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The neural substrates of rapid-onset Dystonia-Parkinsonism

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Although dystonias are a common group of movement disorders, the mechanisms by which brain dysfunction results in dystonia are not understood. Rapid-onset Dystonia-Parkinsonism (RDP) is a hereditary dystonia caused by mutations in the *ATP1A3* gene. Affected individuals can be free of symptoms for years, but rapidly develop persistent dystonia and Parkinsonism-like symptoms after a stressful experience. Using a mouse model, we found that an adverse interaction between the cerebellum and basal ganglia can account for the symptoms of these individuals. The primary instigator of dystonia was the cerebellum, whose aberrant activity altered basal ganglia function, which in turn caused dystonia. This adverse interaction between the cerebellum and basal ganglia was mediated through a di-synaptic thalamic pathway that, when severed, alleviated dystonia. Our results provide a unifying hypothesis for the involvement of cerebellum and basal ganglia in the generation of dystonia and suggest therapeutic strategies for the treatment of RDP.

Dystonias, characterized by prolonged co-contraction of the opposing agonist and antagonist muscles, comprise the third most common movement disorder after Parkinson's disease and essential tremor[1]. Although most dystonias are idiopathic[2], mutations in at least 17 genes have been implicated in its hereditary forms[3]. RDP, DYT12, is a hereditary dystonia caused by loss of function mutations in the α3 isoform of the sodium-potassium ATPase pump (sodium pump)[4]. Subjects carrying these mutations show few symptoms before the sudden onset of the disease, which is often triggered by an extremely stressful event[4,5]. The stressful event rapidly produces a combination of dystonia and Parkinsonism (primarily akinesia) that is frequently accompanied by dysarthria, dysphagia, slurred speech, postural instability and wide stance[5]. The symptoms are permanent, but can, in some cases, improve slightly with time[4,5].

There is currently no treatment for RDP[5]. Moreover, despite our detailed appreciation of the role of the sodium pump in the generation and maintenance of intracellular ionic gradients, how and why the mutations cause dystonia are not understood. Even the identities of the brain regions that are affected remain elusive. Scrutiny of hereditary dystonia and exploration of their therapeutic options in general have been limited by the fact that their genetic animal models have routinely failed to reproduce their pathophysiology[2,6]. In the case of RDP, neither of the two available genetic mouse models display dystonia or dyskinesia[7–9], although rodents are fully capable of manifesting dystonia. The reason for the inability of available genetic animal models of dystonia to fully capture the human symptoms is not known, although it may stem from differences in compensatory mechanisms during brain development in rodents compared with humans.

In contrast with most hereditary dystonias in which the function of the mutated protein is poorly understood, in the case of RDP, the role of the sodium pump in the generation and maintenance of intracellular ionic gradients is well established. Moreover, the function of the sodium pump can be pharmacologically manipulated using its high affinity and exquisitely selective blocker, ouabain[10], which has ~1000-fold higher selectivity for the mutated α3 isoform than the other neuronal α1 isoform[11]. This allowed us to generate a pharmacologic animal model of RDP, bypassing the concerns and complications associated with compensatory mechanisms in the genetic models. We found that dysfunction of sodium pumps in both the cerebellum and the basal ganglia was required to replicate the salient features of RDP. Mice whose cerebellum and basal ganglia were simultaneously perfused with ouabain showed mild symptoms that rapidly transformed to persistent dystonia and rigidity after stress. We found that involuntary dystonic movements were caused by aberrant cerebellar activity and that both pharmacologically reducing cerebellar activity and silencing cerebellar output with selective electrical lesions of its output nuclei were effective at alleviating dystonia. Finally, to reconcile the fact that dystonia is primarily associated with basal ganglia function, we tested the hypothesis that aberrant cerebellar activity adversely affects basal ganglia function, which in turn causes dystonia. Consistent with this hypothesis, we found that severing the di-synaptic link between the cerebellum and basal ganglia by selectively lesioning the centrolateral nucleus of the thalamus effectively alleviated cerebellar-induced dystonia. These data provide a unifying hypothesis that accounts for the involvement of cerebellum and basal ganglia in the generation of dystonia and suggest therapeutic approaches for the treatment of RDP.

RESULTS

To identify the neural substrates of RDP, we stereotaxically implanted guide canula into select brain regions of mice and chronically or acutely perfused low amounts of ouabain to partially block sodium pumps. This allowed us to pharmacologically replicate the loss-of-function sodium pump mutations that afflict individuals with RDP and to examine the consequences for motor function.

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Basal ganglia sodium pump dysfunction causes Parkinsonism

We first bilaterally targeted the basal ganglia ($n = 35$). These subcortical structures are involved in the selection and execution of voluntary movements and their dysfunction is the main cause of Parkinsonism[12] and nonidiopathic dystonias[1]. The ouabain-perfused region contained the caudate, putamen, globus pallidus and nucleus accumbens (**Fig. 1a–c** and **Supplementary Data 1**). Ouabain perfusion into the basal ganglia reduced locomotor activity and induced rigidity, hunched posture, postural instability and tremor (**Fig. 1d–j** and **Supplementary Video 1**).

We quantified the effect of partial dysfunction of basal ganglia sodium pumps on locomotion by adapting a locomotion disability rating scale used for Parkinson's disease in humans[13] for use in mice. Perfusion of ouabain into the basal ganglia reduced locomotion (**Fig. 1e**) in both a time- and concentration-dependent manner ($F = 6.7$, $P = 0.0043$). This reduced mobility manifested as decreases in all of the motor parameters examined (**Fig. 1f–h**): the number of steps taken in 30 s ($F = 15.07$, $P < 0.001$), the average step size ($F = 7.04$, $P = 0.0018$) and the locomotion speed ($F = 9.8$, $P < 0.0001$). In contrast, perfusion of vehicle into the basal ganglia for extended periods (up to 8 d) did not produce any detectable symptoms.

The symptoms produced by perfusion of ouabain into the basal ganglia (**Fig. 1j**), such as rigidity, akinesia and tremor, are hallmarks of Parkinsonism-like symptoms in mice[14] and thus mimic a number of symptoms seen in individuals with RDP[4,5]. Concurrent with manifestation of these symptoms, the mice also showed a pronounced hunched posture. This hunched posture closely resembled the forward flexion of the thoracolumbar spine (camptocormia) seen in individuals suffering from Parkinson's disease. In humans, Parkinsonism camptocormia is not typically considered to be caused by dystonia[15].

Despite producing clear Parkinsonism-like symptoms and the hunched posture, perfusion of ouabain into the basal ganglia did not

