition, amplitude reduction and action potential broadening
863 during 200-Hz trains of action potentials in ZD7288 treated axons were more
864 pronounced than under control conditions.
865 (F) Average delay between the peak of the action potentials (AP) and the stimulation
866 during 200 Hz trains of 20 action potentials normalized to the delay of the first
867 action potential for control conditions (*black*) and in the presence of 30 μ M ZD7288
868 (*grey*).
869 (G) Average delay of the 20th normalized to the delay of the 1st action potential during
870 trains of 20 action potentials at frequencies ranging from 100 to 1000 Hz. The
871 provided P-values based on simple t-test are mostly much smaller than the
872 Bonferroni-corrected significance left of $0.05/6 = 0.008$, indicating a highly
873 significant slowing of the conduction velocity during high-frequency trains.

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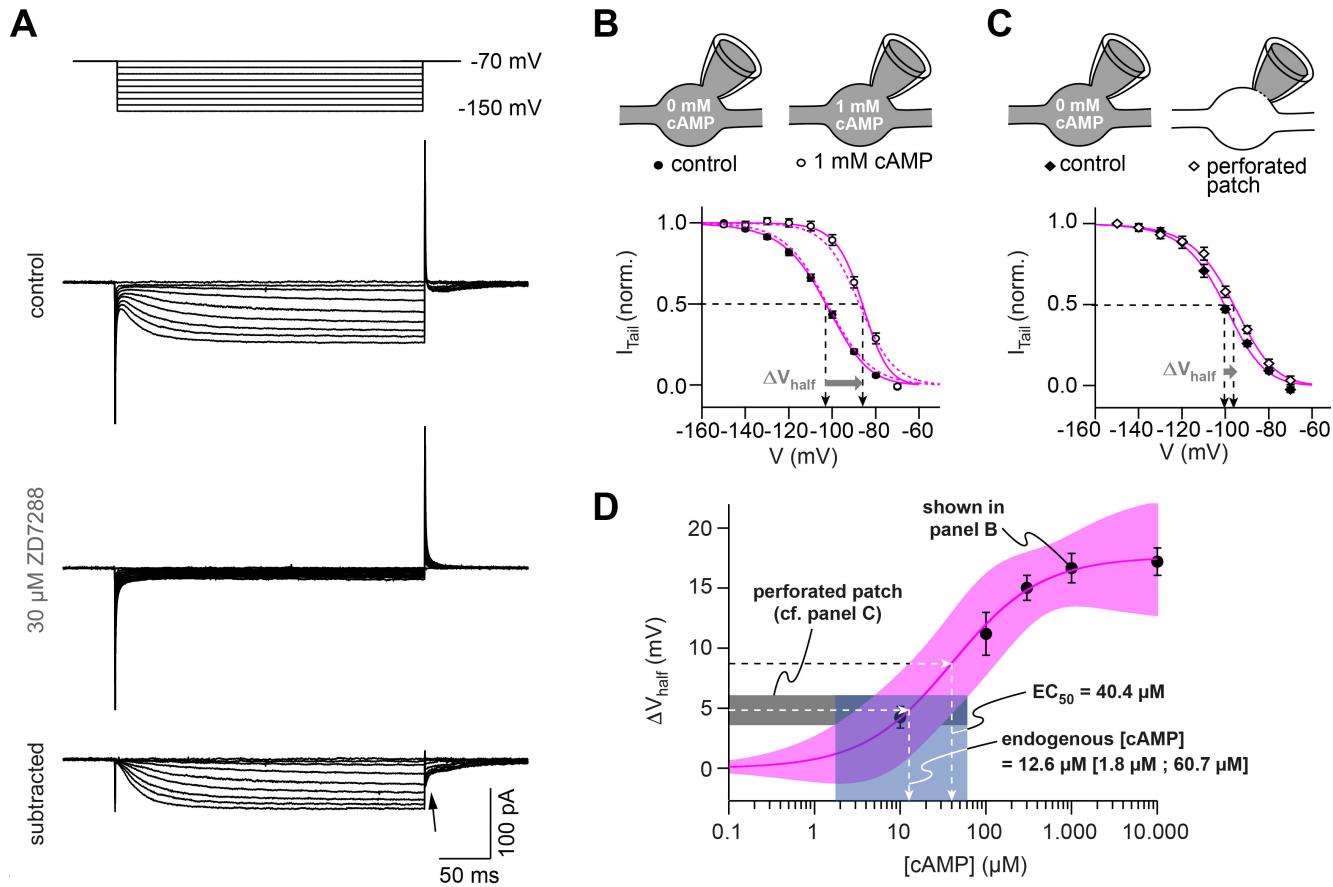
892

893 **Figure 7. HCN2 is uniformly distributed in mossy fiber axons and boutons**

894 (A) Electron microscopic image showing a cMFB (magenta) labeled for HCN2. Many
895 particles are diffusely distributed along the plasma membrane of the cMFB, some of
896 them being clustered. Arrows mark synapses between the cMFB and dendrites of
897 adjacent GCs.

898 (B) Another cMFB, showing similar labeling density for HCN2 in a proximal part of the
899 mossy fiber axon.

900 (C) Reconstructed cMFB (red) with identified synapses (blue) and HCN2 labeled with
901 gold particles (yellow).

