Ectopic expression of the striatal-enriched GTPase Rhes elicits cerebellar degeneration and an ataxia phenotype in Huntington's disease

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**Abstract**

Huntington's disease (HD) is caused by an expansion of glutamine repeats in the huntingtin protein (mHtt) that invokes early and prominent damage of the striatum, a region that controls motor behaviors. Despite its ubiquitous expression, why certain brain regions, such as the cerebellum, are relatively spared from neuronal loss by mHtt remains unclear. Previously, we implicated the striatal-enriched GTPase, Rhes (Ras homolog enriched in the striatum), which binds and SUMOylates mHtt and increases its solubility and cellular cytotoxicity, as the cause for striatal toxicity in HD. Here, we report that Rhes deletion in HD mice (N171-82Q), which express the N-terminal fragment of human Htt with 82 glutamines (Rhes−/−/N171-82Q), display markedly reduced HD-related behavioral deficits, and absence of lateral ventricle dilatation (secondary to striatal atrophy), compared to control HD mice (N171-82Q). To further validate the role of GTPase Rhes expression would elicit a pathology in a brain region normally less affected in HD. Remarkably, ectopic expression of Rhes in the cerebellum of N171-82Q mice, during the asymptomatic period led to an exacerbation of motor deficits, including loss of balance and motor incoordination with ataxia-like features, not apparent in control-injected N171-82Q mice or Rhes injected wild-type mice. Pathological and biochemical analysis of Rhes-injected N171-82Q mice revealed a cerebellar lesion with marked loss of Purkinje neuron layer parvalbumin-immunoreactivity, induction of caspase 3 activation, and enhanced soluble forms of mHtt. Similarly reintroducing Rhes into the striatum of Rhes deleted Rhes−/− Hdh150Q/150Q knock-in mice, elicited a progressive HD-associated rotarod deficit. Overall, these studies establish that Rhes plays a pivotal role in vivo for the selective toxicity of mHtt in HD.

**Introduction**

Huntington's disease (HD) is a Mendelian-dominant condition that, in the majority of HD patients, has symptoms-onset in middle age. In 1993, cloning of the affected gene that codes for the protein huntingtin (Htt) offered the possibility of genetic diagnosis (The Huntington's Disease Collaborative Research Group, 1993). Normal Htt protein contains 11–34 tandem glutamines near the N-terminus of the protein (commencing at the 18th amino acid position). Expansion of glutamines in Htt to more than 37 glutamines (mHtt) causes HD, with a larger numbers correlating with younger age of onset. Early symptoms of HD include psychiatric disturbances, but the predominant symptomatology is motoric and leads to the earlier designation of the disease as Huntington's chorea. The motor disabilities stem from massive damage to the corpus striatum, which can shrink to as little as 10% of its normal volume in advanced disease. Cortical abnormalities are seen in early Huntington's disease (Nopoulou et al., 2010). Surprisingly, the cerebellum is fairly impervious to damage from HD (Gutekunst et al., 2002; Jackson et al., 1995; Tabrizi et al., 1999). Within the striatum, most of the medium spiny neurons (MSN) are destroyed, but small interneurons, enriched in neuronal nitric oxide synthase (nNOS), remain normal, even in advanced disease (Ferrante et al., 1985). Despite the ubiquitous expression of mHtt in all organs of the body, why the pathology and the symptoms of HD appear to be predominantly limited to the brain and its specific regions such as striatum remains unclear.

Over the last few years, several important findings about how mHtt might kill MSN have emerged. Both cell-autonomous and non-autonomous mechanisms contribute to striatal damage in HD (reviewed, (Ehrlich, 2012)). Numerous investigators have sought proteins that might interact uniquely with mHtt to cause damage. Many Htt protein interactors and a number of mechanisms linking these interactors to HD pathogenesis have been described (Kaltenbach et al., 2007; Landles and Bates, 2004; Li and Li, 2004; Ross, 2004). mHtt forms aggregates in tissues and were first thought to be the cause of the cellular toxicity. However, several studies have now discovered that the soluble, non-aggregated (or oligomeric) forms of mHtt are most toxic culprits (Arrastae et al., 2004; Kitamura et al., 2006; Poirier et al., 2005; Ratovitski et al., 2009; Sadowa et al., 1998). Despite such increased knowledge about the mechanisms, it is less clear why selective regions such as the corpus striatum, but not the cerebellum, are vulnerable to damage.
Previously, we have implicated Rhes as a mediator of mHtt toxicity. Rhes, a 266-amino acid protein, contains a GTP-binding domain and a SUMO E3 ligase domain, and is highly expressed in the striatum with much lower levels in other regions, such as the cortex, which has little or no expression in the cerebellum (Falk et al., 1999; Jiang et al., 2013; Usui et al., 1994). Human expression data from Allen Brain atlas confirms with striking enrichment in the striatum with little to no expression in cerebellum. Although its role in the striatum is not yet clear, several studies have demonstrated a role for Rhes in regulating the G-protein–coupled receptor signaling of thyroid hormones and dopamine (Errico et al., 2008; Vargiu et al., 2004). Behavioral analysis of Rhes knockout mice, using dopamine-related drugs, suggests that Rhes may have a role in motor coordination (reviewed, (Harrison, 2012)). We found that Rhes binds to mHtt and promotes cellular death in cell culture models by acting as SUMO-E3 ligase, which is confirmed by a recent study (O’Rourke et al., 2013; Subramaniam et al., 2010; Subramaniam et al., 2009). In addition, the role of Rhes in HD has also been supported by other recent reports (Baiamonte et al., 2013; Lu and Palacino, 2013; Mealer et al., 2013; Okamoto et al., 2009; Sbodio et al., 2011; Seredenina et al., 2011), yet its definitive role in HD pathogenesis in vivo remains unclear. Here, using knockout and overexpression strategies, we show that Rhes plays a major role in phenotypic and pathological deficits of transgenic and knock-in HD mouse models being pivotal for the selective regional toxicity of mHtt.

