The histone deacetylase HDAC3 is essential for Purkinje cell function, potentially complicating the use of HDAC inhibitors in SCA1

Anand Venkatraman¹,†, Yuan-Shih Hu¹,†, Alessandro Didonna¹,†, Marija Cvetanovic¹,3,†, Aleksandar Krbanjevic¹,†, Patrice Bilesimo¹ and Puneet Opal¹,2,∗

¹Davee Department of Neurology and 2Department of Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA and 3Department of Neuroscience, University of Minnesota, Minneapolis, MN, USA

INTRODUCTION

Spinocerebellar ataxia type 1 (SCA1) is an incurable neurodegenerative disease caused by a pathogenic glutamine repeat expansion in the protein ataxin-1 (ATXN1). One likely mechanism mediating pathogenesis is excessive transcriptional repression induced by the expanded ATXN1. Because ATXN1 binds HDAC3, a Class I histone deacetylase (HDAC) that we have found to be required for ATXN1-induced transcriptional repression, we tested whether genetically depleting HDAC3 improves the phenotype of the SCA1 knock-in mouse (SCA1154Q/2Q), the most physiologically relevant model of SCA1. Given that HDAC3 null mice are embryonic lethal, we used for our analyses a combination of HDAC3 haploinsufficient and Purkinje cell (PC)-specific HDAC3 null mice. Although deleting a single allele of HDAC3 in the context of SCA1 was insufficient to improve cerebellar and cognitive deficits of the disease, a complete loss of PC HDAC3 was highly deleterious both behaviorally, with mice showing early onset ataxia, and pathologically, with progressive histologic evidence of degeneration. Inhibition of HDAC3 may yet have a role in SCA1 therapy, but our study provides cautionary evidence that this approach could produce untoward effects. Indeed, the neurotoxic consequences of HDAC3 depletion could prove relevant, wherever pharmacologic inhibition of HDAC3 is being contemplated, in disorders ranging from cancer to neurodegeneration.

INTRODUCTION

Spinocerebellar ataxia type 1 (SCA1) is a dominantly inherited neurodegenerative disorder characterized by progressive motor incoordination (1). Resulting from a CAG nucleotide repeat expansion in the encoded protein, SCA1 is pathogenically related to eight other neurologic diseases that share this mutational mechanism, the most well known of which is Huntington’s disease (1). These so-called polyQ diseases typically have a mid-life onset; a tendency for the repeats to expand over generations with a progressively more severe phenotype; and widespread expression of the disease-causing protein in the face of relatively circumscribed pathology.

In SCA1, the repeat expansion occurs in the protein ataxin-1 (ATXN1), named after the hallmark ataxia resulting from degeneration of the cerebellar Purkinje cells (PCs) (2). Cerebellar degeneration is inexorable and is accompanied by progressive involvement of other neuronal groups that complicates the clinical picture and adds to the travails of the patient. For instance, degeneration of hippocampal and cortical neurons results in cognitive and dysexecutive symptoms along with spasticity, while that of neurons in the brainstem ultimately leads to death by interfering in vital functions, such as swallowing and breathing (1). There is currently no treatment to halt, let alone reverse this disease; hence the pressing need for translational research.

In recent years, we have been intrigued by the possibility of treating SCA1 by reversing transcriptional alterations in gene
expression. There are several reasons for pursuing this therapeutic approach: first, changes in gene expression are the earliest detectable pathologic alteration in SCA1 animal models (3–7). Secondly, genetic studies in mice demonstrate that ATXN1 must have access to the nucleus for it to engender toxicity, a finding consistent with the notion that disruption of a nuclear process such as transcription might well be playing a pathogenic role (8). Thirdly, neurodegeneration can be prevented in SCA1 mouse models by delaying mutant ATXN1 expression beyond the time window when transcriptional derangements first occur (5). Fourthly, both wild-type (WT) and mutant ATXN1 tether to chromatin and modulate transcription in luciferase assays (7,9,10); moreover, ATXN1 binds a slew of transcriptional modulators, whose levels when altered also alter the phenotype of SCA1 in cellular, Drosophila and mouse models (5,9–12). Fifthly, mutant ATXN1 causes a decrease in histone acetylation at the promoters of genes, a post-translational modification of histones that would be expected to turn off gene expression (7,10). Finally, replenishing the low levels of at least one gene whose promoter is hypoacetylated and repressed in SCA1—the angiogenic and neurotrophic factor, Vascular endothelial growth factor (VEGF)—improves the SCA1 phenotype (7).

