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Aberrant Purkinje cell activity is the cause of dystonia in a shRNA-based mouse model of Rapid Onset Dystonia-Parkinsonism

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Abstract

Loss-of-function mutations in the $\alpha 3$ isoform of the sodium pump are responsible for Rapid Onset Dystonia-Parkinsonism (RDP). A pharmacologic model of RDP replicates the most salient features of RDP, and implicates both the cerebellum and basal ganglia in the disorder; dystonia is associated with aberrant cerebellar output, and the parkinsonism-like features are attributable to the basal ganglia. The pharmacologic agent used to generate the model, ouabain, is selective for sodium pumps. However, close to the infusion sites in vivo it likely affects all sodium pump isoforms. Therefore, it remains to be established whether selective loss of $\alpha 3$ -containing sodium pumps replicates the pharmacologic model. Moreover, while the pharmacologic model suggested that aberrant firing of Purkinje cells was the main cause of abnormal cerebellar output, it did not allow the scrutiny of this hypothesis. To address these questions RNA interference using small hairpin RNAs (shRNAs) delivered via adeno-associated viruses (AAV) was used to specifically knockdown $\alpha 3$ -containing sodium pumps in different regions of the adult mouse brain. Knockdown of the $\alpha 3$ -containing sodium pumps mimicked both the behavioral and electrophysiological changes seen in the pharmacologic model of RDP, recapitulating key aspects of the human disorder. Further, we found that knockdown of the $\alpha 3$ isoform altered the intrinsic pacemaking of Purkinje cells, but not the neurons of the deep cerebellar nuclei. Therefore, acute knockdown of proteins associated with inherited dystonias may be a good strategy for developing phenotypic genetic mouse models where traditional transgenic models have failed to produce symptomatic mice.

Introduction

Dystonia is thought to affect as many as 250,000 people in the United States alone (Stacy, 2006). Although dystonia is both common and devastating its etiology remains poorly understood, in part because of the absence of good animal models of the disorder. The

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majority of dystonias are idiopathic and many are thought to have a genetic component (Phukan et al., 2011, Balint and Bhatia, 2014). Over the last 20 years more than 30 genes have been identified which are associated with dystonia (Phukan et al., 2011, Balint and Bhatia, 2014, Moghimi et al., 2014). In many of the cases that have been closely scrutinized the dystonic syndrome is thought to be associated with loss of function of the associated gene (De Carvalho et al., 2004, Goodchild et al., 2005, Esapa et al., 2007, Fuchs et al., 2013). Unfortunately, to date the majority of transgenic models made to replicate mutations of these causative genes in the mouse have not generated dystonic animals (Richter and Richter, 2014), making examination of the neural and neuronal circuitry which contribute to dystonia challenging.

Rapid-Onset Dystonia Parkinsonism (RDP) is an inherited dystonia caused by loss of function mutations in the $\alpha 3$ isoform of the Na^+/K^+ -ATPase pump (sodium pump) (De Carvalho et al., 2004); a protein whose function is widely known and well-studied (Sweadner, 1989, Lingrel, 1992). Since this discovery, two different genetic animal models of $\alpha 3$ dysfunction have been reported. The first was a knockout of the $\alpha 3$ isoform. Unfortunately, mice with a homozygous knockout died prematurely and could not be studied while heterozygotes exhibited no overt motor phenotype (Moseley et al., 2007, Deandrade et al., 2010). In the second model, the gene coding for the $\alpha 3$ isoform has an inactivating point mutation within it. Mice homozygous for this mutation also exhibit early lethality whereas heterozygotes survive and exhibit seizures and ataxia but no clear dystonia (Clapcote et al., 2009). The failure of these transgenic models in replicating RDP has been attributed to developmental compensatory mechanisms in mice that may be different than those present in humans (Calderon et al., 2011, Fremont and Khodakhah, 2012, Fremont et al., 2014).

In 2011, a model of RDP was generated which took advantage of the fact that the loss-of-function mutations associated with sodium pumps in RDP could be acutely mimicked by pharmacologically blocking the function of the sodium pumps (Calderon et al., 2011). In this model ouabain, an exquisitely selective inhibitor of the sodium pumps, was applied to select brain regions of adult mice (Calderon et al., 2011). This pharmacologic model replicated all of the salient features of RDP including dystonia. An interesting observation made in this report was that infusion of ouabain into the cerebellum was necessary and sufficient to induce dystonia. A follow up study using this model showed that to induce dystonia ouabain disrupts the activity of cerebellar neurons particularly Purkinje cells and deep cerebellar neurons (Calderon et al., 2011, Fremont et al., 2014).

Despite the tremendous progress made using the pharmacologic model, two major questions remain unanswered. The first is whether dysfunction of the $\alpha 3$ -containing sodium pumps alone is sufficient to replicate the symptoms of RDP. Even though ouabain is selective for the $\alpha 3$ -containing sodium pumps, at the infusion site the concentration of ouabain is high enough to affect all isoforms. The second question that cannot be answered using the pharmacologic model is whether the sodium pump mutations affect the intrinsic activity of both Purkinje cells and deep cerebellar nuclei neurons to induce dystonia or whether, as indirectly suggested (Fremont et al., 2014), alterations in the activity of Purkinje cells alone is sufficient to cause abnormal cerebellar output and dystonia. Scrutinizing this question requires examining the activity of Purkinje cells and deep cerebellar nuclei neurons in

cerebellar slices made from dystonic mice with synaptic transmission pharmacologically blocked. However, by virtue of making cerebellar slices and perfusing the tissue with blockers of synaptic transmission the very agent that causes dystonia, ouabain, will be washed out rendering such experiments pointless. Thus a different animal model which selectively and permanently reduces the function of $\alpha 3$ -containing sodium pumps and yet generates dystonic mice would be of tremendous value.

The success of the pharmacologic model was attributed to the fact that any potential developmental compensatory mechanisms were bypassed by acutely reducing sodium pump function with ouabain in adult mice (Calderon et al., 2011, Fremont and Khodakhah, 2012, Fremont et al., 2014). We reasoned that acute, post developmental reduction in the sodium pump function must remain a central feature of the new model. We thus explored the utility of acutely knocking down the $\alpha 3$ -containing sodium pumps using small hairpin RNAs (shRNAs) in select brain regions of adult mice by injecting Adeno-associated viruses (AAVs) containing the shRNAs into the desired targets. We found this approach to be fruitful. In agreement with the pharmacologic model we found that knockdown of the $\alpha 3$ isoform in the substantia nigra yielded mice that demonstrated parkinsonism-like symptoms. Conversely, knockdown of the $\alpha 3$ -containing sodium pumps in the cerebellum was necessary and sufficient to cause dystonia. Similar to that seen with the pharmacologic model, *in vivo* recordings showed that cerebellar neurons fire erratically in dystonic mice. Additionally, we took advantage of this new model to make cerebellar slices from the dystonic mice. Our scrutiny of Purkinje cell and DCN neuronal activity in the presence of blockers of fast synaptic transmission in these cerebellar slices demonstrated that the change in cerebellar output associated with dystonia is primarily the consequence of disruption of the intrinsic pacemaking of Purkinje cells.

Overall these studies provide evidence that dysfunction of Purkinje cells underlie dystonia in RDP. Moreover, these data demonstrate that acute loss of the $\alpha 3$ isoform of the sodium pump is sufficient to replicate the symptoms seen in RDP, and suggest that the shRNA approach used here might be a valuable alternative approach for generation of dystonic mouse models of human hereditary dystonias.

Methods

Experiments were performed on 8 to 10 weeks old male or female C57BL/6 mice in accord with the guidelines set by Albert Einstein College of Medicine.

shRNA sequences and packaging: For knockdown of *Atp1a3* mRNA, two different shRNAs targeted against two different non-overlapping regions of the sequence of this protein were identified. The sequences were originally generated by the RNAi consortium and correspond to TRCN0000349864 (5'-ccggcggagcagattgacgagattcctcgaggaatctcgtaatctgctccgttttg-3') and TRCN0000349819 (5'-ccggtatggcagcagtgacttatctcgagataagtcactgctgcccatttttg-3') (see Figure 1 for a schematic). Plasmid was generated by the Albert Einstein College of Medicine Lentiviral Core and AAV9 compatible plasmids containing this shRNA were generated commercially (Virovek, AAV9-U6-shRNA-CMV-GFP). The same company also generated AAV9 virus with an average titer of 2×10^{13} vg/ml in DPBS buffer containing

0.001% pluronic F-68 which was 0.22 μ m filter sterilized. This virus was stereotactically injected into different brain regions of mice. Control AAV9 containing non-targeted (NT) shRNA under the same promoter which also contained the same CMV driven GFP at an equivalent titer was purchased from Virovek as was AAV9-CMV-GFP (see Figure 1 for schematic). The NT-shRNA used was AAV9-U6-NTshRNA-CMV-GFP (5'-ccaactaccggaactattat tcaagagataatagttcggttagttggcattttt-3', Lot# 13-037, Virovek, see Figure 1 for schematic),

Cortical cultures and infection with shRNAs

Cortical neuron primary cultures were prepared from E16 C57BL/6 mice (Charles River). The pregnant mouse was anesthetized using isoflurane (5%), and embryos were transferred to ice-cold HEPES-Glucose buffer (HEPES 10 mM (Fisher), Glucose 33 mM (Fisher) in PBS (Corning)). Embryos were decapitated and the cortices were dissected from the rest of the brain and incubated in HEPES-Glucose buffer containing Trypsin, at 37 °C, for 15 minutes. Trypsin was washed by HEPES-Glucose buffer, and replaced by DMEM (Dulbecco's Modified Eagle Medium, GIBCO) fortified with 10% FBS (Fetal Bovine Serum, GIBCO). Cortical tissues were fragmented by forceful pipetting using glass Pasteur pipettes, and then plated in 24-well culture plates at a density of 20,000 per cm². Cultures were incubated at 37 °C for 2 hours. After this time, the culture media was replaced with Neurobasal medium (GIBCO) fortified with B27 supplement (2%, GIBCO) and Glutamax (0.25 %, GIBCO). After 5 days *in vitro*, the cultures were transfected with adeno-associated viruses carrying shRNA against the target protein's mRNA. Cultures were then incubated for 10 days at 37 °C. Neuronal lysates were subsequently collected using lysis buffer (SDS 2%, Tris 50 mM, EDTA 2 mM).

