Supplementary Data

The neural substrates of Rapid-Onset Dystonia-Parkinsonism

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Supplementary data 1
The extent of ouabain diffusion with chronic perfusion of the basal ganglia.

To determine the extent of diffusion and the concentration of ouabain in the basal ganglia we used Bodipy-FL-ouabain. Bodipy-FL-ouabain is an active fluorescent analogue of ouabain where every molecule of ouabain is conjugated with a fluorescent tag.

The photographs in the next page show superimposition of the fluorescence emitted by Bodipy-FL-ouabain with bright field images of the corresponding tissue section. The blue schematics show the brain structures at the equivalent level. Bodipy-FL-ouabain was chronically perfused bilaterally at a concentration of 18 ng/h for 72 hours. The sections shown are 300 µm apart and the distance from the center of the canula is denoted in microns at the bottom left of each panel. The scale bar corresponds to 1 mm. Only a single side is shown for clarity.
Supplementary data 2
Selective targeting of the output nuclei of the basal ganglia with acute ouabain injections.

To assess the potential role of individual basal ganglia output nuclei we acutely injected Bodipy-FL-ouabain into the target structures. Three distinct nuclei were bilaterally targeted: the entopeduncular nucleus (Figure Ai; AP: -1.34 mm, ML: 1.5 mm, DV: 4.5 mm), the globus pallidus (Figure Aii; AP: -0.58 mm, ML: 1.9 mm, DV: 4 mm), and the substantia nigra (Figure Aiii; AP: -3.28 mm, ML: 1.5 mm, DV: 4.5 mm). Following at least 1 day of recovery from surgery during which guide canula were implanted, mice were infused with a total of 5 µl of a 100 µM ouabain solution. Animals were assessed behaviorally 30, 60 and 90 minutes after infusion.

Mice with entopeduncular infusions and globus pallidus infusions showed a mild hypokinetic behavior. Substantia nigra infused animals showed circling behavior as well as some backwards walking. None of the animals exhibited any dystonic postures as assessed by four observers blind to the experimental paradigm. Comparable injections into the cerebellum, however, in all cases resulted in severe dystonia. Each experiment was performed in at least 3 mice.

The photographs in column A show superimposition of the fluorescence emitted by Bodipy-FL-ouabain with bright field images of the corresponding tissue section. The blue schematics show the brain structures at the equivalent level. The sections chosen represent the one which shows the largest perfusion. The graphs on the right show average dystonia scores given by four independent observers before and after infusion of ouabain for each target structure. The scale bar corresponds to 1 mm.
A

B

Cerebellum

Dystonia Score

Pre Ouabain  Post Ouabain

Entopeduncular Nucleus

Dystonia Score

Pre Ouabain  Post Ouabain

Globus Pallidus

Dystonia Score

Pre Ouabain  Post Ouabain

Substantia Nigra

Dystonia Score

Pre Ouabain  Post Ouabain
Supplementary data 3
Simultaneous EMG recordings from agonist and antagonist muscles confirm that partial blockade of cerebellar sodium pumps results in dystonia.

Two of the observations made when ouabain was chronically perfused into the cerebellum are noteworthy. First, the cerebellum appears to be more sensitive to dysfunction of the sodium pumps than does the basal ganglia. At the highest concentration of ouabain used (72 ng/h) more than 18 hours bilateral perfusion of the basal ganglia was required to produce detectable motor symptoms, whereas unilateral perfusion of the same concentration in the cerebellum produced clear ataxia within 6 hours. The second finding was that partial dysfunction of sodium pumps in the cerebellum not only causes ataxia, but also produces dystonic-like postures. We scrutinized the latter observation in more detail by examining whether the dystonic-like postures were associated with abnormal brain activity, and whether they were truly dystonia by directly recording the activity of agonist and antagonist muscles.

To monitor brain activity, we performed electroencephalogram (EEG) recordings at the level of the motor cortex in five animals chronically perfused with 36 ng/h of ouabain (and an additional one perfused with the vehicle). An example of one such recording is shown in Figure 1a where no epileptic activity was present in the EEG record at the time when the animal showed severe dystonic-like postures. Similar results were obtained in all five mice examined; in none were the motor symptoms associated with epileptic activity.

A strict traditional definition of dystonia is simultaneous co-contraction of agonist and antagonist muscles. With EMG recordings we explored whether the dystonic-like postures seen with chronic perfusion of ouabain in the cerebellum were indeed caused by co-contraction of the agonist and antagonist muscles. Chronic EMG recordings were obtained from the cranial tibial muscle (agonist) and the gastrocnemial muscle (antagonist) in three mice in which the cerebellum was perfused with 36 ng/h ouabain (Figure 1b). While EMG recordings clearly showed independent contraction of either muscle during normal motor activity, prolonged co-contraction of the two muscles were evident during the dystonic-like postures. We quantitatively examined the extent of muscle co-contraction by calculating the cross-correlation between their EMG signals (Figure 1c). As can be seen in the graphs shown, the EMG signals from the two muscles were highly correlated during dystonia and exhibited little correlation during normal ambulation. These data clearly demonstrate that partial dysfunction of cerebellar sodium pumps can cause dystonia.
Figure 1. Dystonic-like postures are mediated by co-contractions of agonist and antagonist muscles.

(a) Absence of epileptiform activity in the EEG signal recorded from the motor cortex during dystonic-like postures induced by cerebellar perfusion of 36ng/h ouabain. Arrow in the EEG record represents the time of dystonic posture shown in the photograph.