An appealing unifying hypothesis to explain ATXN1 pathogenesis is that the polyglutamine expansion causes a gain of ATXN1’s function as a transcriptional repressor. The gain of function itself can be explained by the build-up of expanded ATXN1 (2Q) (Fig. 1B), suggesting that part of ATXN1’s function as a transcriptional repressor comes from the expanded ATXN1 (with 82Q, Q = glutamine) and unexpanded ATXN1 (2Q) (Fig. 1B), suggesting that part of ATXN1’s function as a transcriptional repressor comes from the gain of function when altered also alter the phenotype of SCA1 in cellular, Drosophila and mouse models (5,9–12). Fifthly, mutant ATXN1 causes a decrease in histone acetylation at the promoters of genes, a post-translational modification of histones that would be expected to turn off gene expression (7,10). Finally, replenishing the low levels of at least one gene whose promoter is hypoacetylated and repressed in SCA1—

the angiogenic and neurotrophic factor, Vascular endothelial growth factor (VEGF)—improves the SCA1 phenotype (7). An appealing unifying hypothesis to explain ATXN1 pathogenesis is that the polyglutamine expansion causes a gain of ATXN1’s function as a transcriptional repressor. The gain of function itself can be explained by the build-up of expanded ATXN1 as it fails to be cleared because it misfolds and defies normal function that is enhanced over time, as mutant ATXN1 normal function(s) due to the accumulation of mutated HDAC3, regardless of its polyglutamine length. This is also consistent with the finding that mutant ATXN1 causes toxicity by preserving its native interactions, leading to a gain of normal function(s) due to the accumulation of mutated protein (22).

To test the functional consequences of the ATXN1/HDAC3 interaction, we turned to transcriptional assays. For these experiments, we took advantage of previous findings that ATXN1’s ability to serve as a transcriptional repressor can be monitored in luciferase assays. For instance, in luciferase assays where transcription is induced by the histone acetyl transferase, CREBBinding protein (CBP), ATXN1 inhibits transcription and curtails luciferase expression (10). It is important to note that in this assay both WT and expanded ATXN1 inhibit transcription, once again consistent with the idea that SCA1 is caused by normal function that is enhanced over time, as mutant ATXN1 fails to be cleared. Using this assay, we tested whether depleting HDAC3 by using short interfering RNA (siRNA) can alleviate transcriptional suppression. We were able to knock down HDAC3 expression in N2A cells by at least 60% (Fig. 1C and E), a level sufficient to significantly reduce ATXN1-mediated transcriptional repression compared with an off-target siRNA control (Fig. 1C and D). These results indicate that the two proteins interact in a functional complex, and that endogenous HDAC3 is required for the full extent of ATXN1-induced transcriptional repression.
Genetic depletion of HDAC3 does not have a significant impact on the SCA1 phenotype

If, as suggested by our in vitro assays, HDAC3 is recruited by mutant ATXN1 to cause too much transcriptional repression, then depleting HDAC3 might be expected to relieve this repression to improve the SCA1 phenotype. To test this prediction, we turned to the SCA1 knock-in mouse (SCA1154Q/2Q, SCA1KI) (23). Engineered to express a single expanded copy of the full-length ataxin-1 gene with 154 repeats, this mouse line displays a robust, highly reproducible and well-characterized behavioral and pathologic phenotype that closely mirrors the human disease. It has thus served as an excellent model to test behavioral, pharmacologic and genetic approaches to modulate the SCA1 phenotype (3,4,23,24).

Using this SCA1 knock-in line, we tested whether genetic depletion of HDAC3 mitigates the disease. Since HDAC3 null mice die in utero before embryonic day E 9.5 (25), we tested our hypothesis by mating SCA1 knock-in mice with heterozygous HDAC3+/- mice, which show no overt phenotype. A similar strategy was used by Moumne et al. (26) in testing for the role of HDAC3 in Huntington disease. As reported earlier, HDAC3 haploinsufficient mice show an ≏50% reduction in HDAC3 mRNA without any compensatory changes in the levels of any of the other HDACs (26). At the protein level, the reduction is more modest: ≏30% less than WT HDAC3 in...
the cytoplasm and 20% less in the nucleus (Supplementary Material, Fig. S2). These results differ slightly from those described by Moumne et al., where HDAC3 heterozygous mice displayed a ~40% reduction in nuclear HDAC3 (with total HDAC3 reduction to 80% of WT levels). This could be a result of differences in experimental methods or mouse background (our mice are on a pure C57 background while Moumne et al. used a mixed CBA/C57 background).

To compare the effects of HDAC3 depletion on the SCA1 phenotype and to control for the effects of HDAC3 haploinsufficiency alone, we performed all our assays on the following experimental genotypes: (i) WT, (ii) HDAC3+/−, (iii) SCA1 KI and (iv) SCA1 KI; HDAC3+/− mice. All these mouse models are in the C57/BL6 background, obviating any concerns arising from background effects.

SCA1 mice show significant weight reduction compared with WT mice (23). We therefore monitored the weight of our experimental mouse models over a 6-month period (Fig. 2A). SCA1 KI mice showed a sustained weight reduction compared with WT mice starting from 1.5 months of age. HDAC3+/− mice do not display any alteration in their weight compared with WT mice. However, we also did not detect any amelioration of the SCA1 weight loss with HDAC3 reduction.

SCA1 knock-in mice show a robust ataxic phenotype that is best quantified by the accelerating rotating rod (rotarod) test (7,10,23). In this test, mice that have cerebellar deficits tend to fall early off the rotating rod as it accelerates, with the time that it takes for a mouse to fall being recorded and graphed. We subjected the four experimental genotypes to this assay at first and then again at 6 months when the disease is more advanced (Fig. 2B and C). As expected, the SCA1 knock-in mice performed poorly compared with mice without the knock-in gene (at 3 months, P = 0.034; at 6 months, P = 0.002, Tukey’s HSD post hoc, repeated-measures two-way ANOVAs). HDAC3 depletion did not ameliorate the phenotype; however, as there was no statistical difference between the performance of the SCA1 KI; HDAC3+/− mice and the SCA1 mice (at 3 months, P = 0.982; at 6 months, P = 0.903, Tukey’s HSD post hoc, repeated-measures two-way ANOVAs).

