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Role of defective calcium regulation in cardiorespiratory dysfunction in Huntington’s disease

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Abstract

Huntington’s disease (HD) is a progressive autosomal dominant neurodegenerative disorder affecting striatal neurons beginning in young adults with loss of muscle coordination and cognitive decline. Less appreciated is the fact that HD patients also exhibit cardiac and respiratory dysfunction including pulmonary insufficiency and cardiac arrhythmias. The underlying mechanism for these symptoms is poorly understood. In the present study we provide insight into the cause of cardiorespiratory dysfunction in HD and identify a novel therapeutic target. We now show that intracellular calcium (Ca\textsuperscript{2+}) leak via post-translationally modified ryanodine receptor/intracellular calcium release (RyR) channels plays an important role in HD pathology. RyR channels were oxidized, PKA phosphorylated and leaky in brain, heart and diaphragm in both HD patients and in a murine model of HD (Q175). HD mice (Q175) with endoplasmic reticulum (ER) Ca\textsuperscript{2+} leak exhibited cognitive dysfunction, decreased parasympathetic tone associated with cardiac arrhythmias, and reduced diaphragmatic contractile function resulting in impaired respiratory function. Defects in cognitive, motor and respiratory functions were ameliorated by treatment with a novel Rycal small molecule drug (S107) that fixes leaky RyR. Thus, leaky RyRs likely play a role in neuronal, cardiac and diaphragmatic pathophysiology in HD and identify RyRs as a potential novel therapeutic target.
Introduction

Huntington’s disease (HD) is a progressive autosomal dominant neurodegenerative disorder affecting striatal neurons in young adults with distinct symptoms of cognitive decline and muscle dyscoordination (1, 2). This devastating disease is usually fatal approximately 10-15 years after onset of symptoms, and there is no disease-modifying treatment. HD is caused by a mutation in the HTT gene on chromosome 4 encoding the Huntingtin protein. CAG repeat expansion results in a polyglutamine region (poly Q) at its N-terminus (3). HD patients exhibit impaired locomotor and respiratory muscle function and digestive tract dysfunction (4).

The leading cause of death in HD patients is aspiration pneumonia (5, 6), due to dysphagia (7, 8). Indeed, diaphragmatic muscle weakness combined with difficulty clearing airway secretions and defective swallowing are prevalent in HD patients (9, 10). However, the mechanisms underlying the respiratory dysfunction remain unknown (11-13). Most HD patients do not report respiratory symptoms until later stages of the disease when impaired swallowing and respiratory muscle weakness cause fatal aspiration pneumonias (7, 11, 14). At the late stages of HD, 44% of patients receive respiratory therapy compared to 2% at the early stages of the disease (12). In a recent study HD patients exhibited reduced respiratory pressure, forced vital capacity, peak expiratory flow and maximal voluntary ventilation (13).

Heart failure occurs in ~ 30% of HD patients (15), in contrast to less than ~ 2% of age-matched individuals in the general population (16-22). Despite the limited number of studies evaluating heart function in HD, epidemiological data identify cardiac disease as the second most common cause of death in HD. Recently Stephen et al reported significant cardiac contractile
dysfunction and ECG abnormalities in a large cohort of early symptomatic HD patients. 25.3% of them exhibit ECGs abnormalities including bradycardia and prolonged Qtc interval (23).

Autonomic nervous system (ANS) dysfunction is a feature of HD and may play a role in the increased risk of cardiac arrhythmias in HD (4, 24, 25). HD patients may exhibit reduced heart rate variability (HRV) and altered sympathetic and parasympathetic activity (26, 27). Decreased cardiovagal regulation was found in HD patients characterized by reduced HRV at rest and during deep respiration (24). It has been suggested that dysautonomia results in fatal cardiac arrhythmias (25, 28), due to impaired cardiac parasympathetic and sympathetic signaling (29). The cardiorespiratory consequences and possible mechanisms of ANS dysfunction in HD patients are poorly understood. Ironically, HD patients with cardiorespiratory manifestations are often excluded from clinical studies.

Neurodegeneration in HD may be associated with impaired synaptic transmission (29, 30), reduced brain-derived neurotrophic factor (BDNF) (31, 32), mitochondrial dysfunction (33, 34) and altered calcium (Ca\textsuperscript{2+}) regulation (35-37). Increased intracellular Ca\textsuperscript{2+} concentration due to N-Methyl-D-aspartate receptor (NMDAR) activity and/or other Ca\textsuperscript{2+} sources including the InsP3R1 (inositol 1,4,5-trisphosphate receptor type 1) and ryanodine receptor (RyR) may play a role in striatal neurodegeneration in HD (38-42).

RyRs are ubiquitous intracellular Ca\textsuperscript{2+} release channels expressed early in development (43) and required for the function of many organs including heart, skeletal muscle and synaptic transmission in the brain (44). Three RyRs mammalian isoforms are known: RyR1 (45, 46), RyR2 (47) and RyR3 (48), which are classified as “skeletal muscle”, “heart” and “brain” types respectively (44), although all three forms are expressed in the brain. In skeletal muscle RyR
activation is linked to voltage-gated calcium channels (49) and in other organs including heart, RyRs are calcium-activated calcium release channels (44). RyRs are a homotetrameric macromolecular protein complexes (50) that include four RyR protomers (565 kDa each), kinases (PKA, CaMKII), phosphatases (PP1, PP2A), calmodulin, and a phosphodiesterase (PDE4D3) (47). The RyR channel-stabilizing subunit calstabin (FKBP12) is critical for stabilizing the closed state of the channel and preventing a pathological leak of calcium (45, 51). Maladaptive cAMP-dependent kinase A (PKA)-mediated phosphorylation and redox-dependent modifications (cysteine nitrosylation and oxidation) (52-54) of RyRs have been linked to a loss of calstabin from the channel macromolecular complex (47, 55-57). RyR remodeling, in turn, results in impaired Ca\textsuperscript{2+} handling due to a pathological Ca\textsuperscript{2+} leak from the SR/ER and is associated with multiple organ dysfunction including cognitive impairment (54, 58), heart failure (59) and respiratory/locomotor muscle weakness (47, 58, 60-65).

Defective RyR function has been reported in HD, leading to elevated intracellular Ca\textsuperscript{2+} levels and reduced endoplasmic reticular Ca\textsuperscript{2+} stores in R6/2 striatal and cortical neurons (40). Moreover, RyR inhibitors have been shown to be neuroprotective in vitro and improve motor behavior in vivo in YAC128 mice (37, 40, 66).

Since defective Ca\textsuperscript{2+} regulation has been well documented in HD, but not understood mechanistically, we hypothesized that leaky neuronal RyR play role especially in the cardiorespiratory pathology. We focused on the type 2 isoform of RyR (RyR2) because it is predominantly expressed in the brain (67). Indeed, we previously showed that oxidation, nitrosylation and PKA phosphorylation of RyR2 results in leaky channels that contribute to the pathophysiology of Post-traumatic stress disorder (PTSD) (54) and Alzheimer disease (AD) (58,
60). The role of RyR2 remodeling in brainstem nuclei remains unknown but might be involved in cardiac and respiratory dysfunction, such as arrhythmias and diaphragm muscle weakness in HD.

