

Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease

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Huntington disease is an autosomal dominant neurodegenerative disease with no effective treatment. Minocycline is a tetracycline derivative with proven safety. After ischemia, minocycline inhibits caspase-1 and inducible nitric oxide synthetase upregulation, and reduces infarction. As caspase-1 and nitric oxide seem to play a role in Huntington disease, we evaluated the therapeutic efficacy of minocycline in the R6/2 mouse model of Huntington disease. We report that minocycline delays disease progression, inhibits caspase-1 and caspase-3 mRNA upregulation, and decreases inducible nitric oxide synthetase activity. In addition, effective pharmacotherapy in R6/2 mice requires caspase-1 and caspase-3 inhibition. This is the first demonstration of caspase-1 and caspase-3 transcriptional regulation in a Huntington disease model.

Despite significant advances in understanding the mechanistic pathways mediating progression of Huntington disease (HD) and cloning the mutant gene, effective pharmacotherapy remains elusive¹. HD is caused by a mutation consisting of an expanded CAG repeat in the 5' coding region of the huntingtin gene¹. Evidence points to the caspases as important mediators of apoptosis^{2,3}. We have demonstrated that caspase-1 is activated in brains of humans and mice with HD⁴. In addition, blocking caspase function either by expression of a caspase-1 dominant negative mutant, or by intracerebroventricular administration of a broad caspase inhibitor, delays disease onset and mortality in a transgenic HD mouse model⁴. Huntingtin is itself a substrate of caspase-1 and caspase-3^{5,6}. As evidence indicates a toxic gain of function of huntingtin cleavage fragments, these data provide a direct link between caspase-1 and caspase-3 and the mechanism of disease progression. However, unlike for caspase-1, direct *in vivo* evidence for a role of caspase-3 in HD has not been demonstrated.

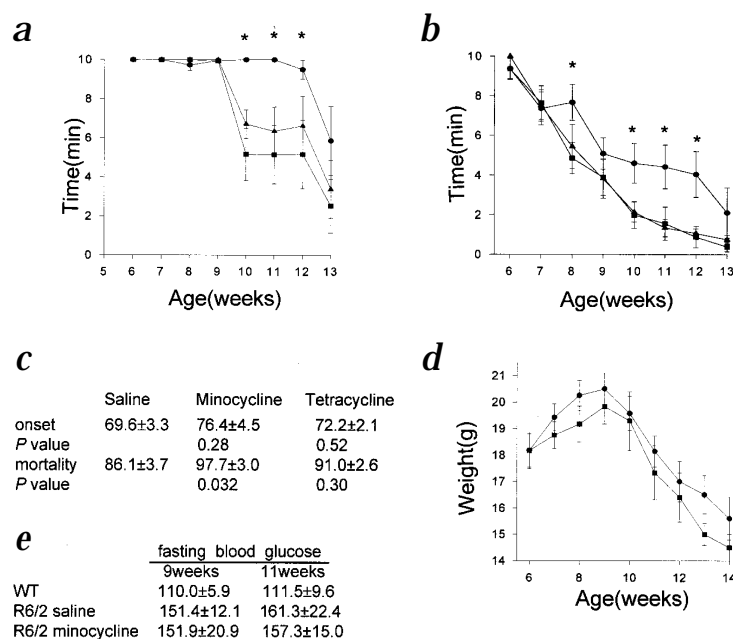
Minocycline is a second-generation tetracycline used in humans, which effectively crosses the blood-brain barrier⁷. After experimental ischemia, minocycline inhibits caspase-1 and inducible nitric oxide synthetase (iNOS) upregulation, and decreases infarct size^{8,9}. As a detrimental role for caspase-1 and iNOS

has been proposed in HD, we evaluated minocycline using the R6/2 HD mouse^{4,10,11}. We report that in R6/2 mice, minocycline delays disease progression and death, as well as inhibits caspase-1 and iNOS activation. In addition, we demonstrate that caspase-1 and caspase-3 gene expression are not only upregulated in this mouse model, but also are remarkably inhibited by minocycline. To provide further relevance to these findings we demonstrate elevated iNOS immunoreactivity in human HD striatum.

