

Decreases in the precision of Purkinje cell pacemaking cause cerebellar dysfunction and ataxia

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Episodic ataxia type-2 (EA2) is caused by mutations in P/Q-type voltage-gated calcium channels that are expressed at high densities in cerebellar Purkinje cells. Because P/Q channels support neurotransmitter release at many synapses, it is believed that ataxia is caused by impaired synaptic transmission. Here we show that in ataxic P/Q channel mutant mice, the precision of Purkinje cell pacemaking is lost such that there is a significant degradation of the synaptic information encoded in their activity. The irregular pacemaking is caused by reduced activation of calcium-activated potassium (K_{Ca}) channels and was reversed by pharmacologically increasing their activity with 1-ethyl-2-benzimidazolinone (EBIO). Moreover, chronic *in vivo* perfusion of EBIO into the cerebellum of ataxic mice significantly improved motor performance. Our data support the hypothesis that the precision of intrinsic pacemaking in Purkinje cells is essential for motor coordination and suggest that K_{Ca} channels may constitute a potential therapeutic target in EA2.

Purkinje cells are the sole output of the computational circuitry of the cerebellar cortex and provide the signals required for motor planning, execution and coordination in their rate of firing and pattern of activity¹. Malfunction of these neurons often results in cerebellar ataxia: a disorder characterized by poor balance, loss of posture and difficulties in performing rapid coordinated movements. Purkinje cells fire spontaneously in the absence of synaptic input^{2–6} and their intrinsically driven pacemaking is very regular^{4,6}. The spontaneous activity of Purkinje cells is shaped by cortical, vestibular and sensory information relayed by over 150,000 excitatory and inhibitory synaptic inputs^{1,4,7–11}. The high precision of intrinsic pacemaking in Purkinje cells may be important for cerebellar function, as it would allow the rate of activity of these cells to accurately reflect the strength and timing of the cells' excitatory and inhibitory synaptic inputs (Discussion). Consistent with this hypothesis, a recent study found that when the firing of Purkinje cells *in vivo* is analyzed with short time windows, it contains periods of activity in which firing is quite regular; further, these periods of regular firing *in vivo* comprise about 50% of the total firing of Purkinje cells with episodes lasting up to several hundred milliseconds (S. Shin, A. Aertsen & E. De Schutter, *Soc. Neurosci Abstr.* **827.11**, 2004). These regular periods may correspond to instances in which the spontaneous activity does not contain synaptic information and simply reflects intrinsic pacemaking.

Conductances best suited for ensuring the precision of intrinsic pacemaking are those whose activity is time-locked with an action potential. The precision of pacemaking in Purkinje cells is maintained mainly by small- (SK) and large-conductance (BK) K_{Ca} channels^{12,13}, which are activated by the calcium influx associated with each action potential^{12–17}. In Purkinje cells, K_{Ca} channels are exclusively activated

by P/Q-type voltage-gated calcium channels¹³ and the coupling ratio between calcium influx and calcium-activated channels is such that the net calcium-dependent current is outward². Blockade of these K_{Ca} channels results in bursting and irregular firing in Purkinje cells^{12,15}, and their knockdown or knockout causes ataxia^{18,19}. Thus, a decrease in the P/Q-type calcium current in Purkinje cells is expected to result in the reduced activation of K_{Ca} channels and hence in irregular firing with adverse consequences for cerebellar function.

EA2 is caused by mutations in P/Q-type voltage-gated calcium channels that result in reduced P/Q-type calcium current density^{20,21}. Because these channels support neurotransmitter release at many central synapses²², it is commonly believed that, in EA2, ataxia is caused by impaired synaptic transmission within the cerebellum. Given the predicted consequences of EA2 mutations on the activity of Purkinje cells, we examined the alternative hypothesis that in these disorders ataxia might be attributed to a reduction in the precision of intrinsic pacemaking.

We evaluated the precision of intrinsic pacemaking by monitoring the activity of Purkinje cells in cerebellar slices from mouse models of EA2. In these mice, we found a gene-dependent loss of the precision of intrinsic pacemaking, which prevented Purkinje cells from accurately encoding the strength and timing of synaptic inputs in their activity. This loss of precision of pacemaking was due to the reduced activity of K_{Ca} channels in Purkinje cells and could be recovered by pharmacologically increasing their activation. K_{Ca} channels proved to be a potent therapeutic target in that their chronic activation *in vivo* significantly improved the motor performance of the ataxic mutant mice. We propose that the high precision of Purkinje cell intrinsic pacemaking allows them to encode synaptic information with high fidelity and that

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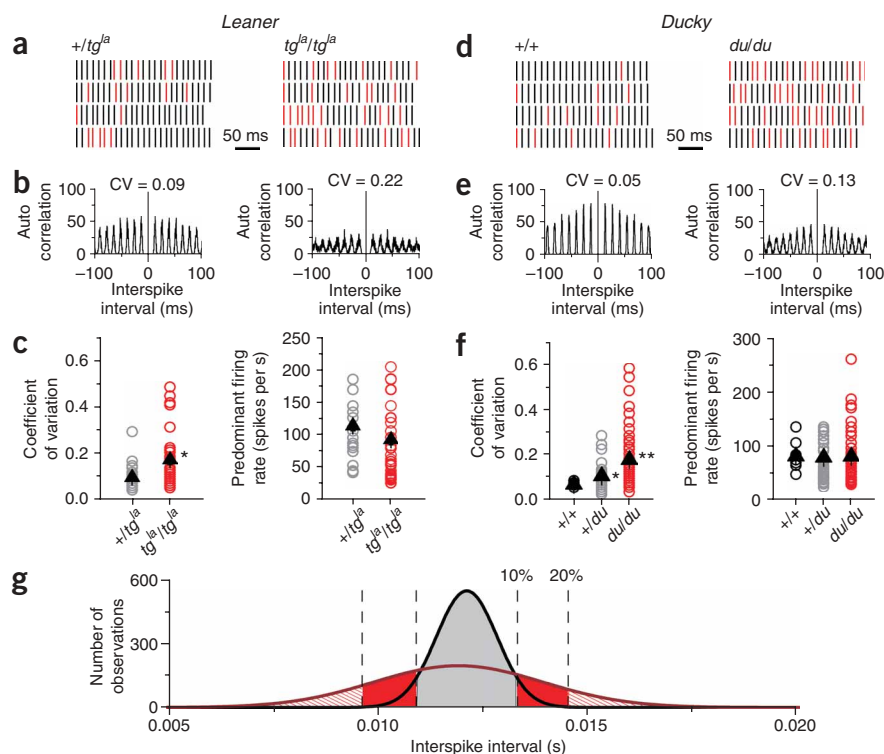