HD is characterized by cognitive dysfunction and involuntary motor movements (68, 69). We sought to determine whether leaky neuronal RyR2 channels play a role in cognitive dysfunction associated with HD. In addition, using a novel Rycal drug (S107), that crosses the blood brain barrier and fixes the leak in RyR2 channels (54, 58, 70), we further determined whether inhibiting RyR2-mediated ER Ca\textsuperscript{2+} leak can improve cognitive function in a murine model of HD. S107 is a small molecule that stabilizes the RyR2-calstabin2 interaction and decreases RyR2 ER Ca\textsuperscript{2+} leak without affecting PKA phosphorylation, oxidation and cysteine nitrosylation of the channel (54, 58, 60).
Results

Neuronal RyR2 are leaky in HD patients and in a murine model of HD

To evaluate the remodeling and functional abnormalities of RyR2 in the brain of HD patients, cortical and hippocampal biopsies from de-identified organ donors (Table S1) with neuropathological grade 3 and 4 HD, 5 female and 3 male (ages 54-66, CAG repeats 36-51) were obtained from the Brain Bank at Columbia University. We compared these specimens to cortical and hippocampal specimens from non-HD controls. Endoplasmic reticulum fractions were purified to analyze the composition of the RyR2 macromolecular complex and post-translational modifications known to be associated with channel leak (71) in the cortex and hippocampus (Figure. 1A, B and C). HD RyR2 exhibited PKA hyperphosphorylation (on RyR2-Ser2808), oxidation, cysteine nitrosylation and were depleted of calstabin2 compared to controls. This is the “biochemical signature” of leaky RyR2 channels (47, 72). Single channel recordings of cortical RyR2, reconstituted into planar lipid bilayers, revealed an increased open probability ($P_o$) in the presence of low non-activating [Ca$_{\text{2+}}$]$_{\text{cis}}$ conditions under which normal RyR2 channels are tightly closed (Figure. 1D-G). This elevated $P_o$ is consistent with pathological ER Ca$_{\text{2+}}$ leak (54, 58). Indeed, neuronal microsomes from HD cortex and hippocampus exhibited increased RyR-mediated ER Ca$_{\text{2+}}$ leak compared to controls (Figure. S1A-D).

We evaluated the time course (at 2, 6 and 10 months of age) of RyR2 remodeling in three different regions of the brain (striatum, cortex and hippocampus) using two different HD murine models (Q111 and Q175) compared to controls. We observed RyR2 remodeling in time dependent
manner with optimal onset of oxidation, phosphorylation and calstabin2 depletion being at 10 months of age (Figure S2).

Then we chose the Q175 mice model at 10 months old to evaluate RyR2 remodeling in with and without S107 treatment compared to WT controls (littermates). As in HD patients’ samples, RyR2 exhibited the biochemical signature of leaky channels in the cortex and in the hippocampus of Q175 mice compared to WT. RyR2 was PKA phosphorylated on Ser2808, oxidized, cysteine nitrosylated and depleted of the stabilizing subunit calstabin2 (Figure. 2A-C). This RyR2 remodeling was associated with evidence of leaky RyR2 channels based on increased $P_o$ recorded at low non-activating $[\text{Ca}^{2+}]_{\text{cis}}$ in both cortex and hippocampus of Q175 mice (Figure. 2D-K). Indeed, isolated brain mitochondria from Q175 mice exhibited increased reactive oxygen species (ROS) production which may explain in part the oxidation of RyR2 channels (Figure S3A). S107 administered in the drinking water reduced calstabin2 dissociation from the RyR2 macromolecular complex and decreased ER Ca$^{2+}$ leak (Figure. 2A-K).

**Leaky RyR2 causes cognitive impairment in a murine model of HD**

We assessed the effects of leaky RyR2 channels on spatial learning and memory using the Morris water maze (MWM) as previously described (54, 58). Q175 mice exhibited increased escape latency in the MWM at days 3 and 5 (Figure. 3A-B), spent less time in the target quadrant, and had a slightly reduced number of target crossings compared to WT mice (Figure. 3C and D). These findings are consistent with deficits in learning and/or memory. S107 treatment of Q175 mice significantly improved the escape latency and the time spent in the target quadrant compared
to untreated Q175 mice suggesting that RyR2 channel leak likely contributes to the cognitive deficits observed in the Q175 mice. Leaky RyR2 channels were also associated with increased anxiety determined using the elevated plus maze (EPM) test. Q175 mice spent more time and made more entries into the open arms of the EPM (Figure. 3E). In the tail suspension test Q175 mice exhibited increased immobilization duration consistent with increased stress, which was normalized by treatment with 107 (Figure. 3F). The Q175 mice exhibited also significant impaired coordination in movement, suggesting deficits of motor neuron function. S107 treatment significantly improve the dysregulated movement coordination. Kyphosis testing and gait analysis did not reach significance, suggesting that the impaired motor neuron function is moderate (Table 2). To confirm our results, we evaluated the RyR2 remodeling and the subsequent cognitive dysfunction in a second murine model of HD (R/6 mice) and observed similar results (Figure S4 and Table 3).

RyR2 phosphorylation in autonomic brainstem areas affecting the heart

Although described as a disease of the central nervous system, studies have revealed abnormalities in non-neuronal organs in patients with HD (4). Whether these defects are a direct consequence of mutant huntingtin protein, or secondary to neurological dysfunction, is poorly understood. Cardiac function is regulated centrally via both parasympathetic and sympathetic activities. Cardiac vagal preganglionic neurons are located in the dorsal motor nucleus of the vagus (DMNV) and in the nucleus ambiguus (NA) in the medulla oblongata. A major source of cardiac sympathetic drive comes from adrenergic pre-sympathetic C1 neurons located in the rostral
ventrolateral medulla (73). C1 neurons are hyperactivated in multiple cardiovascular diseases, including heart failure, which might contribute to their etiology.

