S-Nitrosylation of Dynamin-Related Protein 1 Mediates Mutant Huntingtin-Induced Mitochondrial Fragmentation and Neuronal Injury in Huntington's Disease

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Abstract

Aims: Dynamin-related protein1 (Drp1) is a large GTPase that mediates mitochondrial fission. We recently reported in Alzheimer's disease (AD) that S-nitrosylation of Drp1 (forming S-nitroso [SNO]-Drp1) results in GTPase hyperactivity and mitochondrial fragmentation, thus impairing bioenergetics and inducing synaptic damage and neuronal loss. Here, since aberrant mitochondrial dynamics are also key features of Huntington's disease (HD), we investigated whether formation of SNO-Drp1 contributes to the pathogenesis of HD in cell-based and animal models. **Results:** We found that expression of mutant huntingtin (mutHTT) protein in primary cultured neurons triggers significant production of nitric oxide (NO). Consistent with this result, increased levels of SNO-Drp1 were found in the striatum of a transgenic mouse model of HD as well as in human postmortem brains from HD patients. Using specific fluorescence markers, we found that formation of SNO-Drp1 induced excessive mitochondrial fragmentation followed by loss of dendritic spines, signifying synaptic damage. These neurotoxic events were significantly abrogated after transfection with non-nitrosylatable mutant Drp1(C644A), or by the blocking of NO production using an nitric oxide synthase inhibitor. These findings suggest that SNO-Drp1 is a key mediator of mutHTT toxicity, and, thus, may represent a novel drug target for HD. **Innovation and Conclusion:** Our findings indicate that aberrant S-nitrosylation of Drp1 is a prominent pathological feature of neurodegenerative diseases such as AD and HD. Moreover, the SNO-Drp1 signaling pathway links mutHTT neurotoxicity to a malfunction in mitochondrial dynamics, resulting in neuronal synaptic damage in HD. *Antioxid. Redox Signal.* 19, 1173–1184.

Introduction

HUNTINGTON'S DISEASE (HD) is an autosomal, dominant, and inherited neurodegenerative disorder that is characterized by loss of striatal and cortical neurons, leading to both motor and behavioral symptoms. Aberrant expansion of a CAG repeat beyond a critical threshold (>36) in the *huntingtin* gene leads to formation of soluble toxic oligomers and insoluble aggregates of mutant huntingtin (mutHTT) protein,

and it underlies the development of this devastating disease. Although insoluble aggregates of mutHTT were historically thought to be associated with neuronal damage, more recent studies have found that insoluble mutHTT aggregates may, in fact, represent a molecular sink for soluble toxic mutHTT species (1). Such soluble mutHTT is believed to cause neurodegeneration in HD by influencing multiple cellular events, including oxidative/nitrosative stress, excitotoxicity, transcriptional regulation, the ubiquitin-proteasome pathway,

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Innovation

Our data reveal that dynamin-related protein 1 (Drp1) is S-nitrosylated in both human Huntington's disease (HD) brains and animal models of HD. We further demonstrate that S-nitroso (SNO)-Drp1 is a key mediator of mutant huntingtin-induced mitochondrial fragmentation and resulting dendritic spine loss. These findings link aberrant nitric oxide production to mitochondrial and synaptic dysfunction. Moreover, this study suggests that SNO-Drp1 may represent a new therapeutic target for protecting synapses and neurons in HD.

and autophagic progression (8, 16, 29, 44). Furthermore, increasing evidence suggests that mitochondrial dysfunction may be central to the pathogenesis of HD (6, 12, 15, 48). For instance, treatment of rodents with the potent irreversible inhibitor of mitochondrial complex II, 3-nitropropionic acid, causes a selective loss of striatal neurons, resembling a critical feature of HD (7). Moreover, aberrant changes in mitochondrial morphology are seen in both animal models of HD and human HD patients (14, 15, 45, 50). Usually, mitochondria continuously undergo fission and fusion (a process known as mitochondrial dynamics) to generate smaller organelles or elongated, tubular structures, respectively. This normal mitochondrial fission and fusion can facilitate formation of new mitochondria (biogenesis), repair of defective mitochondrial DNA through mixing, and redistribution of mitochondria to sites requiring high-energy production (9, 25, 32). In neurons, mitochondrial biogenesis is especially important at synapses, which demand high concentrations of ATP; the normal distribution of mitochondria at the nerve terminal can influence synaptic transmission and maintain synaptic structure (9, 35, 36). Conversely, an imbalance in fission or fusion can lead to abnormal mitochondrial morphology and bioenergetics, and may, thus, contribute to synaptic damage and neuronal loss during neurodegeneration (2, 5, 32, 57). The large GTPase, dynamin-related protein 1 (Drp1), is a pro-mitochondrial fission protein that catalyzes mitochondrial fission with its mitochondrial adapter proteins such as Fis1 and mitochondrial fission factor. Intriguingly, excessive activation of Drp1 has been linked to fragmented and small mitochondrial morphology, impaired bioenergetics, and neuronal injury in HD (14, 15, 45, 50).

