# A Rapid Neurobehavioral Assessment Reveals that FK506 Delays Symptom Onset in R6/2 Huntington's Disease Mice

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*Numerous drugs improve survival of R6/2 Huntington's disease mice by 10%–20%. The use of survival as the primary endpoint for these preclinical trials is problematic because one would not wish to prolong the symptomatic phase of Huntington's disease. Additional measures of weight maintenance, rotarod performance, and pathologic leg clasping capture only a fraction of the information about the well-being of mice. To improve the ability to identify drugs that delay onset of neurologic symptoms, we developed a rapid neurobehavioral phenotype assessment that scores for tremors, grooming, spontaneous activity, and locomotor activity in addition to rotarod performance and pathologic clasping. Prospective utilization of this assessment as the primary endpoint for preclinical efficacy studies revealed that the neuroprotective agent, FK506, delayed symptom onset in R6/2 mice without prolonging the symptomatic phase of disease.*

## INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expanded CAG repeat in the *HD* gene (1,2). *HD* encodes the huntingtin protein, and the CAG repeat encodes a polyglutamine stretch in the amino terminus of huntingtin. Normally the repeat length is up to 35 glutamines with larger repeats causing HD. The expanded polyglutamine stretch contributes to neuronal dysfunction and degeneration that are clinically manifested as chorea, cognitive decline, psychiatric and affective abnormalities, and death. Currently there are no effective treatments.

Yeast, *Caenorhabditis elegans, Drosophila,* and mammalian cell HD models are now being used for high- or moderate-throughput drug screening (3). Mouse models of HD serve as an important filter between these drug discovery efforts and human clinical trials. The most widely used mouse model for preclinical drug evaluation is the R6/2 model, which transgenically expresses exon 1 of the *Hdh* gene that contains approximately 150 CAG repeats (4). Previous preclinical studies have shown that Congo red, creatine, minocycline, zVAD-FMK (caspase inhibitor), combination YVAD-CMK and DEVD-FMK (caspase inhibitors), remacemide, cystamine, lipoic acid, dichloroacetate, coenzyme Q, and a number of unpublished compounds increase survival of these mice, typically by 7%–20% (5–12). Environmental enrichment, such as adding a toilet paper roll, also improves survival as well or better than these pharmacologic agents (13,14).

Using survival as a measure of drug efficacy is problematic because the cause of death in R6/2 mice is unknown and because it could lead to the identification of drugs that prolong end-stage disease as opposed to those that delay disease onset or improve quality of life in early disease. In addition, some countries with highly active HD research programs prohibit the use of survival as an end point, limiting data comparison between labs. Assessing motor activity by rotarod performance (duration for which mice are able to remain on an accelerating dowel) offers an opportunity to evaluate neurologic function in all stages of disease. This measure alone is insufficient since it requires a complex mixture of muscle strength, agility, grip, weight, balance, proprioception, cardiopulmonary aerobic capacity, peripheral nerve function, and multiple circuits in the central nervous system. As such, improvements in one of these could be masked by persistent impairments in any of the others. The cause of pathologic clasping, another traditional secondary end point, is unknown. This

| Neurobehavioral Assessment | Physical Phenotype Assessment |
|---|---|
| **Tremor**<br>1 = none/mild<br>2 = marked<br><br>**Grooming**<br>1 = slow/occasional<br>2 = none/excessive<br><br>**Spontaneous Activity**<br>1 = moderate: covers all quadrants<br>2 = slow: covers 1 to 3 quadrants<br>3 = none or darting/circling<br><br>**Locomotor Activity**<br>Count the number of times the mouse places at least one paw on the side of the cage during a 2-min period. The mouse must place its paws on the floor before another touch is scored.<br><br>**Clasping**<br>1 = none or forepaw tuck<br>2 = hind limb clasp<br><br>**Rotarod**<br>Record the number of seconds the mouse can stay on the accelerating (3 to 30 rpm) rotarod for 5 min. | **Palpebral Closure**<br>1 = wide open<br>2 = flattened, swollen lids, squinty<br><br>**Piloerection** (evaluate fur on back of mouse)<br>1 = none<br>2 = erected, scruffy<br><br>**Body Position** (evaluate while mouse is moving)<br>1 = elongated<br>2 = hunched, rounded<br><br>**Tail Position** (evaluate while mouse is moving)<br>1 = horizontally extended<br>2 = dragging/straub |

**Note:** clasping and rotarod are scored separately from the 2-min assessment.

**Figure 1. Neurobehavioral assessment and physical phenotype assessment score sheet.**

abnormal phenotype in the R6/2 mouse is shared by many genetically modified mice as are weight loss and early death by unknown mechanisms. Additional neurobehavioral end points are needed to identify drugs that improved cognitive, affective, or other quality of life impairments that debilitate HD patients.