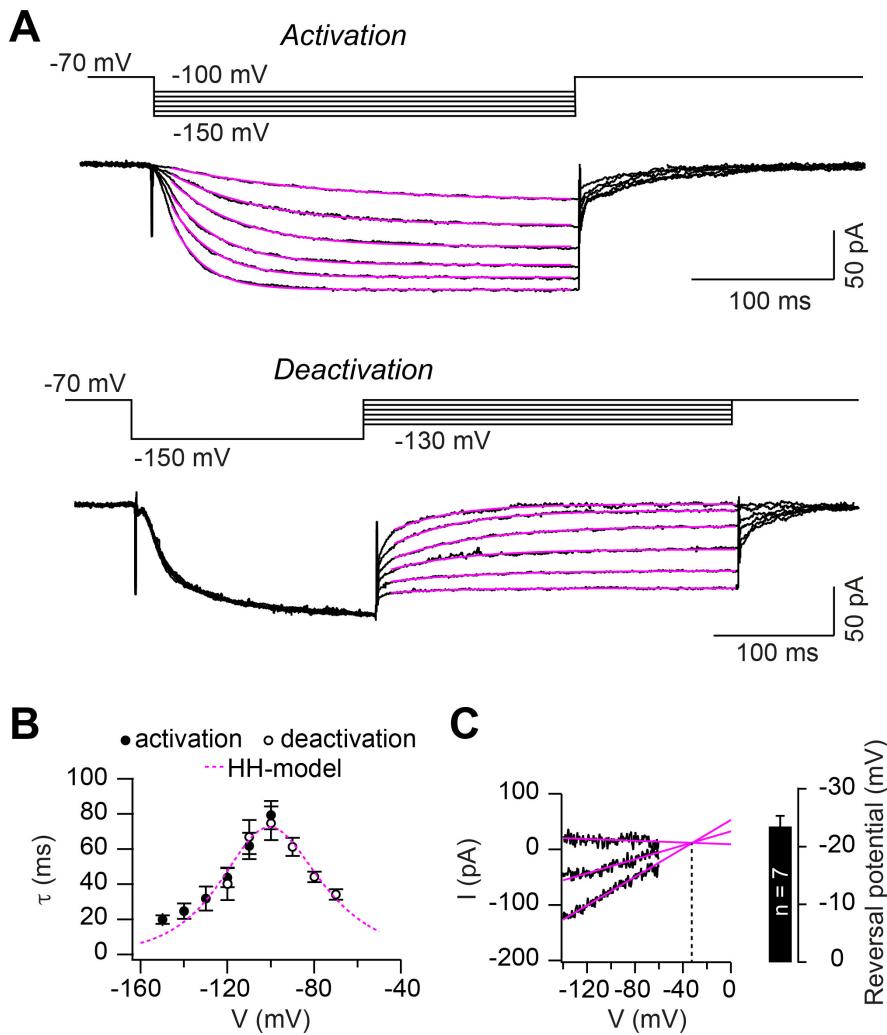


903 **Figure 8. HCN channels in cMFB are strongly modulated by cAMP**

904 (A) Example currents elicited hyperpolarizing voltage steps (-70 mV and then stepped
905 to conditioning pulses between -80 mV and -150 mV). *Top*, the control current,
906 *middle*, remaining transients in the presence of $30 \mu\text{M}$ ZD7288 and, *bottom*, the
907 subtracted currents. The ZD7288-sensitive current is slowly activating, non-
908 inactivating and shows inward tail currents (arrow).
909 (B) Activation curve of I_h determined as the normalized tail current of ZD7288
910 sensitive currents obtained after the end of the conditioning voltage pulse (arrow in
911 *A*) plotted versus corresponding voltage pulse with 0 mM cAMP (filled circles, $n =$
912 36) and 1 mM cAMP (open circles, $n = 15$) in the intracellular solution. Sigmoidal
913 fits (continuous magenta lines), yielding midpoints of I_h activation ($V_{\text{½}}$, arrows). The
914 steady-state activation curves produced by the Hodgkin-Huxley models (dotted
915 magenta line) are superimposed. *Inset on top*: Illustration of the whole-cell
916 recording configuration with 0 and 1 mM cAMP in the intracellular solution.

917 (C) Activation curve obtained with perforated-patch recordings show a shift in the I_h
918 activation curve by 4.8 ± 1.2 mV compared to recordings from the same cell after
919 rupture of the perforated membrane patch ($n = 10$). *Inset on top*: Illustration of the
920 whole-cell recording configuration with 0 mM cAMP in the intracellular solution
921 and in the perforated patch configuration, where the intracellular cAMP
922 concentration is unperturbed.

923 (D) Shift in I_h $V_{1/2}$ versus the corresponding cAMP concentration (mean \pm SEM). Fitting
924 the data with a Hill equation revealed an EC_{50} of 40.4 μ M. Superposition of the
925 68% confidence band of the fit (straight line and light magenta area) with the
926 average voltage shift observed in perforated patch recordings (4.8 ± 1.2 mV, $n =$
927 10, dotted black line and grey area) results in an estimated cAMP-concentration in
928 the non-perturbed presynaptic boutons of 12.6 μ M with a range of 1.8 to 60.7 μ M
929 cAMP (dotted line and light blue area).