Minocycline slows progression and delays mortality in R6/2 mice To evaluate a potential role for minocycline in HD, we used the R6/2 transgenic model¹². R6/2 mice express exon-1 of huntingtin with an expanded polyglutamine repeat under the control of its native promoter. R6/2 mice develop a progressive neurological phenotype with features of HD, neuronal intranuclear inclusions (NII), decreased neurotransmitter receptor binding and early mortality^{13,14}. At 6 weeks of age, we began daily minocycline administration. At this age, R6/2 mice are in the late presymptomatic stage, and early histopathology can be demonstrated¹²⁻¹⁴. Minocycline significantly delayed the characteristic decline of Rotarod performance (Fig. 1a and b), and extended survival by 14% when compared to saline-treated mice (Fig. 1c). As a control, we evaluated whether tetracycline, which does not effectively

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Fig. 1 Progression of disease in R6/2 mice is inhibited by minocycline. Mice were evaluated on the Rotarod at **a**, 5 and **b**, 15 rpm. Testing was terminated either when the mouse fell from the rod or at 10 min if it remained on the rod (minocycline = ● [$n = 12$], saline = ■ [$n = 12$] and tetracycline = ▲ [$n = 6$]). **c**, Onset and mortality in days are recorded as mean \pm s.e.m. (minocycline [$n = 7$], saline [$n = 7$] and tetracycline [$n = 6$]). **d**, Body weight (minocycline = ●, saline = ■). (e) Fasting blood glucose recorded as mean \pm s.e.m. (R6/2 minocycline [$n = 15$], R6/2 saline [$n = 15$] and wild-type [$n = 6$]).



cross the blood-brain barrier, affected disease progression⁷. Tetracycline-treated mice did not demonstrate neuroprotection or significantly increased survival (Fig. 1a, b, and c). The characteristic weight loss and fasting blood glucose elevation observed in R6/2 mice were not altered by minocycline (Fig. 1d and e). Lack of protection by tetracycline, and lack of alteration of the systemic phenotype (that is, weight and blood glucose), indicate that the protective effect of minocycline does not result from a systemic effect, but rather from direct action on the brain.

Caspase-1 is inhibited in minocycline-treated R6/2 mice

Caspase-1 is activated in brains of humans with HD and of R6/2 mice, and caspase-1 inhibition correlates with increased survival in mice⁴. As caspase-1 is required in mice for processing of pro-interleukin-1 β (pro-IL-1 β), measurements of mature IL-1 β provide direct evidence of caspase-1 activation^{4,15-18}. Mature IL-1 β levels in brains of minocycline-treated mice were 43.1% lower than in saline-treated mice, demonstrating that minocycline inhibited caspase-1 activation (Fig. 2a). As huntingtin is cleaved *in vitro* by caspase-1 and caspase-3, and caspase inhibition blocks cleavage of endogenous huntingtin in R6/2 mice, we evaluated whether minocycline would inhibit endogenous huntingtin cleavage^{4,6}. We reported that endogenous huntingtin is cleaved in brain samples of 9-week-old R6/2 mice and not in samples from wild-type littermates⁴. Generation of the endogenous huntingtin cleavage fragment is significantly inhibited in nine-week old minocycline-treated R6/2 mice (Fig. 2b). As huntingtin is important for normal development, depleting endogenous huntingtin likely has a detrimental role on cellular homeostasis¹⁹.

Minocycline does not affect NII formation or receptor-binding

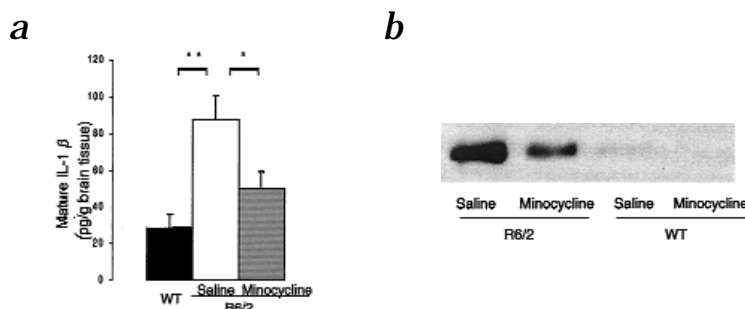
Similar to humans with HD, R6/2 mice develop NII and a progressive decrease in neurotransmitter receptor binding^{4,13,14,20}. Minocycline treatment did not inhibit the formation of NII or the decrease of adenosine A2a, forskolin, dopamine D1 and dopamine D2 neurotransmitter receptor binding in nine-week old R6/2 (data not shown). These results indicate that minocycline-mediated