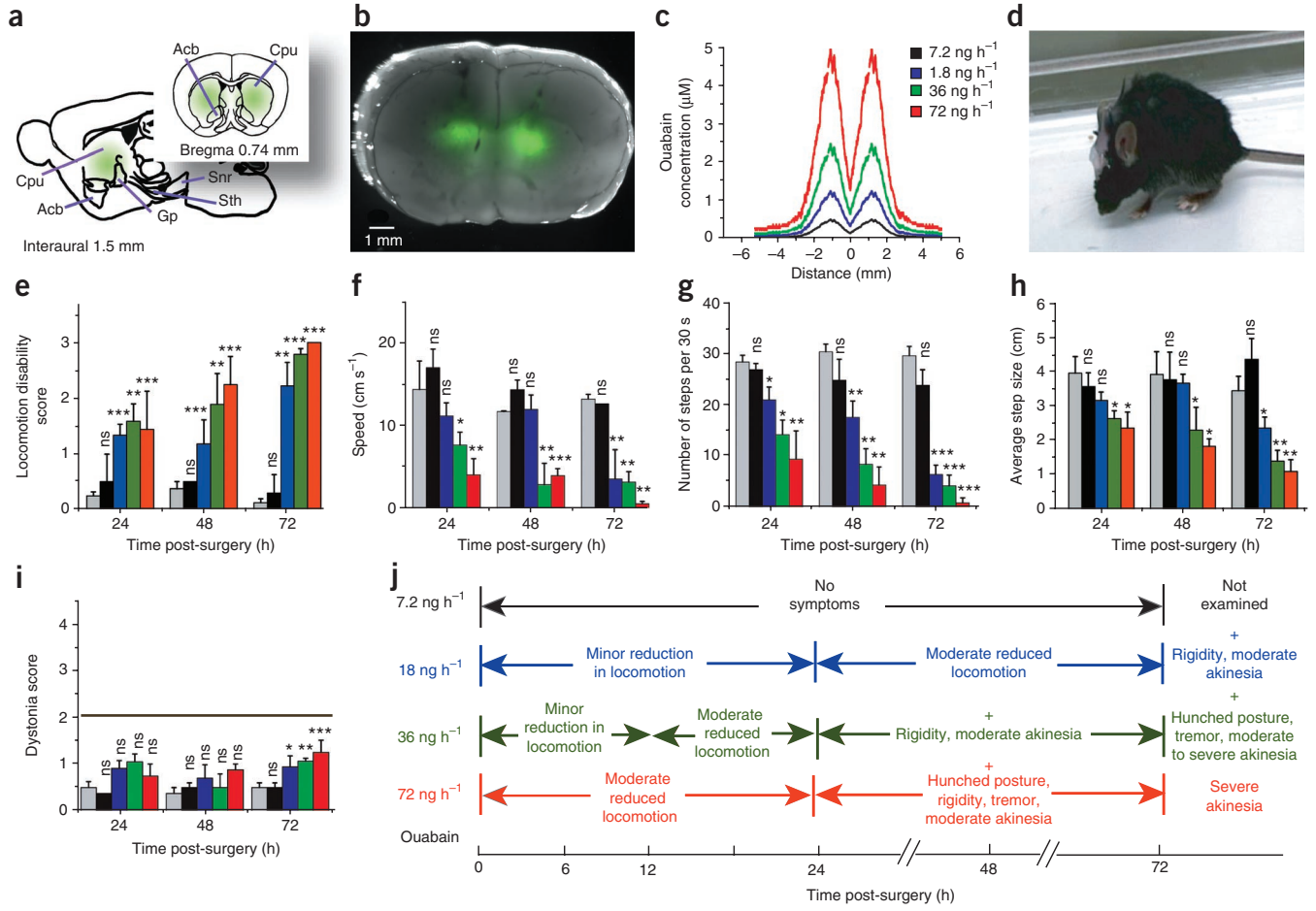

Figure 1 Chronic partial blockade of basal ganglia sodium pumps induces Parkinsonism-like symptoms. (**a**) Sagittal and coronal schematics of the basal ganglia showing the anatomical structures targeted by chronic bilateral perfusion of ouabain. Use of bodipy-FL-ouabain, a fluorescent analog of ouabain, allowed determination of the extent of its diffusion *in vivo*. Acb, accumbens; Cpu, caudate/putamen; Gp, globus pallidus; Snr, substantia nigra; Sth, subthalamic nucleus. (**b**) Merge of the bright field picture of a coronal section of the basal ganglia with the fluorescence intensity profile obtained after perfusion of bodipy-FL-ouabain (18 ng h^{-1}) for 72 h. (**c**) The concentration of ouabain in the tissue was estimated by quantitative fluorescence microscopy using the fluorescence emitted by bodipy-FL-ouabain. The highest concentration of ouabain was obtained close to the canula and dropped monotonically as a function of lateral distance. The key applies to the whole figure. (**d**) Hunched posture, rigidity and akinesia in a mouse after 72 h chronic perfusion of ouabain (36 ng h^{-1}). (**e–h**) Effects of chronic perfusion of 0–72 ng h^{-1} ouabain into the basal ganglia on overall locomotion (**e**), speed (**f**), average number of steps (**g**) and step size (**h**) as a function of time after start of ouabain perfusion (mean ± s.e.m.; $n = 4$ for vehicle, $n = 4$ for 7.2 ng h^{-1} ouabain, $n = 13$ for 18 ng h^{-1} ouabain, $n = 11$ for 36 ng h^{-1} ouabain and $n = 3$ for 72 ng h^{-1} ouabain). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and ns indicates not significant ($P > 0.05$). (**i**) Assessment of the effect of bilateral perfusion of ouabain into the basal ganglia on motor function using a dystonia rating scale for the animals described above. With the scale used, only a score of 2 or more denotes dystonia. Error bars indicate mean ± s.e.m. (**j**) Summary of symptoms associated with chronic bilateral perfusion of ouabain into the basal ganglia for 24, 48 and 72 h.

cause involuntary dystonic-like movements in any of the animals examined at any time or concentration, even when monitored for >8 d ($F = 3.06$, $P = 0.064$; **Fig. 1i,j**). Similarly, chronic perfusion of high amounts of ouabain into the lateral ventricles for several days (360 ng h^{-1}) or primarily targeting individual basal ganglia output nuclei (globus pallidus, substantia nigra and the entopeduncular nucleus) did not produce any form of dystonic postures (**Supplementary Data 2**).

Cerebellar dysfunction produces ataxia and dystonia

We next examined the potential role of the cerebellum in RDP, as a role for the cerebellum in producing dystonia is not unprecedented[16,17] and its principal neurons, the Purkinje cells, exclusively express the α3 isoform of the sodium pumps that is mutated in RDP[18]. Without affecting the neighboring brain regions, cerebellar sodium pumps were selectively targeted by midline chronic perfusion of ouabain *in vivo* ($n = 51$; **Fig. 2a–c**). Cerebellar perfusion of ouabain resulted in ataxia and clear dystonic-like postures (**Fig. 2d** and **Supplementary Video 2**) in a time- and concentration-dependent manner. This was quantitatively reflected as reduced locomotion ($F = 25.43$, $P < 0.0001$) and as high dystonia scores ($F = 7.8$, $P < 0.0003$; **Fig. 2e,f**). The motor symptoms appeared initially as ataxia, which in time transformed into dystonic-like postures and (with a higher concentration of ouabain) generalized dystonia (**Fig. 2d,g**).

Electromyogram (EMG) recordings in agonist and antagonist muscles of the hind limb and examination of the corresponding cross-correlations of the EMG signals confirmed that the dystonic-like postures were caused by prolonged co-contraction of the opposing muscle groups ($n = 3$; **Supplementary Data 3**). These dystonic episodes were not caused by seizures, as electroencephalogram (EEG) recordings revealed that they were not accompanied by epileptic activity in the motor cortex ($n = 5$; **Supplementary Data 3**). Moreover, targeting perfusion of ouabain to only one of the cerebellar hemispheres generated comparable, but unilateral, symptoms.

Stress-induced dystonia

These data are consistent with the involvement of both the basal ganglia and cerebellum in generation of RDP symptoms. However, a hallmark of RDP is that subjects carrying the defective gene do not fully manifest the symptoms until a very stressful event, which then abruptly produces the disease phenotype. We sought to determine whether we could replicate this aspect of the disorder.

With concomitant perfusion of low (18 ng h^{-1}) concentrations of ouabain into both the basal ganglia and the cerebellum, the mice showed mild, but stable, dyskinesia, but not dystonia ($n = 7$; **Supplementary Data 4** and **Supplementary Video 3**). This is somewhat similar to the pre-stress condition in individuals with RDP. Individuals carrying the mutated gene are not always completely free of symptoms and show, to a varying extent, Parkinsonism, cramping and mild focal dystonic spasms in arms and limbs[5]. We then tested whether subjecting these mice to stress could precipitate the permanent RDP symptoms.