Protein lysates from the brain

At 5–10 weeks after injection, mice were anesthetized with isoflurane then decapitated. The brain was quickly removed from the cranium and placed in bubbling ice-cold artificial cerebro-spinal fluid (aCSF: in mM NaCl 125, KCl 2.5, NaHCO₃ 26, NaH₂PO₄ 1.25, MgCl₂ 1, CaCl₂ 2, glucose 10, pH 7.4 when gassed with 5%CO₂:95%O₂). The whole cerebellum or whole basal ganglia was dissected from the brain and homogenized in lysis buffer. Lysates were then incubated at room temperature for 30 minutes before being sonicated and then frozen and stored at –20°C.

Western Blotting

The protein concentrations of samples were determined by BCA assay (Pierce). Samples were then run on 10% Bis-Tris gel (Biorad) to separate proteins and transferred to PVDF membrane (Biorad). Western blots/membranes were incubated for 2 hours or less at room temperature on a shaker with the primary antibody and then for 30 minutes with the secondary antibody. PVDF membranes were developed using chemiluminescence and signals were quantified using ImageJ to perform densitometry. Primary antibodies used were Na⁺/K⁺ ATPase α 3 (C-16, 1:1000, Santa Cruz), beta-tubulin (T8328, 1:40,000, Sigma), Na⁺/K⁺ ATPase α 1-6F (1:1000, graciously provided by Dr. Kathleen Sweadner), and Na⁺/K⁺ ATPase α 2-UBI (1:1000, graciously provided by Dr. Kathleen Sweadner).

Testing the efficacy of selected shRNA sequences *in vitro* and *in vivo*

Virus containing the two shRNAs targeting different regions of the $\alpha 3$ isoform was used to separately infect mouse cortical cultures at 5 days *in vitro*. After 10 days of infection, lysates were prepared from the cultures and western blots were used to determine the level of knockdown. There was little difference between the knockdown (KD) achieved by either shRNA (AAV- $\alpha 3$ shRNA-GFP KD: $75.13 \pm 4.8\%$, AAV- $\alpha 3$ shRNA2-GFP KD: $70.5 \pm 19.7\%$, $p=0.82$) ($N=3$ for each sequence, Figure 1 B, C). For both shRNAs we estimated protein knockdown in the mouse cerebellum *in vivo* (see below) and found it to be comparable ($N=4$ for each sequence, AAV- $\alpha 3$ shRNA-GFP KD: $55.4 \pm 2.0\%$, AAV- $\alpha 3$ shRNA2-GFP KD: $59.4 \pm 2.0\%$). It is likely that this knockdown primarily reflects loss of the protein in neurons since glial cells do not express *Atp1a3* (McGrail et al., 1991).

Injections of AAV9-shRNA

For all AAV9-shRNA injections in initial experiments the virus was titrated to determine the volume and number of injection sites needed to observe robust expression of the AAV and adequate knockdown of the $\alpha 3$ isoform in the region of interest. Cerebellum: AAV9-shRNA-GFP was injected into 4 sites in the cerebellum of 6–8 weeks old mice. AAV9 has been shown to infect multiple cell types in the cerebellum including Purkinje cells and DCN neurons (Huda et al., 2014). Two microliters of the viral solution was injected at a rate of 0.1–0.15 μ l per minute at each site. After each injection, the syringe was left in place for a minimum of 10 minutes before slowly being withdrawn. The coordinates for the sites used were: (AP: –6 mm, ML: 0 mm, DV: –1.5 mm), (AP: –6.96 mm, ML: 0 mm, DV: 1.5 mm), (AP: –6 mm, ML: 1.8 mm, DV: 2.3 mm), (AP: –6 mm, –1.8 mm, DV: 2.3 mm). Striatum and globus pallidus: Injections were performed at 4 sites for mice with basal ganglia injections. Two microliters was injected at each site at rates identical to that used for cerebellar injections. Sites used were: (AP: 0.5 mm, ML: 2 mm, DV: 2.5 mm), (AP: 0.5 mm, ML: –2 mm, DV: 2.5 mm), (AP: –0.5 mm, ML: 2.5 mm, DV: 3.5 mm), (AP: –0.5 mm, ML: –2.5 mm, DV: 3.5 mm). Injections into the substantia nigra were performed at 4 sites with 0.5–1 μ l of solution at each site at a rate of 0.05 μ l per minute. Sites of infusion were: (AP: –3 mm, ML: 1.5 mm, DV: 4 mm), (AP: –3 mm, ML: –1.5 mm, DV: 4 mm), (AP: –3.6 mm, ML: 1 mm, DV: 4 mm), (AP: –3.6 mm, ML: –1 mm, DV: 4 mm).

Staining

For all staining mice were trans-cardially perfused with 1X phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Brains were removed, incubated at 4 degrees overnight in 4% paraformaldehyde and then switched into a 30% sucrose solution before embedding them in Tissue-Tek OCT compound. Brains were then sectioned into 50 μ m slices. Sections were either Nissl stained or stained with an anti-GFP antibody (primary: 1:500, A11122, molecular probes; secondary: 1:1000, A11008, molecular probes) and Hoechst 3342 (1:1000, H3570, molecular probes). Bright field and fluorescent images were taken on a Zeiss Axioskop 2 plus.

Dystonia Scale

The presence of dystonia and its severity were quantified using a previously published scale (Calderon et al., 2011). Briefly, 0 = normal behavior; 1 = abnormal motor behavior, no dystonic postures; 2 = mild motor impairment, dystonic-like postures when disturbed; 3 = moderate impairment, frequent spontaneous dystonic postures; 4 = severe impairment, sustained dystonic postures. Assessment was made by four independent observers blinded to the animal's condition. Observers were trained using a set of videos demonstrating representative mice for each score and highlighting key characteristics.

EMGs

Under anesthesia, electrodes were inserted into the gastrocnemius muscle (plantar flexion) and anterior tibialis muscle (dorsiflexion) and wires were routed under the skin to a connector fixed to the skull. Recordings were performed on animals exhibiting a dystonia score greater than 2 as determined by four observers blinded to the animal's condition. Immediately prior to recordings, a head stage for the Pinnacle EEG/EMG recording system 4100 was secured to the connector. Recordings of muscle activity were performed in the open field for 1–10 minutes while mice were videotaped.

In vivo electrophysiology

Mice were implanted with an L-shaped bracket which was fixed onto the skull with bone screws and dental cement. A recording chamber was drilled in the skull on top of the cerebellum, surrounded with dental cement and covered with surgifoam and bone wax. Single-unit neural activity was recorded extracellularly using a tungsten electrode (Thomas Recording, 2–3 M Ω), which was advanced into the cerebellum until either the Purkinje cell layer or the deep cerebellar nuclei were reached. Purkinje cells were identified by location, characteristic firing rate, the presence of complex spikes, and post-hoc histology. DCN neurons were identified by location, firing rate, and post-hoc histology. Signals were filtered (200 Hz–20 kHz) and amplified (2000x) using a custom built amplifier and then digitized (20 kHz) using a National Instruments PCI MIO 16 XE. Waveforms were sorted offline using characteristics of the spikes such as amplitude and energy as well as those determined by principal component analysis (Plexon).

In vitro electrophysiology

Adult mice were anesthetized with isoflurane and then decapitated. Sagittal slices of 300 μ m thickness were cut from the cerebellum at a temperature of 35°C. After a one hour incubation period at 35°C, slices were then transferred to room temperature. Extracellular recordings were obtained from single Purkinje cells and DCN neurons using a custom-built amplifier and glass pipette electrodes filled with extracellular solution (in mM NaCl 125, KCl 2.5, NaHCO₃ 26, NaH₂PO₄ 1.25, MgCl₂ 1, CaCl₂ 2, glucose 10, pH 7.4 when gassed with 5%CO₂:95%O₂). Slices were recorded on an upright microscope (Zeiss) and perfused with 35°C extracellular solution at a rate of 1.5 ml/min during recording sessions. Perfusion solution contained picrotoxin (10 μ M) and CGP55845 (1 μ M) to block inhibitory synaptic transmission and kynurenic acid (5 mM) to block excitatory transmission. Data was sampled

at 10 kHz using an analog to digital converter (National Instruments PCI MIO 16 XE) and analyzed using custom-written LabView software.