Figure 1 Intrinsic pacemaking in *leaner* and *ducky* mutant mice is highly irregular. **(a)** Raster plots of the intrinsic spontaneous activity of Purkinje cells in the *leaner* mice. Each line represents an action potential. The red lines indicate a previous interspike interval that was not within 2 s.d. of the mean control interspike interval. Spontaneous activity was more irregular in the Purkinje cells of tg^{la}/tg^{la} mice than in those of $+/tg^{la}$ mice. **(b)** Interspike interval autocorrelograms of the cells shown in **a**. Discrete peaks were readily detectable in $+/tg^{la}$ mice, whereas they were absent in the tg^{la}/tg^{la} mice. **(c)** Individual and average values for the interspike interval coefficient of variation and the predominant firing rate in Purkinje cells of the *leaner* mice. $*P < 0.01$. **(d-f)** Same as **a-c**, but for *ducky*, instead of *leaner*, mice. $*P < 0.01$; $**P < 0.001$. **(g)** Distribution of the interspike intervals for wild-type (black line) and *du/du* (red line) mice, based on the average parameters shown in **f**. The dashed lines represent boundaries for interspike intervals that reside within $\pm 10\%$ and $\pm 20\%$ of the mean interspike interval.

the loss of this precision results in impaired cerebellar function and motor performance.

RESULTS

Irregular pacemaking in P/Q-mutant Purkinje cells

There are several strains of mice with ataxia in which spontaneous mutations in P/Q-type voltage-gated calcium channels result in reduced P/Q-type calcium channel current density²³. These mice provide an ideal animal model for studies of P/Q channel-related hereditary ataxias. We examined the intrinsic spontaneous activity of Purkinje cells in two such mice, *leaner* (tg^{la}) and *ducky* (*du*). In both mice, the P/Q-type calcium current density is decreased in cerebellar Purkinje cells^{24–26}. In *leaner*, the spontaneous mutation in the *Cacna1a* gene affects the α_{1A} subunit and alters P/Q channel function in all neurons that express the channel. In the *ducky* mice, however, the mutation is within the *Cacna2d2* gene, which encodes the $\alpha_{2\delta 2}$ auxiliary subunit and thus only affects cells that express this subunit^{24,27}.

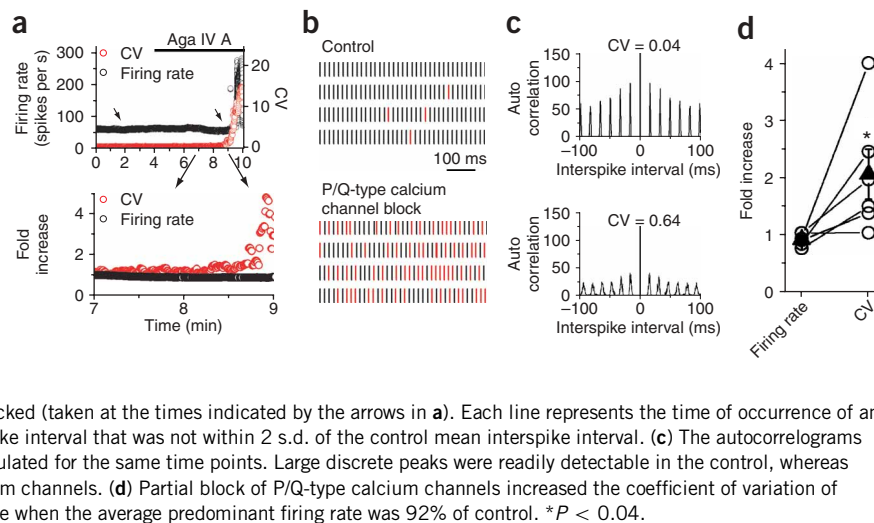
By pharmacologically blocking fast excitatory and inhibitory synaptic transmission, we ensured that the recorded activity of Purkinje cells in acutely prepared cerebellar slices was intrinsically driven. We found that in both mutant mice, the precision of intrinsic pacemaking was significantly reduced (**Fig. 1**). Purkinje cells of the tg^{la}/tg^{la} mice showed highly irregular intrinsic firing, whereas the firing of $+/tg^{la}$ was more regular (*leaner* is bred on oligosyndactylism (*Os*) background, and *Os/Os* is embryonic lethal) (**Fig. 1a**). The difference in the precision of pacemaking can be appreciated by comparing the interspike interval autocorrelogram of tg^{la}/tg^{la} with that of $+/tg^{la}$ (**Fig. 1b**). The autocorrelogram of $+/tg^{la}$ mice showed discrete peaks, whereas these were diminished in the tg^{la}/tg^{la} mice. We also estimated the accuracy of pacemaking by calculating the coefficient of variation of interspike intervals (s.d./mean interspike interval). The average coefficient of variation of interspike intervals was almost twofold higher in the tg^{la}/tg^{la} mice compared with the $+/tg^{la}$ ($+/tg^{la}$ 0.10 ± 0.01 , $n = 28$;

tg^{la}/tg^{la} 0.18 ± 0.02 , $n = 38$; $P = 0.0053$), and the tg^{la}/tg^{la} coefficients of variation covered a larger range of values (**Fig. 1c**). The average firing rates, however, were not significantly different between the two groups ($+/tg^{la}$ 116 ± 9 , $n = 26$; tg^{la}/tg^{la} 95 ± 8 , $n = 42$; $P = 0.14$). These data showed that in the ataxic *leaner* mice, the precision of intrinsic pacemaking was significantly reduced but the average firing rate was not altered.

Similarly, we found that the precision of pacemaking in the *ducky* mutant mice was significantly reduced (**Fig. 1d,e**). The severity of the loss of the precision in pacemaking was dependent on the number of defective genes. The average coefficient of variation of interspike intervals was 2.8 times higher in the *du/du* mice compared with the wild type (*du/du* 0.17 ± 0.02 , $n = 59$; vs $+/+$ 0.06 ± 0.003 , $n = 11$; $P = 0.0045$), demonstrating a significant decrease in the precision of pacemaking. Also, the coefficients of variation of interspike intervals in $+/du$ and *du/du* had a larger range compared with that in the wild-type littermates (**Fig. 1f**). The average predominant firing rate, however, was not significantly ($P = 0.90$) different in *du/du* mice compared with their wild-type counterparts (*du/du* 82 ± 7 spikes per s, $n = 82$; $+/du$ 74 ± 5 spikes per s, $n = 43$; $+/+$ 84 ± 4 spikes per s, $n = 11$), although there was a larger spread in the firing rates of *du/du* and $+/du$ Purkinje cells (**Fig. 1f**).