Previously, in Alzheimer's disease (AD), we had described a redox-based mechanism through which nitric oxide (NO) S-nitrosylates Cys644 of Drp1 (forming S-nitroso [SNO]-Drp1), causing mitochondrial fragmentation, bioenergetic failure, and synaptic injury (10). This finding is emblematic of an emerging theme in both genetic and sporadic neurodegenerative disorders that nitrosative stress caused by excessive generation of NO can contribute to neuronal cell injury and death (28, 37, 39). Consistent with this notion, recent studies have provided evidence for an effect of NO on mitochondrial dynamics in HD (2, 27, 46, 52). Hence, elucidating the molecular mechanism underlying NO-mediated mitochondrial fragmentation may lead to a new therapeutic intervention in HD. In this study, we sought to determine whether activation of Drp1 *via* S-nitrosylation is of pathological relevance to HD. Here, we demonstrate that mutHTT expression induces generation of NO, resulting in formation

of SNO-Drp1 in a mouse model of HD. We also show similar levels of SNO-Drp1 in human brains from HD patients. In addition, we found that S-nitrosylation of Drp1 at Cys644 engenders excessive mitochondrial fission and dendritic spine loss in mutHTT-expressing neurons. These results implicate SNO signaling as a critical mediator of mutHTT toxicity and highlight the SNO-Drp1 pathway in the pathogenesis of HD.

Results

NO has been shown to be increased in the brains of both human HD and HD transgenic mice (17, 19). Here, to directly demonstrate that expression of mutHTT increases NO, we employed a cell-based model of HD by transfecting primary cortical neurons with the N-terminal fragment of HTT encoding either 18 (Q18; wtHTT) or 148 (Q148; mutHTT) polyQ repeats. To monitor NO levels, we employed the fluorescent NO probe, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate), which fluoresces proportionally to its binding of NO (33). Transfection of rat cortical neurons at 14 days *in vitro* (DIV14) with mutHTT resulted in significantly increased intensity of DAF-FM compared with transfection with wtHTT (Fig. 1). The increased DAF-FM intensity after transfection with mutHTT indicates that higher levels of NO were produced compared with wtHTT.

Since mutHTT expression elicited neuronal production of NO, we next asked whether SNO-Drp1 formation is increased in response to polyQ expansions in the *htt* gene *in vivo*. For this purpose, we employed a modified Western blot termed the biotin-switch assay to detect SNO-Drp1 in the brains of BACHD transgenic model mice, which express full-length human Q97 mutHTT (Fig. 2). Similar to HD patients, these mice develop neuronal dysfunction in both the striatum and cortex (26). When brain lysates from the striatum of these mice *versus* wild-type controls were subjected to the biotin-switch assay, we found significantly increased levels of SNO-Drp1 in the striatum of the BACHD mice (Fig. 2A, B), which is consistent with the notion that expression of mutHTT resulted in increased NO production and subsequent formation of SNO-Drp1. In contrast, we observed comparable levels of SNO-Drp1 in the cerebellum of BACHD and control mice (Fig. 2C, D). Although mutHTT influences expression of a similar pattern of genes in both the striatum and cerebellum of an HD mouse model (24), the cerebellum is less affected pathologically, and, thus, our finding of increased SNO-Drp1 in the BACHD striatum compared with control striatum is consistent with the hypothesis that formation of SNO-Drp1 is associated with the pathological changes of HD. To extend these findings to humans, we next examined brains obtained shortly after death from patients manifesting HD (Table 1). Similar to our transgenic mice data, we found significantly increased SNO-Drp1 levels in human HD brains, but not in brains of control patients who died of non-central nervous system (CNS) causes (Fig. 3A, B). Furthermore, the ratio of SNO-Drp1 (determined by the biotin-switch assay) to total Drp1 (from immunoblots) in BACHD mice and human HD brains (Figs. 2B and 3B) was comparable to that encountered in AD transgenic mouse models and human brains manifesting excessive mitochondrial fission and neuronal damage (10). These data are consistent with the notion that pathophysiologically relevant amounts of SNO-Drp1 are present in both BACHD and human HD brains. In addition, these results

FIG. 1. MutHTT increases NO production in rat cortical neurons. Neurons at DIV14 were co-transfected with ptdTomato and the N-terminal fragment of either wild-type HTT (wtHTT; top, white arrow) or mutant HTT (mutHTT; bottom, dotted arrow), and stained with the fluorescent NO probe, DAF-FM. (A, D) Fluorescent images of tdTomato show transfected cells. (B, E) Images of DAF-FM fluorescence 7h after transfection. (C, F) Overlay of the tdTomato and DAF-FM fluorescent images. (G) Quantitative analysis of DAF-FM intensity normalized to control (ctrl; non-transfected cells); $p < 0.05$ by Student's *t*-test, $n = 16$ for wtHTT, $n = 19$ for mutHTT. DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; DIV14, 14 days *in vitro*; HTT, Huntingtin; NO, nitric oxide. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

suggest that S-nitrosylation of Drp1 may play an important role in the pathogenesis of HD.