It is interesting to note that HDAC3 haploinsufficiency seemed to improve performance in mice without the SCA1 gene, but the value did not reach statistical significance (P = 0.584 at 3 months, P = 0.569 at 6 months, Tukey’s HSD post hoc, repeated-measures two-way ANOVAs).

SCA1 mice, like SCA1 patients, have quantifiable cognitive deficits that are readily quantified by the Morris Water Maze test. This is a test of spatial learning and is a well-established assay to document hippocampal involvement in SCA1 mice (23,27). We tested our mice between the ages of 9 and 12 weeks, when they are known to show well-characterized difficulties (27). This test has two parts: the first involves mice having to learn the location of a visible platform. All four experimental genotypes learnt this task by the end of 4 days of training (significant days effect) as evidenced by the decreased time the mice take to reach the platform \(F_{(3, 120)} = 86.015, P < 0.0001\), the shorter distance travelled \(F_{(3, 120)} = 63.902, P < 0.0001\) and an increase in the swim speed \(F_{(3, 123)} = 43.710, P < 0.0001\), repeated-measures two-way ANOVAs (Fig. 2D–F). There was no difference in any of these parameters based on the genotype; therefore, selective motor impairment in SCA1 mice would not be a confounding factor in the assessment of spatial learning.

The second task involves testing the ability of mice to recall the location of the platform when the platform is hidden under water. Here, mice must use various visual cues outside the pool and relate these cues to the platform’s location. As has been described before (23), SCA1 mice perform poorly in this test compared with the WT mice (P = 0.012, Tukey’s HSD post hoc, repeated-measures two-way ANOVAs), with significant variation also as a result of the number of days of training \(F_{(3, 120)} = 11.81, P < 0.0001\). HDAC3 depletion did not improve this phenotype in SCA1 mice (P = 0.525, Tukey’s HSD post hoc, repeated-measures two-way ANOVAs) (Fig. 2G).

After the hidden platform trials, a single probe trial was performed where the mice were allowed to swim around in the pool, in the absence of any platform. In this trial, the number of times the mice cross the location of the platform records their memory of its previous location. Here as well, SCA1 KI mice display deficits compared with WT mice (P = 0.01, Tukey’s post hoc test, ANOVA). Depleting HDAC3 in SCA1 mice did not improve the phenotype (P = 0.715). Interestingly, HDAC3 depletion alone appears to have a deleterious impact on the performance of mice without the SCA1 gene (P = 0.01) (Fig. 2H).

We next examined the effects of HDAC3 reduction on SCA1 neuropathology. Because SCA1 neurodegeneration is most pronounced in the cerebellum because of PC involvement, we focused on evaluating cerebellar histopathology. We stained PCs and their neurites with a calbindin antibody, an excellent method to document PC number and size, cellular heterotopia, and alterations in dendritic arborization (28). As expected, we found that calbindin staining intensity was significantly reduced in SCA1 mice compared with WT (P < 0.001, Tukey’s post hoc test, ANOVA), but we did not observe any significant improvement upon HDAC3 depletion (Fig. 3A–E).