To place the 2.8-fold increase in the average coefficient of variation of interspike intervals in the *ducky* mutant mice in the context of accuracy of pacemaking, the following consideration is informative. In the wild-type Purkinje cells, 90% of interspike intervals were within $\pm 10\%$ of the mean interspike interval, and virtually all of them were accurate to within $\pm 20\%$ (**Fig. 1g**). In contrast, in the *du/du* *ducky*, $< 45\%$ of interspike intervals were accurate within $\pm 10\%$ of the mean interspike interval, and $> 35\%$ of interspike intervals were less accurate than $\pm 20\%$ of the mean interspike interval. Thus, the 2.8-fold increase in the average coefficients of variation of interspike intervals in the *ducky* mice represented a large and significant decrease in the precision of pacemaking in Purkinje cells.

Figure 2 Partial pharmacological blockade of P/Q-type voltage-gated calcium channels reduces the precision of intrinsic pacemaking in Purkinje cells. **(a)** The coefficient of variation of interspike intervals and instantaneous firing rate ($1/\text{interspike interval}$) before and after application of 100 nM ω -agatoxin IVA. Blockade of P/Q channels increased the firing rate and simultaneously made it erratic, as evident from the increase in the coefficient of variation of interspike intervals. Bottom, the initial effects of ω -agatoxin IVA on an expanded timescale. As the toxin washed in, partial blockade of P/Q-type calcium channels made the firing irregular without increasing the firing rate. **(b)** Raster plots of the activity of the cell under control conditions, and when a fraction of P/Q-type calcium channels was blocked (taken at the times indicated by the arrows in **a**). Each line represents the time of occurrence of an action potential. Red lines indicate a previous interspike interval that was not within 2 s.d. of the control mean interspike interval. **(c)** The autocorrelograms and coefficient of variation of interspike intervals calculated for the same time points. Large discrete peaks were readily detectable in the control, whereas these diminished after partial block of P/Q-type calcium channels. **(d)** Partial block of P/Q-type calcium channels increased the coefficient of variation of interspike intervals to 210% of control values at a time when the average predominant firing rate was 92% of control. * $P < 0.04$.



Irregular pacemaking with partial block of P/Q channels

To directly demonstrate that the reduction in the precision of intrinsic pacemaking in Purkinje cells was a consequence of reduced P/Q-type calcium current density, we partially blocked P/Q-type voltage-gated calcium channels in wild-type Purkinje cells with submaximal concentrations of its selective antagonist ω -agatoxin IVA. Low concentrations of ω -agatoxin IVA decreased the precision of intrinsic pacemaking (coefficient of variation of interspike intervals increased 2.1 ± 0.4 times; $P < 0.04$, $n = 6$) without appreciably increasing the firing rate of Purkinje cells (**Fig. 2**). These data suggested that the erratic firing seen in *leaner* and *ducky* Purkinje cells was because of the reduced P/Q calcium current density in these neurons.

Precision of pacemaking determines signal-to-noise ratio

By increasing the noise of the baseline firing rate, erratic intrinsic pacemaking is likely to reduce the accuracy of a Purkinje cell in encoding and transmitting the strength and timing of the synaptic inputs it receives. Such a loss in information processing is likely to adversely affect cerebellar function. To directly test this possibility and evaluate the extent to which irregular pacemaking affects signaling by Purkinje cells, we examined the accuracy of a Purkinje cell in encoding and transmitting the timing and strength of parallel fiber synaptic inputs. Within the cerebellum, the P/Q channels that are affected by the $\alpha 2\delta 2$ subunit mutation in *ducky* mice are restricted to Purkinje cells^{24,27}. As expected from the unaltered P/Q calcium current density in granule cells of these mice^{24,28}, parallel fibers were as effective in increasing the maximum firing rate of Purkinje cells in the *du/du* mice as they were in the wild type (**Fig. 3a** and **Supplementary Fig. 1** online). Despite the comparable efficacy of parallel fiber synaptic inputs in *ducky* mice, the firing rate of *du/du* Purkinje cells less accurately encoded the timing and strength of the parallel fiber synaptic inputs (**Fig. 3b**). Computer simulations showed that all of the changes seen could be the consequence of the reduction in the precision of intrinsic pacemaking and could be reproduced by matching the coefficient of variation of interspike intervals to those obtained experimentally (**Fig. 3c,d** and **Supplementary Fig. 2** online). Two factors contributed to this reduced information content in the *ducky* mice. First, compared with the wild type, the signal-to-noise ratio was markedly reduced because of the increase in the baseline noise. Second, because of the increase in the coefficient of variation of interspike intervals, the response of a *du/du* Purkinje cell to the same parallel fiber synaptic

input was highly variable from trial to trial, covering a large range of firing rates and often overlapping with the noisy baseline (**Fig. 3b–e**). Moreover, because of the large variability in the magnitude of the parallel fibre-evoked increase in the firing rate, the timing of the increases in the firing rate following the activation of parallel fibres was also quite variable (**Fig. 3b–e**). Clearly, a reduction in the precision of intrinsic pacemaking in Purkinje cells significantly affects signaling and the transfer of synaptic information from the cerebellar cortex to neurons of deep cerebellar nuclei (DCN).

The firing of Purkinje cells *in vivo* in the ataxic P/Q channel mutant *tottering* mice is highly erratic and this irregular firing contributes to the poor sensorimotor information processing in these mice²⁹. Further, little synaptic information is conveyed from Purkinje cells to DCN neurons in these mice *in vivo*. However, it has not been determined where the erratic firing of Purkinje cells and the loss of synaptic information originate and, specifically, whether they are caused by a mechanism intrinsic to Purkinje cells or brought about by altered synaptic transmission²⁹. Our data suggest that an intrinsic mechanism makes a significant contribution to the irregularity of firing of Purkinje cells and to the loss of transfer of synaptic information from Purkinje cell to DCN neurons observed in *tottering* mice *in vivo*.