In our earlier report on SNO-Drp1 in AD, we showed that S-nitrosylation of Drp1 at Cys644 increases its oligomerization and GTPase activity, leading to excessive mitochondrial fragmentation. We, therefore, asked whether these findings might hold true in HD, as recent reports had suggested that mutHTT directly binds to and stimulates Drp1 activity to increase mitochondrial fission, but the mechanism of HTT stimulation of Drp1 has remained unclear (49, 50). Since we

identified SNO-Drp1 as a potential mediator of mutHTT toxicity, we evaluated whether S-nitrosylation of Drp1 affects its affinity for HTT. To test this possibility, we used anti-Drp1 antibody to co-immunoprecipitate Drp1 from BACHD striatum, where we had found increased SNO-Drp1 (Fig. 2), and then examined the Drp1 immunocomplex for the presence of wt and mutHTT. Consistent with previous findings, our co-immunoprecipitation assay in BACHD brain confirmed the interaction of Drp1 and mutHTT, although in our hands wtHTT also bound to Drp1 (Fig. 4A, left panel, lanes 1–3). To

FIG. 2. S-nitrosylation of Drp1 in the striatum of BACHD mice. (A, C) Striatum and cerebellum of transgenic BACHD mice and wild-type mice (Ctrl) were subjected to the biotin-switch assay to detect SNO-Drp1. Levels of mutHTT and wtHTT were detected by immunoblotting (note the very faint band representing mutHTT in the striatum of the BACHD mouse). (B, D) Relative ratio of SNO-Drp1 to total Drp1 (Drp1) was determined by densitometric quantification of biotin-switch and immunoblot analyses ($**p < 0.01$ by *t*-test, $n = 6$). Drp1, dynamin-related protein 1; SNO, S-nitrosothiols.

TABLE 1. ATTRIBUTES OF BRAINS FROM HUMAN SUBJECTS IN THIS STUDY

Subjects	Diagnosis	Brain region	PMI (h)	Age at time of death	Gender
Control 1	Pneumonia	Temporal cortex	N/A	87	M
Control 2	N/A	Medial frontal cortex	9	102	F
Control 3	Abdominal aortic aneurysm repair	Temporal cortex	2	71	M
Patient 1	HD	Medial frontal cortex	6	67	F
Patient 2	HD	Medial frontal cortex	8	55	F
Patient 3	HD	Medial frontal cortex	2	46	M
Patient 4	HD	Medial frontal cortex	N/A	37	M
Patient 5	HD	Medial frontal cortex	N/A	42	M

HD, Huntington's disease; N/A, not available; PMI, postmortem interval.

examine the effect of S-nitrosylation, we incubated the Drp1 immunocomplex with the S-nitrosothiol-specific reducing agent, ascorbate (30, 38), and observed rapid dissociation of Drp1 from HTT protein, suggesting that S-nitrosylation facilitates Drp1 binding to HTT (Fig. 4A, left panel, lanes 4–6). Consistent with the notion that NO induced by mutHTT enhances Drp1/HTT complex formation, the interaction of Drp1 and HTT was absent in control mouse striatum (Fig. 4A, right). Interestingly, we found that mutHTT, to a greater degree than wtHTT, can also be S-nitrosylated by physiological NO donors (Fig. 4B). Moreover, since we found significant levels of SNO-Drp1 in human HD brain, this finding raised the possibility that SNO-mutHTT might transfer its NO group to Drp1, resulting in transnitrosylation of Drp1, as previously reported for other protein complexes (42). In any event, our data show that S-nitrosylation facilitates interaction of Drp1 and HTT and results in formation of SNO-Drp1, a reaction that we previously have shown can stimulate Drp1 hyperactivity in models of AD (10).

Given the dramatic increase of SNO-Drp1 levels in HD mouse models and human brain, we next sought to determine whether formation of SNO-Drp1 could result in increased mitochondrial fragmentation in HD, which is similar to our findings in AD (10). Therefore, we transiently co-transfected rat cortical neurons at DIV14 with the mitochondrial marker mito-DsRed2 (to detect mitochondrial morphology) and the N-terminal fragment of wtHTT(Q18) or mutHTT(Q148), as well as with Drp1. Morphological changes in mitochondria were monitored by 3D-reconstruction of the mito-DsRed2

signal under deconvolution fluorescence microscopy 1 day after transfection. After transfection with wtHTT, we observed elongated, interconnected mitochondria in the dendrites of transfected neurons (Fig. 5A, B). In contrast, after transfection with mutHTT, we found smaller, fragmented mitochondrial morphologies (Fig. 5C, D). Strikingly, this effect was blocked by addition of the nitric oxide synthase (NOS) inhibitor *N*-nitro-L-Arginine (NNA), resulting in more elongated, filamentous mitochondria (Fig. 5G, H). In addition, transfection with the nitrosylation-resistant mutant Drp1(C644A) reversed the effects of mutHTT on mitochondrial fission (Fig. 5E, F), which is consistent with the notion that formation of SNO-Drp1 contributes to mutHTT-induced mitochondrial fragmentation.