Cell death analysis

Mice were injected into the cerebellum with AAV containing either NTshRNA or $\alpha 3$ shRNA as described above. Animals in which the $\alpha 3$ isoform of the sodium pump was knocked down began exhibiting dystonia ~2–3 weeks after injection. At 8 weeks post-injection animals were trans-cardially perfused with 1X PBS followed by 4% paraformaldehyde. Brains were removed, incubated at 4°C overnight in 4% paraformaldehyde and then switched into a 30% sucrose solution. Brains were then embedded in Tissue-Tek OCT compound. 30 μ m brain slices were made from the cerebellum at –20°C using a cryostat.

Slides were imaged using a Zeiss Axioskop 2 microscope and the 4 injection sites in each cerebellum were identified. Slides containing the injection sites and up to 1 mm lateral to the injection sites were then stained with TUNEL (Roche) to mark dead cells and Hoechst to determine the total number of cells. Some slides were also co-stained with anti-GFP as described following TUNEL staining to allow for co-localization of TUNEL and GFP. After staining, a 300 $\times$ 300 μ m region of interest was outlined around the injection site and the number of TUNEL positive and Hoechst positive cells were counted and the ratio of dead/total cells was determined. This region of interest was also used to count the number of TUNEL positive cells and to compare cell loss in the cerebellar cortex and DCN. Additional 300 $\times$ 300 μ m regions were selected at different distances from the injection sites and the number of TUNEL positive and Hoescht positive cells were quantified. In each animal, measurements were made from up to 1 mm lateral, anterior, and posterior from the lateral injection sites; up to 1 mm anterior and lateral from the anterior central injection site; and up to 1 mm posterior and lateral from the posterior central injection site.

Statistics

Unless otherwise stated in the text, a one-way anova (when three or more groups were compared) or student t-test (when two groups were compared) was used to determine significance for electrophysiological recordings. When a one-way anova was used it was followed by Dunnet's multiple comparison's test to determine significance. Unless otherwise stated, when determining statistics for dystonia scores, a Friedman test with post-hoc Dunn's multiple comparison's test was used to determine significance. In all cases data was considered statistically different from control when $P < 0.05$.

Results

Knockdown of the $\alpha 3$ isoform in the substantia nigra replicates Parkinsonism

One of the hallmarks of RDP is Parkinsonism, characterized in patients by postural instability, bradykinesia, and slow gait (De Carvalho et al., 2004). In rodents, partially blocking sodium pumps in the basal ganglia has been shown to result in Parkinsonism-like symptoms (Calderon et al., 2011). Reports suggest that the sodium pump plays a uniquely important role in controlling the firing of dopaminergic neurons of the substantia nigra (Johnson et al., 1992, Shen and Johnson, 1998). Therefore, it is possible that knockdown of

the $\alpha 3$ isoform in the substantia nigra may be sufficient to replicate some aspects of Parkinsonism. To test whether this could be the case, an AAV containing shRNA against the $\alpha 3$ isoform and separately driven GFP (AAV- $\alpha 3$ shRNA-GFP, Figure 1A) was injected bilaterally targeting the substantia nigra of adult mice (N=5). Injected mice displayed abnormally slowed movement as early as two weeks after surgery. To quantify the Parkinsonism-like behavioral changes in these animals the distance traveled in 5 minutes, average speed of movement, number of steps taken in 30 seconds, and average step size were calculated. In mice with injection of AAV- $\alpha 3$ shRNA-GFP into the substantia nigra, all of these parameters were reduced over time (Figure 2A). Histology demonstrated that mice with features of Parkinsonism had robust expression of the shRNA as evidenced by the intense GFP staining in the substantia nigra (Figure 2B). These results suggest that loss of $\alpha 3$ -containing sodium pumps in the substantia nigra contributes to Parkinsonism-like symptoms in the rodent.

To confirm that the behavioral effects seen in injected mice were due to knockdown of the $\alpha 3$ protein and not off-target effects of the shRNA, another cohort of mice (N=3) was injected with a second shRNA (AAV- $\alpha 3$ shRNA2-GFP, Figure 1A) targeting a different region of the protein than the first. Mice injected with AAV- $\alpha 3$ shRNA2-GFP exhibited similar symptoms to those injected with AAV- $\alpha 3$ shRNA-GFP (Figure 2A). This corroborated the finding that knockdown of the $\alpha 3$ isoform underlies symptoms in these animals, rather than an off-target effect of the shRNA. As a control, another group of mice (N=3) was injected with an AAV containing a non-targeted shRNA and separately driven GFP (AAV-NTshRNA-GFP, Figure 1A). These mice had no behavioral deficits (Figure 2A) suggesting that non-specific toxicity caused by shRNA expression or infection with the virus did not contribute to Parkinsonism-like symptoms (McBride et al., 2008, Ehler et al., 2010, Martin et al., 2011).

Dystonia has canonically been considered to be a disorder of the basal ganglia (Bhatia and Marsden, 1994, Mink, 2003). While knockdown of the $\alpha 3$ isoform in the substantia nigra did not cause dystonia, it is possible that knockdown in other regions of the basal ganglia such as the striatum and globus pallidus may be sufficient to do so. In fact, a recent post-mortem study demonstrated that there is increased neuronal cell loss in the globus pallidus in patients with RDP compared to a non-affected family member (Oblak et al., 2014). Therefore, it is possible that the striatum and globus pallidus may be involved in RDP. To determine whether knockdown of the $\alpha 3$ isoform in other regions of the basal ganglia causes symptoms, injections of AAV- $\alpha 3$ shRNA-GFP or AAV- $\alpha 3$ shRNA2-GFP were performed targeting primarily the striatum and globus pallidus (N=7 mice total, AAV- $\alpha 3$ shRNA-GFP: N=4, AAV- $\alpha 3$ shRNA2-GFP: N=3). Observers blinded to the condition of the animals rated their behavior on a previously published dystonia scale (Calderon et al., 2011) to determine whether the mice exhibited symptoms consistent with dystonia or Parkinsonism. On this scale a score at or above two is considered dystonic (Calderon et al., 2011). Mice with knockdown of the $\alpha 3$ isoform primarily in the striatum and globus pallidus demonstrated no motor abnormalities with an average score of <1 throughout the post-injection period (Figure 2C). Post-hoc staining of these brain regions in injected mice revealed strong expression of GFP (Figure 2B) and knockdown of the $\alpha 3$ isoform ($\alpha 3$ KD) was confirmed by western blots (N=5 animals, average knockdown = $56.3 \pm 1.0\%$, Figure 2D). Injection of

control AAV-NTshRNA-GFP into the striatum and globus pallidus (N=4) similarly had no effect on behavior (Figure 2C). Notably, mice with $\alpha 3$ KD in the substantia nigra exhibited an average dystonia score >1 but <2 , which is consistent with the observation that these animals exhibit a Parkinsonism-like movement disorder but not dystonia (Figure 2C).

Knockdown of the $\alpha 3$ isoform in the cerebellum of adult mice is sufficient to induce dystonia

One of the most debilitating aspects of RDP is the severe and often generalized dystonia exhibited by patients with this disorder. Calderon et al. (2011) demonstrated that infusion of ouabain to the cerebellum was necessary and sufficient to cause dystonia in mice. To corroborate their findings AAV- $\alpha 3$ shRNA-GFP was injected into the cerebellum of mice. Mice with cerebellar injections developed abnormal limb postures (N=7), similar in appearance to dystonia (Figure 3A). To quantify these symptoms, injected mice were assessed on the dystonia scale (Calderon et al., 2011). Symptoms consistent with dystonia began between two and three weeks after injection in most mice and persisted thereafter (Figure 3A). Electromyograms (EMGs) performed on the gastrocnemius and anterior tibialis of a subset of these mice (N=3) demonstrated that the abnormal postures were caused by co-contraction of agonist and antagonist muscle pairs (Figure 3B), a hallmark of dystonia (Frucht, 2013). Immunohistochemistry performed on dystonic animals with $\alpha 3$ knockdown demonstrated expression of GFP throughout the cerebellum (Figure 3C). As controls, additional cohorts of mice received injections of AAV containing only GFP (AAV-GFP, N=7) or AAV-NTshRNA-GFP (N=5). These mice exhibited normal motor behavior (Figure 3A). To confirm that the behavioral effects seen with injection of AAV- $\alpha 3$ shRNA-GFP into the cerebellum were due to knockdown of the $\alpha 3$ isoform and not off-target effects of the shRNA, another cohort was injected with AAV- $\alpha 3$ shRNA2-GFP (N=7). Injection of AAV- $\alpha 3$ shRNA2-GFP into the cerebellum resulted in the induction of dystonia (Figure 3A) with similar severity and time to onset as mice injected with AAV- $\alpha 3$ shRNA-GFP (Figure 3A). By 3 weeks post-injection, animals in both cohorts with $\alpha 3$ KD exhibited dystonic postures with an average dystonia score that was 2 (average dystonia score of mice injected with AAV- $\alpha 3$ shRNA-GFP at 3 weeks post-injection: 2.0 ± 0.2 , average dystonia score of mice injected with AAV- $\alpha 3$ shRNA2-GFP at 3 weeks post-injection: 2.6 ± 0.4), (Calderon et al., 2011). The fact that injection with AAV- $\alpha 3$ shRNA-GFP or AAV- $\alpha 3$ shRNA2-GFP resulted in a similar phenotype suggests that dystonia in these animals is primarily due to knockdown of the $\alpha 3$ isoform rather than off-target effects, since each shRNA has different off-target binding.