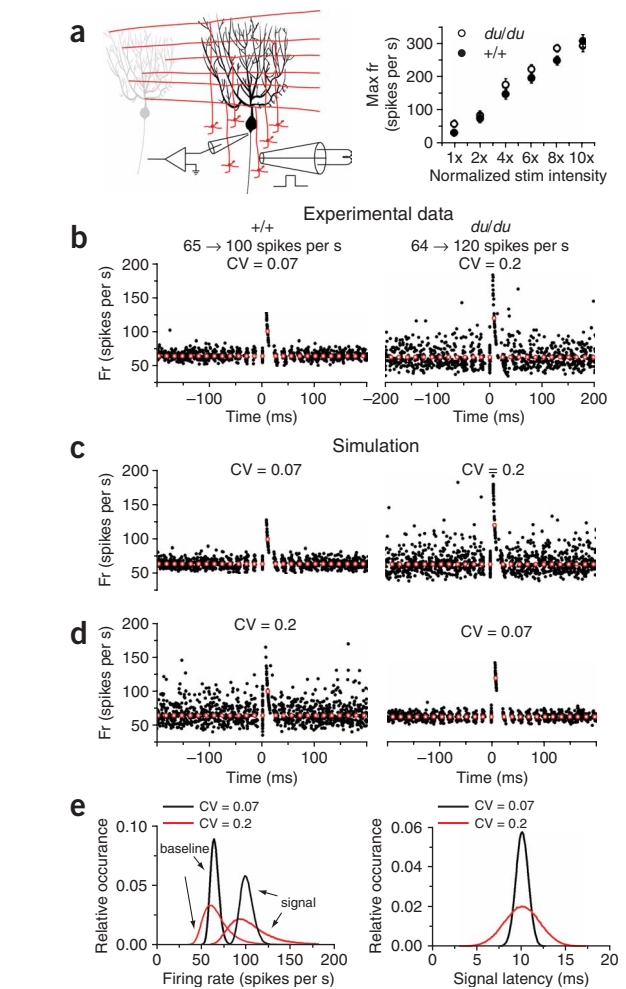
Increasing K_{Ca} channel activity recovers regular firing

When the P/Q-type calcium current density is pharmacologically or genetically decreased in Purkinje cells, K_{Ca} channels are activated to a lesser extent¹³. K_{Ca} channels, particularly the SK type, play a crucial part in regulating the precision of intrinsic spike timing in Purkinje cells^{12,13}. The reduced activation of K_{Ca} channels may thus be the main cause of irregular firing in Purkinje cells when the P/Q-type calcium current density is reduced. Given such a scenario, it might be possible to pharmacologically recover regular firing with compounds that increase the activity of K_{Ca} channels. The SK channel activator EBIO (ref. 30) increases the affinity of SK channels for calcium³⁰ and is effective in regulating SK channel function in wild-type Purkinje cells¹². We examined the usefulness of EBIO in recovering regular firing in Purkinje cells in which firing was made irregular by the partial blockade of voltage-gated calcium channels and also in Purkinje cells of *ducky* mice. We used low concentrations of cadmium (2–5 μM) to block calcium channels and to increase the average coefficient of variation of interspike intervals from a control level of 0.06 ± 0.004 to 0.13 ± 0.018 (mean \pm s.e.m., $n = 11$, $P = 0.0013$). Subsequent addition of EBIO

Figure 3 The activity of mutant *ducky* Purkinje cells less accurately encodes the strength and timing of its synaptic input. **(a)** Parallel fiber-evoked increases in firing rate of *du/du* and *+/+* Purkinje cells ($n = 5$). **(b)** Parallel fibers were stimulated at time 0 coincident with a spontaneous action potential in the Purkinje cell in *+/+* (left) and *du/du* (right) cerebella. Black dots represent the instantaneous firing rate and open red circles show the average firing rate. Forty trials are superimposed. **(c,d)** Baseline firing rate and response to parallel fiber synaptic input (signal) were simulated using the experimentally determined data and pacemaking coefficients of variation from **b** (left *+/+*, right *du/du*). The simulated data accurately reproduce the experimental observations. **(e)** Baseline and signal firing rate distributions (left) and signal latency distributions (right) for the *+/+* Purkinje cell presented in **b** assuming pacemaking coefficients of variation of 0.07 (black) and 0.2 (red). When intrinsic pacemaking was made more irregular by increasing the interspike interval coefficient of variation to 0.2 (red curves), there was a noticeable and significant increase in the extent of overlap of the signal with the baseline firing rate, resulting in a marked decrease in the information content. For the same cell, the right panel shows a loss in the accuracy of encoding the timing of arrival of the synaptic input when the interspike interval coefficient of variation was increased from 0.07 to 0.2.

significantly reduced the average coefficient of variation of interspike intervals to 0.07 ± 0.029 ($n = 11$, $P = 0.0024$), a value that was statistically indistinguishable ($P = 0.28$) from control levels (**Fig. 4a,b**). At the concentrations used to recover regular firing, EBIO had an insignificant effect on the firing rate (**Fig. 4a**). The application of comparable concentrations of EBIO to Purkinje cells that were not exposed to cadmium affected neither the firing rate nor the regularity of firing (data not shown).

We similarly examined the efficacy of EBIO in recovering regular firing in Purkinje cells of *+/du* mice (**Fig. 4c,d**). Bath perfusion of EBIO reduced the average coefficient of variation of interspike intervals in *+/du* mice from 0.14 ± 0.023 to 0.07 ± 0.009 ($n = 5$, $P = 0.026$), a level comparable to that seen in their wild-type littermates (0.06 ± 0.003 , $P = 0.37$ **Fig. 4c,d**). Consistent with the hypothesis that the decreased calcium current in *ducky* mice activates fewer K_{Ca} channels, the average amplitude of action potential after-hyperpolarization (AHP) in the Purkinje cells of *+/du* mice (-53.3 ± 0.4 mV, $n = 6$) was significantly smaller than that seen in the Purkinje cells of the wild-type mice (-58.1 ± 0.4 mV, $n = 4$, $P = 0.00004$) (**Fig. 4e,f**). Although EBIO at concentrations that recovered regular firing did not affect the input resistance of Purkinje cells in the *+/du* mice (control 59.7 ± 13.1 M Ω versus 58.6 ± 12.1 M Ω in 20 μ M EBIO, $n = 4$, $P = 0.79$), it increased



their AHP amplitude to -59.2 ± 0.7 mV ($n = 6$), a value comparable ($P = 0.24$) with that in the Purkinje cells of the wild type (**Fig. 4e,f**). Thus, our data supported the hypothesis that EBIO recovers the precision of pacemaking by transiently increasing the SK conductance after each action potential and thus compensating for the reduced calcium current density.