**Depleting HDAC3 in PCs results in progressive neurodegeneration**

As shown above, HDAC3 insufficiency did not improve the defining behavioral or pathologic features of the SCA1 knock-in mouse model. It is entirely possible that what is required for amelioration is an even greater reduction of HDAC3 in the context of SCA1. However, this approach would first require that neurons withstand progressively limiting levels of HDAC3 without deleterious effects. To address the issue of neuronal reliance on HDAC3, we decided to deplete all HDAC3 in PCs, the most relevant cell type in SCA1. We mated a floxed HDAC3 mouse line (25,29) to a Cre driver line under the control of the pcp-2 promoter. This promoter turns on ~6 days after birth in PCs, with additional activity in the inferior olive that is also affected in SCA1 (30,31). Cre expression is fully established by 2–3 weeks after birth in mice, close to the time point when transcriptional derangements in SCA1 mice commence (3–7). To monitor the activity of the pcp-2 promoter, we mated these mice to the beta-galactosidase reporter mice, where we can clearly see robust beta-galactosidase activity in
Figure 2. HDAC3 haploinsufficiency does not rescue SCA1 behavioral phenotype. (A) One-way ANOVA revealed significant influence of the SCA1 KI gene on mouse weight starting at 1.5 months, but no significant impact of HDAC3 depletion and no interaction between the two genes. Note that HDAC3 haploinsufficiency by itself does not have any effects on the growth curves of mice. (B and C) HDAC3 haploinsufficiency does not rescue the SCA1 cerebellar motor phenotype. WT, HDAC3<sup>+/−</sup>, SCA1 KI and SCA1 KI; HDAC3<sup>+/−</sup> mice were tested on a rotarod at 3 months (B) and 6 months. (C). SCA1 knock-in mice performed poorly compared with mice without the knock-in gene, as noted by their inability to stay on the rotarod (3 months P = 0.034; 6 months P = 0.002; Tukey’s HSD post hoc test, repeated-measures two-way ANOVA). However, no significant improvement was discernible in SCA1 KI; HDAC3<sup>+/−</sup> mice compared with SCA1 KI mice alone (3 months P = 0.982; 6 months P = 0.903; Tukey’s HSD post hoc test, repeated-measures two-way ANOVA). Data indicate mean ± SEM. * P < 0.05. (D–H) HDAC3 haploinsufficiency does not rescue the SCA1 hippocampal phenotype. Spatial learning and memory in 9- to 12-week-old mice were assessed by the Morris Water Maze test. The visible platform part of the test showed all four genotypes improved in this task over the course of 4 days (significant day effects), as determined by (D) time to platform [F<sub>3,120</sub> = 86.015, P < 0.0001], (E) swim distance [F<sub>3,120</sub> = 63.902, P < 0.0001] and (F) swim speed [F<sub>3,123</sub> = 43.710, P < 0.0001], with no significant difference between genotypes (time to platform [F<sub>3,40</sub> = 0.367, P = 0.777; swim distance [F<sub>3,40</sub> = 1.368, P = 0.266; swim speed [F<sub>3,41</sub> = 0.923, P = 0.438]). In part two of the test, when the platform was hidden by submerging, as expected the SCA1 KI mice took significantly longer to reach the platform than WT mice (P = 0.012, Tukey’s HSD post hoc, repeated-measures two-way ANOVA). However, depletion of HDAC3<sup>+/−</sup> in SCA1 KI mice did not rescue the learning and memory deficits of SCA1 KI mice (P = 0.525, Tukey’s HSD post hoc, repeated-measures two-way ANOVA). In a 60-s probe trial given after the hidden platform tests, WT mice crossed the exact location where the platform had rested significantly more often than SCA1 KI mice and also more than HDAC3<sup>+/−</sup> mice, but depletion of HDAC3 did not improve performance of SCA1 KI mice (H). Values indicate mean ± SEM. * P < 0.05.
This efficient deletion of the floxed gene in PCs is consistent with previous reports and occurs across all the lobules of the cerebellum (30–32).

Deleting HDAC3 in cerebellar PCs did not affect the general health of the mice as evidenced by body weight \[F(1,8) = 2.757, P = 0.135\], two-way ANOVAs (Fig. 4B). We next subjected these mice to detailed cerebellar testing by the rotarod. Since it was difficult \textit{a priori} to predict the phenotype, we performed rotarod testing at monthly intervals starting at weaning. We found significant progressive deterioration in rotarod performance in the HDAC3\textsuperscript{flox/flox}; pcp2 Cre\textsuperscript{+} mice beginning at 2 months. Note that the pcp2 allele does not affect the rotarod phenotype (Fig. 4H; rotarod at 3 month is shown as an example).

To evaluate cerebellar histopathology, we sectioned mouse cerebella and stained PCs and their neurites for calbindin (28). We quantified the degree of degeneration by semi-quantitative immunofluorescence using the confocal microscope, documenting the thickness of the molecular layer and the fluorescence intensity profile (Fig. 5). Staining revealed significant PC pathology, demonstrable by a thinning of the molecular layer, an associated decrease in the calbindin staining noticeable in 4- to 6-month-old mice and a loss of PCs (Fig. 5A–F). In the most affected lobules, there was significant loss of PCs, with only a few scattered neurons remaining (Fig. 5G–J). We also performed Nissl staining as an independent method to document the loss of PC (Fig. 5K and L). Because different regions of the cerebellum were variably affected, we performed our analyses on three cerebellar regions (Fig. 5M shows a schematic): the anterior (between lobules III and IV), the border between the anterior and posterior cerebellum (between lobules V and VI) and the border between the posterior cerebellum and flocculonodular lobe (between lobules IX and X) (33,34). Intriguingly, the anterior lobules appeared to be affected more than the posterior lobules, even though Cre excision appeared to be uniform across all lobules (Fig. 4A). There was no clear correlation to the pattern of degeneration seen in SCA1: most of the PC degeneration in SCA1 mice was seen in lobules IX and X, which are characteristically spared in the HDAC3 conditional knock-out line (Fig. 5 and data not shown). This accords with mounting evidence that PCs have topographically complex patterns of cell loss in different disease situations because of differential expression of key molecules, such as Zebrin II, HSP 25 and glutamate transporters (35,36). It would be interesting to discern whether HDAC3 modulates the transcription of these molecules (37).

Regardless, depleting HDAC3 in PCs has significant deleterious consequences, both pathologically and behaviorally.

Finally, we performed several experiments to discern whether cerebellar Purkinje neurons die by apoptosis. TUNEL staining failed to reveal apoptosis (even though a positive control of cerebella treated with DNase I to introduce DNA breaks showed significant TUNEL positivity) (Supplementary Material, Fig. S3). We performed these stainings at several time points, including at 2 and 5 months, when the majority of neuronal loss is observed. It is possible that apoptosis still occurs but at a rate below the detection of our techniques, but it is also possible that neuronal loss occurs by a non-apoptotic mechanism, has been described in several neurodegenerative conditions including polyglutamine diseases (38–41).
One aspect of SCA1 pathogenesis is driven by mutant ATXN1’s exaggerated ability to repress gene transcription. In this study, we tested whether we can tamp down this particular gain of function and thus ameliorate the SCA1 phenotype by depleting the ATXN1 interactor, HDAC3. Somewhat to our surprise, a moderate lowering of HDAC3 levels did not mitigate the disease.