Materials and methods

Reagents and chemicals

Unless otherwise specified, chemicals and reagents were purchased from Sigma (St. Louis, MO), EM-48 antibody was obtained from Millipore (ABM5374). Antibodies against mTOR (2972S), pS6K T389 (5656S) were from Cell Signaling Technology, Inc. Antibody for actin (9234S), S6K (9202S), Cleaved Caspase-3 (9661S) and huntingtin (MAB5374). Antibodies against mTOR (2972S), pS6K T389 (5656S) and huntingtin (MAB5374) were from Santa-Cruz. Pre-made adenoviral-control (null), adenoviral-CMV-Rhes (0.5 μl/injection, 5.6 × 10⁹ opu/μl) and adenoviral-CMV-GFP (0.8 μl/injection, 1 × 10⁹ opu/μl) were from Cell Signaling Technology, Inc. Antibody for actin was from Santa-Cruz. Pre-made adenoviral-control (null), adenoviral-GFP, and adenoviral-Rhes particles were purchased from Applied Biologicals (Abm good, British Columbia, Canada).

Generation of Rhes-deleted N171-82Q mice

Homozygous Rhes KO mice, used in our earlier work (Mealer et al., 2014; Subramaniam et al., 2010), were crossed with N171-82Q mice from Jackson Laboratories and maintained by crossing C57BL6 × C3H/Reagents and chemicals (Abm good, British Columbia, Canada).

Generation of Rhes-deleted Hdh150Q knock-in mice (Rhes<sup>−/−</sup>/Hdh150Q<sup>150Q</sup>)

Homozygous Rhes KO mice, used in our early work (Mealer et al., 2014; Subramaniam et al., 2010), and wild-type (WT) littermates were crossed with heterozygous Hdh150Q (B6.129P2-Htt<sup>tm2Def</sup>/150J, 004595) mice from Jackson Laboratories and maintained in our state-of-the-art animal breeding facility. We obtained in first-generation three heterozygous male mice positive for Rhes<sup>−/−</sup>/Hdh150Q<sup>150Q</sup>−/, which were backcrossed Rhes<sup>−/−</sup>/Hdh150Q<sup>150Q</sup>−/ X Rhes<sup>−/−</sup>− and resulted in 5 heterozygous (Rhes<sup>−/−</sup>/Hdh150Q<sup>150Q</sup>−/) mice (3♂ and 2♀). Rhes-deleted Hdh150Q heterozygous mice were crossed (Rhes<sup>−/−</sup>/Hdh150Q<sup>150Q</sup>−/ X Rhes<sup>−/−</sup>/Hdh150Q<sup>150Q</sup>−/) to obtain Rhes deleted Hdh150Q homozygous mice (Rhes<sup>−/−</sup>/Hdh150Q<sup>150Q</sup>−) along with littermate controls. Genotyping was confirmed for Hdh150Q as per genotyping protocol from Jackson Laboratories and for Rhes as per the standardized protocol as described before (Errico et al., 2008).

Stereotoxic injection of Rhes into the cerebellum of N171-82Q mice

The N171-82Q mice and wild-type littermates were injected in deep cerebellar nuclei of cerebellum with either adenovirus-CMV-null (abmgood) or adenovirus-CMV-Rhes (4 μl/injection, 1 × 10⁹ opu/μl) (n = 7/group/mixed sex), using coordinates (anteriorposterior, −5.75 from bregma; mediolateral, −1.8 from bregma; dorsoventral, −2.6 from dura; incisor bar, 0.0), with a 10 μl Hamilton syringe at a rate of 0.5 μl/min (Dodge et al., 2005) for a total of 4 μl (1 × 10⁹ opu/μl) per injection site. The stereotoxic coordinates were validated using a fast green dye, showing an effective distribution throughout the cerebellum (Supplementary Fig. 1).

Table 1

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Behavioral analysis

Behavioral testing was performed as described in our previous work (Pryor et al., 2014) during the light phase of the light–dark cycle between 8:00 am and 12:00 pm. For Rhes KO/transgenic N171-82Q mice, behavior was recorded at 8, 12, and 16 weeks. Each set of behavioral testing included body weight measurement and open-field test on the first day, and rotarod on the second. For cerebellar injections, seven-week old animals were pre-trained to stay on an accelerating rotarod starting with 2–4 rpm and increased up to 40 rpm in 300 s (cut-off time) before surgery. On the 4th, 9th, and 14th day post injection/surgery, all the behavioral tests were, sequentially carried out, including body weight and open field tests in the morning, and rotarod and behavioral scoring in the evening (Pryor et al., 2014).