In order to validate our findings regarding mitochondrial fragmentation, we employed a new parameter to monitor mitochondrial surface area (termed the mitochondrial index) that Yu *et al.* have developed recently to characterize mitochondrial morphology (58). To measure the relative mitochondrial surface area to dendritic area, we transfected cortical neurons at DIV14 with mito-DsRed2 (to label mitochondria) and green fluorescent protein (GFP) (to visualize total surface area of transfected neuronal dendrites). We found that mutHTT expression lowered the mitochondrial index, suggesting that mitochondrial size is decreased due to mitochondrial fragmentation; whereas mutant non-nitrosylatable Drp1 and NNA blocked this effect of mutHTT on mitochondrial morphology (Fig. 6). These findings are consistent with our result on mitochondrial fission shown in

FIG. 3. Increased SNO-Drp1 in human HD patient brain. (A) Biotin-switch analysis of postmortem brains obtained from human HD patients and patients deceased for non-CNS-related causes (Ctrl, see Table 1). (B) Densitometric quantification of SNO-Drp1 levels in postmortem brains of human HD patients and control patients. Intensity of SNO-Drp1 band normalized to total Drp1 level (* $p < 0.05$ by *t*-test). CNS, central nervous system; HD, Huntington's disease.

FIG. 4. S-nitrosylation promotes Drp1 binding to HTT. (A) HTT was detected in Drp1 co-immunoprecipitates (IP) prepared from BACHD mouse striatum (left). The co-immunoprecipitation assay was performed in triplicate. After co-immunoprecipitation, the Drp1-HTT complex was incubated with ascorbate to reduce SNO on Drp1 *via* denitrosylation. Lack of the HTT and Drp1 interaction in WT mouse striatum (right). (B) HEK293-nNOS cells transfected with either full-length wtHTT or mutHTT were exposed to the physiological NO donor SNOC and assayed for SNO-wtHTT or SNO-mutHTT by the biotin-switch assay. The NOS inhibitor NNA was included in the culture medium to block endogenous NO production. Arrows indicate either mutHTT (transfected) or wtHTT (transfected and endogenous). NNA, *N*-nitro-L-arginine; NOS, nitric oxide synthase; SNOC, S-nitrosocysteine.

Figure 5 that NO generated in response to mutHTT triggers SNO-Drp1 formation and subsequent mitochondrial fragmentation.

Next, we monitored the effect of SNO-Drp1 on synaptic density. Synapses have high-energy demands, met by functional mitochondria, in order to maintain their structure and

function in neuronal signaling (32, 33). Dendritic spines represent postsynaptic sites on dendrites, which we monitored here (Fig. 7A, B). Impaired mitochondrial dynamics is known to result in an undersupply of ATP, leading to loss of synapses and, thus, a decrease in dendritic spine density (2, 10). In HD, synaptic dysfunction precedes neuronal loss in the cerebral

FIG. 5. SNO-Drp1 mediates mutHTT-induced mitochondrial fragmentation. Representative 3D-deconvolution fluorescent images were obtained 1 day after transfection. Cortical neurons were co-transfected at DIV14 with the mitochondrial marker mito-DsRed2 plus wtHTT and wtDrp1 (A, B), mutHTT and wtDrp1 (C, D, G, H), or mutHTT and S-nitrosylation-resistant mutant Drp1(C644A) (E, F). For some experiments, the NOS inhibitor NNA (1 mM) was included in the culture medium to block NO production (G, H). (B, D, F, H) Magnified insets corresponding to boxed areas in (A, C, E), and (G), respectively. (I) Quantification of mitochondrial fragmentation. Neurons with fragmented mitochondria were scored in a masked fashion (See "Materials and Methods" for details). Data presented as mean $\pm$ SEM (** p < 0.01, n = 30–36). SEM, standard error of the means. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

A

B

FIG. 6. S-nitrosylation of Drp1 decreases relative mitochondrial SA in neurons. Cortical neurons (DIV14) were co-transfected with N-terminal fragments of HTT (wt or mut) and Drp1 (wt or C644A). The ratio of mitochondrial SA (labeled with mito-DsRed2) to neuronal SA (represented by GFP) was calculated for each condition, as described in the “Materials and Methods” section. **(A)** Fluorescence microscopy images of mito-DsRed2, GFP, and overlay of both fluorescence channels. **(B)** Quantification of mitochondrial fragmentation by SA analysis 1 day after neuronal transfection. Data presented as mean $\pm$ SEM. (** $p < 0.005$, $n = 30-36$). GFP, green fluorescent protein; SA, surface area. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

cortex and striatum, and is associated with cognitive and psychiatric manifestations (43). Since SNO-Drp1 formation resulted in aberrant mitochondrial dynamics in mutHTT-transfected cells, we hypothesized that increased mitochondrial fragmentation would lead to dendritic spine loss, possibly mediated by energy depletion at synapses (2, 10). We observed

that cortical neurons co-transfected with the N-terminal fragment of mutHTT(Q148) and wtDrp1 displayed significantly decreased spine density compared with transfection with wtHTT(Q18) and wtDrp1 (Fig. 7). Consistent with our finding that transfection with the non-nitrosylatable mutant Drp1(C644A) or incubation in NNA abrogated mitochondrial