We evaluated the levels of RyR2 phosphorylation in NA and C1 neurons by immunohistochemistry in HD mice. Choline acetyltransferase and tyrosine hydroxylase staining were used as markers of NA and C1 neurons, respectively. Compared to WT, there was increased RyR2 phosphorylation at Ser-2808 in the NA (Figure. 4A, panel a). There was also increased RyR2 PKA phosphorylation in C1 neurons, to a lesser extent than in the NA (panel b). This finding was confirmed by immunoprecipitation. Brainstem RyR2 exhibited increased Ser-2808 phosphorylation, oxidation, cysteine nitrosylation and depletion of the stabilizing subunit calstabin2 (Figure. 4B and C). We treated the HD mice with S107 (BBB permeant) and ARM036 (BBB non permeant) Rycals to understand the link between brainstem RyR2 abnormalities and autonomic dysfunction. S107 treatment, but not ARM036, prevented calstabin2 dissociation from the RyR2 macromolecular complex in the brainstem, demonstrating a central origin of these defects. In addition, cardiac RyR2 exhibited increased Ser-2808 phosphorylation, oxidation, cysteine nitrosylation and depletion of the stabilizing subunit calstabin2 (Figure. 5A and B). This cardiac RyR2 remodeling was associated with a significant increase in RyR2 open probability (P0) in heart samples from Q175 mice consistent with leaky channels (Fig. 5C-F). Both S107 and ARM036, prevented calstabin2 dissociation from cardiac RyR2.
Remodeled RyR2 in brainstem contribute to altered heart rate variability (HRV) and cardiac arrhythmias in a murine model of HD

To evaluate the ANS outflows, we monitored cardiac electric activity (ECG) using a telemetric system over 24 hours in freely-moving conscious WT, Q175, Q175 animals treated with S107 or ARM036. Heart rate (HR) rate is controlled by the activity of the pacemaker and modulated by the sympathetic and parasympathetic limbs of the ANS. We hypothesized that the RyR2 remodeling in NA and C1 neurons could induce a sympathovagal imbalance contributing to cardiac arrhythmias. During the awake period, we observed a slight but not significant increase in HR in Q175 mice (Figure 6A and B). During the rest period, HR was significantly increased in Q175 mice compared to WT suggesting a reduction in the parasympathetic tone during rest in line with the nucleus ambiguus and RyR2 remodeling observed in the brainstem slices. Moreover, fixing the calcium leak with S107 but not ARM036 significantly reduced HR level at rest (Figure 6A and B).

Hexamethonium, which blocks both sympathetic and parasympathetic limbs of the ANS (74), caused a significant reduction in HR in both Q175 and Q175+ARM036 mice. These data suggest that the increased HR observed in Q175 mice is driven by decreased parasympathetic activity and/or increased sympathetic activity (Figure 6C).

We then analyzed the activity of the ANS manifested by beat-to-beat HRV (Figure 6D-I). The low frequency (LFr) bands reflect mostly sympathetic modulation of heart rhythm, and oscillations in high frequency (HFr) bands reflect exclusively parasympathetic vagal activity. Thus, the LFr-to-HFr ratio reflects sympathovagal balance (75). The LFr was similar between all the
groups during active and rest periods suggesting that sympathetic regulation of cardiac activity is normal in the Q175 HD mouse model. In contrast, there was a significant decrease in the HFr during the rest period in Q175 mice compared to WT consistent with impaired parasympathetic activity. Q175 mice exhibit a lower LFr-to-HFr ratio compared to WT during the active period and a higher LFr-to-HFr ratio during the rest period (Figure. 6H and I). These data suggest that RyR2-mediated ER Ca\(^{2+}\) leak in the brainstem alters sympathovagal balance, through a reduction in parasympathetic activity that may promote cardiac arrhythmias. Indeed, ventricular extrasystoles (VES) were increased in Q175 mice during the rest period but did not change during awake period. The increased number of extrasystoles was abolished by both S107 and ARM036 suggesting that these VES are triggered in the heart by diastolic SR Ca\(^{2+}\) leak as previously described (76). The remodeling of RyR2 in the heart described above accounts for diastolic SR Ca\(^{2+}\) leak and subsequent VES (Figure. 6J and K). Next we evaluated cardiac function using echocardiography to measure the left ventricular ejection fraction (LVEF) and speckle tracking imaging that allows earlier detection of left ventricular systolic dysfunction, a strong predictor of HF and mortality (77). The LVEF in Q175 mice was normal, however the left ventricular global longitudinal strain was reduced indicating a predisposition to heart failure (Figure S5A-C).

**Remodeled leaky RyR2 in brainstem contributes to respiratory dysfunction**

Respiratory dysfunction including chest muscle rigidity, respiratory muscle weakness, difficulty in clearing airway secretions and swallowing abnormalities have been reported in patients suffering from neurodegenerative disorders including HD (9, 10).
We evaluated ex-vivo and in-vivo the respiratory function in Q175 HD mice. In order to distinguish between centrally mediated versus peripherally mediated effects on respiratory function we used two Rycals drugs that fix leaky RyRs channels S107 and ARM036.

To evaluate the intrinsic properties of the respiratory muscle we compared the ex-vivo diaphragmatic function in Q175 versus WT mice. Force production was significantly reduced (Figure. 7A-E) at different simulation frequency (p<0.05) in Q175 mice compared to their control littermate. Both S107 and ARM036 treatments restored diaphragmatic force generation in Q175 mice (Figure. 7C, D and E). Interestingly, EDL and soleus contractile function were not affected in HD mice (Figure S5. H-Q) suggesting that the muscle dysfunction is more severe in the diaphragm. One possibility is that the severity of the muscle dysfunction in the diaphragm is due to defective central regulation of respiratory function.

We used whole-body plethysmography to compare the respiratory function of Q175 versus WT mice with room air or hypercapnia (8% CO2) (Figure. 7F, G and H). With room air WT and Q175 mice had equivalent Tidal Volumes (VT), respiratory frequency (F), Minute ventilation (MV) and inspiration and expiration times (Figure. 7F-H and Figure S5. P-R). With hypercapnia (8% CO2), Q175 mice exhibited significantly reduced MV compared to the WT mice and decreased TV (p<0.05) (Figure. 7F and H).

S107 and ARM036 administration had no effect on respiratory function in mice breathing room air (Figure. 7F, G and H). In contrast, S107 treatment, but not ARM036 rescued respiratory function in Q175 mice under hypercapnia. These data suggest that leaky neuronal RyR2 alters central respiratory control in Q175 HD mice. In addition, voluntary activity as determined using
running wheel was significantly reduced in terms of time spent on the wheel in Q175 mice compared to control without any difference in the mean speed (Figure. 7I-K). Both S107 and ARM036 treatment similarly rescue the decreased voluntary activity observed in our HD mice.

**Diaphragmatic dysfunction in HD is associated with impaired excitation contraction coupling**

Diaphragmatic dysfunction can occur in response to increased beta-adrenergic signaling which causes PKA-mediated RyR1 hyper-phosphorylation and leaky channels (78). RyR1 Ser-2844 phosphorylation, oxidation, cysteine nitrosylation were increased and calstabin1 was depleted from RyR1 in Q175 diaphragm (Figure. 8A and B). RyR1 remodeling was associated with a significant increase in RyR1 P0 under non-activating conditions ([Ca2+]cyt = 150 nM) consistent with leaky channels that were fixed by treatment with either S107 or ARM036 (Figure. 8C-F). Moreover, diaphragmatic dysfunction was not due to reduced cross sectional area (Figure. 8G and H).
Discussion

Calcium is a second messenger that regulates activity of numerous cellular processes including activation of protein kinases and phosphatases, proteases, ion transporters and channels, neurotransmitter vesicle release and gene transcription. Ca$_{2+}$ is stored in the endo/sarcoplasmic reticulum compartment and can be released via inositol 1,4,5-trisphosphate receptors (IP3Rs) and/or ryanodine receptors (47, 79). In pathological conditions, remodeled RyR channels have been shown to mediate ER/SR Ca$_{2+}$ leak into the cytosol at rest, leading to local increases of $[\text{Ca}_{2+}]_{\text{cyt}}$ and activation of pathological signals (60). In neurons, such increases in $[\text{Ca}_{2+}]_{\text{cyt}}$ can alter synaptic plasticity, survival/growth, and other essential signals (80). Interestingly, it has been shown in R6/2 HD mice that increased $[\text{Ca}_{2+}]_{\text{cyt}}$ is linked to locomotor dysfunction (81).