In RDP, the stressful event can be both psychological and physical in nature, with physical exertion and elevated body temperature being common triggers[5]. Following brief chronic perfusion of 18 ng h^{-1} ouabain (typically for 10–24 h) mice were stressed for 2 h by random exposure to electric foot shocks in a warm environment (≈38 °C). In 10 out of 14 animals, examined stress immediately increased the severity of dyskinesia and resulted in generation of mild to severe dystonia ($P < 0.001$; **Fig. 3** and **Supplementary Video 4**). Similar to what is seen in individuals with RDP, behavioral observations and EMG recordings confirmed that the dystonia in these mice persisted for as long as monitored, although the severity sometimes lessened (**Fig. 3d**). In 4 of the 14 animals, examined stress did not affect the dyskinesia even when repeated a second time.

We also performed experiments in mice in which the cerebellum and basal ganglia were concomitantly perfused with a higher concentration of ouabain (36 ng h^{-1}, $n = 9$). In these cases, the mice

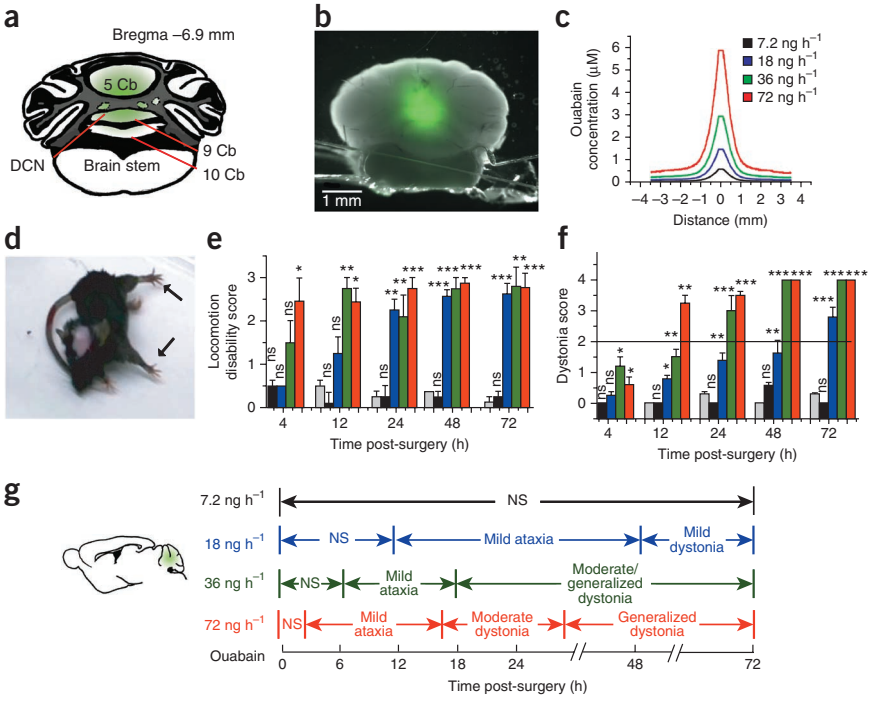

Figure 2 Chronic partial blockade of cerebellar sodium pumps results in ataxia and dystonic-like postures. (**a**) Coronal schematic of the cerebellum showing the regions affected by chronic perfusion of ouabain. Cb, cerebellum; DCN, deep cerebellar nuclei. (**b**) Merge of the bright field picture of a coronal section of the cerebellum with the fluorescence intensity profile obtained after perfusion of bodipy-FL-ouabain (18 ng h^{-1}) for 18 h. (**c**) Estimated concentration of ouabain in the cerebellum as a function of lateral distance from the center of the cannula with different concentrations of perfused ouabain. (**d**) Dystonic postures in a mouse caused by perfusion of ouabain (36 ng h^{-1}) into the cerebellum for 18 h. Arrows point to the commonly observed hyperextensions of anterior and posterior limbs. (**e**) Effects of chronic perfusion of 0–72 ng h^{-1} ouabain into the cerebellum on locomotor activity in mice at 24, 48 and 72 h. The decreases in locomotion reflected in the locomotion disability scores were a reflection of the ataxia and dystonia in these mice and not akinesia and rigidity as seen when ouabain was perfused into the basal ganglia. Error bars indicate mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and ns indicates not significant ($P > 0.05$). (**f**) Severity of ouabain-induced dystonia in mice chronically perfused with 0–72 ng h^{-1} ouabain into the cerebellum at 4, 12, 24, 48 and 72 h. Error bars indicate mean ± s.e.m. (**g**) Symptoms associated with chronic perfusion of ouabain into the cerebellum. NS, no symptoms.

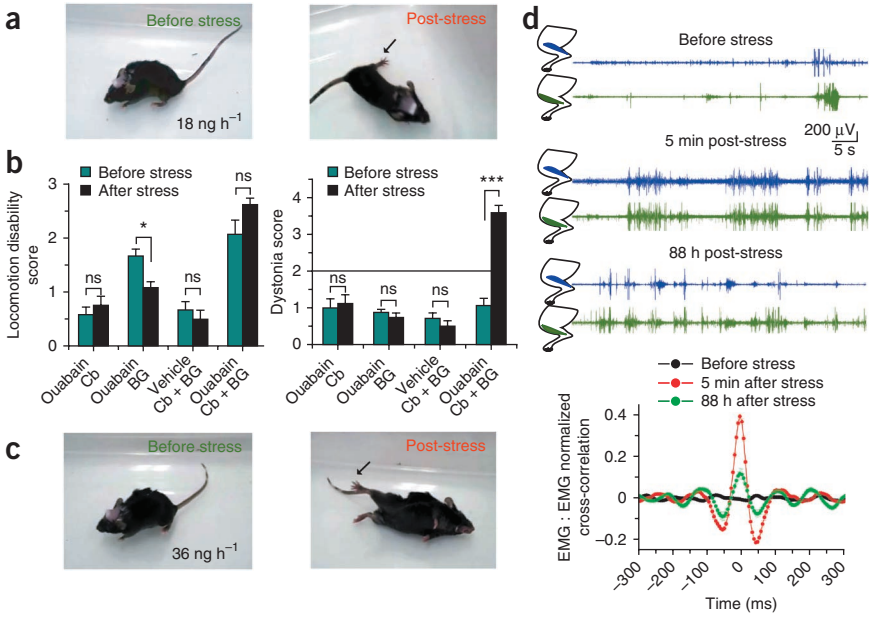

Figure 3 Stress-induced dystonia in mice requires interaction between cerebellar and basal ganglia motor control loops. (**a**) Concomitant perfusion of ouabain (18 ng h^{-1}) into the cerebellum and basal ganglia for 24 h produces reduced locomotion and mild gait disturbance (before stress). Immediately after exposing the animal to severe stress, the mouse developed persistent dystonic postures. Arrow points to hyperextension of posterior limb. (**b**) The effect of stress on locomotion and dystonia in mice chronically perfused with ouabain (18 ng h^{-1}) only in the cerebellum or basal ganglia (BG), or concomitantly in both structures. Stress induced dystonia only in animals in which both the cerebellum and basal ganglia were concomitantly perfused with ouabain. $*P < 0.05$, $***P < 0.001$ and ns indicates not significant ($P > 0.05$). (**c**) EMGs recorded from agonist and antagonist anterior cranial tibial and gastrocnemius muscles in a mouse in which the cerebellum and basal ganglia were concurrently perfused with ouabain (18 ng h^{-1}) for 24 h. Before exposure to stress, the mouse showed reduced locomotion but rarely showed co-contraction of the two muscles. Persistent co-contraction of the two muscles could be seen for several seconds 5 min post-stress. These co-contractions were reduced in frequency and intensity but were nonetheless notable even 3 d later. The graph at the bottom shows normalized cross-correlation of the two EMG signals (mean ± s.e.m.). Significant cross-correlation in the activity of the two muscles was observed after but not before stress. Note that the EMG traces used for analysis correspond to times at which significant activity in at least one muscle was detected. (**d**) More severe symptoms in the form of reduced locomotion and gait disturbance/mild ataxia in a mouse concomitantly perfused with ouabain (36 ng h^{-1}) into the cerebellum and basal ganglia for 5 h (before stress). Subjecting the mouse to the stress procedure resulted in generalized dystonia (post-stress), including the distortion of the lower jaw.

were subjected to the stress procedure 4–6 h after perfusion. Prior to the stressful episode, the mice showed gait disturbance, mild ataxia and reduced locomotion, but no detectable signs of dystonia. Immediately after exposure to the stress procedure, the mice showed severe generalized dystonia (**Fig. 3** and **Supplementary Video 5**). These symptoms did not show signs of improvement for the duration of the observation (in one case up to 30 h).