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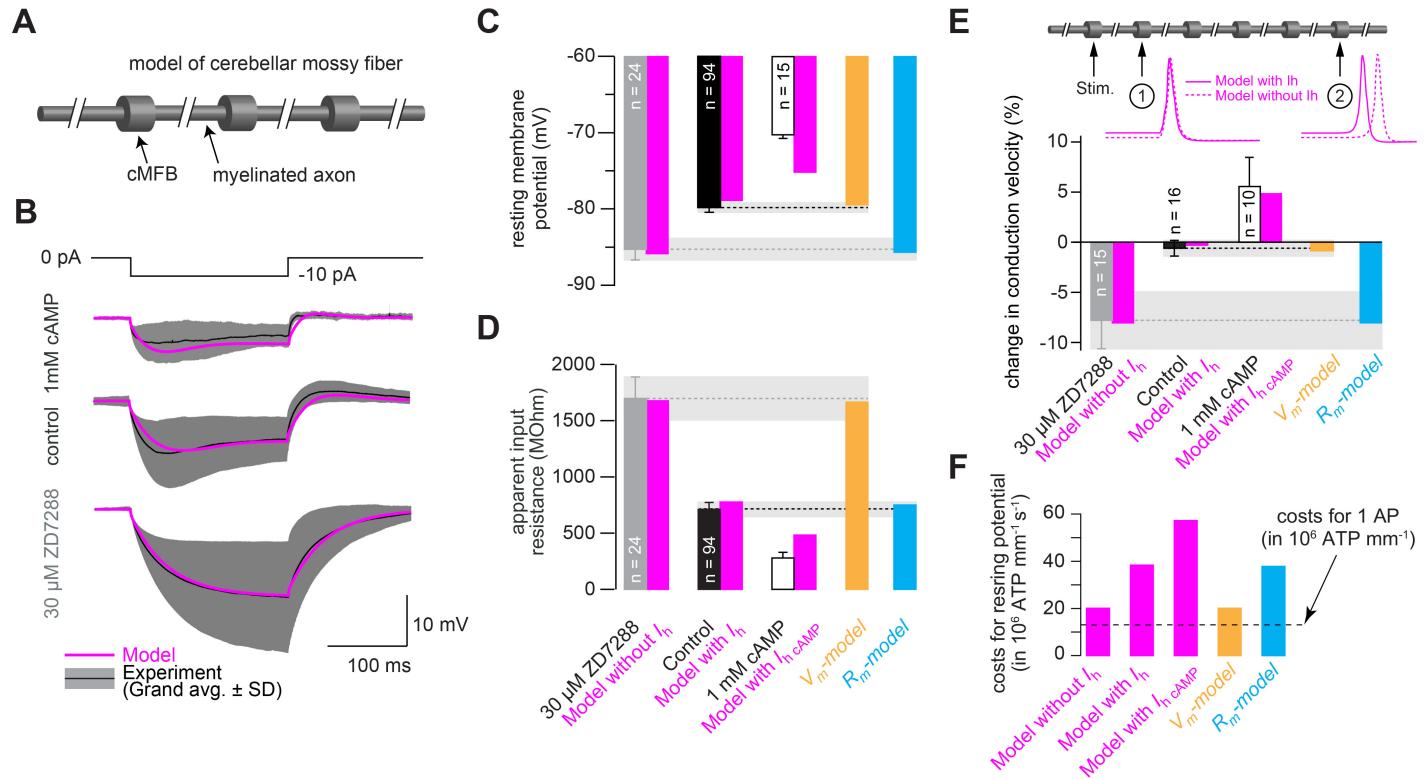
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932 **Figure 9. Hodgkin-Huxley model describing HCN2 channel gating**

933 (A) Example of ZD7288 sensitive currents (black) elicited by the illustrated activation
934 (top) and deactivation voltage protocols (bottom) superimposed with a mono-
935 exponential fits (magenta).

936 (B) Average time constants of activation (filled circles) and deactivation (open circles;
937 mean \pm SEM). The dotted blue line represents the prediction of I_h activation and
938 deactivation time constant based on the Hodgkin-Huxley model.

939 (C) Example of linear extrapolation (magenta lines) of leak subtracted currents evoked
940 by fast (10 ms) voltage ramps generated from a range of holding potentials that
941 extended across the activation range of I_h . The reversal potential was found to be
942 -36 mV in this example. *Inset:* average reversal potential of 7 independent
943 experiments.



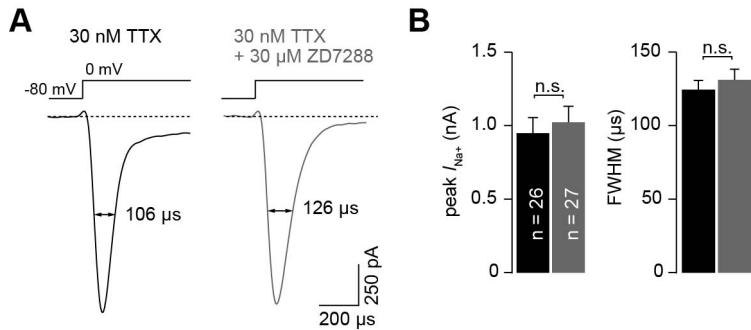
945 **Figure 10. Mechanism of conduction velocity-control and metabolic costs of HCN
946 channels**

947 (A) Illustration of cerebellar mossy fiber model consisting of 15 connected cylindrical
948 compartments representing cMFBS and the myelinated axon.
949 (B) Grand average voltage response (black) and standard deviation (grey area) of
950 cMFBS in response to a -10 pA hyperpolarizing current pulse with 1mM cAMP
951 included in the patch pipette (top), under control conditions (middle) or treated with
952 ZD7288 (bottom), superimposed with the predicted voltage response from the
953 model (magenta).
954 (C) Average resting membrane potential of cMFBS measured under control conditions
955 (black), with I_h blocked by ZD7288 (gray), or enhanced by 1 mM intracellular
956 cAMP (open bar; data from Fig. 6B) and compared to the predictions from the
957 corresponding models (magenta). Furthermore, the resting membrane potential of
958 two models is shown that simulate only the membrane depolarization (V_m -model;
959 light brown) or only the decreased membrane resistance (R_m -model; blue) caused by
960 open HCN channels.

961 (D) Same comparison between measured values and predictions from the models for the
962 apparent input resistance of cMFBs.

963 (E) Measured changes of conduction velocity in mossy fibers compared to the
964 predictions by the different models (as in C and D). *Inset top*: Illustration of the
965 model of a mossy fiber with the stimulation positions and the action potentials at
966 two different positions with (magenta line) and without (dashed magenta line) the
967 HH model of HCN channels.

968 (F) The calculated metabolic costs for maintaining the resting membrane potential are
969 shown for each model as the number of required ATP molecules per mm of mossy
970 fiber axon and per s. The metabolic cost for the firing of a single action potential
971 (AP) is indicated by the dashed line as the number of required ATP molecules per
972 mm of mossy fiber axon (this number was very similar for all models).