It is likely that the decrease in the precision of pacemaking by the reduced activation of SK channels will make Purkinje cells much more susceptible to synaptic noise. Small inputs that may have been 'sub-threshold' in the wild-type Purkinje cells may significantly alter the

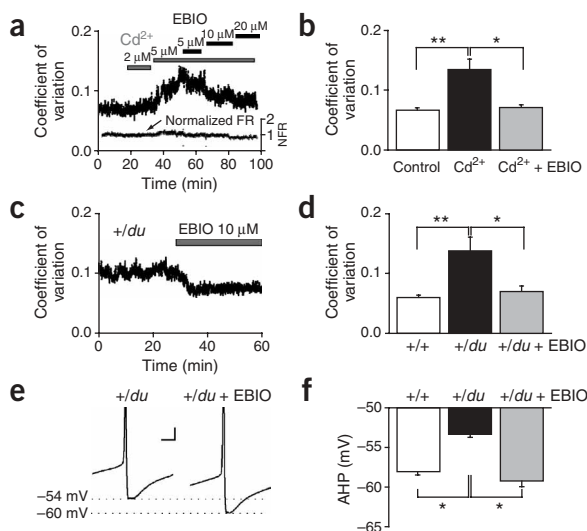


Figure 4 Activation of SK channels with EBIO recovers the precision of intrinsic pacemaking in Purkinje cells by increasing the AHP. **(a)** Bath application of low concentrations of cadmium chloride to block calcium channels in a dose-dependent manner made the firing of the cell irregular as judged by the increase in the coefficient of variation of interspike intervals without a significant increase in the firing rate. Subsequent application of EBIO recovered regular firing to levels comparable with baseline. **(b)** Cadmium significantly increased the irregularity of firing and EBIO restored the precision of firing to levels comparable with baseline ($n = 11$). $*P < 0.005$; $**P < 0.001$. **(c,d)** EBIO (5–20 μ M) reduced the coefficient of variation of *+/du* Purkinje cells (left) to levels observed in *+/+* mice (right). $*P < 0.03$; $**P < 0.01$. **(e,f)** EBIO (20 μ M) increased AHP amplitudes in *+/du* Purkinje cells to *+/+* levels (left, truncated action potentials). $*P < 0.00005$. Firing rates of all groups were comparable (*+/+* 71.7 ± 7.0 spikes per s, *+/du* 63.7 ± 3.5 spikes per s, *+/du* plus EBIO 60.2 ± 5.7 spikes per s, $n = 6$). Scale bar, 5 mV and 2 ms.

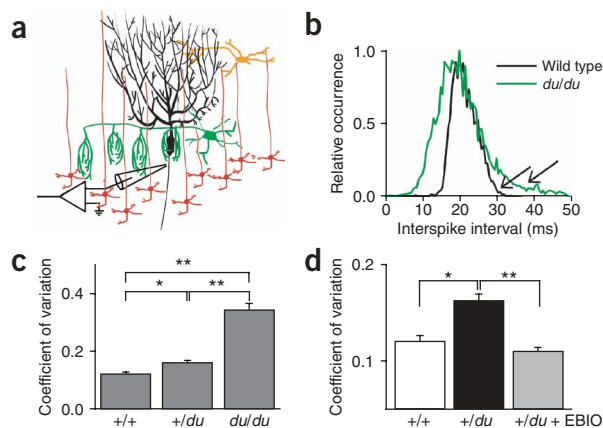


Figure 5 P/Q channel mutations affect the precision of pacemaking in the presence of inhibitory synaptic transmission. **(a,b)** Interspike interval histograms of wild-type and $+/+$ *ducky* Purkinje cells in the absence of blockers of inhibitory and excitatory synaptic transmission. Arrows denote interspike intervals associated with inhibitory synaptic inputs. **(c)** The coefficient of variation of interspike intervals of *ducky* Purkinje cells in the absence of blockers of synaptic transmission ($+/+$ $n = 26$, $+/du$ $n = 58$, du/du $n = 27$). $*P < 0.002$; $**P < 0.0001$. **(d)** The coefficient of variation of interspike intervals of the $+/du$ and $+/+$ *ducky* mice in the presence or absence of 20 μM EBIO ($+/+$ $n = 26$, $+/du$ $n = 58$, $+/du$ plus EBIO $n = 24$).

firing of P/Q channel mutant Purkinje cells. Thus synaptic noise is likely to further exaggerate the irregularity of firing of the mutant Purkinje cells *in vivo* and further decrease the signal-to-noise ratio of synaptic information.

Inhibitory inputs do not mask irregular pacemaking

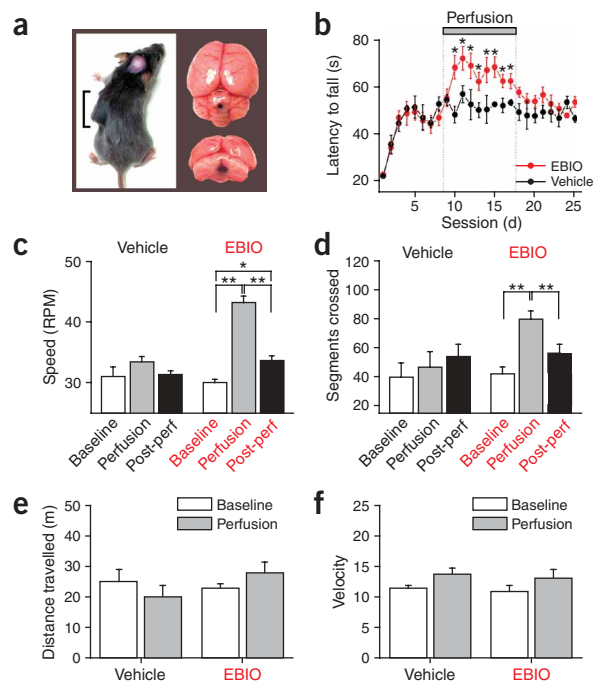
Purkinje cells receive inhibitory input from spontaneously active interneurons within the cerebellar cortex (Fig. 5a). These interneurons are strongly driven by excitatory synaptic inputs³¹ and provide information to Purkinje cells in the form of feed-forward inhibition^{32,33}. In acutely prepared slices *in vitro*, these interneurons are not synaptically driven and, because of their spontaneous activity, provide random inhibitory inputs to Purkinje cells, thus making the firing of Purkinje cells less regular⁴. We examined whether, under these conditions, the spontaneous activity of mutant Purkinje cells remained less regular as compared to that in the wild-type littermates. Consistent with previous findings⁴, the interspike interval histograms of the Purkinje cells in the wild-type and *du/du* mice were not symmetrical Gaussian distributions and demonstrated a prominent tail at higher interspike intervals, corresponding to intervals coincident with the arrival of inhibitory synaptic inputs (Fig. 5b). Despite the presence of inhibitory synaptic