A

B

FIG. 7. mutHTT significantly decreases dendritic spine density via formation of SNO-Drp1. Cortical neurons were transfected with N-terminal fragments of wtHTT or mutHTT plus either wtDrp1 or mutant Drp1(C644A). Co-transfection with GFP permitted visualization of morphological changes in dendritic spines. **(A)** Representative fluorescence microscopy images of dendritic spines were obtained from neurons 2 days post transfection of wtHTT plus wtDrp1, mutHTT plus wtDrp1, or mutHTT plus Drp1 C644A. **(B)** Dendritic spine density in neurons expressing the indicated plasmid constructs. The NOS inhibitor NNA (1 mM) was added to the culture medium to inhibit NO production (** $p < 0.005$, $n = 4$). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

fragmentation, we observed that transfection with mutHTT plus Drp1(C644A) significantly rescued spine density. A similar effect on synaptic spines was observed with NNA (Fig. 7). These data suggest that mutHTT-induced S-nitrosylation of Drp1 contributes to synaptic loss in HD.

Discussion

Excessive generation of NO is thought to contribute to the etiology of a number of neurodegenerative disorders, including AD and HD, *via* modulation of multiple intracellular signaling pathways. Consistent with this notion, increased production of NO has been observed in human HD brain and in HD transgenic mouse brain, and is accompanied by mitochondrial dysfunction and synaptic loss (17, 19, 52). In addition, several reports have attributed the increased neuronal NO production, at least in part, to up-regulation of neuronal NOS (nNOS) expression (18, 46). Interestingly, the length of polyQ repeats in mutHTT has been shown to correlate with the increased production of NO (22), suggesting a relationship between the level of NO and severity of disease. In the current study, we mount evidence that aberrant S-nitrosylation of Drp1 is of pathological relevance to HD. In agreement with previous reports, we observed elevated production of NO in mutHTT-expressing neurons. We then discovered that SNO-Drp1 levels are elevated in an animal model of HD as well as in HD patient brains compared with controls. It is currently thought that excessive mitochondrial fragmentation and consequent synaptic injury play a critical role in the pathogenesis of HD, and our finding that SNO-Drp1 mediates excessive mitochondrial fission and loss of dendritic spines may, therefore, contribute to this damage (Fig. 8). Interestingly, as a compensatory mechanism against oxidative and nitrosative

stress, there is a significant induction of antioxidant defense proteins in both human HD brains and in HD transgenic mouse models (8, 13, 51). However, our finding of aberrant formation of SNO-Drp1 in HD brains suggests that this nitrosylation reaction can overcome the antioxidant defensive system.

Moreover, we provide several lines of evidence, suggesting that SNO-Drp1 initiates mitochondrial fragmentation and synaptic damage at an early stage of HD pathogenesis. Our findings initially revealed that expression of N-terminal mutHTT significantly increases neuronal NO production within hours of transfection in cell-based models. Second, using transgenic BACHD mice expressing full-length mutHTT at a pre-symptomatic stage (≤ 28 weeks old) (11), we found significantly increased formation of SNO-Drp1 in the striatum compared with controls. In contrast, S-nitrosylation of Drp1 was not increased in the cerebellum of these transgenic mice. At this stage of the disease, the primary site of pathological manifestations in BACHD mice is the striatum; the cortex and cerebellum are affected only at later stages (34). This finding, therefore, suggests that SNO-Drp1 increases in the brain region where mutHTT displays early neurotoxicity. In addition, we found increased levels of SNO-Drp1 at later stages of the disease in other brain regions, as they were affected, for example, in the cortex of human postmortem HD brains.

In an analogous fashion to our current findings in HD, we had previously shown that amyloid- β peptide ($A\beta$), considered a key mediator of AD pathogenesis, induces excessive generation of NO, S-nitrosylation of Drp1, and consequent fragmentation of mitochondria. Furthermore, excessive mitochondrial fission in AD or HD brains can lead to increased generation of superoxide, hydrogen peroxide,

FIG. 8. Proposed mechanism by which SNO-Drp1 mediates mutHTT-induced mitochondrial fragmentation and synaptic injury in HD. Under physiological conditions, Drp1 basal activity contributes to the equilibrium of fission and fusion events in mitochondrial dynamics. The normal mitochondrial fission and fusion cycle is important for maintaining synaptic plasticity *via* effective distribution of functional, bioenergetically competent mitochondria to synaptic sites. In HD, expansion of the polyQ repeat in mutHTT results in increased NO production. High levels of NO species cause aberrant S-nitrosylation of Drp1 by reacting at Cys644, with resultant increased GTPase activity and excessive mitochondrial fragmentation. In addition, S-nitrosylation increases the binding affinity of Drp1 and HTT, and Drp1 may possibly be transnitrosylated from SNO-mutHTT. Consequently, excessively fragmented mitochondria contribute to the synapse loss and neuronal damage in HD.