(b) EMG signals recorded from anterior cranial tibial and gastrocnemius muscles in a mouse chronically perfused with 36 ng/h ouabain into the cerebellum. The first two pairs of traces present normal contraction of one muscle vs. the other in the absence of dystonia. The last pair of traces shows clear co-contraction of the agonist and antagonist muscles during a dystonic posture.

(c) An analysis of the normalized cross correlation between the EMG signals from the agonist and antagonist muscle during normal ambulation and while the mice showed dystonic-like postures.
Supplementary data 4
Evidence for an interaction between dysfunctional basal ganglia and cerebellum

The data presented suggest that dysfunction of sodium pumps in the basal ganglia produces parkinsonism-like symptoms, and their dysfunction in the cerebellum causes ataxia and dystonia. In RDP the mutated sodium pump is distributed throughout the brain wherever the α3 isoform is expressed and thus the dysfunctional sodium pumps are simultaneously present both in the basal ganglia and the cerebellum. We thus explored the consequences of concurrent perfusion of both structures with ouabain. Two concentrations of ouabain were perfused, 18 ng/h or 36 ng/h. As before, the basal ganglia were perfused bilaterally whereas the cerebellum was perfused with a single canula.

The symptoms of animals in which the basal ganglia and cerebellum were concomitantly perfused with ouabain were markedly different from those that received ouabain selectively in one of the two regions (Figure 1). Whereas perfusion of 18 ng/h ouabain into the cerebellum (n=23) or the basal ganglia (n=12) alone produced very mild symptoms during the first 24 hours, clear pronounced motor dysfunction was observed within 4 hours in animals that received ouabain into both structures (n=7; Figures 1b-d). Intriguingly, the symptoms were not simply the sum of the symptoms seen when individual structures were perfused with ouabain. Instead, the mice exhibited reduced locomotion and gait disturbance/mild ataxia (Figure 1d, Supplementary video 3).

We similarly examined the consequence of perfusion of both structures with a higher (36 ng/h) concentration of ouabain (n=11). In all animals examined the symptoms were more severe, earlier to occur and were markedly different when both the basal ganglia and the cerebellum were perfused compared to perfusion of either structure in isolation (Figure 1b,b,e). Within three hours of perfusion of both structures reduced locomotion and gait disturbance/mild ataxia was noticeable whereas mice that received ouabain only into a single structure were relatively symptom free for at least 12 hours. The motor dysfunction in the mice that received ouabain into both structures progressed to focal dystonia which by 60 hours post perfusion turned into severe generalized dystonia, rigidity and a hunched posture (Figure 1c and e).

Collectively these data point to an interaction between the cerebellar and basal ganglia motor loops. Not only did symptoms onset earlier and were more severe when both structures were concurrently perfused, but they also had distinct aspects which were not present when one of the structures was perfused. This could be because the two structures perform complementary tasks and can, if needed, compensate for the other when one is made dysfunctional. Alternatively, the much earlier time of onset and the exaggerated severity and distinctness of the symptoms may be suggestive of a somewhat adverse interaction between these two structures when they both malfunction.
Figure 1. Symptoms of animals in which the basal ganglia and cerebellum were concurrently perfused with ouabain suggest an interaction between these two structures.

(a) Photographs of mice perfused for ≈48 hours with 36 ng/h ouabain only in the basal ganglia (i), cerebellum (ii), or concomitantly in both structures (iii).

(b) Consequences of concomitant perfusion of ouabain into the basal ganglia and the cerebellum on locomotion.

(c) Severity of ouabain-induced dystonia in the same mice.

(d and e) Summary of symptoms observed in mice in which the basal ganglia or cerebellum was chronically perfused with ouabain separately, or concurrently.
Supplementary data 5
Inactivation of the motor cortex does not eliminate cerebellum-induced dystonia.

To examine the role of motor cortex in cerebellum-induced dystonia the motor cortex was acutely silenced by infusion of tetrodotoxin (TTX) in mice in which dystonia was induced by acute injection of ouabain into the cerebellum.

In a single surgery mice were implanted with guide canula for acute cerebellar injection of ouabain (AP: 6.96 mm, ML: 0, DV: 2.5 mm) and acute bilateral injection of TTX into the motor cortex (AP: 2.00 mm, ML: 1.5 mm, DV: 2 mm). The guide canula used for injection of TTX into the motor cortex were also used as EEG electrodes to monitor the activity of the motor cortex. After at least 1 day of recovery, the cerebellum was infused with 5 µl of a 100 µM ouabain solution over 15 minutes. These infusions resulted in moderate to severe dystonia within 15-30 minutes which persisted for at least two hours and often for 6-12 hours. Half an hour after cerebellar infusion of ouabain, the motor cortex was bilaterally infused with 5 µl of a 50 µM TTX solution. Within 30 minutes of termination of TTX infusions the motor cortex was silenced. This was evident in the disappearance of the high frequency activity in the EEG records present before TTX infusions (note the raw EEG records of one such experiment before and after infusion of TTX shown in panel A of the attached figure, and also the reduction in the power of the higher frequencies in the corresponding power spectrum graph shown in panel B).

Behaviorally, while the mice became very flaccid, significant dystonia remained in all 8 animals examined. The graph shown in panel C depicts the average dystonia scores given by four observers who were blind to the paradigm used.
Supplementary data 6
Selective bilateral electrical lesions of the centrolateral (CL) nucleus of the thalamus.

The CL nucleus of the thalamus was electrically lesioned in mice. The photographs on the right show Nissl stained brain slices. The blue schematics show the brain structures at the equivalent level. The sections shown are 100 μm apart. The scale bar corresponds to 500 μm.