Figure 6 Chronic *in vivo* activation of cerebellar SK channels improves motor performance in *ducky* mice. **(a)** An implanted cannula (pinkish circle on head) and the osmotic pump ('I') used for chronic perfusion. Perfusion sites were limited to spheres of diameter 2–3 mm within the vermis. **(b)** *Ducky* mice ($+/du$) were trained to proficiency on an accelerating rotarod. A cannula and pump were surgically implanted to chronically perfuse the cerebellum with either 80 ng h^{-1} EBIO or the vehicle during the time indicated. The performance of the mice receiving EBIO significantly improved on the rotarod. $*P < 0.0001$. **(c)** Averages of 6 d of performance of $+/du$ mice on rotarod before (white bar), during (gray bar), and after (black bar) pumps were implanted. $*P < 0.005$; $**P < 0.0005$. **(d)** The average performance of the same *ducky* mice on a balance beam test. $*P < 0.02$; $**P < 0.0001$. **(e,f)** Average distance traveled and the velocity of movement of $+/du$ mice measured in an open field test.

transmission, the interspike interval histograms of Purkinje cells in the *du/du* mice were significantly broader than those of the cells in the wild type (Fig. 5b). Therefore, consistent with the findings when inhibition was blocked, the spontaneous firing of the mutant Purkinje cells was less regular than that of the wild type. As a semiquantitative measure of irregularity, we also determined the coefficient of variation of interspike intervals and found a gene-dependent increase in the average value (Fig. 5c). It is important to note, however, that the coefficient of variation is a mathematical term defined for a Gaussian distribution and is not a very appropriate measure of the spread of nonGaussian distributions. Its utility here was somewhat warranted because of the gene-dependent increase in the width of the distribution for values within 1 or 2 s.d. of the mean (Fig. 5b). Nevertheless, because these coefficients of variation were obtained from nonGaussian distributions, they should not be directly compared with those obtained in the absence of inhibitory synaptic transmission which were calculated from Gaussian distributions. We also found that concentrations of EBIO that restored regular pacemaking when inhibition was blocked were also effective when inhibition was intact (Fig. 5d).

In vivo perfusion of EBIO improves *ducky* motor performance

If the loss of precision in intrinsic pacemaking in Purkinje cells makes a significant contribution to ataxia, then increasing the precision of pacemaking should reduce ataxic symptoms. We thus examined whether *in vivo* chronic perfusion of EBIO into the cerebellum of *ducky* mice reduces ataxia. Because *du/du* mice are too small for the implantation of osmotic pumps and also too ataxic to perform behavioural tests that allow quantification, we used $+/du$ mice for these experiments. These mice were trained on an accelerating rotarod until they reached their maximum proficiency. They then underwent surgery during which a cannula was stereotaxically implanted into the vermal region of the cerebellum (Fig. 6a). The cannula was connected to an osmotic pump that locally delivered either the vehicle (Methods) or EBIO at a rate of 0.25 $\mu\text{l h}^{-1}$. $+/du$ mice that received EBIO showed a significant increase in their performance compared with those that received the vehicle (Fig. 6b,c). The performance of the $+/du$ mice that



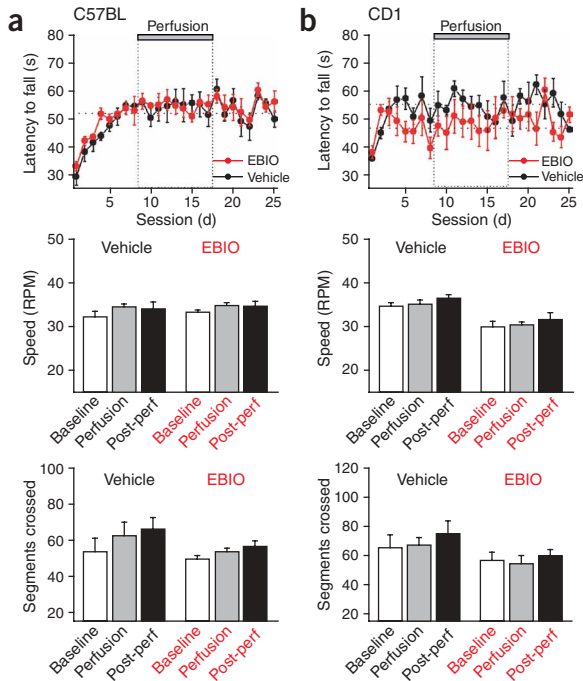


Figure 7 Chronic perfusion of EBIO does not affect the motor performance of C57BL or CD1 mice. **(a)** The cerebella of C57BL mice were chronically perfused with EBIO as in the experiments reported in **Figure 5**, and their performance on the rotarod and balance beam were evaluated. On both tests, the performance of the mice was not affected by surgery or by the perfusion of the vehicle or EBIO ($n = 12$). **(b)** The same experiments shown in **a** are repeated in 12 CD1 mice. EBIO did not affect the performance of the CD1 mice on either test.

mance of either CD1 or C57BL wild-type mice, demonstrating that its efficacy in improving motor performance was related to the reduced calcium current density in the *ducky* mice (**Fig. 7a,b**).

EBIO improves dyskinesia and ataxia in *tottering* mice

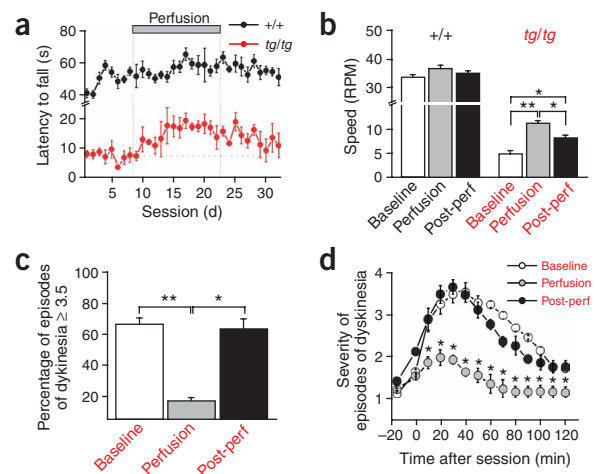
To study the generality of the therapeutic potential of EBIO in alleviating motor dysfunction in P/Q channel-related ataxias, we also examined its efficacy in the *tottering* (*tg*) mice. *Tottering* is perhaps the most and best studied P/Q channel mutant strain; in these mice, a single amino acid substitution in the pore-lining region of the second domain of the α_{1A} subunit of P/Q-type voltage-gated calcium channels significantly reduces the P/Q channel current density throughout the brain³⁴. The mutation results in ataxia and, as in humans with EA2, episodes of severe paroxysmal dyskinesia that are triggered by stress²⁷. Compared with their wild-type littermates, performance of the *tg/tg* mice on the accelerating rotarod was extremely poor (4.9 ± 0.55 r.p.m. in *tg/tg* versus 33.7 ± 0.96 r.p.m. in +/+; **Fig. 8a,b**). Chronic *in vivo* perfusion of EBIO into the cerebellum of *tg/tg* mice more than doubled their performance in the accelerating rotarod protocol (4.9 ± 0.55 r.p.m. in baseline versus 11.2 ± 0.53 r.p.m. in EBIO, $P < 0.00001$). In contrast, the same treatment had no significant ($P = 0.10$) effect on the motor performance of the wild-type littermates (33.7 ± 0.96 r.p.m. at baseline versus 36.6 ± 1.29 r.p.m. in EBIO; **Fig. 8a,b**). Chronic perfusion of EBIO not only reduced the frequency of the episodes of dyskinesia by a factor of 4, but also significantly decreased the severity of attacks (**Fig. 8c,d** and **Supplementary Video** online). Given the effectiveness of EBIO in reducing motor deficits in the *ducky* and *tottering* mice, two mouse strains with markedly different mutations in P/Q-type calcium channels, our data suggested that K_{Ca} channels may constitute an appropriate therapeutic target in EA2.