Here we report the development of a rapid neurobehavioral assessment that scores for tremors, grooming, locomotor activity, and spontaneous activity in addition to rotarod performance and clasping. Prospective application of this assessment to R6/2 mice treated with FK506 demonstrated that FK506 reduced symptoms in the early phase of disease without prolonging the symptomatic phase.

## RESULTS

### A Rapid, Reliable Phenotype Assessment

Our initial goal was to develop a rapid phenotype assessment with a scoring system that minimized inter-investigator variability. We applied an existing phenotype assessment tool (SHIRPA) (see http://www.mgu.har.mrc.ac.uk/mutabase/shirpa_summary.html) to R6/2 and wild-type mice at age 11 weeks and extracted the neurobehavioral and physical parameters that distinguished the genotypes (15). For subsequent trials, mice were placed in an open field for 2 min while the following traits were scored: tremor, grooming, spontaneous activity, and locomotor activity for the neurobehavioral assessment and palpebral closure, piloerection, body position, and tail position

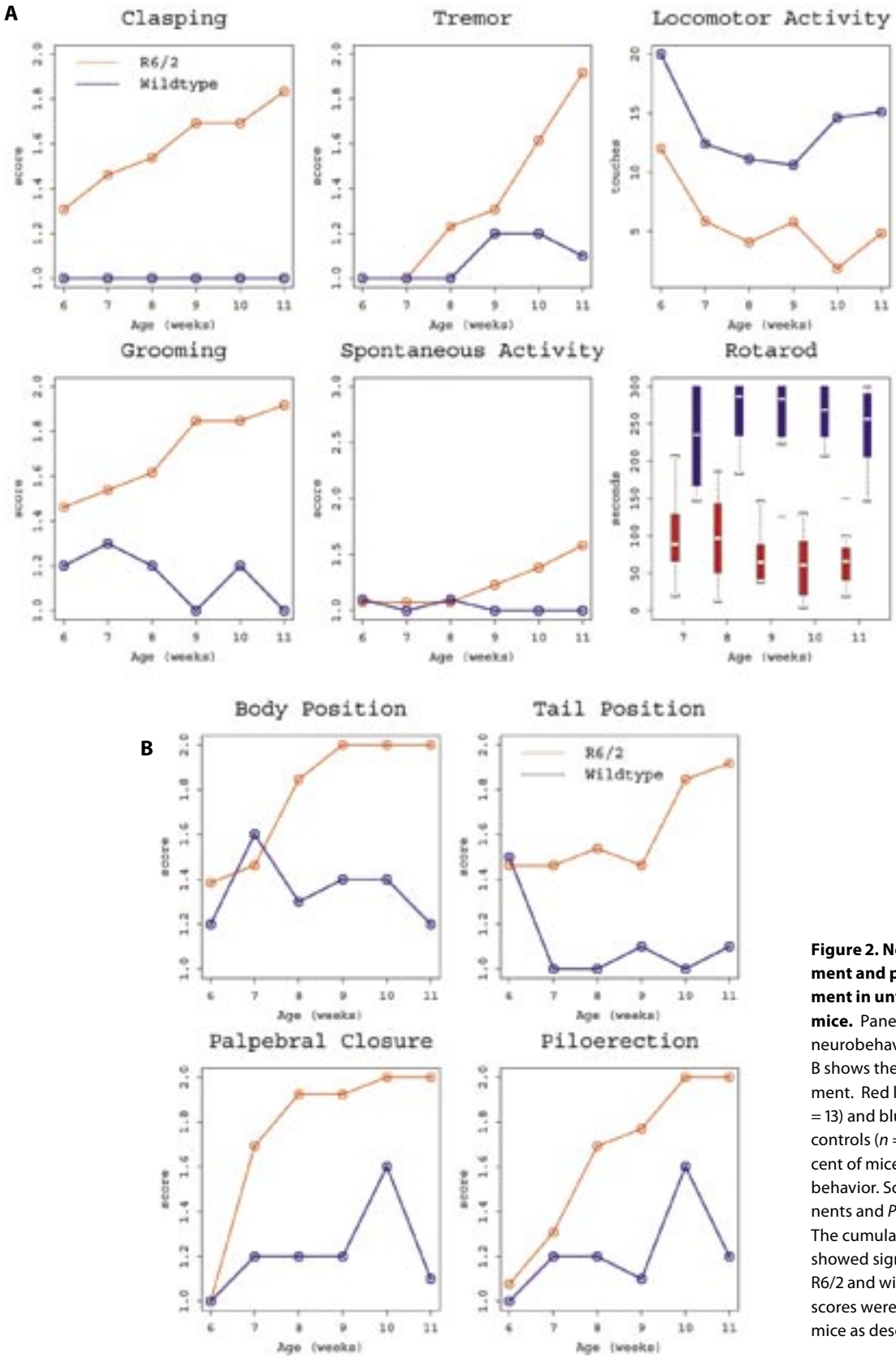


**Figure 2. Neurobehavioral assessment and physical phenotype assessment in untreated R6/2 and control mice.** Panel A shows components of the neurobehavioral assessment and panel B shows the physical phenotype assessment. Red lines represent R6/2 mice ($n$ = 13) and blue lines represent wild-type controls ($n$ = 10). Graphs show the percent of mice that demonstrate abnormal behavior. Scores for individual components and $P$ values are shown in Table 1. The cumulative $z$ score for all measures showed significant difference between R6/2 and wild-type mice ($P < 0.001$). $z$ scores were based on 7–11-week-old mice as described in text.

**Table 1. Neurobehavioral Assessment and Physical Phenotype Assessment in Wild-Type and R6/2 Mice**

| | Wild Type (mean ± SD) | R6/2 (mean ± SD) | *P* Value |
|---|---|---|---|
| **Neurobehavioral Assessment** | | | |
| Clasping | 1.00 ± 0.00 | 1.58 ± 0.29 | < 0.001 |
| Grooming | 1.15 ± 0.22 | 1.70 ± 0.31 | < 0.001 |
| Locomotor activity | 14.0 ± 4.50 | 5.74 ± 3.97 | < 0.001 |
| Rotarod | 249.2 ± 46.8 s | 88.51 ± 31.7 s | < 0.001 |
| Spontaneous activity | 1.03 ± 0.11 | 1.23 ± 0.24 | < 0.001 |