**DISCUSSION**

Figure 4. Selective depletion of HDAC3 in Purkinje cells causes progressive motor impairment. (A) The pcp2 Cre transgenic line is effective in inducing Cre-driven excision in Purkinje cell-conditional manner as shown by PC X-gal staining of the floxed beta-galactosidase transgenic reporter mouse line. Scale bar = 1 mm. (B) Mice with HDAC3 selectively depleted in the PCs (HDAC3flox/flox; pcp2 Cre+) do not show any significant difference in body weight from WT age-matched controls. (C–H) Mice with HDAC3 selectively depleted in the PCs (HDAC3flox/flox; pcp2 Cre+) show a cerebellar motor phenotype noticeable by the rotarod at 2 months [$F_{(1,34)} = 5.601$, $P = 0.024$, repeated-measures two-way ANOVAs] with progression as seen by rotarod performance at 3, 4, 5 and 6 months [3 months, $F_{(1,23)} = 8.105$, $P = 0.008$; 4 months, $F_{(1,28)} = 21.183$, $P < 0.0001$; 5 months, $F_{(1,23)} = 19.839$, $P < 0.0001$; 6 months, $F_{(1,23)} = 34.77$, $P < 0.0001$, repeated-measures two-way ANOVAs]. Note that the pcp2 Cre expression does not affect rotarod performance as shown at 3 months [$F_{(1,18)} = 3.397$, $P = 0.082$] (H). All values are mean ± SEM. *$P < 0.05$. 
phenotype, and complete ablation of HDAC3 in PCs was deleterious both behaviorally and pathologically.

Acetylation of histone tails has two important consequences vis-à-vis transcription: first, histone acetylation reduces the overall negative charge on DNA to promote an open chromatin conformation for transcriptional machinery binding and processivity; secondly, the residues themselves provide docking sites for transcriptional activators that read these marks (42). By keeping histones deacetylated, HDACs, including HDAC3, are crucial enzymes regulating gene expression (15). Their role

Figure 5. Pathologic phenotype of HDAC3 depletion in PCs. (A–F) Parasagittal sections of cerebella from 2- and 6-month-old mice of the HDAC3flox/flox; pcp2 Cre+ genotype and age-matched WT littermate controls were stained with calbindin. (A) and (B) show the length of molecular layer in the border between lobules III/IV, V/VI and IX/X at 2 and 4–6 months, respectively. (C) and (D) show the quantification of intensity of calbindin staining in these lobules. (E and F) show the number of PCs in comparable 200 μm segments from the apex in the relevant lobules normalized to WT as a percentage. Data represent mean ± SEM, at least three mice were used per genotype per time point, and six sections were stained per mouse. *P < 0.05 by unpaired t-test. (G–J) Representative images of calbindin immunostaining on cerebellar sections at 6 months of age are shown for WT (G and I) and HDAC3flox/flox; pcp2 Cre+ mice (H and J). (E and F): scale bar = 500 μm. High magnification images depict lobule V/VI in WT (G) and HDAC3flox/flox; pcp2 Cre+ (H) cerebella. Scale bar = 100 μm. (K–L) Nissl staining confirms loss of PCs in 6-month-old HDAC3flox/flox; pcp2 Cre+ mice (L) when compared with WT control (K). Arrowhead points to a PC in the control section. Scale bar = 100 μm. (M) Schematic of the parasagittal sections of the cerebellar vermis relating the lobule nomenclature to cerebellar anatomy.
has been best studied in the context of cancer, where they play a role in proliferation, preserving an undifferentiated state and promoting proliferation (15); they are thus being increasingly targeted by drugs called HDAC inhibitors. HDACs have been grouped into four distinct classes based on their primary structure and catalytic properties. HDAC3 belongs to the Class 1 family of HDACs that function as part of repressor complex, and includes the closely related proteins HDAC 1 and 2; the proteins that HDAC3 complexes with, however, are completely distinct. For instance, HDAC 1 and 2 interact with NuRD, Sin3 and CoREST, but HDAC3 complexes with NCoR and SMRT, both of which also bind ATXN1 (9), providing a further justification for interest in HDAC3-mediated transcription in SCA1.

Not much is known about the role of HDAC3 in the nervous system, despite the fact that it is one of the most widely expressed HDACs in the brain (19) (Allen Mouse Brain Atlas: http://mouse.brain-map.org/experiment/show/71232781), and that it is one of the HDACs consistently thought to play a role in several other neurodegenerative syndromes, including Huntington disease (43), SCA3 (44) and SCA7 (45). There are, however, some hints that it might play a role in neuroplasticity. Neuronal activity is thought to generate a signaling cascade that increases histone acetylation to promote gene expression responsible for learning and memory (46). HDACs, such as HDAC3, have been shown to be powerful negative regulators of these processes. For instance, depleting HDAC3 in the CA1 region of the hippocampus—either by delivering a pharmacologic HDAC inhibitor, or by conditionally depleting HDAC3 by viral Cre-delivery to conditional HDAC3 null mice—improves the ability of a mouse to remember the location of an object (47). Interestingly, we found that HDAC3 depletion in isolation had a deleterious effect on mice in the memory portion of the Morris Water Maze test (Fig. 2H).