Notably, in none of the mice examined when the cerebellum or basal ganglia were perfused in isolation with 18 ng h^{-1} ouabain (even for as long as 36 h) did stress produce dystonia or worsen the motor symptoms ($n = 23$ for cerebellum and 12 for basal ganglia; **Fig. 3** and **Supplementary Videos 6** and **7**). In some animals, the stress procedure was repeated twice. Thus, concomitant perfusion of both the cerebellum and the basal ganglia is absolutely required for stress to produce dystonia, suggesting that an interaction between the two dysfunctional structures is essential for stress-induced dystonia in RDP. An adverse interaction between dysfunctional cerebellum and basal ganglia has been reported previously[19].

Dystonia is associated with aberrant cerebellar activity

These results indicate that concurrent perfusion of low concentrations of ouabain into the cerebellum and basal ganglia replicates the salient features of RDP; the mice had few motor symptoms before a stressful event and stress rapidly triggered persistent dystonia. As perfusion of higher concentrations of ouabain into the cerebellum alone caused dystonia, we initially used this procedure to explore the mechanism by which dysfunction of cerebellar sodium pumps can cause dystonia. Because several lines of evidence suggest that aberrant cerebellar activity can cause dystonia[16,20,21], we examined whether the dystonic postures in our animals were accompanied with abnormal cerebellar activity.

We first simultaneously recorded EEGs from the motor cortex and the cerebellum of mice in which the cerebellum alone was perfused with concentrations of ouabain that produced dystonia. In the absence of motor dysfunction, EEGs from the cerebellum and motor cortex showed no sign of overt activity. However, once the mice developed intermittent dystonic postures, we found a tight correlation between dystonic postures and abnormal cerebellar EEG activity, but not between dystonic postures and cortical EEG activity ($n = 4$; **Fig. 4**). During dystonic postures, the mean cerebellar EEG signal amplitude increased by 138% compared with its average in the absence of dystonia ($P < 0.001$; **Fig. 4c**). In contrast, during the same dystonic episodes, there was little hyperactivity present in the simultaneously recorded motor cortex EEG (**Fig. 4a**) and on average its mean amplitude during dystonia was only ~33% higher than its average in the absence of dystonia ($P = 0.023$; **Fig. 4c**). To ascertain accurate electrode positioning and to examine the relative sensitivities of the cortical and cerebellar EEG electrodes, we induced status epilepticus in the same mice with pilocarpine. Both the cortical and cerebellar EEGs reported significant activity during epilepsy, with the average cortical EEG signal amplitude increasing by more than 175% ($P < 0.001$; **Fig. 4b**,**c**). We next examined whether the stress-induced dystonia when both the cerebellum and basal ganglia were perfused with ouabain was also accompanied with abnormal cerebellar activity and in all cases found it to be so ($n = 3$; **Fig. 4d**).

Modest role of motor cortex in cerebellar-induced dystonia

The small increase in the amplitude of the cortical EEG signal during cerebellar-induced dystonic postures could, in part, be accounted for by feedback in the cortico-cerebellar and cortico-striatal loops and it is therefore plausible that motor cortex has only a modest role in the generation of dystonia. To directly test this possibility, we bilaterally silenced motor cortex by blocking voltage-gated sodium channels with tetrodotoxin (TTX) and examined the consequences for cerebellar-induced dystonia. The efficacy of TTX in silencing the

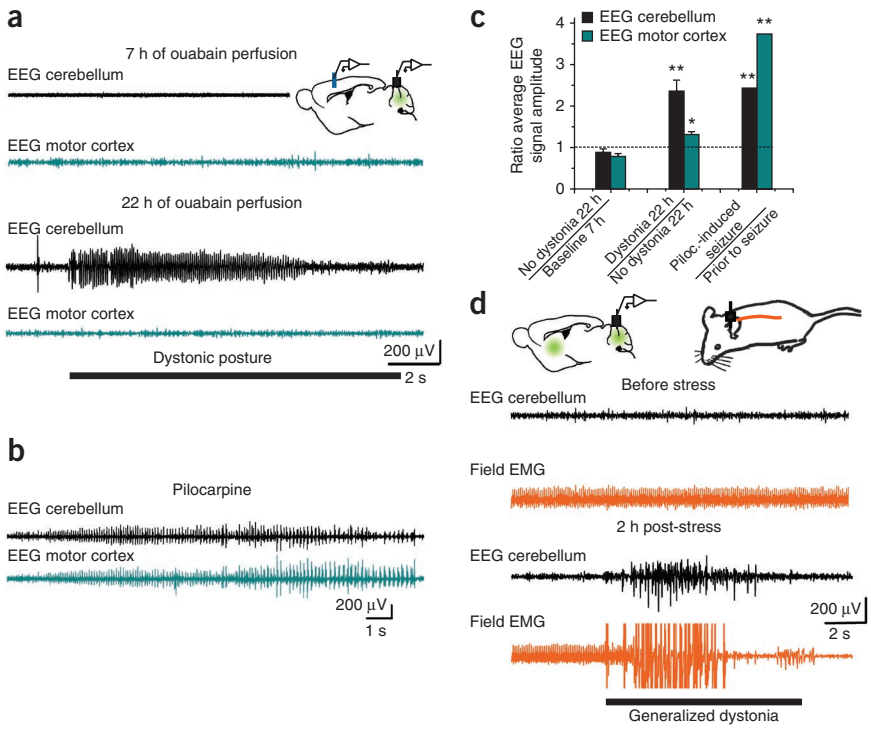

Figure 4 Dystonic postures correlate with abnormal cerebellar activity. (**a**) EEG recordings from the motor cortex and the cerebellum of a mouse whose cerebellum was chronically perfused with ouabain (36 ng h^{-1}). The first pair of traces (7 h perfusion) were obtained before any noticeable dystonic postures. Neither the cerebellar EEG (black) nor the motor cortex EEG (cyan) showed abnormal activity. The second pair of traces (22 h perfusion) were recorded concurrent with a dystonic posture in the mouse and showed an abnormal cerebellar EEG signal, whereas the motor cortex EEG was unremarkable. (**b**) Abnormal electrical activity in both the cerebellum and the motor cortex of the same mouse during status epilepticus induced by 300 mg per kg of body weight pilocarpine. (**c**) The ratios of average cerebellar and motor cortex EEG signal amplitude under various conditions. The average cerebellar EEG signal amplitude was about 2.5-fold larger when the animal manifested dystonic postures compared with time periods straddling dystonia. At exactly the same time periods, the corresponding change in the average motor cortex EEG signal amplitude was much smaller. Both the cerebellar and motor cortex EEG signal amplitudes increased substantially during pilocarpine-induced seizures. Error bars represent mean ± s.e.m. $*P < 0.05$, $**P < 0.01$. (**d**) EEG activity in the cerebellum (black) and field EMG signals from the back muscles (orange) were recorded in a mouse in which the cerebellum and basal ganglia were concomitantly perfused with ouabain (36 ng h^{-1}). The low impedance of the electrode used for field EMG recording permitted the detection of concerted activity of a large group of muscle fibers as an accurate marker of the episodes of generalized dystonia. Prior to stress, the mouse showed mild ataxia/gait disturbance but no dystonic postures. The heart rate was noted in the field EMG with no apparent signs of abnormal activity. The cerebellar EEG signal also did not show any abnormal activity. Exposure of the same mouse to stress (the pair of traces labeled as 2 h post-stress) precipitated repeated dystonic postures, the timings of which were reflected in the field EMG signal. The cerebellar EEG showed abnormal hyperactivity of the cerebellum concurrent with the dystonic postures. In this instance, the episode of generalized dystonia partly dislodged the field EMG wire, which reduced the amplitude of the EKG to within noise levels.