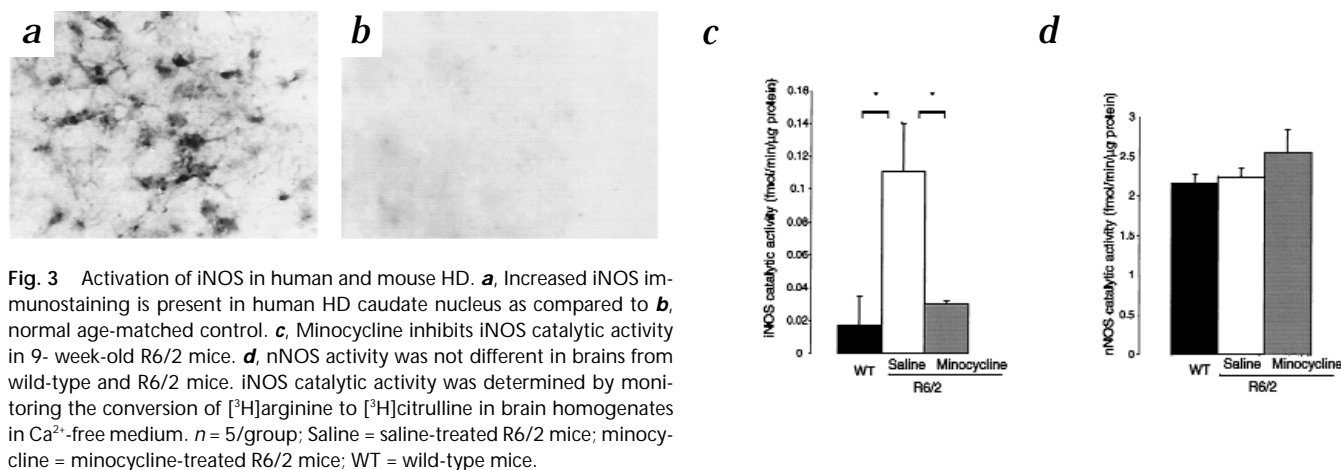
neuroprotection is not related to the effect that NII or decrease in neurotransmitter receptor binding have on the disease. We demonstrated a delay of NII formation and neuroreceptor down-regulation in R6/2 mice mediated by expression of the caspase-1 dominant negative transgene⁴. NII are detected in R6/2 mice as early as three weeks of age (R.J.F., unpublished data). NII detection is delayed by the caspase-1 dominant negative transgene, and, as expression of this transgene is detected during embryogenesis, it indicates that an early caspase-dependent pathway is required for the initial formation of the NII²¹. This caspase-dependent step may be cleavage of full-length huntingtin and, thereafter, aggregation of the mutant huntingtin fragment for generation of NII. Once NII begins to form, then their growth in R6/2 mice is caspase-independent, as the transgene fragment does not require, as well as lacks sites for, caspase cleavage. Minocycline probably did not inhibit NII formation, as it was administered after the caspase-dependent step of NII generation at 6 weeks of age.

Minocycline inhibits iNOS activation in R6/2 mice

Elevated iNOS expression has been demonstrated in R6/2 mice resulting in pathologic protein nitration¹⁰. As in brains of R6/2 mice, human HD brains also demonstrate elevated iNOS expression when compared with normal age-matched controls (Fig. 3a and b). Marked increased iNOS immunoreactivity is present in glial, neuronal and vascular elements of HD striatum. Elevation

Fig. 2 Minocycline inhibits caspase-1 activation as well as endogenous huntingtin cleavage in R6/2 mice. **a**, Mature IL-1 β levels in 12-week-old mice ($n = 7-9$ /group). Error bars indicate SEM * $P < 0.05$, ** $P < 0.01$. **b**, Cleavage of endogenous huntingtin in 9-week-old R6/2 mice is inhibited by minocycline. A large N-terminal cleavage fragment (approx. 210 kDa) was observed in lysates from R6/2 mice using an antibody directed against amino acids 181-810 of mouse huntingtin (results are representative of 5 mice/group).