Previous reports have suggested that widespread expression of constructs delivered via AAV *in vivo* takes approximately 1–2 weeks (Kaplitt et al., 1994, Lo et al., 1999, Kaspar et al., 2002, Kaplitt et al., 2007). Knockdown of protein using shRNA delivered via AAV has been shown to occur on a similar timescale (Hommel et al., 2003). Therefore, the behavior of mice at one week after injection should not be affected by the expression of the shRNA. Indeed, one week after injection the behavior of mice injected with AAV- $\alpha 3$ shRNA-GFP, AAV- $\alpha 3$ shRNA2-GFP, and AAV-NTshRNA-GFP was comparable (Figure 2A and Figure 3A). To confirm that knockdown of $\alpha 3$ followed a similar pattern, a time-course experiment was performed where the knockdown of $\alpha 3$ was examined by Western blot analysis of

lysates from the cerebellum of AAV- injected mice one week, three weeks, and eight weeks after injection (Figure 3D). While there was little knockdown one week after injection of the virus, by three weeks after injection the protein levels were significantly reduced (Figure 3D, N=3 WT, N=3 NTshRNA, N= > 4 for $\alpha 3$ shRNA at each time point. Average knockdown at one week = $19.0 \pm 5.9\%$, three weeks = $57.0 \pm 6.3\%$). Western blots of lysates from the cerebellum of AAV- $\alpha 3$ shRNA-GFP injected mice obtained eight weeks after injection showed that the significant knockdown of the $\alpha 3$ protein seen after three weeks persisted (N=9, Average knockdown = $55.6 \pm 4.1\%$; Figure 3D). To confirm that the virus used was specific to the $\alpha 3$ isoform of the sodium pump, Western blots were also probed for ATP1A1 and ATP1A2, which exhibited near-normal levels (ATP1A1 KD: $14.1 \pm 4.0\%$, ATP1A2 KD: $-7.8 \pm 7.4\%$, Figure 3E). Altogether, these findings suggest that acute knockdown of the $\alpha 3$ isoform in the cerebellum results in dystonia without affecting the expression of $\alpha 1$ and $\alpha 2$ isoforms of the sodium pump.

Knockdown of the $\alpha 3$ isoform of the sodium pump in the cerebellum causes minimal cell death in mice exhibiting dystonia

Some limited cell death has been identified post-mortem in the cerebellum of RDP patients (Oblak et al., 2014). However, previous studies in the pharmacologic model of RDP suggest that while cell death does occur, dystonia is primarily caused by changes in the activity of cerebellar neurons (Fremont et al., 2014). To determine the extent of cell death in mice with $\alpha 3$ KD in the cerebellum, TUNEL staining was performed on cerebella from dystonic mice (N=3) and compared to that of non-dystonic animals with injection of AAV-NTshRNA-GFP (N=3). The percentage of cell death in several areas at a range of distances from the injection sites was determined by performing Hoechst staining to obtain total cell number and TUNEL to define apoptotic nuclei (Figure 4A). Animals with $\alpha 3$ KD exhibited significantly more cell death than animals expressing NTshRNA (percent cell death at the injection site for mice with injection of AAV- $\alpha 3$ shRNA-GFP: $11.4 \pm 2.0\%$, percent cell death at the injection site for mice injected with AAV-NTshRNA-GFP: $2.5 \pm 0.8\%$, $p=0.0094$, Figure 4B). However, even in animals with $\alpha 3$ KD, the percentage of cell death at sites of injection was low. In fact, the percentage of cell death was lower than that seen in the pharmacologic model of RDP where dystonia has been shown to be due to changes in the activity of cerebellar neurons rather than neuronal loss (Fremont et al., 2014). Therefore, the amount of cell death identified in animals with $\alpha 3$ KD in the cerebellum does not appear to be sufficient to be a major cause of dystonia.

Although the cell loss due to $\alpha 3$ KD does not appear to be sufficient to cause dystonia, cerebellar cell death may still be a common feature of the models and patients (Oblak et al., 2014). Oblak et al. noted that some limited cell death occurred in the Purkinje cell layer, granule cell layer, and in the deep cerebellar nuclei (DCN) of patients with RDP. The most cell death in patients was noted in the DCN (Oblak et al., 2014). We find that apoptotic cells are located throughout the cerebellar cortex and the DCN of dystonic mice with $\alpha 3$ KD (Figure 4C). At an equal distance from injection sites, the number of TUNEL positive neurons in DCN was two times higher than that in the cerebellar cortex (Average number of TUNEL positive cells 300 μ m from injection site in DCN: 48.2 ± 6.4 , average number TUNEL positive cells 300 μ m from injection site in cerebellar cortex: 14 ± 4.2 , $p=0.0011$,

Figure 4C). The increased cell death in the DCN compared to the cerebellar cortex was not seen in mice injected with AAV-NTshRNA-GFP (Average number of TUNEL positive cells from AAV-NTshRNA-GFP injected mice 300 μ m from injection site in DCN: 10.5 ± 5.0 , average number of TUNEL positive cells from the same mice 300 μ m away from the injection site in the cerebellar cortex: 6.5 ± 2.9 , $p=0.51$, Figure 4B). Therefore, the location and pattern of cerebellar cell death seen in mice with $\alpha 3$ KD closely mirrors that which has been identified in RDP patients (Oblak et al., 2014).

One possible reason for the greater cell death in the DCN may be that these neurons are more sensitive to knockdown of the $\alpha 3$ isoform of the sodium pumps. If this were the case then the shRNA expressing DCN neurons would be expected to be more likely to undergo apoptosis and exhibit TUNEL staining compared to non-infected DCN cells. We used GFP to assay for shRNA expressing neurons and found that they could be easily distinguished from non-infected cells (Figure 4D). We found that DCN neurons co-labelled for both GFP and TUNEL staining were scattered sparsely in the DCN (Figure 4D, top three panels, white arrows). In contrast, GFP-positive cells without TUNEL staining appeared throughout the DCN (Figure 4D, middle three panels, white arrows). Moreover regions that exhibited strong TUNEL staining, but had minimal GFP-positive neurons, very readily identified (Figure 4D, bottom three panels). The relatively modest overlap of GFP and TUNEL staining provides little support for the notion that DCN neurons are more sensitive to sodium pump KD compared with say Purkinje cells. Instead, other factors such as their relatively poor calcium handling ability (discussed in detail in the Discussion) might be the cause of the more produced cell death in the DCN.

Both DCN neurons and Purkinje cells burst erratically in dystonic mice with knockdown of the $\alpha 3$ isoform in the cerebellum

Previous studies using the pharmacologic RDP model demonstrated that dystonia was caused by abnormal cerebellar output (Fremont et al., 2014). Specifically, neurons of the DCN fired in high-frequency bursts in dystonic animals (Fremont et al., 2014). To determine whether DCN neurons in the cerebellum of dystonic mice with $\alpha 3$ KD exhibited similar activity, *in vivo* recordings from the DCN of awake dystonic mice were performed. The neurons of the DCN comprise the majority of projection neurons that leave the cerebellum. These neurons fire regularly with an average firing rate of ~ 40 Hz (Jahnsen, 1986). DCN cells recorded *in vivo* from awake non-dystonic animals with injection of AAV-GFP (N=3, n=14) or AAV-NTshRNA-GFP (N=4, n=22) exhibited firing similar to wild type mice (WT, N=6, n=17, Figure 5A). In contrast, DCN neurons in dystonic mice with $\alpha 3$ KD (N=3, n=23) exhibited irregular high-frequency burst firing (Figure 5A). Despite this irregular activity, there was no significant difference between the average firing rate of DCN neurons from dystonic mice and those recorded in controls (WT: 46.63 ± 4.83 spikes/second (sp/s), GFP: 42.26 ± 5.89 sp/s, NTshRNA: 53.13 ± 4.74 sp/s, $\alpha 3$ shRNA: 45.5 ± 7.92 sp/s, Figure 5B). This finding was not unexpected since previous studies suggested that neurons of the cerebellum can have dramatic changes in their firing pattern but no change in the average number of spikes overall (Walter et al., 2006).