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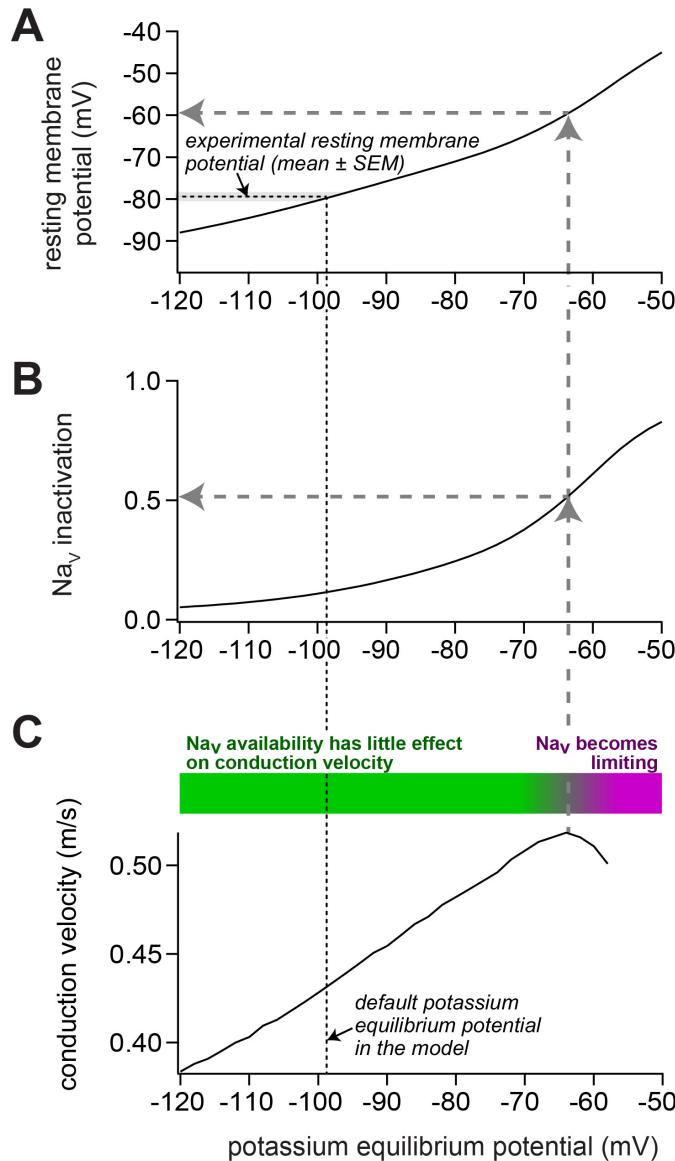
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975 **Supplemental Figure S1. ZD7288 does not alter Na^+ currents in cMFBs**

976 (A) Example whole-cell Na^+ currents measured under control conditions (30 nM TTX)
977 and in the presence of additional 30 μM ZD7288 elicited by voltage steps from -80
978 mV to 0 mV.

979 (B) With 30 nM TTX present in the extracellular solution (see methods), the average
980 Na^+ current amplitude was 947.9 ± 107.5 pA ($n = 26$). In the presence of 30 nM
981 TTX and 30 μM ZD7288, the amplitude was not different compared with the
982 currents recorded in 30 nM TTX only (1022.5 ± 109.0 pA; $n = 27$; $P_{\text{T-Test}} = 0.63$).
983 The average half-duration was 124.3 ± 6.2 μs and 131.5 ± 7.2 μs ($P_{\text{T-Test}} = 0.45$) for
984 control recordings and those performed in the presence of 30 μM ZD7288,
985 respectively.

986



987

988 **Supplemental Figure S2. Impact of depolarization on Na_V availability and on**
989 **conduction velocity in our model of a mossy fiber axon**

990 (A) To change the resting membrane potential, the potassium equilibrium potential was
991 varied between -120 and -50 mV. In the default model, the experimentally
992 observed resting membrane potential (indicated by horizontal black dashed line and
993 grey bar; mean \pm SEM) was obtained with a potassium equilibrium potential of -98
994 mV (vertical black dashed line).

995 (B) Upon depolarization, the inactivation of voltage-dependent sodium (Na_V) channels
996 increased. For the control model, the Na_V inactivation was 12%. For the model
997 reproducing the experiments with ZD7288 and 1 mM intracellular cAMP, the Na_V
998 inactivation was 6 and 17%, respectively. Because the steady-state Na_V availability

999 depends mostly on the resting membrane potential, the relation between resting
1000 membrane potential and Na_v availability was identical for all three models (data
1001 not shown).

1002 (C) Upon depolarization, the conduction velocity increased up to a potassium
1003 equilibrium potential of about -65 mV (vertical grey dashed arrow), corresponding
1004 to an Na_v availability of about 50% (horizontal grey dashed arrow in panel B) and
1005 a resting membrane potential of about -60 mV (horizontal grey dashed arrow in
1006 panel A). Thus, despite increasing Na_v inactivation, the conduction velocity
1007 increased in this range (illustrated by green bar). With stronger depolarization, the
1008 conduction velocity declined, indicating that the Na_v availability becomes limiting
1009 for conduction velocity (magenta bar).

1010

1011 **Supplemental video 1 (video1.mp4). Reconstructed cMFB with labelled synapses**
1012 **and HCN2 channels**

1013 3D rendering of a part of a reconstructed cMFB (red) with identified synapses (blue)
1014 and HCN2 labeled with gold particles (yellow) based on immune-gold electron
1015 microscopic images.

1016

1017 **References**

1018

1019 Aponte, Y., Lien, C.C., Reisinger, E., and Jonas, P. (2006). Hyperpolarization-activated
1020 cation channels in fast-spiking interneurons of rat hippocampus. *J Physiol* 574, 229-
1021 243.

1022 Bacskaï, B.J., Hochner, B., Mahaut-Smith, M., Adams, S.R., Kaang, B.K., Kandel,
1023 E.R., and Tsien, R.Y. (1993). Spatially resolved dynamics of cAMP and protein
1024 kinase A subunits in Aplysia sensory neurons. *Science* 260, 222-226.

1025 Baginskas, A., Palani, D., Chiu, K., and Raastad, M. (2009). The H-current secures
1026 action potential transmission at high frequencies in rat cerebellar parallel fibers. *Eur J
1027 Neurosci* 29, 87-96.

1028 Baker, M., Bostock, H., Grafe, P., and Martius, P. (1987). Function and distribution of
1029 three types of rectifying channel in rat spinal root myelinated axons. *J Physiol* 383.

1030 Baker, M.R., and Edgley, S.A. (2006). Non-uniform olivocerebellar conduction time in
1031 the vermis of the rat cerebellum. *J Physiol* 570, 501-506.

1032 Ballo, A.W., Keene, J.C., Troy, P.J., Goeritz, M.L., Nadim, F., and Bucher, D. (2010).
1033 Dopamine modulates Ih in a motor axon. *J Neurosci* 30, 8425-8434.