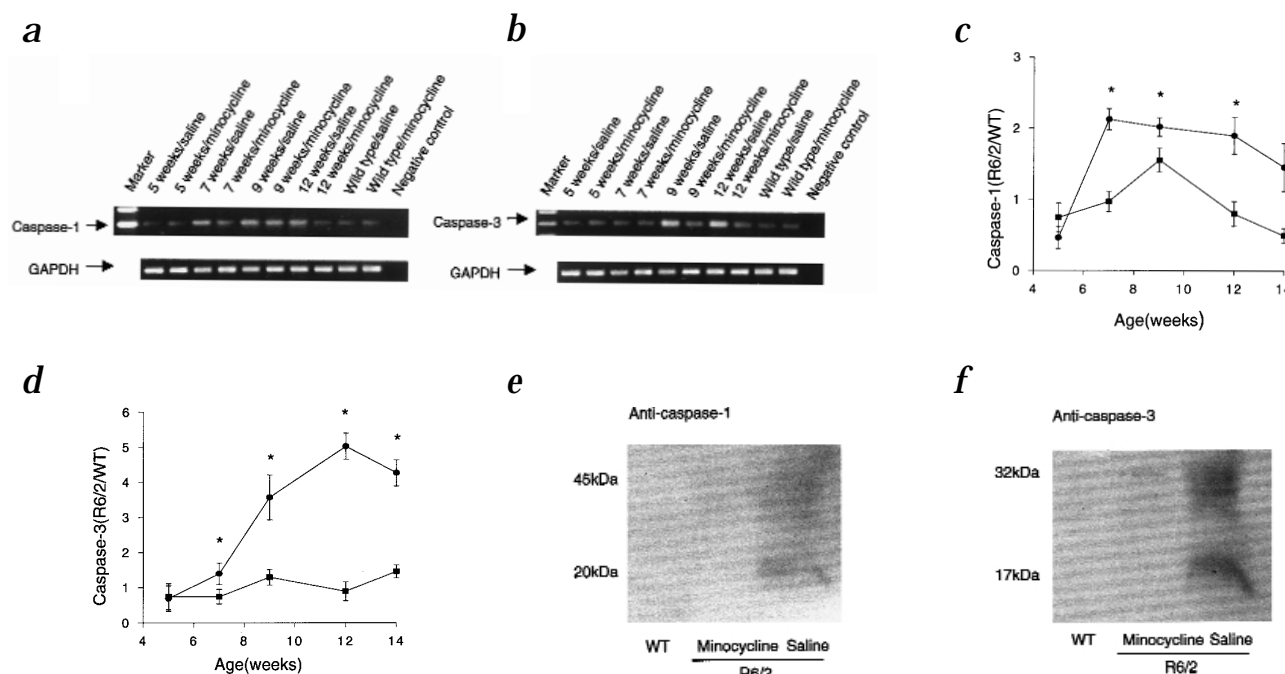




of brain iNOS immunoreactivity in humans with HD and in R6/2 mice provides further validation for this transgenic model as relevant to the human disease. As minocycline inhibits cerebral ischemia-mediated iNOS upregulation, its effect on catalytic iNOS activity in brains of R6/2 mice was evaluated. Ca²⁺-independent NOS activity, representing iNOS activity, was elevated 6.5-fold in R6/2 mice when compared with wild-type mice. Minocycline treatment resulted in a 72% inhibition of iNOS activity in brains of R6/2 mice when compared to saline-treated controls (Fig. 3c). Constitutive Ca²⁺-dependent NOS activity, representing nNOS activity, was not different in brains from wild-type or saline- and minocycline-treated mice R6/2 mice (Fig. 3d). These results indi-

cate that minocycline-mediated neuroprotection results in part from inhibition of iNOS activity, likely resulting in decreased free-radical damage.