motor cortex was ascertained by EEG recordings (**Supplementary Data 5**). In all eight mice examined, silencing the motor cortex made the animals flaccid and reduced the occurrence of spontaneous dystonia. However, sometimes spontaneously and more often with physical perturbation, the mice showed clear dystonia, although the severity was reduced (average dystonia score reduced from 3.7 ± 0.1 to 2.8 ± 0.3, $P = 0.041$, $n = 8$; **Supplementary Video 8**). These findings suggest that, although cortical activity clearly contributes to the severity and frequency of cerebellar-induced dystonic postures, dystonia can nonetheless manifest in the absence of overt motor cortical activity.

Curbing aberrant cerebellar output alleviates dystonia

If ouabain-induced aberrant cerebellar activity is the main cause of dystonia then reducing aberrant activity or eliminating cerebellar output should lessen dystonia. The sodium pump is electrogenic and contributes to the resting membrane potential. We reasoned that the primary cause of the aberrant cerebellar activity when sodium pumps were partially blocked was depolarization of cerebellar neurons. If this were the case, pharmacologically hyperpolarizing the cells with GABA should partially restore their activity to normal and one would therefore predict that acute perfusion of GABA into the cerebellum of ouabain-perfused mice might lessen dystonia. To test this hypothesis, we induced dystonia in mice by chronic cerebellar perfusion of ouabain using a canula that also permitted acute perfusions using a secondary port. Acute perfusion of GABA into the cerebellum of dystonic mice lessened the frequency and severity of dystonic postures and improved their performance on a treadmill ($n = 4$; **Fig. 5a** and **Supplementary Video 9**).

We also examined whether silencing cerebellar output was capable of preventing cerebellar-induced dystonia. The majority of the cerebellar output is routed through the cerebellar nuclei. Consistent with our working hypothesis, we found that bilateral lesioning of cerebellar nuclei significantly reduced the severity of the dyskinesia produced by chronic perfusion of ouabain into the cerebellum ($n = 7$, $F = 4.01$, $P = 0.03$, **Fig. 5b**,**c** and **Supplementary Video 10**). These data are consistent with the hypothesis that aberrant cerebellar activity in RDP makes a major contribution to the generation of involuntary dystonic movements.

Role of cerebellum→basal ganglia di-synaptic connection

Given the predominance of reports implicating basal ganglia as the primary instigator in most dystonias[1], it would be of value if a unifying hypothesis could be formulated to account for the potential contribution of each of these two brain regions, the basal ganglia and the cerebellum, in the induction of dystonia. In an attempt to put forth such a hypothesis, we postulated that in cerebellar-induced dystonia the aberrant cerebellar activity may cause dystonia by dynamically forcing the dysfunction of the basal ganglia. This could be the case, for example, if the cerebellar output has a substantial effect on the activity and function of the basal ganglia. In support of such a supposition, it has been reported that altering the activity of the cerebellar output nuclei alters neuronal firing rates[22,23] and dopamine levels in the basal ganglia[19,24]. Indeed, a direct substrate for an interaction between the cerebellum and the basal ganglia motor loops might be the di-synaptic pathways that connect the two structures[25,26]. One approach to test

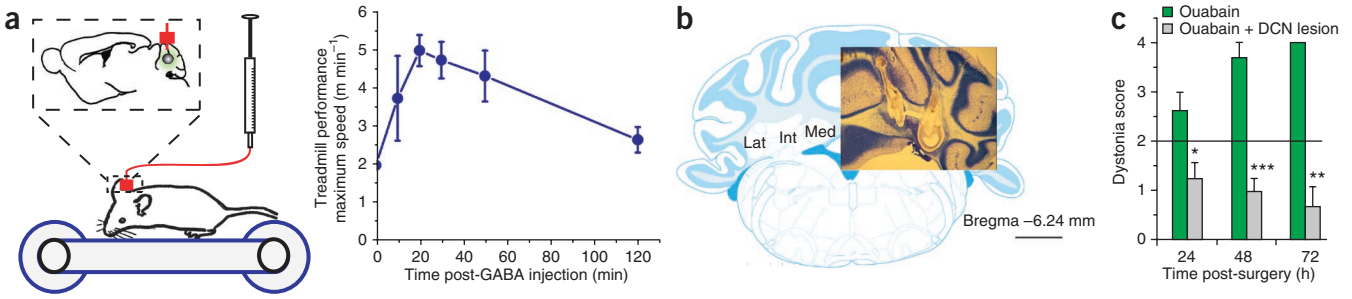

Figure 5 Reducing aberrant cerebellar activity or silencing cerebellar output lessens dystonia. (**a**) Mice whose cerebella were chronically perfused with ouabain (36 ng h^{-1}) showed clear signs of dystonia and were unable to walk on a treadmill at its lowest speed setting of 2 m min^{-1}. Acute injection of GABA into their cerebellum using the auxiliary port of the same cannula used for ouabain perfusion reduced the severity of their dystonic postures such that, on average, the mice could walk on the treadmill at a pace of 5 m min^{-1} ($n = 4$, mean ± s.e.m.). (**b**) Deep cerebellar nuclei were electrically lesioned in both cerebellar hemispheres at either one or two sites (the two-site lesion is shown here). Comparable data was obtained with both approaches. Scale bar, 1 mm. (**c**) Chronic perfusion of ouabain (36 ng h^{-1}) into the cerebellum of deep cerebellar nuclei–lesioned mice did not produce dystonia ($n = 7$, mean ± s.e.m.). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

this hypothesis would be to sever the link between the cerebellum and the basal ganglia; if the cerebellum induces dystonia by altering basal ganglia function then breaking the connection should alleviate dystonia. We sought to experimentally test this hypothesis.

One of the most prominent di-synaptic connections from the cerebellum to the basal ganglia is routed via the centrolateral nucleus of the thalamus[26]. We optimized the parameters needed for selective electrical lesioning of the centrolateral nucleus (**Fig. 6a**) and found that its selective ablation (confirmed by histology post-mortem) significantly reduced the motor symptoms associated with chronic perfusion of ouabain into the cerebellum ($n = 14$; **Fig. 6b**). The centrolaterally lesioned animals in general did not show dystonia, even when monitored for more than 90 h after start of cerebellar ouabain perfusion ($F = 3.51$, $P = 0.023$; **Fig. 6b** and **Supplementary Video 11**). We found that, if the lesions were off target and affected other thalamic nuclei, the dystonia did not improve and in most cases worsened. This suggests that the beneficial effects of centrolateral nucleus lesions were not simply the consequence of generalized thalamotomy, which can sometimes improve dystonia[27]. On the basis of these results, it is plausible that inactivation of the centrolateral nucleus of the thalamus or the output of the cerebellum, either by lesioning or perhaps by deep brain stimulation, might constitute plausible therapeutic approaches for individuals with RDP. Indeed, such an approach might be of value in other cerebellar-induced dystonias.