1034 Ballo, A.W., Nadim, F., and Bucher, D. (2012). Dopamine modulation of Ih improves
1035 temporal fidelity of spike propagation in an unmyelinated axon. *J Neurosci* 32, 5106-
1036 5119.

1037 Basheer, R., Strecker, R.E., Thakkar, M.M., and McCarley, R.W. (2004). Adenosine
1038 and sleep-wake regulation. *Prog Neurobiol* 73, 379-396.

1039 Battefeld, A., Tran, B.T., Gavrilis, J., Cooper, E.C., and Kole, M.H. (2014).
1040 Heteromeric K_v7.2/7.3 channels differentially regulate action potential initiation and
1041 conduction in neocortical myelinated axons. *J Neurosci* 34, 3719-3732.

1042 Beaumont, V., and Zucker, R.S. (2000). Enhancement of synaptic transmission by
1043 cyclic AMP modulation of presynaptic Ih channels. *Nat Neurosci* 3, 133-141.

1044 Bender, K.J., Ford, C.P., and Trussell, L.O. (2010). Dopaminergic modulation of axon
1045 initial segment calcium channels regulates action potential initiation. *Neuron* 68,
1046 500-511.

1047 Biel, M., Wahl-Schott, C., Michalakis, S., and Zong, X. (2009). Hyperpolarization-
1048 activated cation channels: from genes to function. *Physiol Rev* 89, 847-885.

1049 Birch, B.D., Kocsis, J.D., Di Gregorio, F., Bhisitkul, R.B., and Waxman, S.G. (1991). A
1050 voltage- and time-dependent rectification in rat dorsal spinal root axons. *J
1051 Neurophysiol* 66, 719-728.

1052 Börner, S., Schwede, F., Schlipp, A., Berisha, F., Calebiro, D., Lohse, M.J., and
1053 Nikolaev, V.O. (2011). FRET measurements of intracellular cAMP concentrations
1054 and cAMP analog permeability in intact cells. *Nature protocols* 6, 427-438.

1055 Boyle, P.J., Scott, J.C., Krentz, A.J., Nagy, R.J., Comstock, E., and Hoffman, C. (1994).
1056 Diminished brain glucose metabolism is a significant determinant for falling rates of
1057 systemic glucose utilization during sleep in normal humans. *J Clin Invest* 93, 529-
1058 535.

1059 Braitenberg, V., Heck, D., and Sultan, F. (1997). The detection and generation of
1060 sequences as a key to cerebellar function: Experiments and theory. *Behav Brain Sci*
1061 20, 229-&.

1062 Bruce, J.I.E., Straub, S.V., and Yule, D.I. (2003). Crosstalk between cAMP and Ca²⁺
1063 signaling in non-excitable cells. *Cell Calcium* 34, 431-444.

1064 Burke, K.J., Jr., Keeshen, C.M., and Bender, K.J. (2018). Two forms of synaptic
1065 depression produced by differential neuromodulation of presynaptic calcium
1066 channels. *Neuron* 99, 969-984.

1067 Buzsáki, G., Logothetis, N., and Singer, W. (2013). Scaling brain size, keeping timing:
1068 evolutionary preservation of brain rhythms. *Neuron* 80, 751-764.

1069 Canto, C.B., Onuki, Y., Bruinsma, B., van der Werf, Y.D., and De Zeeuw, C.I. (2017).
1070 The Sleeping Cerebellum. *Trends Neurosci* 40, 309-323.

1071 Caporale, N., and Dan, Y. (2008). Spike timing-dependent plasticity: a Hebbian
1072 learning rule. *Annu Rev Neurosci* 31, 25-46.

1073 Cardona, A., Saalfeld, S., Schindelin, J., Arganda-Carreras, I., Preibisch, S., Longair,
1074 M., Tomancak, P., Hartenstein, V., and Douglas, R.J. (2012). TrakEM2 software for
1075 neural circuit reconstruction. *PLoS One* 7, e38011.

1076 Carp, J.S., Tennissen, A.M., and Wolpaw, J.R. (2003). Conduction velocity is inversely
1077 related to action potential threshold in rat motoneuron axons. *Exp Brain Res* 150,
1078 497-505.

1079 Chadderton, P., Margrie, T.W., and Häusser, M. (2004). Integration of quanta in
1080 cerebellar granule cells during sensory processing. *Nature* 428, 856-860.

1081 Chaplan, S.R., Guo, H.-Q., Lee, D.H., Luo, L., Liu, C., Kuei, C., Velumian, A.A.,
1082 Butler, M.P., Brown, S.M., and Dubin, A.E. (2003). Neuronal hyperpolarization-
1083 activated pacemaker channels drive neuropathic pain. *J Neurosci* 23, 1169-1178.

1084 Chéreau, R., Saraceno, G.E., Angibaud, J., Cattaert, D., and Nägerl, U.V. (2017).
1085 Superresolution imaging reveals activity-dependent plasticity of axon morphology
1086 linked to changes in action potential conduction velocity. *Proc Natl Acad Sci USA*
1087 114, 1401-1406.

1088 Cheung, U., Atwood, H.L., and Zucker, R.S. (2006). Presynaptic effectors contributing
1089 to cAMP-induced synaptic potentiation in *Drosophila*. *J Neurobiol* 66, 273-280.

1090 Chevaleyre, V., and Castillo, P.E. (2002). Assessing the role of I_h channels in synaptic
1091 transmission and mossy fiber LTP. *Proc Natl Acad Sci U S A* 99, 9538-9543.

1092 Cuttle, M.F., Rusznák, Z., Wong, A.Y.C., Owens, S., and Forsythe, I.D. (2001).
1093 Modulation of a presynaptic hyperpolarization-activated cationic current (I_h) at an
1094 excitatory synaptic terminal in the rat auditory brainstem. *The Journal of Physiology*
1095 534, 733-744.

1096 Debanne, D., Campanac, E., Bialowas, A., Carlier, E., and Alcaraz, G. (2011). Axon
1097 physiology. *Physiol Rev* 91, 555-602.

1098 Delvendahl, I., and Hallermann, S. (2016). The cerebellar mossy fiber synapse as a
1099 model for high-frequency transmission in the mammalian CNS. *Trends Neurosci* 39,
1100 722-737.