Minocycline inhibits caspase-1 and -3 upregulation in R6/2 mice
We then evaluated whether transcription of caspase-1 and caspase-3 were regulated in R6/2 mice. Using RT-PCR we detected a peak of caspase-1 mRNA elevation at 7 weeks of age, which was 2.1-fold higher than wild-type levels (Fig. 4a). Thereafter, caspase-1 mRNA levels remained stably elevated throughout the disease. Caspase-3 mRNA elevation began 2 weeks after caspase-1 at 9 weeks, being 3.6-fold of wild-type levels, and peaking at 5.5-fold at



0.05, *n* = 5/group. Caspase-1 (**e**) and caspase-3 (**f**) western blot analysis of 12-week-old wild-type and R6/2 mouse brain lysates. R6/2 mice show a minocycline-sensitive increase in activated caspase-1 (p20) and activated caspase-3 (p17) subunits. There is faint pro-caspase-1 (p45) band in saline-treated brain lysates of R6/2 mice. As with RT-PCR results, there is increased amount of pro-caspase-3 (p32) in the R6/2 samples. Each lane 50 μg protein.

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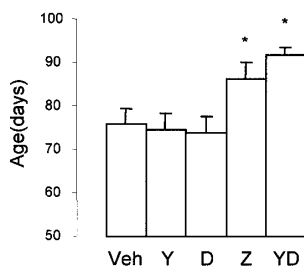


Fig. 5 Caspase-1 and caspase-3 inhibition are required to delay mortality of R6/2 mice. Beginning at 7 weeks of age, caspase inhibitors were intracerebroventricularly administered for 4 weeks in R6/2 mice. Veh = vehicle ($n = 13$); Y = YVAD-cmk ($n = 6$); D = DEVD-fmk ($n = 8$); Z = zVAD ($n = 15$); YD = combined YVAD-cmk and DEVD-fmk ($n = 5$). * $P < 0.05$.

12 weeks of age (Fig. 4b). Unlike caspase-1, caspase-3 mRNA elevation was progressive throughout the disease. These results are consistent with *in vitro* evidence demonstrating early caspase-1 and delayed caspase-3 activation during apoptosis²². Similarly, in a mouse model of amyotrophic lateral sclerosis, caspase-1 mRNA is elevated before caspase-3 mRNA²³. Caspase-1 and caspase-3 mRNA elevation in R6/2 mice were remarkably inhibited in minocycline-treated mice (Fig. 4c and d). Despite almost complete inhibition of elevation of caspase-3 mRNA, the disease continues to progress likely due to the less thorough caspase-1 inhibition, as well as due to additional cell death mediators. By western blot we confirmed a minocycline-sensitive elevation of caspase-1 and caspase-3 protein, in particular, elevation of activated subunits in brain lysates of 12-week old R6/2 mice (Fig. 4e and f). Fluorogenic caspase-1 and caspase-3 assays were done using HeLa cell-free extracts to rule out a direct enzymatic effect of minocycline. Caspase-1 and caspase-3 activities were not directly inhibited by minocycline (data not shown). As a control, it was evaluated whether minocycline inhibited expression of the R6/2 transgene. RT-PCR demonstrated that minocycline did not alter R6/2 transgene expression (data not shown).

Inhibition of both caspase-1 and -3 required for neuroprotection
So that the specific functional contribution of caspase-1 and caspase-3 to disease progression could be evaluated, R6/2 mice were treated with intracerebroventricular administration of either zVAD-fmk (Val-Ala-Asp-fluoromethyl ketone) (a broad caspase inhibitor), Tyr-Val-Ala-Asp-chloromethylketone (YVAD-cmk [a caspase-1-like inhibitor]), Asp-Glu-Val-Asp-aldehyde-fmk (DEVD-fmk, a caspase-3-like inhibitor), or with a combination of YVAD-cmk and DEVD-fmk. As we reported, zVAD-fmk extended survival of R6/2 mice⁴. Neither YVAD-cmk nor DEVD-fmk demonstrated neuroprotection. However, the combination of YVAD-cmk and DEVD-fmk resulted in improved Rotarod performance and significantly extended survival comparable to zVAD-fmk treated mice (17.3% and 12.2% respectively) when compared to vehicle-treatment (Fig. 5). To rule out that neuroprotection mediated by the combination of YVAD-cmk and DEVD-fmk is not a result of simply doubling the dose of caspase inhibitors, we independently evaluated higher doses of YVAD-cmk and DEVD-fmk. Higher concentrations of YVAD-cmk or DEVD-fmk were toxic causing earlier death than in vehicle-treated mice (data not shown). As caspase-1 and caspase-3 are upregulated, and their combined inhibition is required for therapeutic efficacy, effective therapy for HD (and likely other chronic neurodegenerative dis-

eases) may ultimately require inhibition of both caspase-1 and caspase-3. However, as YVAD-cmk and DEVD-fmk inhibit caspases in addition to caspase-1 and caspase-3, albeit with lower affinities, we can not rule out that the neuroprotection mediated by these drugs might result from inhibiting other caspases in addition to caspase-1 and caspase-3.