In the present study we show that RyR2 channels in the brain are oxidized, PKA phosphorylated, nitrosylated and depleted of the stabilizing subunit calstabin in both human HD patients and in three models of HD (R6/2, Q111 and Q175). This biochemical signature of leaky RyR2 is consistent with previous reports of increased $[\text{Ca}_{2+}]_{\text{cyt}}$ in HD neurons (37, 81). Moreover, the observation that fixing RyR2-mediated ER Ca$_{2+}$ leak with S107 improves cognitive and locomotor function in a murine model of HD (Q175 mice) suggests that leaky RyR2 play a heretofore crucial role in HD pathogenesis. This result is consistent with the in vitro neuroprotection and improved motor function previously reported using dantrolene in YAC128 mice (66, 80). Indeed, increased cytosolic Ca$_{2+}$ concentrations can activate a number of kinases Ca$_{2+}$ dependent such the Ca$_{2+}$/Calmodulin which may contributed to further RyR2 remodeling and Ca$_{2+}$ leak (82). This has been reported in cardiac tissues, cortex and striatum of BACHD and R6/2 murine of HD where the expression of the Ca$_{2+}$/Calmodulin IV was increased (83, 84) . Of note,
HD likely shares common features with Alzheimer disease in which enhanced RyR-mediated ER Ca\textsuperscript{2+} leak has been linked to pathological post-translational modifications (e.g. PKA phosphorylation, oxidation/nitrosylation and calstabin depletion) (58).

While the mechanisms involved in HD remain unclear, several hypotheses have been put forward: mutated huntingtin aggregates form inclusion bodies inside neurons and insoluble huntingtin causes mitochondrial dysfunction, as well as Ca\textsuperscript{2+} dyshomeostasis, defective protein-protein interactions and vesicular transport of proteins including neurotransmitter receptors, ultimately leading to neuronal death. Moreover, mutated huntingtin micro aggregates increase ROS production in the brain (85). Here we show increased ROS production in Q175 brain and increased RyR2 oxidation which causes ER Ca\textsuperscript{2+} leak. (Figure. S3A).

**Beyond the brain: widespread pathology in HD**

In addition to the classic symptoms, HD is complicated by weight loss, heart failure, respiratory and swallowing muscle dysfunction. These features can appear early in the disease course and can eventually contribute substantially to both morbidity and mortality (4). Heart failure occurs in about 30% of patients with HD and is a leading cause of death (86). Moreover, many patients with HD report respiratory symptoms at late stages of the disease when the impaired motor control of the swallowing and respiratory muscle increases the risk of aspiration pneumonia (7, 14, 87). However, little is known about the pathophysiological mechanism underlying the cardiopulmonary dysfunction in HD.
We found significant remodeling of RyR2 in the cortex and the hippocampus, likely related to cognitive function impairment, and in brainstem areas involved in autonomic regulation of cardiac activity (88). RyR2 remodeling includes PKA hyper-phosphorylation, oxidation, nitrosylation and calstabin depletion. These changes represent the biochemical signature of leaky RyR2. ER Ca\textsuperscript{2+} leak may contribute to neurodegeneration (54) and could explain in part the dysregulation of the ANS resulting in decreased heart rate variability that has been observed in HD patients and in murine HD models (25, 27, 88). Signaling from the central autonomic network is mediated through the preganglionic sympathetic and parasympathetic neurons. The most widespread cranial parasympathetic output is carried through the vagus nerve which originates from the DMVN and NA and regulates cardiac activity. The increase in heart rate in Q175 mice is consistent with reduced parasympathetic activity. Our observations are consistent with the reduced heart rate variability in HD patients (89) including presymptomatic HD mutation carriers and mildly disabled HD patients (90). Further evidence of sympathovagal dysfunction includes symptoms such as orthostatic dizziness and tachycardia (25).

It has been suggested that sympathovagal dysautonomia in favor of sympathetic drive could result in fatal cardiac arrhythmias and/or promote heart failure in HD (28). Indeed, augmented sympathetic outflow and decreased vagal activity are considered to be pro-arrhythmic (91).

A complex network of neurons present mainly in the ventrolateral medulla oblongata and dorsolateral pons forms the respiratory central pattern generator(92). We found an overall brainstem remodeling of RyR2, and decreased diaphragmatic force production measured ex-vivo in Q175 mice. In addition respiratory function was impaired at 10 months of age manifested as a decreased tidal volume and minute ventilation response to hypercapnia, indicative of a central
respiratory defect. Interestingly, in a murine model of Duchenne Muscular Dystrophy, \textit{mdx} mice, there is normal breathing at rest despite diaphragmatic weakness (93, 94). Our results are in accordance with previous data obtained in HD patients where respiratory pressure, forced vital capacity, peak expiratory flow and maximum voluntary ventilation were reduced compared to controls (13). HD symptoms have been suggested to be due to neurological dysfunction or secondary to a systemic illness due to mutated Huntingtin protein expressed in the peripheral tissues (2, 95, 96). To our knowlge, this is the first study evaluating the effects of huntingtin mutation in the brain, the heart and the diaphragm and central versus peripheral pathophysiological mechanisms involved in HD. Since only S107 but not ARM036 prevented cardiorespiratory dysfunction we conclude that leaky RyRs in the brainstem play an important role in HD pathology.
Methods

S107 and ARM036 Treatment

The Rycal S107 [blood brain barrier (BBB) permeant] was administered in drinking water at 75 mg/kg/day for 1 month as previously described (54). In order to differentiate between central CNS effects vs peripheral muscle effects the Rycal ARM036 (non-BBB permeant) was administered in drinking water at 20 mg/kg/day. Standard food was provided ad libitum throughout the experiments. Mouse weights and brain drug levels were as described in the Figure. S3B and C.