| Tremor | 1.08 ± 0.16 | 1.34 ± .14 | < 0.001 |
| **Composite** | | | **< 0.001** [a] |
| **Physical Phenotype Assessment** | | | |
| Body position | 1.38 ± 0.30 | 1.86 ± 0.25 | < 0.001 |
| Palpebral closure | 1.26 ± 0.31 | 1.91 ± 0.24 | < 0.001 |
| Piloerection | 1.26 ± 0.28 | 1.75 ± 0.33 | < 0.001 |
| Tail position | 1.04 ± 0.12 | 1.64 ± 0.31 | < 0.001 |
| **Composite** | | | **< 0.001** |

[a]Composite scores are determined as described in the Methods section.

for the physical phenotype assessment. Extensive observational studies defined the range of normal and abnormal behaviors, and these were typically converted to a binary scoring system (1 point for normal, 2 points for abnormal) (Figure 1 and data not shown). Investigators independently scored the same mice to determine inter-investigator reliability. Multiple pairs of investigators assessed mice over a 6-month period and showed less than 5% scoring discordance (data not shown). Pathologic clasping and rotarod performance were conducted separately from the open field test and included in the neurobehavioral assessment.

To assess the feasibility of using the neurobehavioral assessment and physical phenotype assessment for future drug efficacy studies, 13 R6/2 and 10 wild-type mice from age 6 to 11 weeks were prospectively evaluated. For most measures, R6/2 mice were indistinguishable from littermate controls at 6 weeks of age but showed significant neurologic decline by 8 weeks (Figure 2). For all measures, the gap between R6/2 and wild-type mice increased as the mice aged. Rotarod performance and clasping could not be evaluated in the open field. These studies were assessed by traditional methods, and scores were included in the neurobehavioral assessment.

## Statistical Basis for Subsequent Drug Efficacy Studies

The neurobehavioral assessment was developed with the intention of replacing survival as the primary outcome measure for R6/2 drug efficacy studies. Since R6/2 mice are poorly distinguished from wild-type mice at 6 weeks of age and since R6/2 mice must be euthanized at approximately 12 weeks of age in Great Britain, we conducted power analyses on the window of 7–11 weeks to determine how many mice would be needed for subsequent prospective efficacy trials.