Discussion

It is demonstrated for the first time in R6/2 mice that caspase-1 and caspase-3 are transcriptionally regulated. As caspase expression in HD is regulated rather than constitutive, caspase transcriptional modulation provides a new target for therapeutic manipulation. The mechanism of mutant huntingtin-mediated toxicity is at present not clearly understood. A role for mitochondrial dysfunction and toxic nitric oxide metabolites have been proposed^{10,11,24,25}. Beginning in the late presymptomatic stage of the disease mutant huntingtin-mediated intracellular toxicity induces caspase-1 upregulation and activation, resulting in mature IL-1 β production. As disease progresses, caspase-3 is upregulated, further exacerbating toxicity. Interestingly, caspase-1 activates caspase-3 *in vitro*²⁶. The early upregulation of caspase-1 with respect to caspase-3 provides evidence that caspase-1 is not merely an inflammatory mediator, but rather an important early trigger of the apoptotic cascade. Minocycline inhibits caspase-1 and caspase-3 upregulation, although the later more thoroughly. As caspase-1 and caspase-3 are upregulated, an effective therapeutic intervention in HD will likely require inhibition of at least both of these caspases. Our data are consistent with a detrimental role of iNOS in HD. Minocycline has been used in humans for extended periods of time with relatively few side effects^{27,28}. With the described results, and with the relatively low toxicity, minocycline represents a new potential therapeutic agent for the treatment of HD.

Methods

Mice and treatment regimen. R6/2 mice (Jackson Laboratories, Bar Harbor, Maine) were randomly assigned to three groups. At 6 weeks of age, mice were treated with daily intraperitoneal injections of either saline, minocycline hydrochloride (5 mg/kg, Sigma, St. Louis, Missouri), or with tetracycline hydrochloride (5 mg/kg, Sigma) in 0.5 ml of saline. Experiments were in accordance with protocols approved by the Harvard Medical School Animal Care Committee.

Mature IL-1 β determination. Mature IL-1 β quantification was done as described using an enzyme-linked immunosorbent assay kit specific for the mature form of the cytokine (R&D Systems, Minneapolis, Minnesota)¹⁸.

Assay of NOS catalytic activity. NOS activity was measured by conversion of L-[³H] arginine to [³H]citrulline as described by Bredt and Snyder²⁹ and Yoshida *et al.*³⁰ with modifications. Brain samples were homogenized in 10 vol Tris buffer (25 mM; pH 7.4; 4 °C) containing 1 mM ethylenediaminetetraacetic acid and 1 mM ethyleneglycolcoltetraacetic acid and centrifuged (20,000g for 5 min at 4 °C). For total NOS or iNOS activity, 10 μ l aliquots of the supernatant were incubated with or without 0.6 mM Ca²⁺, respectively, in 25 mM Tris (pH 7.4), 3 μ M tetrahydrobiopterin, 1 μ M flavin mononucleotide, 1 μ M flavin adenine dinucleotide, 1 mM nicotinamide adenine dinucleotide phosphate, 0.1 μ M calmodulin and 22 μ M L-[2,3,4-³H] arginine (specific activity 45.2 Ci/mmol; NEN, Boston, Massachusetts) for 15 min at 37 °C. The assays were terminated by addition of 400 μ l N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid buffer (50 mM; pH 5.5; 4 °C) containing 5 mM ethylenediaminetetraacetic acid and 1 mM ethyleneglycoltetraacetic acid and centrifuged (20,000g, applied to 100 μ l Dowex AG50WX-8 resin (Na⁺ form; Bio-Rad, Hercules, California) in spin cups (Sigma, St. Louis, Missouri) and centrifuged (20,000g for 30 s at 4 °C). [³H]citrulline was quantitated by liquid scintillation counting of the eluate

(LS 6000; Beckman Instruments, Palo Alto, California). Protein concentration in the reaction mix was measured with a Bio-Rad assay.