Immunoprecipitation

RyR1 and RyR2 were immunoprecipitated from diaphragm, heart and brain using an anti-RyR1 or anti-RyR2 specific antibody (2 μg) in 0.5 ml of a modified radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.2, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na3VO4, 1% Triton X-100, and protease inhibitors) overnight at 4 °C. 1) RyR1-specific antibody was RyR1-1327, an affinity-purified rabbit polyclonal antibody raised against a KLH-conjugated peptide with the amino acid sequence CAEPDTDYENLRRS, corresponding to residues 1327–1339 of mouse skeletal RyR1, with an additional cysteine residue added to the amino terminus, and affinity purified with the unconjugated peptide (78). 2) RyR2 specific antibody was an affinity-purified polyclonal rabbit antibody using the peptide CKPEFNNHKDYAQEK corresponding to amino acids 1367-1380 of mouse RyR2 with a cysteine residue added to the amino terminus (60). The immune complexes were incubated with protein A-Sepharose beads (Sigma) at 4 °C for 1 h, and the beads were washed three times with radioimmune precipitation assay buffer. The immunoprecipitates were size-fractionated on SDS-PAGE gels (4-20 % for RyR1/RyR2 and calstabin) and transferred onto
nitrocellulose membranes for 2 h at 200 mA. Immunoblots were developed using the following primary antibodies: anti-RyR1/2 (Affinity Bioreagents, 1:2000), anti-phospho-RyR-Ser(P)-2808 (Affinity Bioreagents 1:5000), anti-calstabin (FKBP12 C-19, 1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Cys-NO (1: 1,000, Sigma). To determine channel oxidation, the carbonyl groups in the protein side chains were derivatized to DNP by reaction with 2,4-dinitrophenylhydrazine. The DNP signal associated with RyR was determined using a specific anti-DNP antibody according to the manufacturer's instructions (Millipore, Billerica, MA). All immunoblots were developed using an Odyssey system (LI-COR Biosciences, Lincoln, NE) with IR-labeled anti-mouse and anti-rabbit IgG (1: 10,000 dilution) secondary antibodies.

**Sarcoplasmic Reticulum Vesicle preparation**

Brain, heart and diaphragm were homogenized on ice in 300 mM sucrose, 20 mM Pipes (pH 7.0) in the presence of protease inhibitors (Roche), and centrifuged at 8,000 rpm (5,900 × g) for 20 min at 4 °C. The supernatant was ultracentrifuged at 32,000 rpm (100,000 × g) for 1 h at 4 °C. The final pellet containing microsomal fractions enriched in SR vesicles was resuspended and aliquoted in 300 mM sucrose, 5 mM Pipes (pH 7.0) containing protease inhibitors. Samples were frozen in liquid nitrogen and stored at −80 °C.

**Single channel data using planar lipid bilayers**

Planar lipid bilayers were formed using a 3:1 mixture of phosphatidylethanolamine and phosphatidylcholine (Avanti Polar Lipids) suspended (30 mg/mL) in decane by painting the lipid/decane solution across a 200-µm aperture in a polysulfonate cup (Warner Instruments).
separating two chambers. The trans chamber (1 mL) representing the intra-ER/SR (luminal) compartment was connected to the headstage input of a bilayer voltage clamp amplifier (BC-525D, Warner Instruments) and the cis chamber (1 mL), representing the cytoplasmic compartment, was held at virtual ground. Solutions in both chambers were as follows: 1 mM EGTA, 250/125 mM Hepes/Tris, 50 mM KCl, 0.64 mM CaCl2, pH 7.35 as cis solution and 53 mM Ca(OH)2, 50 mM KCL, 250 mM Hepes, pH 7.35 as trans solution. The concentration of free Ca2+ in the cis chamber was calculated using the WinMaxC program (version 2.50; www.stanford.edu/~cpatton/maxc.html). SR vesicles were added to the cis side, and fusion with the lipid bilayer was induced by making the cis side hyperosmotic by the addition of 400–500 mM KCl. After the appearance of potassium and chloride channels, the cis compartment was perfused with the cis solution. Single-channel currents were recorded at 0 mV by using a Bilayer Clamp BC-535 amplifier (Warner Instruments), filtered at 1 kHz, and digitized at 4 kHz. All experiments were performed at room temperature. Data acquisition were performed using Digidata 1440A and Axoscope 10.2 software, recordings were analyzed using Clampfit 10.2 (Molecular Devices). Open probability was identified by 50% threshold analyses using a minimum of 2 min of continuous record. At the conclusion of each experiment, ryanodine (5 µM) was added to the cis chamber to confirm channels as RyR.

**Brain immunohistochemistry**

Mice were perfused transcardially with 30 ml phosphate-buffered saline followed by 30 ml of 4% formaldehyde. Brainstems were removed and post-fixed for 12 h in 4% formaldehyde at 4°C. All brainstems were cut coronally (50 µm sections) using a vibratome. Fluorescence immunohistochemistry was performed as previously described (97, 98). Sections were
immunostained for detection of tyrosine hydroxylase (TH), choline acetyltransferase (ChAT) and either RyR2 or P*RyR2. The primary antibodies used were mouse anti-TH (1:500, Merck, Germany, MAB318), goat anti-ChAT (1:200, Merck, Germany, AB144P), rabbit anti-RyR2 (60) (1:400) and rabbit anti-P*RyR2 (Affinity Bioreagents 1:400). The secondary antibodies used were AlexaFluor-647 donkey anti-mouse (1:500, Invitrogen, Carlsbad, USA, A31571), AlexaFluor-555 donkey anti-rabbit (1:500, Invitrogen, Carlsbad, USA, A31572) and AlexaFluor-488 donkey anti-goat (1:500, Invitrogen, Carlsbad, USA, A11055). The presence of RyR2- or P*RyR2-immunoreactivity in pre-sympathetic adrenergic C1 neurons of the rostral ventrolateral medulla oblongata (TH-immunoreactive) and cardiac parasympathetic preganglionic neurons of the nucleus ambiguus (ChAT-immunoreactive) was examined using a Zeiss Axioplan two microscope with an Apotome module. Brainstem sections were sampled every 100 µm, and bilateral images (20x objective) of typically 2-3 sections per animal were acquired using 9 tiles of Z-stacks composed of 8 optical sections (8 µm focal spacing). For Figure 3A, a maximum intensity projection of optical sections was performed. Semi-quantitative analyses of colocalization between RyR2- or P*RyR2-immunoreactivity and TH- or ChAT-immunoreactivity were performed in 4 WT and 4 Q175 mice, using the following code: - = 0 colocalized neurons; + = 1-25% colocalized neurons; ++ = 26-50% colocalized neurons; +++ = 51-75% colocalized neurons; ++++ = 76-100% colocalized neurons.

**Measurement of diaphragm fibers cross sectional areas**

Frozen diaphragm muscle strips from the left middle segment of costal diaphragm were embedded in OCT for cryosections. Cryosections (10 µm) were taken precisely perpendicular to the fibers by a previously described technique (99), fixed with cold isopentane for 2 minutes, and immunostained with murine anti-α myosin heavy chain, (Sigma Co., St. Louis, Missouri 1:2000),
Muscle membrane was counterstained with rabbit anti-dystrophin antibodies (Abcam, 1:1000) using standard procedures. Alexa 555 anti-mouse and Alexa 488 anti-rabbit secondary antibody were then applied. Slides were mounted in Prolong Gold anti-fading reagent (Invitrogen, Carlsbad, California) and imaged by confocal fluorescent microscopy. Cross Sectional Area (CSA) was measured using Fiji (http://fiji.sc/wiki/index.php/Fiji) software, an enhanced version of Image J. Two different regions from each section (total ~150 fibers/sample) were processed.