Behavioral scoring was adapted from assessment previously reported (Guynet et al., 2010). The scoring consisted of a ledge test, claspimg, gait, kyphosis, and tremor. An unbiased person who was blind for animal’s genotype performed all the scoring. Individual measures were scored on a scale of 0–3, with 0 representing an absence of the relevant phenotype and 3 representing the most severe manifestation. Each test was
performed in triplicate. To determine tremor, mice were placed in a clean cage and observed for 30 seconds. Each mouse was scored as follows: 0, no signs of tremor, 1, present but mild tremor, 2, severe intervals of tremor or constant moderate tremor, and 3, outrageous chronic tremor. All protocols were approved by The Scripps Research Institute Florida Institutional Animal Care and Use Committee.

**Immunohistochemistry**

Animals were anesthetized with a 250 mg/kg dose of Avertin (2,2,2-Tribromoethanol, T84802, Sigma-Aldrich) [Stock solution: 1 g/ml in Terr-Amyl-Alcohol (2-Methyl-2-butanol, 152463, Sigma-Aldrich); working solution: 25 mg/ml in PBS] and perfused with 4% paraformaldehyde, PFA (Sigma, St Louis, MO) in PBS (pH 7.4) through the left cardiac ventricle, as described. The brains were removed and post-fixed in 4% PFA overnight, before transfer into 20% sucrose in PBS for 48 h at 4 °C. The coronal sections were collected using a cryostat (Leica) on + charged slides (Fisher superfrost plus), 40 μm thick sections at 200 μm intervals for the striatum, and 30 μm thick sections at 150 μm intervals for the cerebellum. Histopathological changes were detected by immunostaining of frozen sections with parvalbumin (P3088, Sigma) at 1:2000 dilutions, NeuroTraceTM Fluorescent Nissl Stains (Green-N21480, Molecular Probes) at 1:50 dilution, and DAPI at a 1:1000 dilution. Alexa Fluor secondary antibodies (Molecular Probes) were used to detect bound primary antibodies. Brain sections were mounted in Vectashield hard-set and images were acquired using VS120 (Olympus) scanner. The number of Parvalbumin-positive cells were manually counted on each confocal image.

**Western blotting**

Mouse brain cerebellum was homogenized in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with a protease inhibitor cocktail (Roche) and phosphatase inhibitor II (Sigma). The homogenate of HEK-293 cells infected with Adeno Rhes virus (5–10 μg of protein) was loaded as a positive control. Then, 50–100 μg proteins were separated by electrophoresis in 4–12% Bis–Tris Gel (Invitrogen), transferred to PVDF membranes, and Western blotting carried out as previously described (Pryor et al., 2014; Shahani et al., 2014). An apparent molecular weight of Rhes is 28-32 kDa. In addition, a prominent Rhes band was seen at 38-42 kDa in WT but not in KO Rhes striatum (data not shown), which is consistent with previous report (Errico et al., 2004). Ectopically expressed Rhes, either in the striatum or cerebellum, also showed a prominent band at 38-42 kDa suggesting that Rhes in vivo may exist in a posttranslationally (presumably SUMO) modified form (Subramaniam, 2009).

**Statistics**

The statistical significance was determined using the two-way ANOVA Newman–Keuls multiple comparisons post-hoc test or in some cases (example, western blots) Student’s-t-test to compare the two groups (**p < 0.001, *p < 0.01 and *p < 0.05).**

**Results**

**Rhes deletion ameliorates HD-related motor deficits in N171-82Q mice**

To elucidate the genetic-basis for the role of Rhes in HD, we crossed Rhes knockout mice (Rhes −/−) (Mealer et al., 2013; Spano et al., 2004; Subramaniam et al., 2010, 2012) with N171-82Q mice, which expresses an N-terminal fragment of human Htt containing 171 amino acids with 82 glutamines (Schilling et al., 1999). We derived six groups of mice, and the distribution in each cohort is indicated in Table 1 (WT, Rhes−/−, Rhes−/−/N171-82Q, Rhes−/−/N171-82Q and N171-82Q). Our previous studies in a toxin-model of HD or L-DOPA Parkinson’s disease model indicated that Rhes−/− mice were protected from striatal-lesions and motoric dysfunction compared to wild-type mice (Mealer et al., 2014; Subramaniam et al., 2012). Also, previously we found no difference in basal behavior/motor function of Rhes−/− mice compared to wild-type controls (Mealer et al., 2014; Subramaniam et al., 2012), consistent with previous reports (Errico et al., 2008; Lee et al., 2011; Quintero and Spano, 2011; Quintero et al., 2008), suggesting that that Rhes−/− or Rhes−/− would also serve as additional controls for N171-82Q mice. This study included both male and female mice as N171-82Q mouse model is known to exhibit gender-dependent phenotypic variations; males show reproducible and an early motor/behavioral deficits, whereas, females display variable and late onset symptoms, consistent with previous reports (Chen et al., 2013a; Duan et al., 2003; Masuda et al., 2008; Orr et al., 2008; Schilling et al., 1999), beginning at 8-weeks of age (Fig. 1A). N171-82Q mice showed a significant weight loss compared to WT, Rhes−/− or Rhes−/− in an age-dependent manner, that weight loss was not significantly protected in homozygous Rhes−/−/N171-82Q mice or heterozygous Rhes−/−/N171-82Q mice (Fig. 1B). Compared to male N171-82Q mice, female N171-82Q mice appear to resist loss of body weight and are comparable to control groups in all ages tested (Fig. 1C).