Histochemical staining. Human postmortem striatal tissue specimens from 6 HD (grades 3 and 4; mean age, 64.8 yr; range, 54–73 yr and 6 neurologic age-matched controls (mean age, 67.5; range 58–76) were dissected fresh and placed in cold (4 °C) 2% paraformaldehyde-lysine-periodate solution for 24–36 h. Brain tissue specimens were received from the Bedford VA Medical Center Brain Tissue Archive (Bedford, Massachusetts). The post-mortem intervals did not exceed 18 h (mean time, 8.3 h; range, 4–18 h). Each HD patient had been clinically diagnosed based upon known family history and phenotypic symptoms of HD. The diagnosis of HD was confirmed by neuropathologic examination and graded by severity. Tissue blocks were rinsed in 0.1 M sodium phosphate buffer and placed in cold cryoprotectant in increasing concentrations of 10% and 20% glycerol/2% dimethyl sulfoxide solution over 36 h. Frozen serial sections of the striatal tissue block were cut at 50 µm intervals in the coronal plane. The cut sections were stored in 0.1 M sodium phosphate buffer/0.08% sodium azide at 4 °C and subsequently immunostained for iNOS (Upstate, dilution 1:500) using the conjugated second antibody method³¹.

Western blot. Huntingtin western blot accomplished as described using an antibody (MAB2166) raised against a mouse huntingtin fusion protein (amino acids 181–810)(Chemicon, Temecula, California)⁴. Antibodies for caspase western blots were purchased from Santa Cruz (Santa Cruz, California).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the brains of R6/2 and wild-type mice was prepared using TRIZOL Reagent (Gibco-BRL, Grand Island, New York) according to the manufacturer's instructions. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm, respectively. First-strand cDNA was synthesized from total RNA using the Superscript Preamplification system with Superscript II RNase H-reverse transcriptase (Gibco-BRL) according to the manufacturer's instructions. Then 1 µl of cDNA template was amplified by PCR in 20 µl of total reaction volume containing 18 µl of Supermix (Gibco-BRL) and 4–10 pmol of each specific primer. Caspase-1 primer sequences were 5'-TGGTCTTGACTTGAGGA-3'(forward) and 5'-TG-GCTTCTATTGGCAGCAT-3' (reverse). Caspase-3 primer sequences were 5'-TGTCATCTCGCTCTGGTACG-3' (forward) and 5'-AAATGACCCCTCAT-CACCA-3' (reverse). As an internal control, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was amplified using primer sequences 5'-AACTTGGCATTGTGGAAGG-3' (forward) and 5'-GGAGACAACCTGGTCTCAG-3' (reverse). GAPDH levels were not altered in R6/2 mice when compared to the wild-type controls. Each PCR cycle consisted of 45 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C. PCR amplification was carried out for 30 cycles for caspase-1, 35 cycles for caspase-3 and 28 cycles for GAPDH. Using these protocols DNA amplification was within its linear range. After amplification, the products were separated in a 1.5% agarose gel containing 0.03% ethidium bromide. The PCR results were analyzed by using a Bio-Rad Imaging Densitometer Quantity One-4.1.0 (Bio-Rad, Hercules, California)

Rotarod test. Motor performance was evaluated weekly from 5 to 13 weeks on a Rotarod (Columbus Instruments, Columbus, Ohio), at 5 and 15 rpm. If the mouse remained on the rod for 10 min the test was completed and scored as 10 min.

Placement of osmotic pumps. Pumps were inserted at 7 weeks of age as described by Ona *et al.*⁴. Concentrations were for zVAD-fmk 100 µg/20 g body weight/28 d, for YVAD-cmk and DEVD-fmk 50 µg/20 g body weight/28 d for the low dose, and 100 µg/20 g body weight/28 d for the high dose. Combined YVAD-cmk and DEVD-fmk were used at 50 µg/20 g body weight/28 d each.

Data analysis. Data are presented as mean ± standard error of the mean (SEM). Statistical comparisons were made by Student's *t*-test.

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