**Contractile Function in Murine Muscle Samples.**

Mice were euthanized by exsanguination and the entire diaphragm EDL or soleus was surgically excised. Isometric contractile properties were assessed as described elsewhere (100). The excised diaphragm strip, EDL and soleus were mounted into jacketed tissue bath chambers filled with equilibrated and oxygenated Krebs solution. The muscles were supramaximally stimulated using square wave pulses (Model S48; Grass Instruments). The force–frequency relationship was determined by sequentially stimulating the muscles for 600 ms at 10, 20, 30, 50, 60, 80, 100, and 120 Hz with 1 min between each stimulation train (53). After measurement of contractile properties, muscles were measured at Lo (the length at which the muscle produced maximal isometric tension), dried, and weighted. For comparative purposes, muscle force production was normalized for total muscle strip cross-sectional area and expressed in N/cm². The total muscle strip cross-sectional area was determined by dividing muscle weight by its length and tissue density (1.056 g/cm³).

**Behavioral Studies:** Elevated plus maze and Morris water maze tests were performed as previously described (54, 58). Novel object recognition test was performed as previously described.
Briefly, the elevated plus maze (EPM) test was performed using the EPM apparatus, which consisted of four arms (two open without walls and two enclosed by 15 cm high walls) 68 cm long and 5 cm wide, was elevated 55 cm off of the floor. Video-tracking system (Noldus Information Technology Inc. VA) with computer interface and video camera were used to automatically collect behavioral data. Each mouse was placed at the junction of the four arms of the maze, numbers of entries and the time spent in each arm were recorded by the examiner and the video-tracking system simultaneously for 5 min. Deodar wiper was used between each tested mouse. The ratio of total time spent in, and the numbers of entry to the open arm versus closed arm of each mouse were analyzed.

Spatial learning and memory function was evaluated using Morris water maze (MWM) task, which consisted of a circular pool (122 cm in diameter, 76 cm in depth) (San Diego Instruments, CA) in which mice were trained to escape from water by swimming to a 10 cm diameter hidden platform (1.0-to-1.5 cm underneath water surface). Water temperature was maintained at 23± 1°C and the water was rendered opaque by the addition of white nontoxic paint (Discount School Supply, CA). The pool was divided into four quadrants by a computerized tracking/image analyzing system (Noldus Information Technology Inc.). The hidden platform was placed in the middle of the northwest (NW) quadrant and remained in the same position during the experiment. The spatial acquisition phase consisted of 15 training trials: 5 training days and 3 trials per day with an inter-trial interval of 40-60 min. Mice were released from different quadrants between trials with their heads facing the pool wall of one of the four compass locations (SW, SE and NE), and allowed to swim and search for the platform for 60 sec. The latency to reach the hidden platform, travel path, and swimming velocity were recorded by the examiner and the video tracking system.
simultaneously. On day-6, memory retention was evaluated by a probe trial 24 hours after the last training session in the absence of the escape platform. Mice were allowed to swim freely for 60 sec. The location where the hidden platform was previously located was defined as the target. The northwest quadrant where the platform was previously hidden was defined as the target quadrant. The number of target crossings and the proportion of swimming time spent in the target quadrant were recorded and analyzed.

The tail-suspension test was used to assess depression-related behavior, as described previously (102). Briefly, on the top of a box, a suspension bar was used to suspend the tail of the mouse. Each mouse was suspended separately in the middle of the box, using black adhesive tape applied to the end of the tail (with 2–3 millimeters remaining outside of the tape), and the free end of the tape was attached to the middle of the suspension bar. The approximate distance between the mouse’s nose and the apparatus floor was 10–15 cm. Every session lasted for 6 min, and was recorded using a video camera placed on a tripod in front of the box. The time that each mouse spent as immobile was measured. Lack of escape-related behavior is considered immobility, which is indicative of depression-like behavior.

**Whole body plethysmography measurement**

Respiratory function was measured in conscious, unrestrained mice using whole body plethysmography and analyzed using iox2 software (EMKA Technologies, Paris, France) as previously described (94, 103). Mice were randomly placed into individual chambers. After one-hour stabilization, each animal was visually monitored and ventilatory parameters were recorded during an inactive phase for 5 min under two experimental conditions. First, room air breathing,
then under hypercapnic condition using 8% CO\textsubscript{2}-enriched airflow to activate respiration. Air volume changes corresponding to spontaneously breathing were obtained using a pressure transducer (EMKA Technologies, Paris, France). This pressure signal was used to calculate ventilatory parameters: tidal volume (TV), respiratory rate (RR), minute volume (MV), inspiratory time (Ti) and expiratory time (Te).

**Running wheel**

Mice were subjected to voluntary aerobic exercise with free access to a running wheel (diameter 11.5 cm) which was connected to a digital counter for 24 hours (light/dark cycle) as previously described (104).

**ECG recording in conscious animals**

Mice were implanted with radio telemetry transmitters (Data Sciences International, Saint Paul, MN, USA) as described in detail elsewhere (105). Briefly, the transmitter (PhysioTel, ETA-F10 transmitter) was inserted in mice subcutaneously along the back under general anesthesia (20% inhaled isoflurane/O\textsubscript{2}, Aerrane,, Baxter, France) coupled with local anesthetic (lidocaine 0.5%), and two ECG electrodes were placed hypodermically in the region of the right shoulder (negative pole) and toward the lower left chest (positive pole) to approximate lead II of the Einthoven surface ECG. During procedure, respiratory and cardiac rhythm, adequacy of anesthetic depth, muscle relaxation, body temperature and analgesia were monitored to avoid anesthesia-related complications. Post-operating pain was considered during one-week post-implantation period and buprenorphine (0.3mg.kg\textsuperscript{-1 sc}) was given. A minimum period of 2 weeks was allowed for recovery from the surgery. Animals were housed in individual stainless steel cages for telemetry recordings.
Environmental parameters were recorded continuously and maintained within a fixed range, room temperature at 15–21°C and 45–65% relative humidity. The artificial day/night cycle was 12 h light and 12 h darkness with light on at 07:00 h. Drinking water was provided ad libitum. Solid diet (300 g) was given daily in the morning. ECG waveforms were continuously recorded at a sampling rate of 2000 Hz using signal transmitter-receiver (RPC-1) connected to data acquisition system (Ponemah system, Data Science International). Continuous digital recording were analyzed off line after to be digitally filtered between 0.1 and 1,000 Hz. ECGs during nocturnal and diurnal periods (2*10-hours) were analyzed with ponemah software using template automatic detection, secondly validated by an operator. The mean RR interval and QT durations were calculated. The QT interval was defined as the time between the first deviation from an isoelectric PR interval until the return of the ventricular repolarization to the isoelectric TP baseline from lead II ECGs.

Presence of ectopic beats were scanned by hand. Then, HRV was evaluated by power spectra analysis (ms-2) using the fast Fourier transformation (segment length of 2048 beats, linear interpolation with resampling to a 20-Hz interbeat time series and Hamming windowing). The cut-off frequency ranges for the low frequency powers (HF:1.5-5Hz) were chosen according to used in the literature (106).