and peroxynitrite, thus amplifying mitochondrial dysfunction, oxidative stress, and bioenergetic catastrophe (*i.e.*, decreased ATP production) (2, 49). The mitochondrial compromise thus engendered can trigger dendritic spine loss that ultimately leads to neuronal damage, as observed in models of AD (10). Importantly, we and others have discovered that the level of S-nitrosylated Drp1 is elevated in the brains of virtually all cases of HD and sporadic AD but not in control brains, suggesting that the formation of SNO-Drp1 represents an aberrant signaling pathway which only appears under neurodegenerative conditions (10, 56 and [Fig. 3]). Moreover, a recent study reported that SNO-Drp1 is also increased in peripheral blood lymphocytes of AD patients (55), suggesting that S-nitrosylation of Drp1 can serve as a biomarker for AD. In contrast, a recent publication from another group reported that S-nitrosylation of Drp1 had little effect on GTPase activity or mitochondrial fragmentation in their *in vitro* experiments (4). We should point out that this contradiction is most likely due to their use of artifactually pre-oxidized Drp1 (as evidenced by formation of disulfide-linked dimers on their immunoblots). Such oxidation of the free thiol group at amino-acid residue 644 on Drp1 renders it difficult or impossible to observe the effects of S-nitrosylation at this site (40). Here and in our previous publications on this subject, virtually all Drp1 used for *in vitro* experiments was present in its native reduced form (*i.e.*, monomer) and thus susceptible to S-nitrosylation. Moreover, consistent with our observations, other laboratories have independently found that S-nitrosylation increases GTPase activity of both dynamin 1 and 2, close homologues of Drp1 (31, 40, 53).

With regard to Drp1 in HD, a number of previous studies have reported a role for Drp1 in excessive mitochondrial fragmentation that may contribute to the pathogenesis of striatal and cortical neuronal loss (6, 49, 50, 54). In particular, recent publications proposed a mechanism for Drp1 activation by which mutHTT directly binds to Drp1, activating its GTPase activity and triggering excessive mitochondrial fission (49, 50). Here, similar to the previous effect we had observed for A β peptide-increasing SNO-Drp1, we found that mutHTT increased S-nitrosylation of Drp1, with consequent increases in GTPase activity and mitochondrial fragmentation. Although previous publications reported the interaction of Drp1 only with mutHTT (50), we found that S-nitrosylation also facilitates interaction of Drp1 with wtHTT in the brains of mice hemizygous for the BACHD transgene. The underlying mechanism for these effects of S-nitrosylation may involve conformational alteration of Drp1. In fact, recent structural studies have pointed to the fact that the GED domain of Dynamin and Drp1 (containing Cys644, which we have shown to be the site of nitrosylation) is important for structure and function (10, 21, 23). The discovery of S-nitrosylation—mediated Drp1/HTT interaction—provides an additional clue to the mechanism of mitochondrial fragmentation in HD, although further studies will be necessary to gain additional insights into this neurodegenerative process.

In the current study, both morphological analysis and surface area analysis confirmed that mutHTT significantly increased mitochondrial fragmentation and subsequent synaptic damage. Importantly, this synaptic phenotype was rescued by non-nitrosylatable mutant Drp1(C644A). The protective effects of nitrosylation-resistant mutant Drp1 as well as of an NOS inhibitor are consistent with the notion that

mutHTT-induced S-nitrosylation plays a crucial role in HD-associated mitochondrial fragmentation and neuronal injury. Considering the mechanism of these effects, we found that in addition to Drp1, mutHTT itself could be S-nitrosylated, increasing the possibility that SNO-mutHTT may transnitrosylate or transfer NO to Drp1 to form SNO-Drp1. In other neurodegenerative conditions, transnitrosylation has been postulated to play a role in several protein complexes, including transfer of NO from Cdk5 to Drp1 (41, 42, 47). Notwithstanding mechanism, our findings increase the possibility that prevention or reversal of the formation of SNO-Drp1 may represent a novel therapeutic target to ameliorate synaptic and neuronal damage in HD.

Materials and Methods

Plasmids and reagents

Cultured cells were transfected with N-terminal fragments of HTT consisting of exon 1 (Myc-wtHTT-N63-18Q or Myc-mtHTT-N63-148Q) as described elsewhere (44). Constructs of Drp1 and the nitrosylation-resistant mutant Drp1 C644A were previously described (10). Mito-DsRed2 and ptdTomato were purchased from Clontech, and pmax-GFP was purchased from Amara. The mouse monoclonal anti-HTT (MAB2166 or MAB5492) was purchased from Millipore, mouse monoclonal anti-Drp1 (8/DLP1) was purchased from BD Bioscience, and rabbit polyclonal anti-Drp1 (H-300) was purchased from Santa Cruz Biotechnology.