Next, we subjected Rhes−/−/N171-82Q mice to a battery of established behavioral tests, including rotarod, behavioral battery and open-field analysis to assess motor and exploratory deficits. These deficits, as described in our recent work (Pryor et al., 2014), are reminiscent of symptoms observed in HD and have been shown to be robust in N171-82Q mice (Andreasen et al., 2001; Cheng et al., 2011; Chiu et al., 2011; Figiel et al., 2012; Masuda et al., 2008; Rose et al., 2010; Schilling et al., 1999; Stack et al., 2010). On the rotarod test, the N171-82Q male mice performed poorly, as expected, with a progressive loss of ability to stay on the rotarod at 12 and 16 weeks, compared to non-HD controls (WT, Rhes−/−, or Rhes−/− mice) (Fig. 1D). However, Rhes−/−/N171-82Q or Rhes−−/− N171-82Q male mice performed significantly better when compared to N171-82Q (Fig. 1D). We did not observe any significant differences on the rotarod tests in female groups in any category at these ages (Fig. 1E), consistent with previous findings (Duan et al., 2003; Jia et al., 2012). Subsequently, we carried out a rapid and sensitive measure of motor deficits in HD mouse models, as previously described (Guyenet et al., 2010). Rhes−/−/N171-82Q or Rhes−−/− N171-82Q mice are also protected from, ledge defect (which measures coordination), clasping, gait abnormality, kyphosis and tremor defects observed in N171-82Q mice, which are most robust in male mice (Fig. 1F) compared to female mice (Fig. 1G).

**Rhes deletion prevents psychiatric-like behavioral deficits in N171-82Q mice**

In the open-field test (OFT), which measures exploratory and anxiety-like behavior (Dedenberg, 1969), the N171-82Q mice at 8 and 12 weeks displayed a significantly higher distance traveled compared to WT, Rhes−/−, or Rhes−−/− male (Fig. 2A) and female mice (Fig. 2B). Such increased activity in N171-82Q mouse model compared to wild-type control groups has been reported earlier (Chen et al., 2013a), and it appears to be a common phenomenon even in other HD mouse model as well (Waldron-Roby et al., 2012). Such enhanced distance travel in OFT was markedly reduced in Rhes−/−/N171-82Q or Rhes−−/−/N171-82Q mice, both male (at 8 and 12 weeks, Fig. 2A) and female groups at all ages (Fig. 2C). Estimation of velocity has revealed that all N171-82Q male groups performed slightly albeit significantly worse than wild-type control groups (Fig. 2C). However, female N171-82Q groups had higher velocity of movement, which is robustly attenuated in Rhes−/−/N171-82Q and Rhes−−/−/N171-82Q female groups (Fig. 2D). Other sex differences are also evident in open field tests; while N171-82Q male mice showed a moderate but significantly (p < 0.05) higher center, sides and corner frequency at 12 weeks (Figs. 2E, G), their female counterparts exhibited highly significant (p < 0.001) higher center, sides and corner frequency even at 8 weeks (Figs. 2F, H). Despite no observable motor
deficits in female mice (such as those measured in the rotarod tests), higher open-field activity may suggest that these mice have increased anxiety-like behaviors. Interestingly, these altered behaviors are significantly lower in $\text{Rhes}^{-/-}/\text{N171-82Q}$ and $\text{Rhes}^{+/+}/\text{N171-82Q}$ mice, indicating that Rhes may have a gender-specific role in altering the anxiety-like symptoms in the N171-82Q mouse model.

**Rhes deletion prevents striatal atrophy and brain weight loss of N171-82Q mice**

Next, we investigated whether the protection against motor deficits in $\text{Rhes}^{-/-}/\text{N171-82Q}$ or $\text{Rhes}^{+/+}/\text{N171-82Q}$ male mice is accompanied by alterations in the anatomical deficits seen in N171-82Q mice. Consistent with previous reports (Duan et al., 2008; Gardian et al., 2005), N171-82Q mice showed an enlargement of the lateral ventricles compared to controls, which is presumably due to striatal atrophy. Ventricular enlargement is largely attenuated in Rhes deleted, $\text{Rhes}^{-/-}/\text{N171-82Q}$ and $\text{Rhes}^{+/+}/\text{N171-82Q}$, mice groups (Figs. 3A, B). Brain weight loss observed in N171-82Q is significantly reduced in Rhes deleted mice (Fig. 3C). Together these results suggest that the embryonic deletion (complete KO) or even a partial lowering (heterozygous knockout) of Rhes is sufficient to protect against the behavioral and anatomical deficits seen in HD. Thus, Rhes GTPase physiologically modulates the striatal-related deficits in the N171-82Q transgenic mouse model.