DISCUSSION

Most principal neurons involved in motor coordination are spontaneously active; it has been suggested that this is because spontaneous

received EBIO improved from an average speed of 30.0 ± 0.5 r.p.m. to 43.1 ± 1.0 r.p.m. during EBIO perfusion ($n = 9$, $P < 0.001$), a $>40\%$ improvement. In contrast, +/*du* mice that received the vehicle achieved an average speed of 33.4 ± 0.86 r.p.m., a level that was not significantly ($P = 0.31$) different from their baseline performance of 31.0 ± 1.53 r.p.m. ($n = 4$). Thus, in agreement with our prediction, chronic *in vivo* cerebellar perfusion of EBIO significantly improved performance on an accelerating rotarod (**Fig. 6c**). When the osmotic pumps were disengaged, the performance of mice receiving EBIO declined (**Fig. 6b,c**). The performance of the mice receiving EBIO also improved when it was evaluated with a balance beam task that determined mobility and balance on an elevated wooden beam (**Fig. 6d**). The +/*du* mice receiving EBIO or vehicle performed comparably in an open field test (**Fig. 6e,f**), in agreement with the notion that the improvements seen on the rotarod and the balance beam were because of reduced ataxia. Limitations in the availability of +/+ *ducky* mice prevented the evaluation of the effect of EBIO perfusion on their motor performance. However, chronic perfusion of EBIO did not affect the motor perfor-

Figure 8 EBIO reduces the severity and the frequency of dyskinesia and improves motor performance in *tottering* mice. **(a)** Wild-type and *tg/tg* mice were trained on the accelerating rotarod. At the time indicated, the mice underwent surgery and a cannula and pump were surgically implanted to chronically perfuse the cerebellum with 200 ng h^{-1} EBIO. The performance of the *tg/tg* mice, but not the wild-type littermates, improved significantly while the pumps were active and declined when the perfusion of EBIO terminated. **(b)** Bar chart of the average rotational speed of the rod at which the mice described in **a** failed the rotarod test. The *tg/tg* mice, but not the wild type, achieved significantly higher speeds (an increase of 125%) while their cerebellum was perfused with EBIO. $*P < 0.005$; $**P < 0.00001$. **(c,d)** The stress and exercise associated with performing the rotarod test resulted in episodes of paroxysmal dyskinesia in the *tg/tg* mice that lasted for several hours. Chronic *in vivo* perfusion of EBIO into the cerebellum of *tg/tg* mice significantly reduced the frequency, severity and duration of the dyskinesia attacks. In **c**, $*P < 0.003$; $**P < 0.0005$. In **d**, $*P < 0.0001$.



activity allows for information to be encoded bidirectionally with high precision and speed³⁵. An intrinsically active neuron can encode information in its pacemaking by serving as a clock similar to myocytes of the sinus node, which provide the intrinsic pacemaking of the heart. The intrinsic pacemaking, however, does not carry any meaningful time-variant information. To control time-variant processes such as movement, the relevant information must be relayed to the intrinsically active neurons by external inputs. To provide the sole output of the computational circuitry of the cerebellar cortex, Purkinje cells integrate time-variant information from a wealth of sensory and cortical synaptic inputs with their intrinsic pacemaking¹. This information, which is relayed by both excitatory and inhibitory synapses, is encoded in the activity of Purkinje cells as rapid and transient changes in their rate of spontaneous activity. Given an ideal signal-to-noise ratio, an increase or decrease in each individual interspike interval from that of the intrinsic Purkinje cell pacemaker will relay to DCN neurons time-variant information relevant to motor coordination. Given that no biological system is free from noise, Eccles recognized that to improve the signal-to-noise ratio, DCN neurons average the same information from several converging Purkinje cells³⁵. Although this averaging improves the signal-to-noise ratio, it is immediately obvious that the signal-to-noise ratio of the averaged time-variant information is dependent on the precision of pacemaking of each individual Purkinje cell. Here we propose that the highly precise intrinsic pacemaking of Purkinje cells^{2,4,6} is essential for an adequate signal-to-noise ratio and thus for cerebellar function. Furthermore, we showed that P/Q voltage-gated calcium channels have an indispensable role in maintaining the precision of intrinsic pacemaking in Purkinje cells.

Paradoxically, P/Q channel mutations associated with EA2 reduced the precision of intrinsic Purkinje cell pacemaking because in the mutant cells, fewer K_{Ca} channels were activated with each action potential, leading to smaller AHPs and a decreased potassium conductance during interspike intervals. In principle, the precision of pacemaking in mutant Purkinje cells can be restored if more K_{Ca} channels are activated with each action potential. Here we demonstrated the utility of this approach by increasing the affinity of SK channels for calcium with EBIO, which rescued the precision of pacemaking in Purkinje cells *in vitro*. Because SK channels in Purkinje cells are activated only by the calcium influx mediated by each action potential^{12,15,16}, EBIO selectively enhanced the SK current associated with the AHP and had little global or tonic effect on the membrane input resistance and did not alter the average rate of activity of Purkinje cells. This selectivity of action of EBIO makes it a potential therapeutic agent, a prediction that was confirmed by its efficacy in improving the motor performance of mutant mice *in vivo*.