Studies on cell-based, Drosophila and mouse models of Huntington disease have shown that HDAC inhibitors can improve the phenotypes (48,49). The notion that these drugs work via HDAC3 inhibition came initially from studies in Caenorhabditis elegans, where a systematic study of individual HDACs in a Huntington model suggested that depleting the C. elegans version of HDAC3 had the most beneficial effects (50). Work in cultured neurons also suggests that neurons are particularly susceptible to the toxic effects of HDAC3 overexpression (51). Indeed, HDAC3 could well be considered a proapoptotic molecule—normally kept in check by prosurvival Akt-mediated signaling—that is unleashed in the context of neurodegeneration (51). These findings have spurred the development of novel HDAC3-specific inhibitors that are showing extremely encouraging results in preclinical studies (52). They also provide the backdrop for our own studies in SCA1.

Our intention, at the start of these experiments, was to reduce HDAC3 by genetic deletion as a prelude to a pharmacologic approach. The results of genetic depletion should, in principle, be easier to interpret compared with pharmacologic studies since there are no confounding off-target effects, often the case with even the most selective drugs. For these experiments, we reduced HDAC3 globally, by mating HDAC3+/− mice with SCA1 knock-in mice. We studied the effects of HDAC3 depletion on the constellation of SCA1 signs (weight loss, hippocampal cognitive deficits and cerebellar motor dysfunction). All in all we did not find significant improvement on the disease phenotype of SCA1 mice. This could well be because of a lack of effect of HDAC3 depletion, but might also be because the depletion was too modest to elicit a phenotypic improvement. These results are reminiscent of a comparable lack of beneficial response using a similar strategy in a mouse Huntington disease model (26).

The next obvious step was to test if further depletion might improve cerebellar physiology that would trump the SCA1 phenotype; however, we observed deleterious effects of HDAC3 depletion, as evidenced by the PC-specific HDAC3 null line. These mice show early-onset ataxia, with pathologic changes including dendritic pruning of the PC arbors and the eventual loss of the neurons themselves. Our results clearly demonstrate a requirement for HDAC3 in the maintenance of postmitotic PCs, and that other HDACs of the same class such as HDAC1 and 2 cannot compensate for its lack.

How might one explain our results in the face of the lack of toxicity from depleting HDAC3 in the hippocampus and nucleus accumbens? There could be several explanations: for one, in those experiments, the effects of HDAC3 depletion were studied after a relatively short period of 2 weeks. This might explain why HDAC3 heterozygous mice in our hands showed spatial memory deficits in the Water Maze task, rather than the beneficial effects described in the relatively short-term studies described to date (47). Indeed, our experiments are the first to study the effects of long-term genetic depletion of HDAC3 in any post-mitotic neuron. It is also possible that the efficiency of Cre-mediated excision is higher in our hands than by adenoviral delivery, the methodological approach used in these reports. Finally, we cannot exclude the possibility that cerebellar PCs are especially sensitive to HDAC3 depletion. For instance, HDAC3 is important for mediating transcriptional repression by unliganded nuclear and thyroid hormone receptors (53). Could it then be that PCs have energy demands that make them especially vulnerable, given the role of these receptors in regulating metabolism (54,55)(29,54,56)? This could help explain the cerebellar ataxia seen in hypothyroid disorders (57,58). To comprehensively explore the possibility of selective PC vulnerability, one would have to generate genetic mouse models where HDAC3 is depleted in distinct sets of post-mitotic neurons to address its role elsewhere in the brain and nervous system. If neuron-specific susceptibility to HDAC3 depletion were to be observed, then it would be important to determine HDAC3-dependent gene networks in different neuronal subtypes. This would require detailed experiments dedicated to profiling genes regulated when HDAC3 levels are modulated in a neuron-specific manner—such as microarray or RNA-Seq—along with techniques such as chromatin immunoprecipitation to determine the direct targets of HDAC3.

Regardless, our results have important implications for therapy aimed at HDAC3 depletion in the context of SCA1, particularly since the side effects overlap with the symptoms of the disease. Similar considerations are relevant not only to other neurodegenerative syndromes, where HDAC 3 inhibitors are being proposed, but also to cancer. For instance, in non-neuronal cancers, the neurologic side effects of HDAC3 depletion might be confused with central nervous system metastases or paraneoplastic syndromes that include ataxia (59). Given the promise of HDAC inhibitors for a wide range of conditions, considerable effort should be expended on discerning the deleterious
consequences of HDAC inhibition and mitigating side effects by delineating the therapeutic window. It is possible that strategies such as pulse-dosing, the use of lower-dose cocktails of several HDAC inhibitors or their use in combination with other agents might also help avoid neuronal side effects of this promising class of epigenetic modifiers.

MATERIALS AND METHODS

Cell lines and culture conditions

Human Embryonic Kidney (HEK) 293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin). Mouse neuroblastoma N2a cells were cultured in DMEM supplemented with sodium pyruvate, non-essential amino acids, 10% FBS and antibiotics.