Each of the neurobehavioral assessment and physical phenotype assessment measures reported highly significant differences between R6/2 and wild-type mice during this time period (Table 1). The composite $z$ score $P$ values for these assessments were even more extreme than for individual measures. The difference in the mean scores between R6/2 and wild-type mice provides an upper bound for the maximal difference achievable for new drugs. Therefore, we express power calculations based on a hypothesized fraction (or

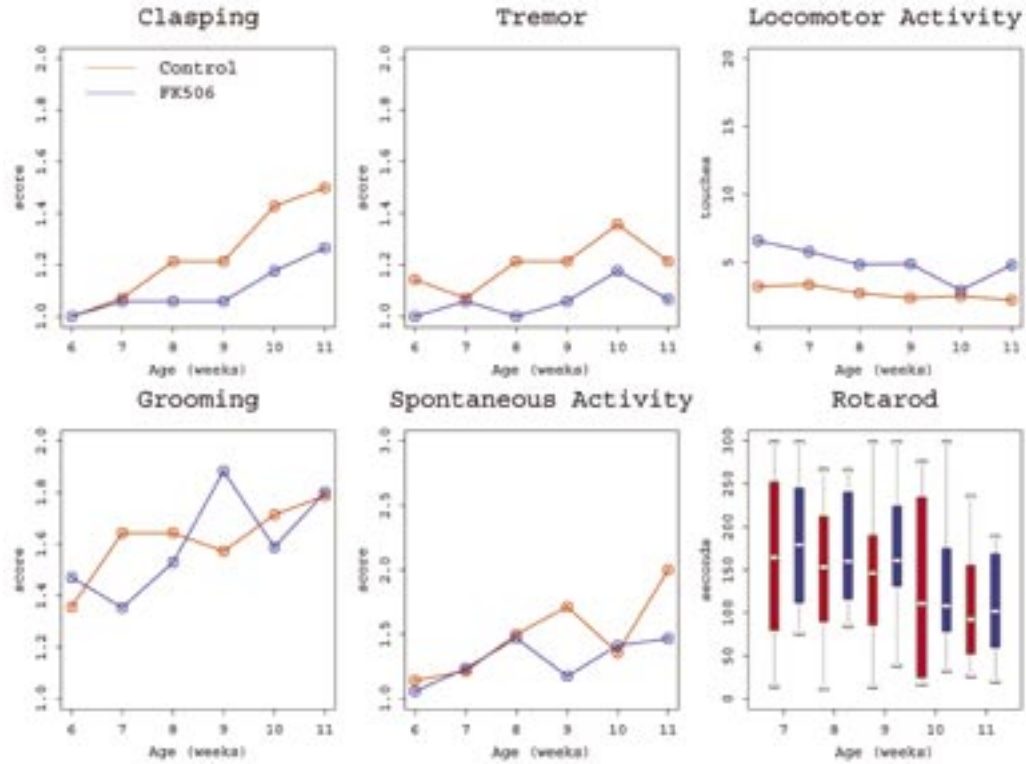


**Figure 3. Improvement in neurobehavioral assessment scores with FK506 treatment.** Graphs of mean data shown in panels ($n$ = 15–19). The rotarod graph shows mean ± SEM. Scores for individual components and $P$ values are shown in Table 2. The cumulative $z$ score for all six measures showed significant improvement in neurologic function of FK506-treated mice ($P < 0.03$). $z$ scores were based on 7–11-week-old mice as described in text.

## Table 2. Neurobehavioral Assessment and Physical Phenotype Assessment in FK506-Treated and Control R6/2 Mice

| | Control (mean ± SD) | FK506-Treated | *P* Value |
|---|---|---|---|
| **Neurobehavioral Assessment** | | | |
| Clasping | 1.28 ± 0.33 | 1.11 ± 0.15 | 0.052 |
| Grooming | 1.67 ± 0.32 | 1.63 ± 0.28 | 0.383 |
| Locomotor activity | 2.63 ± 2.05 | 4.81 ± 3.78 | 0.043 |
| Rotarod | 137.2 ± 72.2 s | 153.4 ± 53.2 s | 0.212 |
| Spontaneous activity | 1.56 ± 0.50 | 1.39 ± 0.35 | 0.097 |
| Tremor | 1.21 ± 0.28 | 1.04 ± 0.08 | 0.057 |
| **Composite** | | | **0.023** [a] |
| **Physical Phenotype Assessment** | | | |
| Body position | 1.34 ± 0.27 | 1.52 ± 0.34 | 0.044 [b] |
| Palpebral closure | 1.63 ± 0.34 | 1.69 ± 0.26 | 0.245 [b] |
| Piloerection | 1.41 ± 0.35 | 1.56 ± 0.34 | 0.089 [b] |
| Tail position | 1.17 ± 0.22 | 1.25 ± 0.32 | 0.238 [b] |
| **Composite** | | | **0.074** [b] |

[a]Composite scores are determined as described in the Methods section.