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**Fig. 1.** Rhes deletion protects HD-related motor deficits in N171-82Q mouse model. (A) Longitudinal behavioral assays of the indicated genotypes/groups. (B and C) Depicts changes in body weight in male and female. (D and E) Rotarod test in male and female mice. (F and G) Behavioral battery – ledge test, clamping, gait, kyphosis and tremor, scored from 0 to 3 (highest) in male and female mice aged 16 weeks. Data are means ± SEM from 6 mice in each groups ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$, two-way ANOVA followed by Newman-Keuls multiple comparisons post-hoc test.
Cerebellar expression of Rhes causes early motor and locomotion deficits in N171-82Q mice

Having established that striatal expression of Rhes promotes HD-related deficits in an HD mouse model, we asked whether the ectopic expression of Rhes in a brain region not associated with HD pathology could elicit a regional-specific deficit. To answer this question, we overexpressed Rhes into the cerebellum, a region usually not affected in HD, and has little or no expression of Rhes (Falk et al., 1999; Figiel et al., 2012; Jiang et al., 2013; Luthi-Carter et al., 2002; Schilling et al., 1999). We chose ~8-week-old WT or N171-82Q mice for such injections because at this age, there are no apparent motor phenotype (rotarod or general motor activity) in any of the N171-82Q mice tested compared to wild-type littermate controls, which is consistent with previous reports.

Fig. 2. Rhes deletion reduces anxiety-like behaviors in N171-82Q HD mouse model. Open-field test (OFT): showing (A–B) total distance traveled by male and female mice, (C–D) velocity for movements in male and female mice, (E–F) thigmotaxis frequency from center point to center of male and female mice, and (G–H) thigmotaxis frequency from center point to sides and corner of male and female mice. Data are means ± SEM from 6 mice in each groups ***P < 0.001, **P < 0.01 and *P < 0.05, two-way ANOVA followed by Newman-Keuls multiple comparisons post-hoc test.
(Chen et al., 2013a; Cheng et al., 2011; Schilling et al., 1999). Stereotaxic coordinates for cerebellar injections were validated using a fast green dye that showed effective distribution throughout the cerebellum (Supplementary Fig. 1). We then injected null control adenovirus (Ad-null) or Rhes expressing adenovirus (Ad-Rhes) into littermate WT control or N171-82Q transgenic mice (4 μl/injection, 1 × 10^10 opu/μl). In a separate cohort of mice, we confirmed Rhes overexpression levels that were comparable between the WT and N171-82Q mice as well as its marked activity measured by mTORC1 (mechanistic Target of Rapamycin)-dependent phosphorylation of S6K (Subramaniam et al., 2012) (Supplementary figure 2A, B). The four groups [wild-type mice injected with adenovirus-Null (WT-Ad Null) or adenovirus-Rhes (WT-Ad Rhes); N171-82Q mice injected with adenovirus-Null (N171-82Q-Ad Null) or adenovirus-Rhes (N171-82Q-Ad Rhes) (n = seven/per group, mixed sex distribution)] were subjected to longitudinal behavioral tests, on the 4th, 9th, and 14th day after injection, and sacrificed for biochemical and histochemical analysis, on day 17 as indicated in Fig. 4A. Within four days of injection, a sudden drop in body weight was observed in Ad Rhes-injected N171-82Q mice compared to Ad Null-injected N171-82Q or Ad Rhes-injected WT mice (Fig. 4B). The body weight did not drop further, but remained significantly low in Ad-Rhes-injected N171-82Q, compared to control-injected mice (Fig. 4B).

Rotarod test revealed that Ad-Rhes-injected HD mice exhibited a progressive loss of ability to stay on the rotating rod, which worsened with time, compared to control groups (Fig. 4C). Also we carried out behavioral tests for ataxia-like defects, as previously described (Guyenet et al., 2010), with multitude of tests including: the ledge test, hind limb clasping, gait analysis, kyphosis and tremor. All of these analyses of cerebellar functions worsened with time in Ad-Rhes injected N171-82Q mice compared to control groups (Fig. 4). The open-field test

**Fig. 3.** Rhes deletion prevents enlargement of lateral ventricles in N171-82Q HD mouse model. (A) Representative image of striatal section of indicated mouse group, stained for cresyl violet. (B) Quantification of ventricle size [area in (μm^2)]. (C) Quantification of brain weight (mg) of indicated mouse groups. Data are means ± SEM from 6 mice in each groups ***P < 0.001, **P < 0.01 and *P < 0.05, two-way ANOVA followed by Newman-Keuls multiple comparisons post-hoc test.
Cerebellar expression of Rhes elicits cerebellar degeneration, an increased level of soluble Htt and caspase-3 activation in the cerebellum of N171-82Q mice

The severe motor deficits with ataxia-like phenotype in Ad-Rhes-expressing N171-82Q mice (in cerebellum) prompted us to investigate the cerebellar integrity. Parvalbumin, which is expressed in Purkinje neurons and molecular layer interneurons (Caillard et al., 2000) as a marker has shown to be reduced both in mouse models and post mortem tissue of human neurodegeneration (spinocerebellar ataxia-1) (Vig et al., 1996, 1998, 2012). In our immunohistochemical analysis, the Nissl and DAPI co-stains clearly differentiated the granule layer and
the molecular layer in cerebellar sections (Fig. 6A), with a clear parvalbumin positive Purkinje-cell layer (P). The immunoreactivity in this layer is markedly reduced in the Ad-Rhes-injected cerebellum of N171-82Q mice compared to control groups (Figs. 6A, B). We found, consistent with previous reports, parvalbumin staining in the molecular layer (M) (Caillard et al., 2000), that also appears to be affected in Ad-Rhes-injected cerebellum of N171-82Q mice, compared to control groups (Figs. 6A, B, compare P and M layers). Of note, the Ad-Null-injected wild type as well as N171-82Q mice displayed comparable and intact Purkinje cell layers, at this time point, implying that the cerebellum is not affected at this age. In addition, Rhes expression alone did not elicit a cerebellar phenotype, indicating that Rhes itself is not toxic to the cerebellum, and that the Rhes/mHtt interaction is required for toxicity.