Indeed, although the average firing rate of DCN neurons from animals with $\alpha 3$ KD was not altered, there was a significant increase in their predominant firing rate compared to DCN cells from wild type, AAV-GFP injected, or AAV-NTshRNA-GFP injected animals (Figure 5B). The predominant firing rate for a neuron is calculated as the inverse of the mode of the interspike intervals (ISIs). DCN cells recorded in dystonic animals exhibited a nearly quadrupled predominant firing rate compared to controls (WT: 55.15 ± 5.43 sp/s, GFP: 60.04 ± 6.50 sp/s, NTshRNA: 65.90 ± 3.45 sp/s, $\alpha 3$ shRNA: 206.39 ± 32.22 sp/s, $p < 0.0001$, Figure 5B). When neurons fire in bursts, clustered spiking can occur at very fast rates punctuated by periods of quiescence. This results in an average predominant firing rate over 200 Hz (representing the firing during bursts) and a normal average firing rate (representing the combined rate of burst periods and intermittent pauses) in dystonic animals.

The irregularity of neuronal firing can be quantified by calculating the coefficient of variation of the interspike intervals (CV ISI). CV ISI is defined as standard deviation of ISIs/ISI mean. Therefore neurons that fire more irregularly will have an increased CV ISI. Since DCN cells from dystonic animals fired in high-frequency bursts, an increase in the CV ISI was expected. Indeed, DCN neurons from dystonic animals had a CV ISI nearly threefold higher than controls, confirming that these neurons fire more irregularly (WT: 0.64 ± 0.05 , GFP: 0.59 ± 0.06 , NTshRNA: 0.61 ± 0.03 , $\alpha 3$ shRNA: 1.24 ± 0.12 , $p < 0.0001$, Figure 5B).

Although DCN neurons are intrinsically active their firing rate is effectively modulated by their synaptic inputs. Therefore, the changes observed in the firing of DCN neurons could be caused by alterations in their intrinsic pacemaking due to knockdown of the $\alpha 3$ isoform or, alternatively, could be synaptically driven. Purkinje cells of the cerebellum which form the sole output of the cerebellar cortex drive DCN neuronal activity via strong inhibitory synaptic connections (Gauck and Jaeger, 2000) and are especially sensitive to loss of sodium pump function (Fremont et al., 2014). Therefore, altered Purkinje cell activity could be the cause of the aberrant DCN firing. To address this possibility, Purkinje neurons were recorded from the same cohorts of mice. Purkinje cells are also intrinsically active neurons which under normal conditions (Figure 5 C) fire tonically at around 50 Hz (Brookhart et al., 1950, Hausser and Clark, 1997, Nam and Hockberger, 1997, Raman and Bean, 1997). In contrast, Purkinje cells in dystonic animals with $\alpha 3$ KD exhibited abnormal high-frequency bursting similar to that identified in DCN neurons of the same animals (Figure 5C).

Quantitatively, Purkinje cells from dystonic animals had an average firing rate comparable to controls (WT: 58.02 ± 3.10 sp/s (N=6, n=12), GFP: 59.07 ± 9.67 sp/s (N=3, n=13), NTshRNA: 63.11 ± 4.65 sp/s (N=4, n=19), $\alpha 3$ shRNA: 55.43 ± 7.30 sp/s (N=3, n=22), Figure 5F) but a predominant firing rate nearly triple that of controls (WT: 71.74 ± 4.54 sp/s, GFP: 80.10 ± 14.44 sp/s, NTshRNA: 77.97 ± 5.81 sp/s, $\alpha 3$ shRNA: 175.30 ± 25.07 sp/s, $p < 0.001$, Figure 5D). Further, the CV ISI of these animals was increased by over two fold compared to controls (WT: 0.51 ± 0.02 , GFP: 0.56 ± 0.03 , NTshRNA: 0.42 ± 0.03 , $\alpha 3$ shRNA: 1.15 ± 0.09 , $p < 0.0001$, Figure 5D). Therefore, knockdown of the $\alpha 3$ isoform in the cerebellum results in abnormal high-frequency burst firing of Purkinje cells. These data are consistent with the hypothesis that abnormal Purkinje cell firing drives changes in DCN activity that underlie dystonia in animals with $\alpha 3$ KD in the cerebellum. These changes in cerebellar physiology

are similar to those previously identified in the pharmacologic model of RDP (Fremont et al., 2014).

Purkinje neurons with knockdown of the $\alpha 3$ isoform exhibit burst firing *in vitro*

In the pharmacologic model of RDP, disruption of Purkinje cell pacemaking likely contributed to the activity changes seen *in vivo* and also to dystonia (Fremont et al., 2014). Indeed, partially blocking sodium pumps of Purkinje cells with ouabain in cerebellar slices was shown to affect their intrinsic firing rate, causing them to burst erratically (Fremont et al., 2014). Therefore, it is possible that acute knockdown of the $\alpha 3$ isoform of the sodium pump in Purkinje cells also alters the intrinsic activity of these neurons which could underlie some of the activity changes identified *in vivo*. Examination of post-mortem tissue from symptomatic mice with $\alpha 3$ KD revealed expression of the construct in cells throughout the cerebellum, including the Purkinje cells of the cerebellar cortex (Figure 6A). This allowed us to test whether knockdown of the $\alpha 3$ isoform of the sodium pump in Purkinje cells affects their intrinsic activity. To this end, cerebellar slices were made from dystonic mice (N=9). The activity of Purkinje cells was recorded extracellularly with fast glutamatergic and GABAergic transmission pharmacologically blocked to isolate the effect of knockdown of the $\alpha 3$ isoform on the intrinsic activity of these neurons. GFP+ Purkinje cells with $\alpha 3$ KD (n=17) exhibited erratic high-frequency burst firing (Figure 6A). In contrast, GFP- Purkinje cells without knockdown of the $\alpha 3$ isoform (n=19) exhibited regular pacemaking similar to that seen in wild type Purkinje cells (N=7, n=25, Figure 6A). As a control, GFP+ (n=18) and GFP- (n=18) Purkinje cells were recorded from mice injected with AAV-NTshRNA-GFP. These Purkinje cells also exhibited normal activity.

To quantify the changes in firing exhibited by Purkinje cells with $\alpha 3$ KD, the average firing rate, predominant firing rate, and CV ISI were calculated. There was no significant difference between the average firing rate of Purkinje cells from dystonic animals and that of Purkinje cells from mice injected with AAV-NTshRNA-GFP or wild type animals (WT: 55.38 ± 5.63 sp/s, NTshRNA GFP-: 50.96 ± 5.39 sp/s, NTshRNA GFP+: 55.52 ± 7.40 sp/s, $\alpha 3$ shRNA GFP-: 75.92 ± 8.26 sp/s, $\alpha 3$ shRNA GFP+: 38.82 ± 7.10 sp/s, Figure 6B). In contrast, both predominant firing rate (WT: 56.22 ± 5.65 sp/s, NTshRNA GFP-: 52.59 ± 5.90 sp/s, NTshRNA GFP+: 55.41 ± 7.46 sp/s, $\alpha 3$ shRNA GFP-: 78.22 ± 8.48 sp/s, $\alpha 3$ shRNA GFP+: 132.4 ± 27.89 sp/s, $p < 0.0001$, Figure 6C) and CV ISI were increased only in GFP+ Purkinje neurons from dystonic animals (WT: 0.11 ± 0.01 , NTshRNA GFP-: 0.14 ± 0.02 , NTshRNA GFP+: 0.12 ± 0.02 , $\alpha 3$ shRNA GFP-: 0.11 ± 0.01 , $\alpha 3$ shRNA GFP+: 1.24 ± 0.20 , $p < 0.00001$, Figure 6D). In fact, the mean CV ISI of GFP+ Purkinje cells from dystonic animals with $\alpha 3$ KD was nearly 10 fold higher than that of control Purkinje cells. This effect did not appear to be due to infection of Purkinje cells with AAV or the expression of shRNAs as Purkinje cells infected with AAV-NTshRNA-GFP exhibited firing which was not significantly different from wild type neurons (Figure 6 B, C, and D). These findings suggest that acute knockdown of the $\alpha 3$ isoform disrupts the intrinsic activity of Purkinje cells, resulting in erratic high-frequency bursting.

In dystonic animals, the average firing rate of GFP- Purkinje cells was slightly higher than that of GFP+ Purkinje cells with $\alpha 3$ KD (Figure 6B, $p < 0.01$). The cause of the small

increase in the firing rate of GFP⁻ cells is likely the increase in extracellular potassium that is caused by the high frequency burst firing of the neighboring GFP⁺ cells (Nicholson et al., 1978, Malenka et al., 1981, Hounsgaard and Nicholson, 1983). An increase in extracellular potassium has been shown to increase the firing rate of Purkinje cells *in vitro* (Hounsgaard and Midtgaard, 1988).

Knockdown of the $\alpha 3$ isoform of the sodium pump in neurons of the deep cerebellar nuclei does not affect their intrinsic activity

While Purkinje cells are thought to solely express the $\alpha 3$ isoform of the sodium pump, neurons of the deep cerebellar nuclei express both $\alpha 3$ and $\alpha 1$ (Brines et al., 1991, Peng et al., 1997). Therefore, these cells are better suited to deal with loss of their $\alpha 3$ -containing sodium pumps. In fact, low concentrations of ouabain, which disrupt Purkinje cell pacemaking *in vitro*, have no effect on the intrinsic activity of DCN neurons (Fremont et al., 2014).