In addition to SK channels, BK channels in Purkinje cells are also activated by the calcium influx with each action potential and contribute to the AHP^{13–15}. Under normal conditions, the voltage dependence of these channels and their extremely low affinity for calcium leads to their transient activation during the AHP^{14,15}; however, agents that increase the channels' affinity for calcium by increasing their mean open time may also provide a means of recovering the precision of pacemaking of mutant Purkinje cells. Note that although carbonic anhydrase inhibitors such as acetazolamide are routinely used for the treatment of episodic ataxia (including EA2), their mode of action is not understood^{36–40}. Therapeutic concentrations of these compounds activate BK channels^{41,42}, and it is tempting to speculate that their therapeutic mode of action may be the recovery of regular pacemaking in Purkinje cells.

Chronic *in vivo* perfusion of EBIO into the cerebellum of *tottering* mice almost completely abolished the episodes of dyskinesia and significantly reduced the severity of the remaining episodes; however, it did not improve the motor performance of the mice on the accelerating rotarod to levels comparable with that of the wild-type littermates. There are at least three factors that could contribute to the lack of full recovery of the *tottering* mice. First, the consequence of the local perfusion was that only a small fraction of the total cerebellum was perfused with EBIO, with the perfused area restricted to the vermis. Second, we did not optimize the concentration of EBIO delivered to the mutant cerebella; it is plausible that such optimization may have further improved the motor performance of the *tottering* mice. Finally, despite the compensatory mechanisms that rescue synaptic transmission when P/Q channels are knocked out^{43–45}, impaired synaptic transmission within the cerebellum could also contribute to the ataxia.

Erratic Purkinje cell activity may also contribute to motor dysfunction in other hereditary ataxias. For example, episodic ataxia type-1 (EA1) is caused by point mutations in the *KCNA1* gene encoding subthreshold voltage-gated potassium channels. In cerebellar slices, pharmacological blockade of Kv1 channels allows for the generation of random dendritic calcium spikes that result in transient bursts of increased activity in Purkinje cells⁴⁶. The firing rate of Purkinje cells during these bursts of activity reaches as high as 500 spikes per s and, although infrequent, the convergence of these random bursts from several Purkinje cells onto individual neurons of the DCN is likely to have severe consequences for motor coordination. It remains to be established whether Purkinje cells express *KCNA1* subunits but, if they do, it is likely that these random bursts contribute to the motor dysfunction associated with EA1.

Collectively, our data support the hypothesis that high precision intrinsic pacemaking by Purkinje cells is essential for the faithful encoding of synaptic information in the activity of Purkinje cells and thus for cerebellar function. Our data suggest that the loss of precision of pacemaking in Purkinje cells is likely to be a major contributing factor to motor performance dysfunction in P/Q channel-related ataxias.

METHODS

All experiments were approved and performed in compliance with the guidelines set by Albert Einstein College of Medicine. Detailed information about the Methods is provided as **Supplementary Methods** online.

Mutant mice. Breeding pairs of *leaner* (+/*tg^{td}*) and *ducky* (+/*du*) mice were obtained from Jackson Labs (Bar Harbor, Maine). The genetic classification of the offspring was determined as described (see **Supplementary Methods**). *Tottering* mice were genotyped and kindly provided by E. Hess (Johns Hopkins University, Baltimore). For electrophysiological experiments, *leaner* mice were taken at postnatal days 13–17 and *ducky* mice at postnatal days 21–31.

Electrophysiological recording and analysis. We prepared 300- μ m-thick sagittal cerebellar slices as described¹³. Slices were visualized using a 40 \times objective and infrared optics (Zeiss Axioskope). Slices were superfused (1.5 ml min⁻¹) with 125 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 1 mM MgCl₂, 2 mM CaCl₂ and 10 mM glucose and gassed with a mixture of 5% CO₂ and 95% O₂ (pH 7.4). Measurements of spontaneous firing rate were made from individually identified Purkinje cells at 35 \pm 0.5 $^{\circ}$ C with extracellular recordings^{6,12,13,47} in the presence of kynurenic acid⁴⁸ and picrotoxin⁴⁹. Whole-cell current-clamp and voltage-clamp recordings were performed as described¹³ with pipettes containing 120 mM potassium methylsulfate, 10 mM KCl, 4 mM MgATP, 1.8 mM MgCl₂, 14 mM sodium creatinine-phosphate, 0.01 mM EGTA and 10 mM HEPES (pH 7.2; 292 mOsmol kg⁻¹). In most of the cases, the recordings were performed with the experimenter blind to the genotype of the mouse.

Parallel fiber stimulation. Parallel fibers were activated by a 200- μ s current pulse (DS3, Digitimer) with a theta pipette positioned in the granule cell layer. A homemade electronic timing device sampled the spontaneous firing of the target Purkinje cell and coordinated the electrical stimulation of parallel fibers to occur concurrent with a spike in the Purkinje cell.

In vivo cerebellar perfusion. An osmotic pump (Model 1002, ALZET) was connected to a cannula (Plastics One) that was stereotaxically positioned within the midline cerebellum of 7- to 8-week-old mice under isoflurane anaesthesia. Pumps were filled with solutions containing either 2 mM or 5 mM EBIO (delivering 80 ng h⁻¹ or 200 ng h⁻¹ of EBIO) or the vehicle (0.01% methylene blue, 0.4% dimethylsulfoxide).

Accelerating rotarod test. The rotarod (Rotamex-5, Columbus Instruments) was 3 cm in diameter and in each trial was accelerated from stationary position at a rate of 0.1 cm s⁻². We recorded the speed of the mice and their latency to fall. Data were averaged over ten trials.

Balance beam test. Mice were placed on a wooden beam, 1.5 cm in diameter and 40 cm in length. The beam was elevated 50 cm from the ground and was divided into four segments. We counted the number of segments crossed by all four paws during a 5-min session.

Open field test. Mice were placed in a white circular tub of diameter 54 cm. They were videotaped for 5 min while they moved freely. The total distance travelled and the average speed in each session was quantified using EthoVision software.

Scoring of dyskinesia. The severity and frequency of these attacks were quantified with the following scale (ref. 50): 0 = normal motor behavior; 1 = slightly slowed or abnormal movements; 2 = mild impairments, limited ambulation unless disturbed; 3 = moderate impairment, limited ambulation even when disturbed, frequent abnormal postures; 4 = severe impairment, almost no ambulation, sustained abnormal postures; 5 = prolonged immobility and abnormal postures.

Because rodents are nocturnal, all behavioral tests were carried out during their dark cycle. All data are reported as mean \pm s.e.m. Data were considered to be statistically significant only at $P < 0.05$, and if so are specifically noted when presented.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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