Western blot analysis revealed that the Rhes-injected cerebellum of N171-82Q elicited robust increase in the level of soluble Htt, compared to Ad-null-injected N171-82Q mice (Figs. 7A, B), and upregulation of cleaved caspase 3 (Fig. 7), an apoptotic marker. All together these data indicate that Rhes expression in the cerebellum of N171-82Q mice promotes an ataxia-like phenotype, which is accompanied by a loss of parvalbumin-immunoreactivity and biochemical upregulation of the cell death pathways, as well as enhanced mHtt solubility. Thus, ectopic Rhes expression can elicit both selective neuronal degeneration and specific behavioral deficits in an N171-82Q mouse model.

Reintroducing Rhes into the striatum of Rhes-deleted Hdh150Q/150Q knock-in mice accelerates motor deficits

Having established that the Rhes plays an important role in HD transgenic N171-82Q mice, we wondered whether Rhes can also play a similar role in HD knock-in mice, expressing full length Htt containing (Hdh150Q/150Q), which develop late onset HD-related motor symptoms. Rhes-deleted Hdh150Q/150Q mice (Rhes−/−/Hdh150Q/150Q) appeared normal, and their rotarod performance was comparable to that of Rhes-deleted WT mice (Rhes−/−/WT) at 6-months of age. This is expected as Hdh150Q/150Q mice develop age-dependent, behavioral phenotype with significant motor abnormalities appearing between 12 and 25 months of age (Heng et al., 2007). Therefore, we hypothesized that if Rhes were necessary for striatal damage in Hdh150Q/150Q mice, an overexpression of Rhes into the striatum of Rhes−/−/Hdh150Q/150Q mice would promote motor deficits. In support of this hypothesis, we found that Ad-Rhes injection, but not Ad-Null or Ad-GFP, into the striatum elicited a progressive motor defects in Rhes−/−/Hdh150Q/150Q mice (Fig. 8). Together these data suggest that Rhes overexpression selectively in the striatum of Rhes-deleted Rhes−/−/Hdh150Q/150Q mice accelerates HD-related deficits. Thus, Rhes plays major role in striatal damage in HD.

Discussion

Three major findings emerged from this report regarding the physiological role of Rhes in HD: 1) genetic deletion of Rhes ameliorates HD-associated behavioral and pathological deficits; 2) ectopic expression of Rhes in the cerebellum of transgenic N171-82Q mice can elicit a robust cerebellar phenotype reminiscent of cerebellar ataxia, and 3) reintroducing Rhes in striatum of Rhes−/−/Hdh150Q/150Q knock-in mice can elicit HD-related motor deficits (see graphical abstract). Together, these studies are consistent with the notion that Rhes confers
striatal vulnerability in HD, and that the cerebellum and other regions, which are normally impervious to damage in HD, can become vulnerable upon Rhes overexpression. Striatal atrophy is an early event in human HD, which is correlated to the length of poly-Q repeats (Graveland et al., 1985; Gutekunst et al., 2002; Jackson et al., 1995; Rosas et al., 2003; Ruocco et al., 2006b; Tabrizi et al., 1999, 2011; Vonsattel et al., 1985), and which is consistent with clinical symptoms, such as involuntary movements and exaggerated reflexes, abnormal gait, and dystonia (Kalkhoven et al., 2014). Yet, damage outside the striatum, including in the cerebellum, can occur in HD. Whether cerebellar damage is an early event or late event in the disease progression remains controversial. While some researchers claim, based on brain MRI studies, that cerebellar damage is an early event others have suggested it is a late event in the disease (Gomez-Anson et al., 2009; Hobbs et al., 2010; Rees et al., 2014; Rosas et al., 2003). Cerebellar atrophy may occur because of retrograde secondary changes due to massive neuronal loss in the striatum, or due to the progressive loss of white matter during the course of the disease (Ruocco et al., 2006b). Early striatal damage occurs in the majority of adult-onset HD, which is correlated with the clinical motor symptoms. However, the clinical features of ataxia, reflecting cerebellar damage, can also occur in juvenile HD, which is characterized by dystonia, rigidity, and early death (Ruocco et al., 2006a). Though, Rhes mRNA expression is abundant in the striatum of human HD (source: Allen brain atlas), (Rhes protein levels in different brain region remains unknown) whether or how Rhes is regulated in different regions of the brain during development or aging remains unknown. In rats, Rhes mRNA expression is transiently upregulated in the cerebellum, during the period of post-natal development (P15-P25) (Harrison et al., 2008). Thus, the possibility that Rhes might also have a role in cerebellar damage in HD cannot be ruled out. Nevertheless, this report—from knockout and ectopic expression of
Rhes in transgenic and knock-in HD mouse models—strongly supports the finding that Rhes GTPase plays an important role in regional-specific damage of HD.