In mice the DCN are organized in clusters and embedded in myelinated fibers (Chan-Palay, 1977, Aizenman and Linden, 1999). We targeted DCN clusters which clearly contained either GFP⁺ (n=7) or GFP⁻ (n=5) neurons in cerebellar slices acutely prepared from dystonic mice (N=4). The activity of the neurons within these clusters was recorded with fast glutamatergic and GABAergic synaptic transmission blocked. DCN neurons recorded *in vitro* from wild type animals (n=8, N=4), those from GFP⁺ (n=9), and GFP⁻ clusters (n=10) from animals infected with AAV-NTshRNA-GFP (N=3) were all used as controls. The activity of DCN neurons from GFP⁺ clusters in slices taken from dystonic animals was similar to that of DCN neurons from GFP⁻ clusters within the same slice and other controls (Figure 7A). There was no significant difference between the average firing rate (WT: 24.18 ± 3.15 sp/s, NTshRNA GFP⁻: 22.34 ± 2.79 sp/s, NTshRNA GFP⁺: 22.03 ± 2.15 sp/s, $\alpha 3$ shRNA GFP⁻: 20.40 ± 3.51 sp/s, $\alpha 3$ shRNA GFP⁺: 15.61 ± 1.63 sp/s, Figure 7B), predominant firing rate (WT: 27.54 ± 3.92 sp/s, NTshRNA GFP⁻: 24.61 ± 4.44 sp/s, NTshRNA GFP⁺: 21.38 ± 2.23 sp/s, $\alpha 3$ shRNA GFP⁻: 21.14 ± 3.60 sp/s, $\alpha 3$ shRNA GFP⁺: 16.20 ± 1.60 sp/s, Figure 7C), or coefficient of variation of interspike intervals (WT: 0.09 ± 0.01 , NTshRNA GFP⁻: 0.11 ± 0.01 , NTshRNA GFP⁺: 0.10 ± 0.01 , $\alpha 3$ shRNA GFP⁻: 0.13 ± 0.03 , $\alpha 3$ shRNA GFP⁺: 0.12 ± 0.01 , Figure 7D) between DCN neurons recorded in GFP⁺ clusters from mice with $\alpha 3$ KD compared to controls. Therefore, we find no evidence that knockdown of the $\alpha 3$ isoform of the sodium pump affects the intrinsic activity of DCN neurons.

Discussion

Loss of function mutations in the $\alpha 3$ isoform of the sodium pump are associated with Rapid-Onset Dystonia Parkinsonism (RDP) (De Carvalho et al., 2004). Even though the causative gene is known, genetic models of RDP have proven unsuccessful (Moseley et al., 2007, Clapcote et al., 2009). The purpose of this study was to determine whether acute selective knockdown of the $\alpha 3$ isoform in different brain regions of the adult mouse could produce the key symptoms of RDP; Parkinsonism and dystonia. We found that knockdown of the $\alpha 3$ isoform in the substantia nigra resulted in Parkinsonism-like symptoms whereas knockdown

in the cerebellum resulted in dystonia. In animals with dystonia, abnormal high-frequency bursting activity of the output neurons of the cerebellum was observed. Dystonia was also associated with aberrant bursting of Purkinje cells and *in vitro* studies demonstrated that knockdown of the $\alpha 3$ isoform altered the intrinsic activity of these neurons. Altogether, these findings show that acute knockdown of $\alpha 3$ containing sodium pumps in the adult mouse is sufficient to replicate symptoms of RDP.

While the data presented are largely in agreement with the findings of a recent pharmacological animal model of RDP, they substantially advance our understanding of the disorder. First, the data clearly show that selective cerebellar loss of $\alpha 3$ containing sodium pumps (rather than all the sodium pump isoforms) is sufficient to cause dystonia. Second, our data show that a primary cause of cerebellar-induced dystonia is aberrant Purkinje cell activity; the affected DCN neurons in our model had seemingly normal intrinsic pacemaking. In addition to the obvious implications for RDP, the ability of the shRNA-based model to replicate the pharmacologic model of RDP and the disorder itself suggests that a shRNA approach might be appropriate for generating models of other hereditary human dystonias which are caused by loss of function mutations.

Post-mortem studies in a small number of patients with RDP show that there is some neuronal loss in the basal ganglia (Oblak et al., 2014). However, in agreement with the findings of the ouabain model of RDP (Calderon et al., 2011) our data also show that acute knockdown of the $\alpha 3$ isoform in the striatum and globus pallidus does not cause dystonia in adult mice. Still, our data does not exclude the possibility that these brain regions contribute to the generation or maintenance of dystonia in RDP. In fact with the pharmacologic model of RDP it was demonstrated that communication between the basal ganglia and cerebellum is required to replicate the stress-induced dystonia seen in RDP patients (Calderon et al., 2011). Moreover, we have previously shown that cerebellar-induced dystonia is likely caused by basal ganglia dysfunction. Specifically, aberrant cerebellar output alters basal ganglia activity via a thalamic disynaptic connection and silencing this pathway alleviates dystonia (Chen et al., 2014).

Acutely targeting the basal ganglia sodium pumps does not cause dystonia. However, the results of this study suggest that Parkinsonism in RDP likely results from loss of $\alpha 3$ isoform sodium pump function in the substantia nigra. It has been shown that sodium pumps contribute to maintaining the resting potential of dopaminergic cells and therefore their dysfunction could disrupt dopaminergic transmission (Kim et al., 2007, Calderon et al., 2011). It is tempting to hypothesize that loss of the $\alpha 3$ isoform in dopaminergic neurons is responsible for the Parkinsonism seen in this model and in RDP. However, the AAV virus used does not discriminate between different cell types and thus the $\alpha 3$ -containing sodium pumps were knocked down in all cells present in the substantia nigra. There is evidence that some non-dopaminergic neurons in the substantia nigra also express the $\alpha 3$ isoform of the sodium pump (Bottger et al., 2011) and thus we cannot rule out the possibility that alterations in their activity might have also contributed to the symptoms.

In the late twentieth century, some evidence was presented in support of the notion that interventions involving the cerebellum may alleviate symptoms in some dystonic patients

refractory to other treatment (Zervas et al., 1967, Cooper and Upton, 1978). Functional imaging studies have also suggested that a number of dystonic patients have changes in cerebellar activity (Eidelberg et al., 1998, Argyelan et al., 2009, Carbon et al., 2013). Further, a number of studies in animal models suggest that abnormal cerebellar function is sufficient to cause dystonia (LeDoux et al., 1993, LeDoux and Lorden, 1998, Campbell et al., 1999, Pizoli et al., 2002, Calderon et al., 2011). This study is the first to show that acute disruption of the $\alpha 3$ isoform, the same protein mutated in RDP, disrupts the intrinsic pacemaking of cerebellar Purkinje cells *in vitro*. Unlike normal Purkinje cells that exhibit tonic pacemaking, the affected neurons exhibited high-frequency bursts of activity punctuated by pauses. A similar change in activity of Purkinje cells is observed when a fraction of sodium pumps are blocked by ouabain (Fremont et al., 2014). This finding suggests that changes in the intrinsic activity of Purkinje cells contribute to dystonia in RDP. In contrast, with extracellular recordings from clusters of predominantly GFP+ or GFP- DCN neurons, we found no evidence for changes in the intrinsic activity of these neurons *in vitro*. These findings are in agreement with prior observations that the intrinsic pacemaking of DCN neurons are far less sensitive to partial loss of sodium pumps than that of Purkinje cells (Fremont et al., 2014). It is therefore reasonable to postulate that aberrant Purkinje cell activity is the main instigator of abnormal cerebellar output in our animal models of RDP, and that the *in vivo* changes in DCN activity in dystonic animals are driven by aberrant Purkinje cell pacemaking. Using a pharmacologic model of RDP we have previously shown that the aberrant cerebellar output caused by dysfunction of the sodium pumps alters the activity of striatal neurons via a disynaptic thalamic pathway (Chen et al., 2014). The extent of aberrant cerebellar output seen here with our shRNA KD approach is comparable to that which is achieved with the pharmacologic model. It is reasonable, therefore, to conclude that in the present work dystonia was also generated to a large extent by cerebellar disruption of basal ganglia firing via the disynaptic thalamic pathway.

In addition to identifying possible mechanisms of pathophysiology in RDP, we also examined whether knockdown of the $\alpha 3$ isoform causes cell death. Indeed, we found that some cell death was seen in dystonic animals. Although there was very little cell death in general, there was more cell death in the DCN than in the cerebellar cortex which is consistent with a recent post-mortem study performed on RDP patients (Oblak et al., 2014). We find little evidence for extensive overlap between TUNEL staining and GFP staining in the DCN of dystonic animals suggesting that knockdown of the $\alpha 3$ isoform in these cells is not the primary cause of cell death. In agreement with this finding, recordings in slices made from dystonic animals revealed little change in the intrinsic activity of DCN neurons recorded in GFP+ clusters. We postulate that changes in the activity of DCN neurons driven by aberrant Purkinje cell firing could result in the death of some DCN cells. Purkinje cells are able to maintain high firing rates in part because they can buffer large amounts of calcium which, in other neurons, would cause cell death (Fierro and Llano, 1996). DCN neurons do not have such specialized calcium buffering but were found in this study to have an average predominant firing rate of around 200 Hz. Therefore, extended periods of high-frequency burst firing could result in their damage and death thus providing a potential explanation as to why more cell death is seen in the DCN than in the cerebellar cortex.