DISCUSSION

Dystonia is a devastating movement disorder. However, our understanding of its pathophysiology remains incomplete. A major handicap in dystonia research is the limited availability of animal models of identified human dystonia that faithfully mimic the dystonic symptoms experienced by patients[2,28]. This shortcoming persists even in genetic mouse models in which the engineered mice express the mutated genes that are known to be the cause of various hereditary dystonias[2]. Although these transgenic mice are informative[2,28], the need for animal models that actually display dystonia and mimic human symptoms cannot be overstated. In this context, RDP presents a challenge, as the individuals with RDP not only have dystonia, but also show Parkinsonism combined with a number of other motor symptoms. Moreover, the symptoms are precipitated by an episode of severe stress.

The ouabain-based pharmacological animal model that we generated reproduces all of the salient features of RDP. We found that, when sodium pumps are partially blocked both in the cerebellum and in the basal ganglia, the mice show mild signs of motor dysfunction. This condition mimics the mild symptoms seen in some subjects affected with the mutated RDP gene *ATP1A3* before experiencing severe stress. Furthermore, in the model stress rapidly caused severe motor dysfunction including dystonia and Parkinsonism-like symptoms such as akinesia similar to that seen in individuals with RDP.

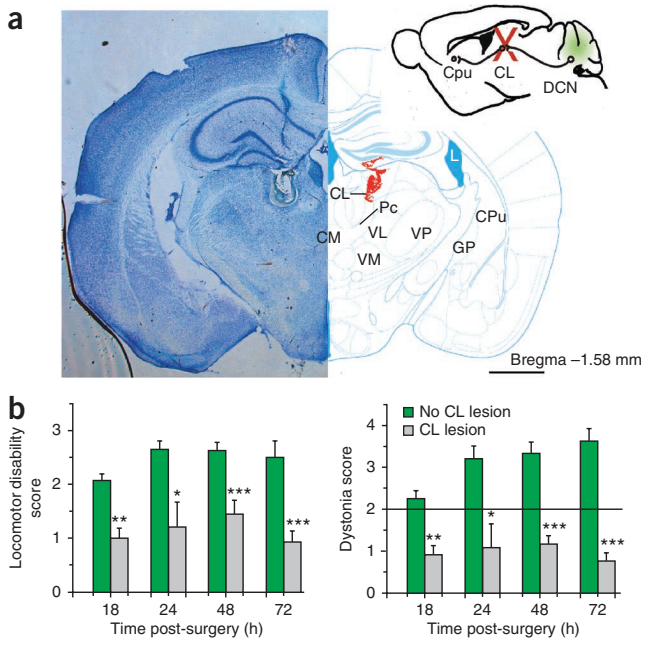

Figure 6 Severing the link between the cerebellum and basal ganglia alleviates dystonia. (**a**) Using bilateral electrical lesions, the centrolateral nucleus of the thalamus was selectively ablated. The photograph on the left shows a Nissl stain of one such lesion and the schematic on the right shows the relevant brain structures with the lesion area marked in red. Scale bar, 1 mm. Nuclei of the thalamus: CL, centrolateral; CM, centromedian; Pc, Paracentral; VL, ventral lateral; VM, ventral medial; VP, ventral posterior. (**b**) The consequences of centrolateral lesions (or sham operations noted as no CL lesion) on the motor symptoms associated with chronic perfusion of ouabain (36 ng h^{-1}) into the cerebellum of mice was determined by assessing their locomotion and dystonia scores. Lesioning the centrolateral nucleus significantly reduced ouabain-induced motor dysfunction and prevented generation of dystonia. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Paralleling the human disorder, these symptoms outlasted the stress and persisted until the animals were killed. Thus, our results suggest that the ouabain-based model is a reasonable animal model of RDP, indicate that the basal ganglia and cerebellum are the primary sites of dysfunction in this disorder, and offer potential therapeutic interventions for alleviating or lessening the severity of the motor symptoms in individuals with RDP.

Our data implicate two structures in the pathophysiology of RDP: the basal ganglia and the cerebellum. A role for the basal ganglia in RDP was not unexpected; individuals with RDP show Parkinsonism-like symptoms, a hallmark of basal ganglia dysfunction. Our data clearly corroborate the hypothesis that a reduction in the overall function of sodium pumps in the basal ganglia, as is likely to occur in RDP, may be the cause of the Parkinsonism-like symptoms seen in individuals with RDP. Considering a wider context, the crouched posture, akinesia and tremor seen in mice following partial blockade of basal ganglia sodium pumps closely resemble the actual symptoms of individuals suffering from Parkinsonism and provide an attractive animal model of this disorder.

What remains to be established is the mechanism by which partial dysfunction of sodium pumps in the basal ganglia causes Parkinsonism. One possibility is that reduced activity of sodium pumps might have affected presynaptic dopaminergic axons and nerve terminals. Because sodium pumps are electrogenic and contribute to maintaining the membrane resting potential, their dysfunction can depolarize nerve endings and render the axon incapable of supporting an action potential or liable to propagation failure[29]. Alternatively, the symptoms may have been caused by reduced expression of the D1 dopamine receptors and altered phosphorylation levels, which can occur with chronic ouabain perfusion[30,31]. The reduced dopamine signaling in the basal ganglia caused by either of these mechanisms may account for the Parkinsonism-like symptoms seen in our animal model and in individuals with RDP, although this issue requires further scrutiny.

Our findings also implicate cerebellar dysfunction in RDP and suggest that it makes a major contribution to dystonia. There are a number of patient case reports that implicate cerebellar dysfunction in dystonia[32–34]. Surgically lesioning the dentate cerebellar nuclei in some of these individuals improved dystonia[32,33], suggesting that, in these and some other cases[35–40], the cerebellum might have been a major contributing factor to dystonia. Indeed, abnormal cerebellar activity has been noted frequently in dystonic individuals, although these observations have routinely been interpreted in the context of cerebellar compensation of dysfunctional basal ganglia[17].

The evidence amassed in patients in support of a direct role of the cerebellum in some dystonias is also corroborated with observations in animal models of dystonia. Injection of kainic acid into the cerebellum results in dystonia[16] and abnormal cerebellar activity has been implicated in several rodent strains afflicted with spontaneous mutations that render them dystonic[21,41–43]. In some of these animals, cerebellectomy, or Purkinje cell degeneration, has been shown to alleviate dystonia alas at the expense of producing ataxia[43,44]. Thus, as summarized recently[17], it might be important and timely to re-evaluate the role of the cerebellum in dystonia in general.

The mechanisms by which partial dysfunction of cerebellar sodium pumps result in aberrant cerebellar activity are not understood. However, it is noteworthy that, in contrast with most neurons that express a combination of different isoforms of the sodium pump, cerebellar Purkinje cells exclusively express the α3 isoform[18] (the isoform affected in individuals with RDP[4]). Thus, although neurons can upregulate other pump isoforms to compensate for a dysfunctional α3 protein, Purkinje cells lack this option. It is plausible that the outward current contributed by the sodium pumps might functionally be an integral part of pacemaking in these neurons. In the absence of any compensatory mechanisms, the reduction in the sodium pump current that occurs as a consequence of the RDP mutations may affect the activity of Purkinje cells and possibly cause the aberrant cerebellar activity that we observed.

Our findings suggest an intricate interaction between the cerebellar and basal ganglia motor control systems. There is a strong body of anatomical and functional evidence in support of reciprocal interactions between these two structures. In the cat, electrical stimulation of the cerebellar output nuclei (dentate) alters the rate of firing of neurons in the caudate nucleus[22] and, to a lesser extent, in globus pallidus[23]. Electrical stimulation of the dentate nuclei was also shown to alter dopamine levels in both the substantia nigra and caudate of the cat[24]. A substantial change in dopamine levels in the basal ganglia of mice after acute cerebellar injection of kainic acid has also been noted[19]. These observations are supported by anatomical studies that found direct and indirect projections from the cerebellum to the basal ganglia[25,26].