**Autonomic blockade**

Complete suppression of autonomic control was achieved with ganglioplegic compound, hexamethonium (20 mg·kg⁻¹, i.p.).
Statistics

Group data are presented as mean ± SD unless otherwise indicated. Statistical comparisons between two groups were tested using an unpaired t test. ANOVA tests with a Bonferroni’s post hoc adjustment were used for multiple comparisons. Values of p < 0.05 were considered statistically significant. All statistical analyses were performed with Prism 8.0.

Study approval

Human Samples

De-identified human hippocampus and cortex samples were obtained from the Brain Bank at Columbia University. The age and gender of these samples were: 64, female; 58, female; 54, female; 61, female; 66, female; 56, male; 58, male; 63, male. Age and gender-matched controls exhibited absence of neurological disorders and plaques and previous experiments using these specific control samples had shown a lack of remodeling and leak in RyR2 (54). Information on the Huntington’s patients is listed in supplement Table1.

Animal model

Ten months old Q175-Z heterozygous (human mHtt allele with the expanded CAG repeat ~179 repeats within the native mouse huntingtin gene) mice and age-matched WT littermates and R6/2 (contains N-terminally truncated mutant Htt (mHtt) with CAG repeat expansion (~125 repeats) within the HTT gene exon 1) were purchased from Jackson laboratory, maintained and studied according to protocols approved by the Institutional Animal Care and Use Committee of Columbia
University (reference no AC-AAAV5455) and Directive 2010/63/EU of the European parliament and the Council of 22 September 2010 for the protection of animals used for scientific purposes and Ethics committee for animals experiments, Languedoc Roussillon, C2EA-36 (agreement: B34-172-38; project APAFIS#13528-2018). Q111 frozen brain tissues were obtained from the CHDI foundation (New York, NY. 350 Seventh Ave, suite 200, New York 10001) for only RyR2 analysis. We chose 10 months old Q175 mice when the mice exhibit remarkable behavioral deficits accompanied by marked brain atrophy, brain metabolite changes and start developing respiratory and autonomic dysfunction (107). Both male and female mice were used in this study. All in vivo animal experiments were performed by investigators blinded to genotype and treatment groups.

Acknowledgments

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References

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**Figure 1:** Cortex and hippocampal RyR2 channel remodeling result in biochemical signature of “leaky RyR2” in human HD brain.

Representative SDS-PAGE analysis and quantification (bands normalized to total RyR2) of RyR2 immunoprecipitated from cortex (A and B) and hippocampus (A and C) from human samples (see supplement, Table 1). “Control” (n=4); “HD patients” (n=4) (band intensity were normalized to total RyR2). D) Single-channel recordings of RyR2 incorporated in planar lipid bilayers with 150 nM Ca2+ in the cis chamber, corresponding to representative experiments performed with Human cortex samples from control and 3 HD patients. RyR2 open probability (Po), mean open time (To) were increased and the mean closed time (Tc) was decreased in HD RyR2 channels (E-G). Po was 0.014±0.005 in control (n=14), in HD patients increased to 0.095±0.01 (n=13). Results are expressed as mean ± SD. Student’s t test; * p <0.05, Control vs HD patient.
Figure 2: Cortical and hippocampal RyR2 channel remodeling result in biochemical signature of “leaky RyR2” in the brain of Q175 mice.

Representative SDS-PAGE analysis and quantification of RyR2 immunoprecipitated from cortex (A and B) and hippocampus (A and C) from mice samples. WT (n=6), Q175 (n=6) and Q175 (n=6) treated with S107, (bands intensities were normalized to total RyR2).

D) Single-channel traces of RyR2 incorporated in planar lipid bilayers with 150 nM Ca2+ in the cis chamber, corresponding to representative experiments performed with mice cortex samples from WT, Q175 and Q175 treated with S107.

E-G) RyR2 Po was increased in HD. Mean Po was 0.005±0.002 in WT (n=4), in Q175 mice increased to 0.17±0.04 (n=3) and restored by S107 treatment to 0.008±0.001 (n=3).

H) Single-channel traces of RyR2 incorporated in planar lipid bilayers with 150 nM Ca2+ in the cis chamber, corresponding to representative experiments performed with mice hippocampus samples from WT, Q175 and Q175 treated with S107. RyR2 Po, T0 were increased and Tc decreased in HD (I-K). Po was 0.004±0.0007 in WT (n=4), in Q175 mice increased to 0.09±0.05 (n=4) and restored by S107 treatment to 0.003±0.001 (n=4). Data (mean ± SD) analysis was performed by one-way ANOVA. Bonferroni post-test revealed *p<0.05 vs.WT, # p<0.05 vs.Q175.
Figure 3: Long-term learning and memory deficits in Q175 mice: S107 treatment targeting RyR2 channels improves cognitive function in HD. A) Learning curves showing the escape latency during a 5-day training period in the Morris water maze (MWM). B) The heat-maps from all the training trials were recorded (Noldus Information Technology Inc.) and downloaded. Representative heat-maps from WT (left column), Q175 (middle column), and S107 treated Q175 (right column) on day-2 (upper panels) and day-5 (lower panels) are shown. C) The time spent in all quadrants. D) The number of target crossing on day 6 probe trial of the MWM. E) The ratio of the number of entries to open arms vs. closed arms of the elevated plus maze (EPM). F) Total immobilization time of mice during 300 sec of tail suspension test. The same groups of mice were used for MWM, EPM and tail suspension. WT (n=8), Q175 (n=8) and Q175+S107 (n=6). Data (mean ± SD) analysis was performed by one-way ANOVA. Bonferroni post-test revealed * p<0.05 vs.WT, # p<0.05 vs.Q175.
**Figure 4:** Brainstem RyR2 remodeling induces nucleus ambiguus disruption.

A) Immunostaining and semi-quantitative analysis of total and phosphorylated RyR2 on serine 2808 in nucleus ambiguus neurons (choline-acetyltransferase (ChAT)-positive), which include cardiac parasympathetic neurons, and in presympathetic RVLM C1 neurons (tyrosine hydroxylase (TH)-positive) in the brainstem of WT and Q175 mice. Panels a and b, white arrows show ChAT/TH neurons with RyR2/P*RyR2 co-localisation, black arrows show ChAT/TH neurons without RyR2/P*RyR2 co-localisation. Semi-quantitative analysis of ChAT/TH and RyR2/P*RyR2 co-localization were made as following: - = 0% co-staining, + = 1 to 25% co-staining, ++ = 26 to 50% co-staining, +++ = 51 to 75% co-staining, ++++ = 76 to 100% co-staining. Analysis was made in 4 WT and 4 Q175 mice, bilaterally in 2-3 sections per animal.

B and C) Representative SDS-PAGE analysis and quantification of RyR2 immunoprecipitated from the brainstem collected from WT (n=6), Q175 (n=6), Q175+ARM036 (n=6) and Q175+S107 (n=6) mice (band intensity were normalized to total RyR2). Data (mean ± SD) analysis was performed by one-way ANOVA. Bonferroni post-test revealed * p<0.05 vs.WT, # p<0.05 vs.Q175. Scal bar is 100 µm in panel A and 50µm in panel a and b.
**Figure 5:** Cardiac RyR2 remodeling in Q175 mice.