The onset of symptoms in RDP patients characteristically occurs after a stressful event (Brashear et al., 2007). However, animals with knockdown of the $\alpha 3$ isoform of the sodium pump exhibited symptoms that were independent of stress. In the previously published pharmacologic model of RDP, infusing low concentrations of ouabain to the cerebellum and basal ganglia replicated the stress-induced aspect of this disorder (Calderon et al., 2011). The same study showed that when higher concentrations of ouabain were infused to the cerebellum or basal ganglia to block a greater fraction of sodium pumps symptoms were observed independent of stress. It was suggested that infusing low concentrations of ouabain to the cerebellum and basal ganglia makes the brain receptive to a second insult, stress, which can then precipitate the symptoms (Calderon et al., 2011). Infusing higher concentrations of ouabain or knocking down the $\alpha 3$ isoform may bypass this state and directly cause symptoms without the need for the additive component of stress. In patients, the RDP-associated mutations of the $\alpha 3$ isoform do not result in a complete loss of function of the sodium pumps. A prediction of the hypothesis presented is that knocking in these mutations may generate mice in which the expression of symptoms only occurs after a stressful event. Future studies should address this possibility.

While some mutations in *ATP1A3* are associated with RDP, others are associated with the early-acquired neurologic disorders alternating hemiplegia of childhood (AHC) and CAPOS (cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss) syndrome (Demos et al., 2014, Heinzen et al., 2014). Although some patients with AHC may exhibit dystonia, most AHC and CAPOS patients exhibit progressive ataxia as their predominant motor symptom (Heinzen et al., 2012). Recently, it has been shown that *myshkin* mice, which exhibit ataxia and seizures but not dystonia, have a mutation in the *Atp1a3* gene at a site that is associated with AHC in humans, suggesting these mice may represent a genetically faithful model of this disorder (Clapcote et al., 2009, Kirshenbaum et al., 2013). We find that knockdown of the $\alpha 3$ isoform in the cerebellum results in the induction of dystonia beginning ~3 weeks after injection. However, at 2 weeks after injection, a number of mice have a dystonia score close to 1 which is consistent with a non-dystonic movement disorder. These mice exhibited a movement disorder characterized by unsteadiness similar in appearance to ataxia. It is possible that the extent of sodium pump dysfunction in the cerebellum dictates whether the predominant motor manifestation is ataxia or dystonia. Indeed, a previous publication suggests that when sodium pumps in the cerebellum are pharmacologically blocked, lower concentrations of the drug result in an ataxic phenotype while higher concentrations result in dystonia (Calderon et al., 2011). Therefore, knocking down the $\alpha 3$ isoform to different extents and in different brain regions in rodents may also represent an approach for modeling related disorders such as AHC and CAPOS.

Modeling human genetic dystonias in rodents has been difficult because many knockout mice die prematurely and heterozygotes tend not to show symptoms similar to patients (Richter and Loscher, 1998, Jinnah et al., 2005). This is also the case for *ATP1A3*, the gene coding for the $\alpha 3$ isoform of the sodium pump (Moseley et al., 2007). The present study shows that acute loss of $\alpha 3$ containing sodium pumps causes symptoms that mimic the disorder, in contrast to what is seen by constitutive loss of the protein from birth in

transgenic mouse models. This finding is not surprising because there is evidence that in mice heterozygous for *Atp1a3* there is an up-regulation of the remaining allele to near-normal levels (Sacino, 2009). Similar differences in the effect of embryonic and adult protein loss have been reported for other proteins (Mukherjee et al., 2010). The present study demonstrates that acute shRNA mediated knockdown of the $\alpha 3$ isoform of the sodium pump in adult mice replicates the symptoms of RDP. The success of this new model suggests that the use of shRNAs can provide novel insights into how regional loss-of-function of a particular protein could cause neurological symptoms. Therefore, it may be possible to use a similar strategy to generate symptomatic rodent models of other inherited dystonias that are associated with loss-of-function mutations.

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Highlights

- Acute knockdown of ATP1A3 in mice replicates the symptoms of Rapid-onset dystonia Parkinsonism
- Acute knockdown of ATP1A3 in the substantia nigra of mice results in Parkinsonism
- Acute knockdown of ATP1A3 in the cerebellum of mice results in dystonia
- In this mouse models of RDP Dystonia is caused by aberrant high frequency burst firing of cerebellar Purkinje cells

Figure 1. The α3 isoform of the sodium pump can be efficiently knocked down in vitro using small hairpin RNAs (shRNAs)

A. Schematics showing the design of constructs used throughout this study which were packaged into adeno-associated virus serotype 9 (AAV9). In the first row is the design for AAV-α3shRNA-GFP/AAV-α3shRNA2-GFP, below is AAV-NTshRNA-GFP, and at the bottom is AAV-GFP. B. Representative western blot showing the *in vitro* knockdown efficiency of the first ATP1A3 shRNA in mouse cortical neurons (NI= non-infected controls, A3 = α3 protein, tubulin = β tubulin control). To the right is a quantification of the

knockdown normalized to non-infected controls (N=3 replicates, *p<0.05, Mean $\pm$ S.E.M.).

C. Representative western blot showing the *in vitro* knockdown efficiency of the second shRNA in mouse cortical cultures. To the right is a quantification of the knockdown efficiency compared to non-infected controls (N=3 replicates, *p<0.05, Mean $\pm$ S.E.M.).

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Figure 2. Knockdown of the $\alpha 3$ isoform of the sodium pump in the substantia nigra of adult rodents results in parkinsonian symptoms

A. Mice with knockdown of the $\alpha 3$ isoform of the sodium pump in the substantia nigra (N=5 for 1st shRNA, N=3 for 2nd shRNA) show a progressive decrease in distance traveled in 5 minutes, average speed, steps taken in 30 seconds, and average step size compared to animals with injection of AAV-NTshRNA-GFP to the substantia nigra (NT SN, N=3). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean $\pm$ S.E.M). B. Representative images of histological sections from a mouse with injection of AAV- $\alpha 3$ shRNA-GFP to the substantia nigra (left) and one with injection to the striatum/globus pallidus (right) stained for GFP and Hoechst.

In the left image, the dotted line delineates the approximate area of the substantia nigra. On the right, the dotted line delineates the border between the cortex and the basal ganglia. Scale bar = 500 μ m. SN = substantia nigra, H = hippocampus, Cx = Cerebral Cortex, St = striatum. C. Knockdown of the α 3 isoform in the striatum and globus pallidus or in the substantia nigra does not cause dystonia (Mean $\pm$ S.E.M). However, knockdown of the α 3 isoform in the substantia nigra does cause abnormal behavior indicated by a score 1 on the dystonia scale. Expression of a non-targeted (NT) shRNA in either of these regions does not cause motor abnormality. D. Representative Western blot from the basal ganglia of a mouse injected with AAV- α 3shRNA-GFP. Compared to the wild type, there is a decrease in the amount of α 3 protein. Below is a quantification of the relative density of α 3 from the basal ganglia of mice injected with AAV- α 3shRNA-GFP (N=4) compared to wild type controls (N=3) (*p<0.05, Mean $\pm$ S.E.M).

Figure 3. Knockdown of ATP1A3 in the cerebellum of adult mice results in dystonia

A. Representative mouse with knockdown of the $\alpha 3$ isoform of the sodium pump in the cerebellum exhibiting a dystonic-like posture of the left hind limb. To the right is a quantification of the dystonia scores in all of the mice. Dystonia scores increase after 2 weeks in mice injected with either AAV- $\alpha 3$ shRNA-GFP or AAV- $\alpha 3$ shRNA2-GFP. This was never observed in mice injected with control viruses. (Mice injected with AAV-GFP (N=7), mice injected with AAV-NTshRNA-GFP (N=5), mice injected with AAV- $\alpha 3$ shRNA-GFP (N=7), mice injected with AAV- $\alpha 3$ shRNA2-GFP (N=7), *p<0.05,