Cell culture and transfection

Cortical cultures were prepared from embryonic day-17 rat pups as previously described (3). Briefly, after dissociation, cerebrocortical cells were plated on poly-L-lysine-coated cover slips and incubated in D10C media. Cells were transfected *via* LipofectAMINE 2000 (Invitrogen) on the 14th day *in vitro* (DIV14). For transfection of cortical neurons, the N-terminal fragment of HTT was employed. In contrast, the human embryonic kidney cell line stably transduced with nNOS (HEK293-nNOS) displayed greater transfection efficiency, enabling us to perform transfections with full-length HTT. All experiments involving primary cultures and animals were performed with the approval of the Animal Care and Use Committee at the Sanford-Burnham Medical Research Institute and the University of California, San Diego (UCSD).

NO detection by DAF-FM staining

DAF-FM diacetate (Invitrogen) is an NO indicator for quantifying micromolar concentrations of NO. Cerebrocortical neuronal cells (DIV14) were co-transfected with ptdTomato (to detect positively transfected neurons) and either N-terminal fragments of wtHTT (Q18) or mutHTT (Q148). After 7 h, coverslips were washed with Earle's Balanced Salt Solution containing 1.8 mM Ca²⁺ (EBSS/Ca²⁺) without serum and incubated immediately with 2.5 μ M of DAF-FM diacetate for 45 min. After incubation, DAF-FM solution was removed, and cells were maintained in EBSS/Ca²⁺/1 mM L-arginine/5 μ M glycine buffer to enable NO production. DAF-FM intensity was monitored at 515 nm emission by taking single images of randomly chosen cells. Images of ptdTomato-expressing cells ($n > 15$) were acquired by deconvolution microscopy using a 40 $\times$ air objective.

DAF-FM intensities in the cell body were determined and normalized to non-transfected cells, as previously described (11).

Brain lysates

The striatum and cerebellum of BACHD transgenic mice expressing human mutant full-length HTT (Q97) under the control of the endogenous human HTT regulatory machinery have previously been described (26). Mouse brains from early to pre-symptomatic stage (28 weeks old) were used for the preparation of brain lysates. Brain cortices from human postmortem HD patients and brains of patients who were deceased for reasons other than CNS-related disorders were provided by Dr. Eliezer Masliah (UCSD). Brain tissue was lysed in 500 μ l of buffer containing 100 mM HEPES, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.1 mM Neocuproine, 1% NP-40, 1% deoxycholate, and 0.1% sodium dodecyl sulfate [SDS], as previously described (20) with minor modifications. In brief, samples were lysed with a glass homogenizer on ice in the dark in order to maintain protein S-nitrosylation for analysis with the biotin-switch assay.

Biotin-switch assay

Analysis of SNO-Drp1 and SNO-mutHTT by the biotin-switch assay was performed as described (30). Briefly, free thiols were blocked with S-methylmethanethiosulfonate, and cell extracts were precipitated with acetone and resuspended in HEN buffer (100 mM HEPES, 1 mM EDTA, and 0.1 mM Neocuproine) with 1% SDS. SNO were selectively reduced by ascorbate to reform the thiol group and subsequently biotinylated with 1 mM N-[6-(Biotinamido)hexyl]-3'-(2'-pyridylthio)propionamide (Soltec Ventures). The biotinylated proteins were pulled down using a high-capacity neutravidin agarose resin (Thermo Scientific) and analyzed by immunoblotting. Relative levels of S-nitrosylated proteins were determined by a comparison of S-nitrosylated proteins to the total amount of protein (input).

Immunoblotting

Samples were extracted in sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. After blocking with skim milk in TBS-T, membranes were incubated with specific primary antibodies and IRDye 680LT secondary antibodies (Li-Cor). A Li-Cor Odyssey infrared imager was used for detection, equipped with the Odyssey software V3.0.21 for densitometric quantification of the blots.

Human brain subjects

Table 1 lists all subjects used in the current study and also includes subject age, postmortem interval, and gender. Human brain samples were analyzed with Institutional permission under California and NIH guidelines. Informed consent was obtained according to procedures approved by the UCSD and Sanford-Burnham Medical Research Review Institutional Review Boards.

Protein complex immunoprecipitation

We analyzed the interaction of Drp1 and full-length HTT using co-immunoprecipitation with Dynabeads Protein A

(Invitrogen) and an antibody to Drp1, followed by western blotting and probing with an antibody to HTT. We lysed BACHD striatal samples in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 1 mM EDTA, and 0.1 mM Neocuproine (RIPA-EN). We then co-immunoprecipitated the brain lysates using an antibody to Drp1 for 1 h at 4°C followed by three washes with RIPA-EN buffer. Ascorbate treatment to facilitate denitrosylation consisted of incubating the co-immunoprecipitated Drp1-HTT complex with RIPA-EN containing 20 mM ascorbate for 1 h.