1101 Delvendahl, I., Jablonski, L., Baade, C., Matveev, V., Neher, E., and Hallermann, S.
1102 (2015). Reduced endogenous Ca^{2+} buffering speeds active zone Ca^{2+} signaling. *Proc
1103 Natl Acad Sci USA* 112, E3075-3084.

1104 DiFrancesco, D. (2006). Funny channels in the control of cardiac rhythm and mode of
1105 action of selective blockers. *Pharmacol Res* 53, 399-406.

1106 Elgueta, C., Köhler, J., and Bartos, M. (2015). Persistent discharges in dentate gyrus
1107 perisoma-inhibiting interneurons require hyperpolarization-activated cyclic
1108 nucleotide-gated channel activation. *J Neurosci* 35, 4131-4139.

1109 Engel, D., and Jonas, P. (2005). Presynaptic action potential amplification by voltage-
1110 gated Na^+ channels in hippocampal mossy fiber boutons. *Neuron* 45, 405-417.

1111 Fledrich, R., Stassart, R.M., Klink, A., Rasch, L.M., Prukop, T., Haag, L., Czesnik, D.,
1112 Kungl, T., Abdelaal, T.A., Keric, N., *et al.* (2014). Soluble neuregulin-1 modulates
1113 disease pathogenesis in rodent models of Charcot-Marie-Tooth disease 1A. *Nat Med*
1114 20, 1055-1061.

1115 Forbes, T.A., and Gallo, V. (2017). All Wrapped Up: Environmental Effects on
1116 Myelination. *Trends Neurosci* 40, 572-587.

1117 Ford, M.C., Alexandrova, O., Cossell, L., Stange-Marten, A., Sinclair, J., Kopp-
1118 Scheinpflug, C., Pecka, M., Attwell, D., and Grothe, B. (2015). Tuning of Ranvier
1119 node and internode properties in myelinated axons to adjust action potential timing.
1120 *Nat Commun* 6, 8073.

1121 Gentet, L.J., Stuart, G.J., and Clements, J.D. (2000). Direct measurement of specific
1122 membrane capacitance in neurons. *Biophys J* 79, 314-320.

1123 George, M.S., Abbott, L.F., and Siegelbaum, S.A. (2009). HCN hyperpolarization-
1124 activated cation channels inhibit EPSPs by interactions with M-type K⁺ channels.
1125 *Nat Neurosci* 12, 577-584.

1126 Geurts, F.J., Schutter, E., and Timmermans, J.-P. (2002). Localization of 5-HT2A, 5-
1127 HT3, 5-HT5A and 5-HT7 receptor-like immunoreactivity in the rat cerebellum. *J*
1128 *Chem Neuroanat* 24, 65-74.

1129 Golding, N.L., and Oertel, D. (2012). Synaptic integration in dendrites: exceptional
1130 need for speed. *The Journal of Physiology* 590, 5563-5569.

1131 Grafe, P., Quasthoff, S., Grosskreutz, J., and Alzheimer, C. (1997). Function of the
1132 hyperpolarization-activated inward rectification in nonmyelinated peripheral rat and
1133 human axons. *J Neurophysiol* 77, 421-426.

1134 Greenberg, S.M., Bernier, L., and Schwartz, J.H. (1987). Distribution of cAMP and
1135 cAMP-dependent protein kinases in Aplysia sensory neurons. *J Neurosci* 7, 291-301.

1136 Hallermann, S., de Kock, C.P., Stuart, G.J., and Kole, M.H. (2012). State and location
1137 dependence of action potential metabolic cost in cortical pyramidal neurons. *Nat*
1138 *Neurosci* 15, 1007-1014.

1139 Hallermann, S., Pawlu, C., Jonas, P., and Heckmann, M. (2003). A large pool of
1140 releasable vesicles in a cortical glutamatergic synapse. *Proc Natl Acad Sci U S A*
1141 100, 8975-8980.

1142 Harnett, M.T., Magee, J.C., and Williams, S.R. (2015). Distribution and function of
1143 HCN channels in the apical dendritic tuft of neocortical pyramidal neurons. *J*
1144 *Neurosci* 35, 1024-1037.

1145 Heck, D., Sultan, F., and Braitenberg, V. (2001). Sequential stimulation of rat cerebellar
1146 granular layer *in vivo*: Further evidence of a 'tidal-wave' timing mechanism in the
1147 cerebellum. *Neurocomputing* 38, 641-646.

1148 Hodgkin, A.L., and Huxley, A.F. (1952). A quantitative description of membrane
1149 current and its application to conduction and excitation in nerve. *J Physiol* 117, 500-
1150 544.

1151 Hore, J., Wild, B., and Diener, H.C. (1991). Cerebellar dysmetria at the elbow, wrist,
1152 and fingers. *J Neurophysiol* 65, 563-571.

1153 Horn, S., Quasthoff, S., Grafe, P., Bostock, H., Renner, R., and Schrank, B. (1996).
1154 Abnormal axonal inward rectification in diabetic neuropathy. *Muscle Nerve* 19,
1155 1268-1275.

1156 Howells, J., Bostock, H., and Burke, D. (2016). Accommodation to hyperpolarization of
1157 human axons assessed in the frequency domain. *J Neurophysiol* 116, 322-335.

1158 Howells, J., Trevillion, L., Bostock, H., and Burke, D. (2012). The voltage dependence
1159 of I_h in human myelinated axons. *J Physiol* 590, 1625-1640.

1160 Huang, H., and Trussell, L.O. (2014). Presynaptic HCN channels regulate vesicular
1161 glutamate transport. *Neuron* 84, 340-346.

1162 Huang, Z., Lujan, R., Kadurin, I., Uebele, V.N., Renger, J.J., Dolphin, A.C., and Shah,
1163 M.M. (2011). Presynaptic HCN1 channels regulate $Ca_{v}3.2$ activity and
1164 neurotransmission at select cortical synapses. *Nat Neurosci* 14, 478-486.

1165 Hummert, S., Thon, S., Eick, T., Schmauder, R., Schulz, E., and Benndorf, K. (2018).
1166 Activation gating in HCN2 channels. *PLoS Comput Biol* 14, e1006045.