Mouse strains

All animal experiments were performed in compliance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and the Northwestern University Institutional Animal Care and Use Committee. The generation and characterization of the SCA1 knock-in mice have been previously described (7,23). HDAC3 constitutive and conditional mice were kindly provided by Dr Scott Hiebert (25). The B6.129-Tg (Pcp2-cre) 2Mpin/J mice expressing a Cre gene under the control of the Pcp2 gene promoter (31), and the B6.129S4-Gt(ROSA)26SorMLZ/J reporter line, which expresses the LacZ gene only in those cells where Cre is expressed (60), were obtained from the Jackson Laboratory. All the mouse lines are in the C57/Bl6 background, obviating any confounding genetic background effects.

Co-immunoprecipitation assays

HEK293 cells were grown at 80% confluence on 10 cm dishes in preparation for transfection and immunoprecipitation. GFP-ataxin-1 (2Q or 84Q) and Flag-HDAC3 expressing plasmids were co-transfected per dish with Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection, the cells were re-seeded onto 12 mm coverslips, and the following day they were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature (RT). Cells

Luciferase assay

Luciferase functional assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Briefly, N2a cells were plated in six-well plates at a density of 500 000 cells/well. Upon reaching 80% cell confluence, N2a cells were co-transfected with the following DNA constructs: pGL4-luc, pGL4-CBP, pEGFP-Ataxin1-84Q or pEGFP-Ataxin1-2Q (300 ng each). Empty vector pcDNA3 was added to normalize the amounts of DNA for transfection to 900 ng per well. Knock down was achieved by transfecting cells with an siRNA pool (mouse HDAC3 Smart Pool or Off-Target Pool as a negative control; Dharmacon) at the final concentration of 100 nM. A CMV promoter-driven renilla luciferase construct, pRL-CMV, was included in each transfection to control for experimental variability arising from differences in transfection efficiency or cell number. All transfections were performed using Lipofectamine 2000 (Invitrogen), and the cells were harvested 48 h post-transfection for luminescence measurements. A total of five independent experiments were performed, wherein each transfection was performed and assayed at least in triplicate. Results were first plotted as the ratio of luciferase-to-renilla activity per condition. Subsequently, the results were normalized to the basal activity of the control samples transfected with pGL4-luc construct alone. Finally, the extent of repression was plotted as a percentage inhibition (calculated relative to ATXN1-induced inhibition on CBP-induced luciferase activity). Statistical analysis was performed using one-way ANOVA followed by a post hoc Tukey’s test. Data were considered statistically significant when P < 0.05. To confirm the expression levels of the transfected ATXN1 constructs and the relative siRNA-induced knock-down of HDAC3, 100 μg of N2a cell lysates were loaded on denaturing SDS gels for analysis by western blotting. The antibodies used were mouse anti-ataxin-1 (11- NQ, Neuro-mab), mouse anti-GFP (A5441, Roche), rabbit anti-HDAC3 (H3034; Sigma) and mouse anti-actin (AC15; Sigma). Protein expression levels were quantified by densitometric analysis using the ImageJ 1.46 software (National Institutes of Health), where the expression of HDAC3 was normalized to the actin loading control. Statistical analysis was performed using unpaired Student’s t-test and data were considered statistically significant when P < 0.05.

Immunofluorescence assay

N2a cells were grown in 12 well plates and transfected with either GFP-ataxin-1 (2Q or 84Q) coding plasmid or empty vector using Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection, the cells were re-seeded onto 12 mm coverslips, and the following day they were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature (RT). Cells
were permeabilized with 0.3% Triton X-100 in PBS for 10 min and then blocked with 5% normal goat serum (NGS) in PBS for 30 min. The cells were then incu...body anti-HDAC3 (F3403; Sigma) diluted in 2% NGS (1:400) for 2 h at RT. Coverslips were washed in PBS-T (0.05% Tween 20) twice before the incubation with a goat anti-rabbit Alexa fluor 594 secondary antibody (Invitrogen). After four washes in PBS-T, coverslips were mounted onto glass slides using Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Cells were imaged using a CTR6500 confocal microscope (Leica) equipped with the Leica LAS AF software.

**Mouse body weight**

Five mice of each experimental genotype were weighed every 2 weeks (between the ages of 1 and 6 months) for the SCA1 KI × HDAC3+/− experiment, and every month (also between the ages of 1 and 6 months) for the HDAC3flox/flox experiment. To avoid spurious variability because of sex differences, only female mice were used for these weight plots.

**Rotarod analysis**

The rotarod assay was performed as previously described (7,10). Briefly, mice were placed on the rotarod apparatus (Ugo Basile) that accelerates from a speed of 4–40 rpm over a 5-min period. The time it takes for a mouse to fall off is recorded, to a maximum of 10 min. Mice were subjected to four trials per day for 4 consecutive days, with at least 10 min of rest between each trial. Mice from the SCA1 KI × HDAC3+/− breedings were sequentially assayed at 3 and 6 months. The average performances for each day were plotted, and statistical differences between the different groups were statistically analyzed using repeated-measures two-way ANOVAs, followed by Tukey’s HSD post hoc test for multiple comparisons. Mice from the HDAC3flox/flox group were assayed sequentially at monthly intervals till they reached 6 months of age. Significance was assumed at $P < 0.05$. All experiments were performed blinded with respect to the knowledge of genotype.