One aspect of our study is that we were able to reproduce the stress-induced dystonia seen in individuals with RDP. We do not understand why dysfunction of both the basal ganglia and the cerebellum are required for stress-induced dystonia, nor do we know the mechanism by which stress results in generation of permanent symptoms. Both of these questions require further examination. Speculatively, one potential mechanism by which stress might exacerbate partial dysfunction of sodium pumps is by increasing endo-ouabain levels in the brain. Endo-ouabain is produced by adrenal cortex and the hypothalamus and is indistinguishable from ouabain[45]. Notably, stress and exercise increase endo-ouabain levels by as much as 18-fold[46,47]. We found that perfusion of higher concentrations of ouabain into the cerebellum and basal ganglia precipitated the symptoms seen in RDP in the absence of stress. Thus, by increasing the concentration of endo-ouabain, stress and activities commonly associated with triggers of RDP are likely to increase the fraction of dysfunctional sodium pumps in the cerebellum and the basal ganglia, thus prompting the symptoms.

Limitations and therapeutic implications

Our model suffers from a number of caveats, one of which is the suitability of rodents as an accurate model of human neurological or behavioral disorders. In this context, it is likely that motor control in quadrupeds is different from that of primates, including humans. This concern, however, is balanced by the preceding discussions which highlight the consistency of our findings with many prior observations in individuals with RDP.

Another caveat is that in our studies we targeted a limited number of specific brain regions with a blocker that, despite its selectivity for the α3 sodium pumps, can nevertheless block other isoforms. In RDP, in contrast, only the α3 sodium pump isoform is dysfunctional and the mutated protein is expressed throughout the brain. At face value, the best pharmacological approach might be to perfuse ouabain into the ventricles. However, our preliminary attempts corroborated the finding that perfusion of ouabain into the ventricles results in seizures[48,49], and we failed to detect any motor symptoms prior and post-stress when we used lower concentrations of ouabain. Because it is tremendously difficult to chronically perfuse two brain regions and simultaneously monitor EEG and EMG signals in mice, it was not practical to survey additional brain regions in a reasonable time frame. Despite this shortcoming, we believe that the choice of the structures that we examined were well justified. Nonetheless, we look

forward to the development of a genetic mouse model of RDP in which to test our findings. Obviously, a genetic RDP mouse model that shows a clear phenotype will be of immense value because, in addition to allowing for confirmation of our findings, it will provide a far less laborious model than ours for exploring potential therapeutic approaches for this disorder.

Two sets of observations made here have potential implications for treatment of RDP. First, our data suggest that lesions or deep brain stimulation of structures that mediate adverse interactions between the basal ganglia and the cerebellum might be of some therapeutic value. Second, our data also suggest that aberrant activity of the cerebellum might be a major contributing factor to dystonia in RDP. Accordingly, pharmacological approaches that restore cerebellar activity to normalcy or lesions/deep brain stimulation of its output nuclei might also lessen associated symptoms in individuals with RDP. Obviously both of these approaches require rigorous scrutiny.

A unifying hypothesis

Our finding that aberrant cerebellar activity can have an adverse effect on the basal ganglia via the thalamic di-synaptic pathways provides a plausible unifying working hypothesis to account for the role of each of these two structures in the generation of dystonia. Concurrently, our findings underscore the importance of basal ganglia dysfunction in the generation of dystonia, even in cases in which the primary instigator is elsewhere in the brain (in this case the cerebellum). It is therefore plausible that aberrant activity of other structures that innervate the basal ganglia might also be capable of disrupting basal ganglia function and causing dystonia.

The fact that a neuron can burst and fire erratically in the absence of any substantial change in its average firing rate aberrant activity does not necessarily translate into hyper- or hypo-activity when assayed with imaging techniques. Thus, it might be fruitful to explore the potential dysfunction of basal ganglia input structures (such as the cerebellum) in other hereditary and idiopathic dystonias even in cases in which imaging data have not specifically pointed to their hypo- or hyperactivity.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

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

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AUTHOR CONTRIBUTIONS

The studies were initiated by K.K. and D.P.C., who designed the bulk of the experiments and wrote the manuscript. D.P.C. performed all of the experiments except those in **Figures 5b,c** and **6**. R.F. designed and carried out the experiments in **Figure 6** and designed and helped F.K. perform the experiments shown in **Figures 5b,c**. R.F. also contributed to the experiments reported in the **Supplementary Information**.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Experiments were performed on 8–10-week-old C57/BL6 mice in accord with the guidelines set by Albert Einstein College of Medicine. The behavior of each animal before and after surgery, perfusion of drugs or exposure to the stress procedure was documented by video recordings. To minimize pain experienced by the animals, we monitored mice that exhibited severe dystonia or Parkinsonism symptoms for only a few days and then killed them. To lessen the pain of these animals, we gave them the long-lasting pain reliever Flunixin. It is important to note that in these instances we did not detect any symptoms aside form the movement disorders described, suggesting that long-term perfusion of ouabain with the methods employed here did not have nonspecific toxic effects on the CNS.

Chronic perfusion of the basal ganglia. To chronically perfuse the basal ganglia, we stereotaxically implanted bilateral canula (Plastics One; anteroposterior (AP), 0.74 mm from bregma; mediolateral (ML), 1.5 mm; dorsoventral (DV), 4 mm) and we connected them to two osmotic pumps (model 1007D, 0.25 µl h^{-1}, Alzet), which were placed under the skin on the back of the mice. The concentration of ouabain solution was set such that 7.2–72 ng of ouabain were dispensed by the pump every hour. Ouabain was dissolved in water and 0.01% methylene blue (wt/vol) was added to the solution to allow visual confirmation of the perfusion site post-mortem. Immediately after each surgical procedure, and every 12 h thereafter, Flunixin was administered subcutaneously.

Chronic perfusion of the cerebellum. The cerebellum was perfused at the midline (AP, −6.90 mm from bregma; DV, 3 mm) following the procedures described above. For some experiments, a perfusion canula with a second port was used to perfuse the cerebellum. This auxiliary port allowed for acute injection of drugs directly into the cerebellum. For acute injections, 5 µl of the desired solution was injected over a period of 15 min using an automated pump.

Chronic perfusion of lateral ventricles. The lateral ventricles were perfused using a single canula with 360 ng h^{-1} of ouabain using the coordinates at −0.5 mm AP, 1.0 mm ML and −1.6 mm DV from bregma.

Concurrent perfusion of the basal ganglia and the cerebellum. For concurrent perfusion of the basal ganglia and the cerebellum, we used the same coordinates delineated above. The procedure for perfusion of the cerebellum was identical. However, because mice can maximally carry only two osmotic pumps, perfusion of the basal ganglia required the use of a 'Y' bifurcation canula (Plastics One) that was connected to a single pump filled with double the concentration of ouabain used for cerebellum.

Acute injections of ouabain into the cerebellum and select basal ganglia nuclei. For acute injections, guide canula were stereotaxically implanted at the target location and 2.5 or 5 µl of the desired solution was injected over a period of 15 min using an automated pump. The same coordinate used for chronic perfusion were used in the acute injection of 5 µl of solution into the midline cerebellum. To target select basal ganglia output nuclei, we bilaterally positioned guide canula and independently injected 2.5 µl of the solution to each side using the following coordinates: the entopeduncular nucleus (AP, −1.34 mm, ML, 1.5 mm; DV, 4.5 mm), the globus pallidus (AP, −0.58 mm; ML, 1.9 mm; DV, 4 mm) and the substantia nigra (AP, −3.28 mm; ML, 1.5 mm; DV, 4.5 mm).