1167 Isope, P., Dieudonne, S., and Barbour, B. (2002). Temporal organization of activity in
1168 the cerebellar cortex: a manifesto for synchrony. *Ann N Y Acad Sci* 978, 164-174.

1169 Jack, J.J.B., Noble, D., and Tsien, R.W. (1983). Electric current flow in excitable cells.
1170 (Oxford: Clarendon Press).

1171 Jankelowitz, S.K., Howells, J., and Burke, D. (2007). Plasticity of inwardly rectifying
1172 conductances following a corticospinal lesion in human subjects. *J Physiol* 581, 927-
1173 940.

1174 Kim, J.H., and von Gersdorff, H. (2012). Suppression of spikes during posttetanic
1175 hyperpolarization in auditory neurons: the role of temperature, I_h currents, and the
1176 Na^{+} - K^{+} -ATPase pump. *J Neurophysiol* 108, 1924-1932.

1177 Ko, K.W., Rasband, M.N., Meseguer, V., Kramer, R.H., and Golding, N.L. (2016).
1178 Serotonin modulates spike probability in the axon initial segment through HCN
1179 channels. *Nat Neurosci* 19, 826-834.

1180 Kole, M.H., Hallermann, S., and Stuart, G.J. (2006). Single I_h channels in pyramidal
1181 neuron dendrites: properties, distribution, and impact on action potential output. *J
1182 Neurosci* 26, 1677-1687.

1183 Koschinski, A., and Zacco, M. (2017). Activation of PKA in cell requires higher
1184 concentration of cAMP than in vitro: implications for compartmentalization of
1185 cAMP signalling. *Scientific reports* 7, 14090.

1186 Lang, E.J., and Rosenbluth, J. (2003). Role of myelination in the development of a
1187 uniform olivocerebellar conduction time. *J Neurophysiol* 89, 2259-2270.

1188 Lin, C.S., Krishnan, A.V., Lee, M.J., Zagami, A.S., You, H.L., Yang, C.C., Bostock, H.,
1189 and Kiernan, M.C. (2008). Nerve function and dysfunction in acute intermittent
1190 porphyria. *Brain* 131, 2510-2519.

1191 Lorenz, C., and Jones, K.E. (2014). I_h activity is increased in populations of slow versus
1192 fast motor axons of the rat. *Front Hum Neurosci* 8, 766.

1193 Magee, J.C. (1999). Dendritic I_h normalizes temporal summation in hippocampal CA1
1194 neurons. *Nat Neurosci* 2, 508-514.

1195 Maiellaro, I., Lohse, M.J., Kittel, R.J., and Calebiro, D. (2016). cAMP signals in
1196 Drosophila motor neurons are confined to single synaptic boutons. *Cell Reports* 17,
1197 1238-1246.

1198 Marder, E., and Bucher, D. (2001). Central pattern generators and the control of
1199 rhythmic movements. *Curr Biol* 11, R986-996.

1200 Mathews, P.J., Jercog, P.E., Rinzel, J., Scott, L.L., and Golding, N.L. (2010). Control of
1201 submillisecond synaptic timing in binaural coincidence detectors by K_v1 channels.
1202 *Nat Neurosci* 13, 601-609.

1203 Matsubara, A., Laake, J.H., Davanger, S., Usami, S.-i., and Ottersen, O.P. (1996).
1204 Organization of AMPA receptor subunits at a glutamate synapse: a quantitative

1205 immunogold analysis of hair cell synapses in the rat organ of Corti. *J Neurosci* 16,
1206 4457-4467.

1207 McKenzie, I.A., Ohayon, D., Li, H.L., de Faria, J.P., Emery, B., Tohyama, K., and
1208 Richardson, W.D. (2014). Motor skill learning requires active central myelination.
1209 *Science* 346, 318-322.

1210 Momin, A., and McNaughton, P.A. (2009). Regulation of firing frequency in
1211 nociceptive neurons by pro-inflammatory mediators. *Exp Brain Res* 196, 45-52.

1212 Nicholas, A.P., Pieribone, V.A., and Hökfelt, T. (1993). Cellular localization of
1213 messenger RNA for beta-1 and beta-2 adrenergic receptors in rat brain: an in situ
1214 hybridization study. *Neuroscience* 56, 1023-1039.

1215 Notomi, T., and Shigemoto, R. (2004). Immunohistochemical localization of I_h channel
1216 subunits, HCN1-4, in the rat brain. *J Comp Neurol* 471, 241-276.

1217 Nusser, Z. (2012). Differential subcellular distribution of ion channels and the diversity
1218 of neuronal function. *Curr Opin Neurobiol* 22, 366-371.

1219 Palay, S.M., and Chan-Palay, V. (1974). *Cerebellar cortex: cytology and organization*
1220 (Springer, Berlin).

1221 Pape, H.C., and McCormick, D.A. (1989). Noradrenaline and serotonin selectively
1222 modulate thalamic burst firing by enhancing a hyperpolarization-activated cation
1223 current. *Nature* 340, 715-718.

1224 Person, A.L., and Raman, I.M. (2012). Synchrony and neural coding in cerebellar
1225 circuits. *Front Neural Circuits* 6, 97.

1226 Porkka-Heiskanen, T., Strecker, R.E., Thakkar, M., Bjorkum, A.A., Greene, R.W., and
1227 McCarley, R.W. (1997). Adenosine: a mediator of the sleep-inducing effects of
1228 prolonged wakefulness. *Science* 276, 1265-1268.

1229 Press, W.H., Teukolsky, S.A., Vetterling, W.T., and Flannery, B.P. (2002). *Numerical
1230 recipes in C++: the art of scientific computing*, 2nd edn (Cambridge, UK: Cambridge
1231 UP).

1232 Rama, S., Zbili, M., Bialowas, A., Fronzaroli-Molinieres, L., Ankri, N., Carlier, E.,
1233 Marra, V., and Debanne, D. (2015). Presynaptic hyperpolarization induces a fast
1234 analogue modulation of spike-evoked transmission mediated by axonal sodium
1235 channels. *Nat Commun* 6, 10163.