Deconvolution fluorescence microscopy

Microscopic image acquisition was performed using a Zeiss AX10 Observer.Z1 microscope equipped with Slide-Book5.0 software for deconvolution of fluorescent images (Intelligent Imaging Innovations, Inc.). Acquired images were deconvolved using the constraint iterative algorithm included in the software package. Objectives used for microscopy included 20 $\times$ /0.8 air, 40 $\times$ /0.75 air, 63 $\times$ /1.4 oil, and 100 $\times$ /1.4 oil.

Mitochondrial fragmentation assay

Rat cortical neurons were cultured for DIV14 before transfection with mito-DsRed2 for mitochondrial visualization and pmax-GFP for identification of positively transfected cells. Neurons were additionally co-transfected with either N-terminal fragments (exon 1) of wtHTT (Q18) or mutHTT (Q148) as well as wtDrp1 or Drp1(C644A). In order to inhibit NO production, neurons were treated with the NOS inhibitor NNA (1 mM) immediately after transfection. Cells were fixed 1 day after transfection in 4% paraformaldehyde for deconvolution fluorescence microscopy analysis. Mitochondrial fragmentation was monitored by morphological changes and by surface area analysis (mitochondrial index). Morphological analysis was performed on randomly selected GFP-expressing neurons in at least three experiments for each condition using a total of 30–36 neurons. Mitochondrial morphology was detected by deconvolution microscopy with 3D reconstruction as previously described (10). Volocity software (Improvision, Inc.) was employed for the 3D rendering of acquired z-stacks. Mitochondrial fragmentation was analyzed by an observer blinded to the condition, and a neuron was scored as containing fragmented mitochondria if >50% of the mitochondria displayed small, rounded morphologies (defined as the length of each mitochondrial segment being <5 μ m). For this analysis, the number of neurons with fragmented mitochondria was counted and expressed as a percentage of all neurons analyzed.

Mitochondrial surface area analysis

Relative mitochondrial surface area was obtained by determining the ratio of mitochondrial surface area to neuronal surface area, as previously described with minor modifications (58). In brief, mitochondrial index (surface area) analysis was performed on the same neurons as morphological analysis (total n =30–36). Fluorescent images were used to make 2D projections. Mitochondrial surface area was measured by tracing the fluorescence of mito-DsRed2 along the dendrites, and the surface area of the dendrite (indicated by pmax-GFP

expression) was determined. To obtain the relative mitochondrial surface area, we divided the summed mitochondrial surface area within the dendrites by the corresponding dendritic area.

Dendritic spine density assay

Neurons were co-transfected after DIV14 with pmax-GFP, N-terminal fragments of either wtHTT or mutHTT, and Drp1 or Drp1 C644A. Cells were fixed 2 days after transfection in 4% paraformaldehyde. Images of GFP-expressing cells were acquired by deconvolution microscopy using 63× or 100× oil objectives. Three distinct fields adjacent to the first dendritic branch were randomly selected in GFP-expressing neurons ($n \geq 4$ for each condition), and a stack of images was acquired in the z dimension. Dendrites were analyzed in a masked fashion to determine the number of spines per μm , as previously described (10).

Statistical analysis

Data are expressed as mean $\pm$ standard error of the means. Pairwise comparisons between control and treatment group were performed by a Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

Acknowledgments

The authors thank Traci Newmeyer for preparation of primary cultures, Joseph Russo and Jeffery Zaremba for analysis of mitochondrial fragmentation, and Eliezer Masliah for kindly providing human brain tissues. This work was supported in part by grants from Deutscher Akademischer Austausch Dienst (to F.H.), the Alzheimer Association (to T.N.), the Michael J. Fox Foundation (to T.N. and S.A.L.), and the NIH (P01ES016738, P01 HD29587, R01 EY05477, and P30 NS076411 to S.A.L., and R01 AG033082 and R01 NS065874 to A.R.L.).

Author Disclosure Statement

No competing financial interests exist.

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Date of first submission to ARS Central, August 30, 2012; date of final revised submission, April 26, 2013; date of acceptance, May 5, 2013.

Abbreviations Used

A β = amyloid- β peptide
 AD = Alzheimer's disease
 CNS = central nervous system
 DAF-FM = 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate
 DIV14 = 14 days *in vitro*
 Drp1 = dynamin-related protein 1
 EBSS = Earle's Balanced Salt Solution
 EDTA = ethylenediaminetetraacetic acid
 GFP = green fluorescent protein
 HD = Huntington's disease
 HTT = Huntingtin
 NNA = N-nitro-L-arginine
 NO = nitric oxide
 NOS = nitric oxide synthase
 PMI = postmortem interval
 RIPA = radioimmunoprecipitation assay
 R-SNO = S-nitrosothiols
 SA = surface area
 SDS = sodium dodecyl sulfate
 SEM = standard error of the means
 SNOC = S-nitrosocysteine