How might Rhes elicit neuronal death in HD? Mechanistically, we predict that Rhes enhances soluble forms of poly-Q expanded Htt, through post-translation SUMO modification, which is toxic to cells. This prediction is based on previous biochemical data, in which depletion of SUMO1 prevents Rhes-mediated poly-Q Htt toxicity in a cell culture model (Subramaniam et al., 2009). This notion is further supported in the Rhes-injected cerebellum of HD mice, which displayed higher soluble forms of Htt (Fig. 7). The downstream signals that are recruited by SUMOylated mHtt remain unclear. These might involve transcriptional dysregulation, mitochondrial stress, and caspase-3 activation [(Steffan et al., 2004; Subramaniam et al., 2009) and (Fig. 7)].

Our recent study implicates mHtt in the promotion of mTORC1 signaling in HD pathogenesis (Pryor et al., 2014). We found that mHtt alters the movement of mTOR kinase and binds to its regulator Rheb GTPase to promote mTORC1 activity. When mTORC1 is genetically upregulated in the striatum of N171-82Q mice, this caused HD pathogenesis and the premature death of HD mice (Pryor et al., 2014). As Rhes is a prominent activator of mTORC1 (Subramaniam et al., 2012), we speculate that Rhes-mediated SUMOylation of mHtt would further deregulate mTORC1 signaling in the striatum, thus leading to early striatal motor deficits, which remains to be investigated.

A bigger question emerges from this study—would Rhes be now beyond doubt an excellent drug target for HD? Although a complete knockout of Rhes is relatively indistinguishable from the wild-type mice at baseline, the behavioral deficits emerged when the mice were challenged with dopamine-related drugs. For example, complete Rhes knockout mice exhibit an increased D1 agonist-initiated locomotor activation and an increased D2 antagonist-initiated catalepsy (Errico et al., 2009). Reintroducing Rhes in the striatum of Rhes-deleted Hdh<sup>150Q/150Q</sup> knock-in mice (Rhes<sup>−/−</sup>/Hdh<sup>150Q/150Q</sup>, 6 months old) elicits early motor deficits. Rotarod test in Ad-null, Ad-GFP or Ad-Rhes, injected in Rhes deleted WT or Hdh<sup>150Q/250Q</sup> mice. Data are means ± SEM from 4 mice in each group. *P < 0.05, two-way ANOVA followed by Newman–Keuls multiple comparisons post-hoc test.

Fig. 7. Rhes expression increases EM-48-positive soluble huntingtin and caspase-3 activation in N171-82Q mice. (A) Western blot for full-length (FL)-Htt, EM-48 positive Htt, Caspase-3 (p19 and p17 fragment), Rhes along with ponceau staining of adenovirus-null (Ad-null) or adenovirus-Rhes (Ad-Rhes) injected wild-type or HD (N171-82Q) mice. (B) Quantification of soluble Htt in adenovirus-null (Ad-null) or adenovirus-Rhes (Ad-Rhes) injected HD mice. Data are means ± SEM from n = 3 (HD) mice. P = 0.0339, Student’s t test.

Fig. 8. Reintroducing Rhes in the striatum of Rhes-deleted Hdh<sup>150Q/150Q</sup> knock-in mice (Rhes<sup>−/−</sup>/Hdh<sup>150Q/150Q</sup>, 6 months old) elicits early motor deficits. Rotarod test in Ad-null, Ad-GFP or Ad-Rhes, injected in Rhes deleted WT or Hdh<sup>150Q/250Q</sup> mice. Data are means ± SEM from 4 mice in each group. *P < 0.05, two-way ANOVA followed by Newman–Keuls multiple comparisons post-hoc test.
2008). Similarly, D1/D2 receptor agonist stimulation induces a stereotypic behavior that is enhanced in Rhes KO mice (Quintero and Spano, 2011; Quintero et al., 2008). Recently, we found that L-DOPA-induced dyskinetic movements were markedly reduced in Rhes KO mice, in a Parkinson’s disease model (Subramaniam et al., 2012). These studies imply that Rhes plays an important role as a negative regulator of dopamine-related movements, which may have implications in HD (Chen et al., 2013b). Thus targeting Rhes might provide beneficial effects on striatal-motor defects associated with both HD and PD. However, due to its diverse yet incompletely understood roles blocking Rhes function in adult animals and the biological consequences on striatal functions remains unclear. Rhes acts as a SUMO-E3 ligase; activates mTORC1 signaling (Subramaniam et al., 2010, 2012); activates Akt (Bang et al., 2012); modulates autophagy through Beclin-1, independent of mTOR (Mealer et al., 2013); and interacts with golgi protein ACBD3 (Sbodio et al., 2013).