1236 Rancz, E.A., Ishikawa, T., Duguid, I., Chadderton, P., Mahon, S., and Häusser, M.
1237 (2007). High-fidelity transmission of sensory information by single cerebellar mossy
1238 fibre boutons. *Nature* 450, 1245-1248.

1239 Ritzau-Jost, A., Delvendahl, I., Rings, A., Byczkowicz, N., Harada, H., Shigemoto, R.,
1240 Hirrlinger, J., Eilers, J., and Hallermann, S. (2014). Ultrafast action potentials
1241 mediate kilohertz signaling at a central synapse. *Neuron* 84, 152-163.

1242 Robinson, R.B., and Siegelbaum, S.A. (2003). Hyperpolarization-activated cation
1243 currents: from molecules to physiological function. *Annu Rev Physiol* 65, 453-480.

1244 Salami, M., Itami, C., Tsumoto, T., and Kimura, F. (2003). Change of conduction
1245 velocity by regional myelination yields constant latency irrespective of distance
1246 between thalamus and cortex. *Proc Natl Acad Sci U S A* 100, 6174-6179.

1247 Sánchez-Soto, M., Bonifazi, A., Cai, N.S., Ellenberger, M.P., Newman, A.H., Ferré, S.,
1248 and Yano, H. (2016). Evidence for noncanonical neurotransmitter activation:
1249 norepinephrine as a dopamine D2-Like receptor agonist. *Mol Pharmacol* 89, 457-
1250 466.

1251 Santoro, B., Chen, S., Luthi, A., Pavlidis, P., Shumyatsky, G.P., Tibbs, G.R., and
1252 Siegelbaum, S.A. (2000). Molecular and functional heterogeneity of

1253 hyperpolarization-activated pacemaker channels in the mouse CNS. *J Neurosci* 20,
1254 5264-5275.

1255 Schmidt-Hieber, C., and Bischofberger, J. (2010). Fast Sodium Channel Gating
1256 Supports Localized and Efficient Axonal Action Potential Initiation. *J Neurosci* 30,
1257 10233-10242.

1258 Schweighofer, N., Doya, K., and Kuroda, S. (2004). Cerebellar aminergic
1259 neuromodulation: towards a functional understanding. *Brain Res Brain Res Rev* 44,
1260 103-116.

1261 Softky, W. (1994). Sub-millisecond coincidence detection in active dendritic trees.
1262 *Neuroscience* 58, 13-41.

1263 Soleng, A.F., Chiu, K., and Raastad, M. (2003). Unmyelinated axons in the rat
1264 hippocampus hyperpolarize and activate an H current when spike frequency exceeds
1265 1 Hz. *J Physiol* 552, 459-470.

1266 Sugihara, I., Lang, E.J., and Llinás, R. (1993). Uniform olivocerebellar conduction time
1267 underlies Purkinje cell complex spike synchronicity in the rat cerebellum. *The
1268 Journal of Physiology* 470, 243-271.

1269 Swadlow, H.A., and Waxman, S.G. (2012). Axonal conduction delays. *Scholarpedia* 7,
1270 1451 doi:1410.4249/scholarpedia.1451.

1271 Tang, Z.Q., and Trussell, L.O. (2015). Serotonergic regulation of excitability of
1272 principal cells of the dorsal cochlear nucleus. *J Neurosci* 35, 4540-4551.

1273 Thon, S., Schmauder, R., and Benndorf, K. (2013). Elementary functional properties of
1274 single HCN2 channels. *Biophys J* 105, 1581-1589.

1275 Townsend, R.E., Prinz, P.N., and Obrist, W.D. (1973). Human cerebral blood flow
1276 during sleep and waking. *J Appl Physiol* 35, 620-625.

1277 Trevisiol, A., Saab, A.S., Winkler, U., Marx, G., Imamura, H., Möbius, W., Kusch, K.,
1278 Nave, K.A., and Hirrlinger, J. (2017). Monitoring ATP dynamics in electrically
1279 active white matter tracts. *eLife* 6.

1280 Wang, J., Chen, S., and Siegelbaum, S.A. (2001). Regulation of hyperpolarization-
1281 activated HCN channel gating and cAMP modulation due to interactions of COOH
1282 terminus and core transmembrane regions. *The Journal of General Physiology* 118,
1283 237-250.

1284 Waxman, S.G., Kocsis, J.D., and Stys, P.K. (1995). *The Axon: Structure, Function, and
1285 Pathophysiology* (Oxford University Press).

1286 Waxman, S.G., and Ritchie, J.M. (1993). Molecular dissection of the myelinated axon.
1287 *Ann Neurol* 33, 121-136.

1288 Wickenden, A.D., Maher, M.P., and Chaplan, S.R. (2009). HCN pacemaker channels
1289 and pain: a drug discovery perspective. *Curr Pharm Des* 15, 2149-2168.

1290 Williams, S.R., and Stuart, G.J. (2000). Site independence of EPSP time course is
1291 mediated by dendritic I_h in neocortical pyramidal neurons. *J Neurophysiol* 83, 3177-
1292 3182.

1293 Wu, X., Liao, L., Liu, X., Luo, F., Yang, T., and Li, C. (2012). Is ZD7288 a selective
1294 blocker of hyperpolarization-activated cyclic nucleotide-gated channel currents?
1295 *Channels (Austin)* 6, 438-442.

1296 Yang, J., Ye, M., Tian, C., Yang, M., Wang, Y., and Shu, Y. (2013). Dopaminergic
1297 modulation of axonal potassium channels and action potential waveform in
1298 pyramidal neurons of prefrontal cortex. *J Physiol* 591, 3233-3251.

1299 Yin, L., Rasch, M.J., He, Q., Wu, S., Dou, F., and Shu, Y. (2017). Selective modulation
1300 of axonal sodium channel subtypes by 5-HT1A receptor in cortical pyramidal
1301 neuron. *Cereb Cortex* 27, 509-521.

1302 Zagotta, W.N., Olivier, N.B., Black, K.D., Young, E.C., Olson, R., and Gouaux, E.
1303 (2003). Structural basis for modulation and agonist specificity of HCN pacemaker
1304 channels. *Nature* 425, 200-205.

1305