**Morris Water Maze test**

Spatial learning in the Morris Water task was tested following a protocol previously described elsewhere (Watase 2002). Briefly, mice were trained to locate a platform in a circular pool (178 cm diameter; Hastings Corp.) connected to a video-tracking system composed of an infra-red USB digital camera equipped with the WaterMaze software (Actimetrics, Inc.). In the first part of the experiment, the mouse had to locate the platform (made visible with black flags and a trim of black around the edges) in eight trials per day in two blocks of four trials each, over 4 consecutive days. In the second part of the experiment, the platform was hidden (submerged 0.5 cm under water) and the mouse was subjected to the same numbers of trials as in the first part. Both phases had a maximum time allowed of 60 s per trial. For probe trials, the platform was removed and each mouse was given 60 s to find the platform. The number of times the mouse crossed over the previous location of the platform was tracked. The relative performances among the different groups of mice were compared using repeated-measures two-way ANOVAs to assess the impact of the genotypes and the number of days of training experienced beforehand, and followed by Tukey’s HSD post hoc test for multiple comparisons whereas stated. Probe trials were analyzed using one-way ANOVA, followed by Tukey’s post hoc test. All experiments were performed blinded with respect to knowledge of genotype. Statistical significance was assumed at $P < 0.05$.

**Histopathologic analysis of cerebellum**

Brains were isolated from mice and fixed with paraformaldehyde 4% in PBS overnight at 4°C. They were subsequently equilibrated in 30% sucrose and embedded in optimal cutting temperature (OCT) medium. Forty micrometer parasagittal sections were cut using a cryostat (Microm M505, Thermo Fisher Scientific). Brain slices were permeabilized with 1% Triton X-100 in PBS (PBS-T) for 10 min and blocked with 5% NGS in PBS-T for 3 h at RT. Slices were then stained with the primary antibody anti-calbindin (C9848, Sigma for the SCA1 KI experiments; EG-20, Sigma for the HDAC3flox/flox experiments) diluted (1:200) in 5% NGS overnight at 4°C. After three washes in PBS, slices were incubated with a goat anti-rabbit Alexa fluor 594 secondary antibody (Invitrogen) diluted (1:400) in PBS-T for 3 h at RT in the dark. Slices were washed four times in PBS and mounted onto glass slides using Vectashield with DAPI (Vector Laboratories). Cerebella were imaged using a CTR6500 confocal microscope (Leica) equipped with the Leica LAS AF software. Calbindin staining intensity was assessed using established techniques (7,23). Nissl stain was performed by the Northwestern University Pathology Core on ~10 μm Paraffin sections using Cresyl violet 0.5% solution. All experiments were performed on littermate controls. We used at least three separate litters for each experimental condition with at least six sections per mouse, with a representative experiment shown. For the quantification of calbindin intensity of the SCA1 mice and the effect of HDAC3 depletion on this phenotype, the images from lobule IX/X that we have found to be most affected in SCA1 mice were quantified. HDAC3flox/flox experiments had calbindin intensity and molecular layer thickness quantified over three distinct cerebellar regions as indicated. PCs were counted in comparable 200 μm regions starting from the apex of each relevant lobular fold. Statistical analyses were performed using one-way ANOVA, followed by Tukey’s test for the SCA1 experiment and unpaired t-test for the HDAC3flox/flox experiments.

**X-gal staining for β-galactosidase activity**

Brains were isolated from mice and fixed with 0.2% paraformaldehyde in PIPES buffer (0.1 M PIPES pH 6.9, 2 mM MgCl2, and 5 mM EGTA) at 4°C overnight. The following day, the brains were equilibrated in 30% sucrose in PBS supplemented with 2 mM MgCl2 and embedded in OCT medium. About 60 μm parasagittal sections were cut using a cryostat (Microm M505, Thermo Fisher Scientific) and post-fixed with 2% paraformaldehyde in PIPES washes in PBS-T, coverslips were mounted onto glass slides using Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Cells were imaged using a CTR6500 confocal microscope (Leica) equipped with the Leica LAS AF software.
(concentrated Rinse buffer containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 1 mg/ml X-gal) at 37°C for 48 h. The stained slices were then rinsed in PBS supplemented with 2 mM MgCl2 and mounted onto glass slides using Vectashield (Vector Laboratories). The sections were imaged using an Axiosvert microscope (Zeiss) equipped with the Axiosvision software. The images of the different portions of the cerebellum were captured using a 4x objective and merged together using the ImageJ software to obtain a composite picture of the whole structure.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank members of the Opal lab for their intellectual input. P.O. thanks Dr Ameet Kini for discussions and critical reading of the manuscript. We thank Jessica Huang for help with histology and mouse genotyping. We also thank the Northwestern University Behavioral Phenotyping Core for help with behavioral assays, and the Northwestern University Mouse Histology and Phenotyping Laboratory for help with staining. We thank Dr Kwang-Youn Kim in the Biostatistics Core for advice on statistical tests.

Conflict of Interest statement. None declared.

FUNDING

This work was funded by the US National Institutes of Health (grant nos R01 NS062051 and 1R01NS082351); with additional funding from the National Ataxia Foundation and the Brain Research Foundation (P.O.).

REFERENCES