A recent study investigated the role of Rhes shRNA in an adult HD mouse model (Lee et al., 2014). In that study, Rhes shRNA, which was injected into seven-week-old N171-82Q or four-month-old BACHD mice, did not alter the results of the rotarod, a widely used test to assess motor defects in HD mouse models (Lee et al., 2014). This suggests that i) depleting Rhes mRNA in the striatum of adult animals has no lethal consequences, and ii) depleting Rhes at seven weeks or later does not alleviate the motor symptoms or the Rhes-mediated HD defects. The latter possibility is particularly important as Rhes expression begins in the striatum as early as postnatal day 4, and gradually reaches a peak at day 15 and 24 (Harrison et al., 2008); thus suggesting that Rhes-mediated striatal dysfunction might have begun before the Rhes shRNA injection. This notion is further strengthened by our rapid cerebellar HD model, in which we found, a robust deficit in motor functions, within four days of Ad-Rhes injection. Moreover in Lee et al. (2014) model it is unclear how much Rhes protein is depleted. As mRNA levels does not usually predict its protein levels (Liu and Tian, 2004; Lundberg et al., 2010; Schwanhausser et al., 2011; Vogel et al., 2010), it is challenging to interpret the data just with reduced Rhes mRNA levels. Nevertheless, in the Lee et al., 2014 model, Rhes mRNA depletion negatively affected mobility in the open-field tests in Rhes shRNA-depleted HD mice (BACHD model). This is similar to our model, in which Rhes-deleted N171-82Q mice but not WT mice were less mobile than control N171-82Q mice (Figs. 2 A–H), which may be attributable to the protective effects of Rhes on anxiety-like hyperactive behaviors in HD. Thus, our data supports the hypothesis that Rhes offers protection against both motor and psychiatric-like defects in HD, but the potential mechanisms by which Rhes differentially regulate motor vs. psychiatric-like defects in HD pathogenesis remain to be investigated.

In another study Lee and colleagues (Lee et al., 2015) found that AAV-Rhes overexpression into the striatum of N171-82Q mice improved the rotarod performance compared to GFP injected N171-82Q mice. This appears to be contradictory with our study in HD-knock-in mice. This appears to be contradictory with our study in HD-knock-in mice. Moreover, we have used untagged rotarod de mice. This appears to be contradictory with our study in HD-knock-in mice. In that study, Rhes shRNA, which was injected into seven-week-old N171-82Q or four-month-old BACHD mice, did not alter the results of the rotarod, a widely used test to assess motor defects in HD mouse models (Lee et al., 2014). In that study, Rhes shRNA, which was injected into seven-week-old N171-82Q or four-month-old BACHD mice, did not alter the results of the rotarod, a widely used test to assess motor defects in HD mouse models (Lee et al., 2014). This suggests that i) depleting Rhes mRNA in the striatum of adult animals has no lethal consequences, and ii) depleting Rhes at seven weeks or later does not alleviate the motor symptoms or the Rhes-mediated HD defects. The latter possibility is particularly important as Rhes expression begins in the striatum as early as postnatal day 4, and gradually reaches a peak at day 15 and 24 (Harrison et al., 2008); thus suggesting that Rhes-mediated striatal dysfunction might have begun before the Rhes shRNA injection. This notion is further strengthened by our rapid cerebellar HD model, in which we found, a robust deficit in motor functions, within four days of Ad-Rhes injection. Moreover in Lee et al. (2014) model it is unclear how much Rhes protein is depleted. As mRNA levels does not usually predict its protein levels (Liu and Tian, 2004; Lundberg et al., 2010; Schwanhausser et al., 2011; Vogel et al., 2010), it is challenging to interpret the data just with reduced Rhes mRNA levels. Nevertheless, in the Lee et al., 2014 model, Rhes mRNA depletion negatively affected mobility in the open-field tests in Rhes shRNA-depleted HD mice (BACHD model). This is similar to our model, in which Rhes-deleted N171-82Q mice but not WT mice were less mobile than control N171-82Q mice (Figs. 2 A–H), which may be attributable to the protective effects of Rhes on anxiety-like hyperactive behaviors in HD. Thus, our data supports the hypothesis that Rhes offers protection against both motor and psychiatric-like defects in HD, but the potential mechanisms by which Rhes differentially regulate motor vs. psychiatric-like defects in HD pathogenesis remain to be investigated.

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Overall, our study demonstrates that Rhes is important for promoting the tissue-specific damage of mHtt in HD. We found that Rhes deletion protects against HD-related deficits in a well-characterized mouse model of HD and ectopic expression of Rhes in the cerebellum, which is normally not affected in HD, or in the striatum of HD-knock-in mice, can elicit robust pathological changes accompanied by biochemical, morphological, and motor-behavioral deficits. Thus this study is consistent with growing number of studies linking toxic role of Rhes in HD cell and mouse models (Baiamonte et al., 2013; Lu and Palacino, 2013; Mealer et al., 2013; Mealer et al., 2014; Okamoto et al., 2009; Sbodio et al., 2013; Seredenina et al., 2011) Rather than depleting Rhes in the adult, which might have untoward side effects due to its association with multiple partner proteins, we hypothesize that selective inhibition of Rhes’ activity or its interaction with mHtt might have better beneficial effects in ameliorating or preventing symptoms in HD (Subramaniam and Snyder, 2011).

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Author contributions

S. Swarnkar and S. Subramaniam designed the study and S. Swarnkar carried out all mice behavioral and brain-analysis work. Y.C. assisted in mouse work and brain analysis. W. P performed a blind analysis of behavioral battery. N.S prepared the graphs and analyzed the data together with S. Swarnkar and S. Subramaniam. D.T.P. provided technical support for brain imaging. S. Subramaniam provided further conceptual input, and wrote the paper with input from all co-authors.

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Abbreviations

Rhes (Ras homolog enriched in striatum)
HD (Huntington’s disease)
Htt (huntingtin)
mHtt (poly glutamine expanded Htt)
mTOR (mammalian target of rapamycin)
SGK (ribosomal S6 kinase)

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