A and B) Representative SDS-PAGE analysis and quantification of RyR2 immunoprecipitated from the heart samples from WT (n=6), Q175 (n=6), Q175+ARM036 (n=6) and Q175+S107 (n=6), (bands intensity were normalized to total RyR2).

C) Single-channel traces of RyR2 incorporated in planar lipid bilayers with 150 nM Ca\(^{2+}\) in the cis chamber, corresponding to representative experiments performed with heart samples from WT, Q175, Q175+ARM036 and Q175+S107 mice.

D, E and F) Single channel analysis: Po was 0.009±0.001 in WT (n=3), in Q175 mice increased to 0.18±0.01 (n=3) and restored by S107 treatment to 0.015±0.003 (n=3) and ARM036 to 0.015±0.003 (n=3). Data (mean ± SD) analysis was performed by one-way ANOVA. Bonferroni post-test revealed * p<0.05 vs. WT, # p<0.05 vs. Q175.
Figure 6: Sympathovagal imbalance and arrhythmias in HD during rest period

A) Representative ECG trace in freely-moving conscious animals allowing heart rate (bpm) measurement in WT and Q175 mice treated or not with S107 or ARM036 during light (rest period) and dark (awake period) cycles. B) Heart rate (bpm) average during awake and rest period in WT (n=5), Q175 (n=8), and Q175 mice treated or not with S107 (n=6) or ARM036 (n=6). C) Heart rate average record after Hexamethonium injection (20 mg/kg), had moderate effects on HR related to suppression of cardiac autonomic control of HR, that is lowering HR during the daylight period in Q175 (n=6), and Q175 mice treated or not with S107 (n=6) or ARM036 (n=6). Data (mean ± SD) analysis was performed by one-way ANOVA. Bonferroni post-test revealed * p<0.05 vs.WT, # p<0.05 vs.Q175 at rest period. Student’s t test; $ p<0.05 rest vs awake period and baseline vs Hexamethonium. D and E) Low frequency spectral power density measured by heart rate variability analysis using fast Fourier transformation (LF:0.15-1.5 Hz) in WT, Q175, Q175+ARM036 and Q175+S107 during rest and awake period (n=5-8 mouse/group). F and G) High frequency spectral power measured by heart rate variability analysis using fast Fourier transformation (HF:1.5-5 Hz) during rest and awake period WT, Q175, Q175+ARM036 and Q175+S107 during rest and awake period (n=5-8 mouse/group). H and I) Low frequency-to-high frequency ratio (LF/HF; n=5-8 mouse/group). J and K) Number of isolated and triplet (three consecutive) ventricular extrasystoles (VES) during
10 hours in WT, Q175, Q175+ARM036 and Q175+S107 during rest and awake period (n=5-8 mouse/group). Representative example of ventricular extrasystoles (VES) in Q175 mice are shown in (S5G). Data (mean ± SD) analysis was performed by one-way ANOVA. Bonferroni post-test revealed * p<0.05 vs.WT, # p<0.05 vs.Q175. Scale bar is 110 ms.

Figure. 7: RyR mediated SR Ca$_2^+$ leak contributes to respiratory dysfunction and reduces voluntary activity in murine model of HD.

A-D) Representative records of diaphragmatic specific force production measured ex-vivo at 20 and 120 Hz in muscle bundles under isometric conditions in WT, Q175, Q175+ARM036 and Q175+S107 treated mice. E) Average force-frequency relationship recorded in WT (n=13), Q175 (n=10), Q175+ARM036 (n=9) and Q175+S107 (n=8) treated mice. Data (mean ± SEM) analysis was performed by Two-way ANOVA. Bonferroni post-test revealed * p<0.05 vs.WT, # p<0.05 Q175 vs. Q175+S107/ARM036. F) Tidal volume (mL/g) at rest and during CO$_2$ stimulation. G) Respiratory frequency (bpm) at rest and during CO$_2$ stimulation H) Minute volume (mL/g) at rest and during CO$_2$ stimulation in WT (n=15), Q175 (n=19), Q175+ARM036 (n=10) Q175+S107 (n=10). The CO$_2$ values were recorded 10 min after the initiation of CO$_2$ stimulation.
Data (mean ± SD) analysis was performed by one-way ANOVA. Bonferroni post-test revealed * p<0.05 vs. WT, # p<0.05 vs. Q175. I-J-K) Active time (s), mean speed (m/min) and running distance (m) for voluntary activity on running wheels, respectively in WT (n=13), Q175 (n=10), Q175+ARM036 (n=10) and Q175+S107 treated mice (n=12). Data (mean ± SD) analysis was performed by one-way ANOVA. Bonferroni post-test revealed * p<0.05 vs. WT, # p<0.05 vs. Q175.

**Figure 8: Diaphragmatic RyR1 remodeling in HD**

A-B) Representative SDS-PAGE analysis and quantification of RyR1 immunoprecipitated from diaphragm samples (band intensity were normalized to total RyR1) of WT (n=6), Q175 (n=6), Q175+ARM036 (n=6) and Q175+S107 mice (n=6). C) Single-channel traces of RyR1 incorporated in planar lipid bilayers with 150 nM Ca²⁺ in the cis chamber, corresponding to representative experiments performed with diaphragm samples from Q175 mice. D, E and F) Increased RyR1 Po, To and decreased Tc in Q175 mice diaphragm. Po was 0.007±0.001 in WT (n=3), in Q175 increased to 0.13±0.017 (n=3), restored to 0.017±0.02 in Q175+S107 (n=3) and to 0.010±0.002 in Q175+ARM036 group (n=3). Data (mean ± SD) analysis was performed by one-way ANOVA. Bonferroni post-test revealed * p<0.05 vs. WT, # p<0.05 vs. Q175. G) Representative immunostaining of fast and slow diaphragm muscle fibers.
of WT (n=6), Q175 (n=6), Q175+ARM036 (n=6) and Q175+S107 mice (n=6). Quantified data are represented as a box-and-whisker plot, with bonds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values. Antibodies against fast (yellow arrows) and slow (white arrows) type myosin ATPase were used to perform immunostaining on cryo-sections of mouse diaphragm. Muscle membrane was counterstained with dystrophin antibodies (green color). H) Quantification of cross-sectional area (in µm²) were calculated using ImageJ software in each condition. Scale bar is 50 µm.

Figure 9: Graphical abstract-Neuronal RyR2 calcium leak in HD contributing to cognitive impairment, cardiac arrhythmia and respiratory dysfunction.

Aggregated huntingtin protein increases mitochondrial ROS production that oxidize ryanodine receptor and dissociated its stabilizing subunit calstabin2 resulting in pathological calcium leak that causes cognitive dysfunction in HD. Increased RyR2 calcium leak in the brainstem leads to respiratory control alteration, and dysregulates the autonomic nervous system leading to cardiac arrhythmia. S107 prevents calstabin2 dissociation from RyR2, reduces ER calcium leak and prevents cognitive impairment and cardiorespiratory dysfunction.