EMG and EEG recordings. For EMG recordings, electrodes were surgically inserted into the gastronemius (extensor) and cranial tibial muscle (flexor), respectively. Thin Teflon-coated EMG wires were routed underneath the skin and attached to a connector secured on the skull. For field EMG recordings, a wire underneath the skin on the back of the animal was positioned to terminate 2/3 of the way between the neck and the tail. The low impedance of the exposed end of the wire permitted pick up of electrical signals over relatively long distances. In addition, also as a consequence of its very low impedance, the wire only registered large electrical changes and thus a detectable signal correlated with concerted activity of large groups of excitable elements (in this case, mainly muscle fibers). This technique is analogous to extracellular field recordings in the CNS in which a low impedance electrode is positioned above a region and registers concurrent activity of a large number of neurons.

To perform EEG recordings, we stereotaxically implanted a bipolar electrode (MS 303, Plastics One) or a screw into the motor cortex at −1 mm AP, 1 mm ML and 1 mm DV from bregma. To record the cerebellar EEG, or in experiments in which motor cortex EEG was recorded while TTX was injected into the cortex, the same canula used to deliver ouabain into the cerebellum or TTX into the cortex was used as the EEG electrode (the canula is conductive throughout its length). EEG, EMG and field EMG signals were monitored by attaching the headstage of a Pinnacle Technology EEG/EMG recording system (4100 USB Data acquisition and conditioning system) to the connection platform secured on the skull just before the recording session. In all cases, the segments of data used for analysis contained at least one channel in which substantial muscle activity was evident. To calculate cross correlation, we normalized signals to their respective s.d. such that two identical input signals or signals that are scaled versions of one another yielded a cross correlation value of 1 (ref. 50). To induce seizures, we subcutaneously injected mice with pilocarpine hydrochloride (300 mg per kg, Sigma). Methyl scopolamine nitrate (1 mg per kg, Sigma) was injected subcutaneously 30 min before pilocarpine to minimize peripheral cholinergic effects.

Stress procedure. Electric foot shocks were delivered via the grid floor of a custom-made plastic box (30 cm × 22 cm × 30 cm). Electric shocks of 250-ms duration (6-mA maximal limiting current) were applied randomly at 10–60-s intervals over a 2-h period and under an elevated temperature of 38–40 °C. Right before and after the stress procedure, animals were video taped and EMGs and EEGs were recorded.

Locomotion disability score. Locomotion disability was quantified using a scale based on the unified Parkinson's disease rating scale in humans, but modified for rodents[13]. With this adapted scale, 0 = normal motor behavior, 1 = slightly slow movements, 2 = limited and slow ambulation even when persistently disturbed, but disturbance rarely resulted in the animal losing balance and falling, and 3 = no ambulation even when repeatedly disturbed with the animal usually losing balance and falling after being disturbed.

The assessment of the severity of locomotion disability (and the dystonia rating) was made independently by four members of the laboratory (none of the authors). All four colleagues were first trained; they were shown a set of exactly the same training videos from mice with movement disorders, asked to identify specific abnormal movement features, and told what score each behavior should receive. Then, the same four colleagues reviewed and scored all of the mice that we examined by viewing video clips of individual mice. All four reviewers saw exactly the same video clips. Moreover, all four reviewers were blind to the procedures that were done. Their scores was averaged and decoded by the authors.

Dystonia rating scale. The presence and severity of ouabain-induced dystonia in mice was quantified using a modification of a previously published scale[16] in which 0 = normal motor behavior, 1 = abnormal motor behavior, no dystonic postures, 2 = mild motor impairment, dystonic-like postures when disturbed, 3 = moderate impairment, frequent spontaneous dystonic postures, and 4 = severe impairment; sustained dystonic postures. As described above, the scores given by four colleagues who were blind to the treatment that each mouse had received were averaged.

Number of steps. We quantified the number of steps taken with the forelimb during a 30-s trial[14]. The analysis was done by two observers that were blind to the treatment that each animal had received.

Average step size. We quantified the average step size by measuring the distance between the toe of the posterior limb and the hill of the forelimb.

Open field test. Mice were placed into an arena of (50 cm length × 35 cm width × 25 cm height) for at least 5 min to monitor spontaneous locomotor activity. The Viewer[2] software (Biobserve) was used to calculate the speed of animals.

Treadmill. Mice were individually placed on a treadmill that was advanced at a constant rate starting from 2 m min^{-1}. The maximum speed that the mice could walk on the treadmill for at least 30 s was noted.

Estimation of ouabain concentration in tissue. To estimate the concentration of ouabain in the tissue, we perfused a fluorescent analog of ouabain, Bodipy-FL-ouabain (Molecular Probes), into the brain instead of ouabain.

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Bodipy-FL-ouabain, is active and clear symptoms were observed in the mice used for these experiments. Examination of the perfused tissue confirmed that the perfusion site could be well described by a sphere centered on the tip of the canula. The intensity profile of bodipy-FL-oubain was quantified using IP Lab software and was then used to calculate the concentration of ouabain as a function of lateral distance from the center of the canula. To estimate the concentration of ouabain, we adjusted the total amount of ouabain delivered during the perfusion period for its clearance in the brain. Because of a lack of data, we conservatively used a $\tau_{1/2}$ of 60 min based on the fact that, after an acute increase in the concentration of brain endo-ouabain, its levels return to baseline in less than 1 h. The actual $\tau_{1/2}$ is likely to be much less than 60 min. Because each molecule of ouabain is conjugated with a fluorescent tag, the total ouabain was taken to be distributed within the tissue based on the intensity profile of bodipy-FL-oubain. Based on this distribution profile, the concentration of ouabain was calculated for concentric spherical shells by dividing its amount in each shell by the volume of each shell. Given the value used for the rate of breakdown of ouabain in the brain the concentration of ouabain is likely to be significantly over-estimated.

Electrical lesion of the centrolateral nucleus. To bilaterally lesion the centrolateral nucleus, we sequentially positioned an electrode at −1.45 mm AP, −0.8 mm ML and 3.5 mm DV from bregma and 300-μA current pulses of 45-s duration were delivered to each side. As a control, the same surgery was done and the electrode was lowered into place, but no current was delivered. The extent and location of lesions were determined histologically using Nissl staining. Only mice in which the location and specificity of the lesion for the centrolateral nucleus were histologically confirmed were included in the data.

Electrical lesion of the cerebellar nuclei and subsequent perfusion of ouabain into the cerebellum. Lesions of the cerebellar nuclei were performed by one of either two methods. Comparable results were obtained with both approaches. With the first method, a single lesion was made bilaterally at −6.24 mm AP, 1.37 mm ML and 3.00 mm DV using a 45-s-long, 0.75-mA current at each site. In the second method, a total of four lesions (two lesions bilaterally) were made using a 45-s, 0.50-mA current at −6.24 mm AP, 1.15 mm ML and 1.75 mm DV on each side. After lesions, many mice exhibited signs of severe ataxia. In time, however, the mice recovered such that little overt motor symptoms were discernable (analogs to published experiments in which the entire cerebellum is removed). Approximately 1 week post lesion surgery, once the gross motor behavior of mice returned to normalcy, the cerebellum of was chronically perfused with ouabain (36 ng h^{-1}) as described earlier.

Statistical analysis. We used a 'mixed-design analysis of variance' model to analyze changes in akinesia and dystonia using the REML estimation method of JMP software (version 8.0.1; SAS software). The statistical analysis scrutinized differences in animals perfused with different ouabain concentrations (0–72 ng h^{-1}), and provided repeated measures of these variables at times 4–72 h post-drug perfusion. In this mixed effects model, one factor is between-subjects variable (or fixed effect) and the other is within-subjects variable (or random effect). This analysis was followed by individual ANOVAs at each time point and a *post hoc* Dunnet's *t* test. Thus, data were considered not to be statistically different from vehicle if $P > 0.05$. For other cases, when appropriate, we used a paired *t* test.

50. Lampl, I., Reichova, I. & Ferster, D. Synchronous membrane potential fluctuations in neurons of the cat visual cortex. *Neuron* **22**, 361–374 (1999).