** $p < 0.01$, *** $p < 0.001$, Mean $\pm$ S.E.M). B. Dystonic-like postures in mice with knockdown of ATP1A3 in the cerebellum are caused by co-contraction of agonist and antagonist muscles. EMGs were performed on the gastrocnemius (Ga, black) and anterior tibialis (AT, red) muscles in the hind-limb of mice with dystonic-like postures due to knockdown of the $\alpha 3$ isoform in the cerebellum (N=3, scale is 2 s (x) by 10 μ V (y)). During normal activity when the mouse was not exhibiting dystonic postures, the muscles tended to have alternating activity (left). However, during dystonic postures of the implanted limb, there was prolonged co-contraction of these two muscles (right). C. There is expression of GFP throughout the cerebellum of mice injected with AAV- $\alpha 3$ shRNA-GFP. In all three images dotted lines delineate the borders of major structures. Cb = cerebellum, Bs = brainstem. There is no marked change in cerebellar morphology as assessed using bright field microscopy and Hoechst staining (scale bar = 500 μ m). D. Knockdown efficiency of the shRNA was determined by collecting lysates from the cerebella of mice injected with AAV- $\alpha 3$ shRNA-GFP (N=4 at 1 wk, N=7 at 3 wks, N=9 at 8+ wks) and comparing the amount of $\alpha 3$ protein to lysates from WT mice (N=3) and mice with injection of AAV-NT shRNA-GFP (N=3). The first and second shRNA showed comparable knockdown and data from both cohorts of mice were included in the graph. On the left is a representative western blot. The graph on the right shows that 1 week after injection of the $\alpha 3$ shRNA into the cerebellum there is no knockdown of the $\alpha 3$ protein, however by 3 weeks after injection $\alpha 3$ protein levels were reduced. The reduced $\alpha 3$ protein levels were also seen at 8 weeks after injection. Quantification of the relative density of each condition was normalized to WT lysates and also NTshRNA which were found to give comparable results (*** $p < 0.001$, **** $p < 0.0001$, Mean $\pm$ S.E.M). E. $\alpha 1$ and $\alpha 2$ are additional isoforms of the sodium pump. Western blots show that the protein levels of both $\alpha 1$ and $\alpha 2$ are not significantly altered by knockdown of $\alpha 3$. Quantification of the relative density of each condition was normalized to WT lysates (ns - non-significant, ** $p < 0.01$, Mean $\pm$ S.E.M).

Figure 4. Knockdown of ATP1A3 in the cerebellum causes some cell death in dystonic mice
 A. TUNEL and Hoechst staining was performed on mice injected with AAV-NTshRNA-GFP (N=3) and dystonic mice injected with AAV-α3shRNA-GFP (N=3) perfused 8 weeks after injection. Representative merged images of TUNEL (red) and Hoechst (blue) from sites close to the infusion and more distant from the cerebellum of mice injected with either AAV-NTshRNA-GFP (black) or AAV-α3shRNA-GFP (green) (scale bar = 200 μm). B. Quantification of the percent cell death at specific distances from the injection site (Mean ± S.E.M). Percentage cell death occurring on 3 axes (A-P, D-V, and M-L) was calculated and then averaged. Although both conditions elicited <15% cell death at the site of infusion, animals with knockdown of the α3 isoform (green) had significantly more cell death than controls (black) (*p<0.05, **p<0.01, Mean ± S.E.M). C. Lower magnification (10x) image

of a representative TUNEL stained section from an injection site in a dystonic mouse with knockdown of the $\alpha 3$ isoform in the cerebellum (scale bar = 200 μm). The arrow indicates site of injection, DCN = deep cerebellar nuclei, Cb Cx = cerebellar cortex. The dotted line indicates the borders of these regions. The inset contains quantification of the cell death (number of TUNEL positive neurons) in the DCN versus the cortex 300 μm from the site of infusion. In mice with knockdown of the $\alpha 3$ isoform, there is more cell death in the DCN than in the cerebellar cortex an equal distance away from the injection site (** $p < 0.01$, Mean $\pm$ S.E.M). D. In the DCN of AAV- $\alpha 3$ shRNA-GFP injected mice, there is little co-localization between GFP positive cells and TUNEL positive cells. Representative images from the DCN of mice injected with AAV- $\alpha 3$ shRNA-GFP and stained for TUNEL (red), Hoechst (blue) and GFP (green). In the top panel the white arrows point to 2 cells showing co-staining of GFP and TUNEL. In the middle panel, the arrows point to GFP labeled cells in DCN without TUNEL staining. The bottom row shows an area in DCN with several TUNEL positive cells but no GFP staining (scale bar = 100 μm in all images in D).

Figure 5. Neurons in the deep cerebellar nuclei and Purkinje cells exhibit high-frequency bursting activity in awake dystonic mice with knockdown of the $\alpha 3$ isoform in the cerebellum
 Deep cerebellar nuclei neurons were recorded *in vivo* from awake head restrained mice which had received no injection (WT, black, N=6, n=17), or injection of either AAV-GFP (dark gray, N=3, n=14), AAV-NTshRNA-GFP (light gray, N=4, n=22), or AAV- $\alpha 3$ shRNA-GFP (green, N=3, n=23) (A, schematic). A. Irregular burst firing of DCN neurons recorded from dystonic animals was identifiable in raw traces (A) compared to those recorded from animals with infusion of a non-targeted shRNA (A) (scale is 500 ms by 100 μ V). B. Average

firing rate defined as (number of spikes/recorded time) did not differ between control and dystonic animals ($p>0.05$, Mean $\pm$ S.E.M). DCN neurons from dystonic (green) mice exhibit an increased predominant firing rate (1/ISI histogram mode) compared to all controls (*** $p<0.0001$, Mean $\pm$ S.E.M). DCN neurons recorded in dystonic animals (green) exhibit a higher ISI coefficient of variation than those from controls (gray) (*** $p<0.0001$, Mean $\pm$ S.E.M). The Purkinje cells of awake dystonic mice with knockdown of the $\alpha 3$ isoform in the cerebellum also fire aberrantly. *In vivo* recordings of Purkinje cells (schematic) were performed in awake head restrained animals which received no injection (WT, N=6, n=12), or injection of either AAV-GFP (N=3, n=13), AAV-NTshRNA-GFP (N=4, n=19), or AAV- $\alpha 3$ shRNA-GFP (N=3, n=22) (C, schematic). C. Raw traces of the activity of Purkinje cells from control animals exhibited normal tonic firing characteristic of this cell type (AAV-NTshRNA) while traces from Purkinje cells in dystonic animals displayed abnormal burst firing (AAV- $\alpha 3$ shRNA, scale bar for both is 250 ms by 100 μ V). D. Similar to DCN neurons recorded *in vivo*, Purkinje cells from dystonic animals do not exhibit a change in average firing rate ($p>0.05$, Mean $\pm$ S.E.M). Purkinje cells recorded from dystonic mice exhibit an increase in predominant firing rate (** $p<0.001$) and an increase in interspike interval coefficient of variation (*** $p<0.0001$) compared to controls (Mean $\pm$ S.E.M).

Figure 6. Purkinje cells are sensitive to knockdown of ATP1A3

A. A representative GFP positive Purkinje cell from a mouse injected with AAV- α 3shRNA-GFP. Scale bar = 25 microns. Raw traces comparing the firing rate of a representative Purkinje cell *in vitro* from a WT animal to a Purkinje cell with α 3 knockdown (α 3shRNA) and a neighboring Purkinje cell from the same slice (Non-infected). B. The average firing rates of Purkinje cells were compared from a wild type animal (WT, N=4, n=25); from an animal injected with AAV-NTshRNA-GFP both infected cells (NTshRNA GFP+, N=5, n=18) and non-infected cells (GFP-, N=5, n=18) and from an animal injected with AAV-

$\alpha 3$ shRNA-GFP both infected cells ($\alpha 3$ shRNA GFP+, N=9, n=17) and non-infected cells (GFP-, N=9, n=19). Although there was no significant difference between the average firing rates of Purkinje cells from control animals compared to dystonic animals overall, there was a difference between GFP- and GFP+ cells from mice injected with AAV- $\alpha 3$ shRNA-GFP (B, $p = 0.01$, Mean $\pm$ S.E.M). The predominant firing rate of GFP+ cells from dystonic animals was significantly higher than all other groups (C, $*p < 0.05$ compared to GFP- from dystonic animals and $***p < 0.001$ compared to all other groups, Mean $\pm$ S.E.M). The coefficient of variation of interspike intervals was also significantly increased in GFP+ neurons from dystonic mice (D, $***p < 0.001$, Mean $\pm$ S.E.M).

Figure 7. DCN neurons are not sensitive to knockdown of ATP1A3

A. Schematic showing how recordings were performed from DCN neurons *in vitro*. The firing rate of a representative DCN neuron from a WT animal compared to a DCN cell recorded from a GFP+ cluster with $\alpha 3$ knockdown and a DCN cell from a GFP- cluster from the same slice (Non-infected). B. The average firing rates of DCN cells from a wild type animal (WT, N=4, n=8), DCN cells from GFP+ clusters (NTshRNA GFP+, N=3, n=9) and GFP- clusters (GFP, N=3, n=10) from an animal injected with AAV-NTshRNA-GFP, and DCN cells from GFP+ clusters ($\alpha 3$ shRNA GFP+, N=4, n=7) and GFP- clusters (GFP-,

N=4, n=5) from an animal injected with AAV- α 3shRNA-GFP were compared. There was no significant difference between the average firing rates of DCN neurons (Mean $\pm$ S.E.M). There was no significant difference found between these neurons with regard to predominant firing rate (C, Mean $\pm$ S.E.M) or the coefficient of variation of interspike intervals (D, Mean $\pm$ S.E.M).

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