[b]Control R6/2 mice scored better than FK506-treated mice on all components of the physical phenotype assessment.

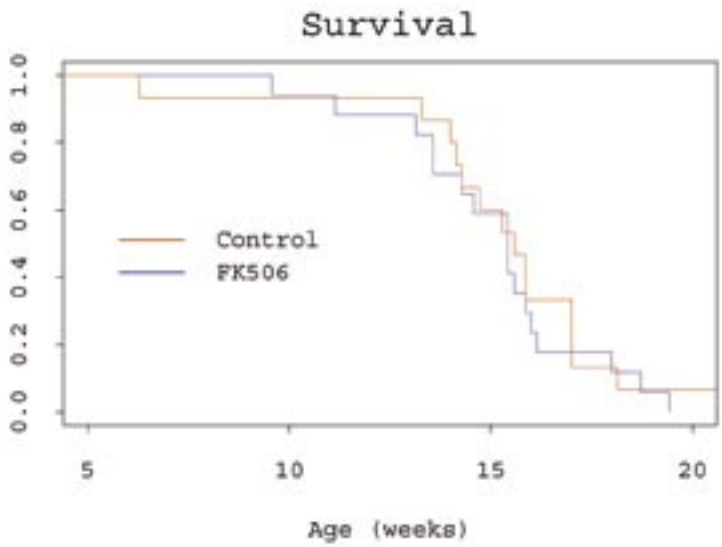


**Figure 4. Kaplan-Meier survival curves of R6/2 mice treated with FK506 and untreated controls.** No difference was observed between the two arms ($n = 16$ per arm).

percentage) of that difference. Because mouse behavior is by nature highly variable, the number of mice required to show an improvement equal to 50% of this difference (with 85% power) in single components of the neurobehavioral assessment ranged as high as 105 mice per arm. However, only 15 mice per arm would be sufficient to show a 25% improvement in the neurobehavioral assessment composite score with power of approximately 92%, assuming the variance of new observations is similar to the R6/2 mouse outcome variation seen in this study.

### FK506 Efficacy Study

FK506 (tacrolimus) is a neuroprotectant and neurotrophic agent that acts through immunophilin receptors (e.g., FKBP12). FK506 (4 mg/kg) was administered as a supplement to powdered food 7 days per week from age 3 weeks until death. A preliminary dose response study using 0.1, 1, 4, and 10 mg/kg was conducted using three R6/2 and three wild-type mice per dose. Mice were on drug from 6 to 13 weeks of age, and weights and behavioral observations were done weekly. Mice treated with 10 mg/kg showed signs of toxicity in wild-type mice, primarily weight loss and tremors. Only mild weight loss and no tremors were observed in mice treated with 4 mg/kg. Serum levels of FK506 were in the immunosuppressive range for mice treated with 10 mg/kg and were below the immunosuppressive range for mice treated with 4 mg/kg. Therefore, for our FK506 efficacy study, we used 4 mg/kg, which was nontoxic and nonimmunosuppressive.

Primary study end points included survival and the neurobehavioral assessment described above. Secondary end points included weight and the physical phenotype assessment. FK506 caused a delay in onset of five neurologic changes measured by the neurobehavioral assessment, including locomotor activity, clasping, spontaneous activity, rotarod performance, and tremors (Figure 3 and Table 2). No improvement was observed in grooming behavior. The cumulative $z$ score for all six measures showed that in FK506-treated animals, neurobehavioral deficits were less severe than in untreated controls ($P < 0.03$, $n = 15$–19 animals per arm). FK506 did not prolong the highly symptomatic phase of disease as shown in the Kaplan-Meier survival curve (Figure 4).

Secondary end point analyses showed that untreated R6/2 mice scored better in the physical phenotype assessment than FK506-treated animals (Figure 5 and Table 2). Weight was decreased by approximately 10% in FK506-treated R6/2 mice compared to untreated controls (Figure 6). The same was true of wild-type mice (not shown). There were no overt infections in FK506-treated animals.

## DISCUSSION

The average time from clinical diagnosis of HD until death is 15 ± 8 years. Although disease progression is variable, over half of HD patients are no longer able to maintain employment within 6 years of onset of illness (16). In the United States, patients require institutional or skilled nursing care for an average of 8 years. As potential therapies for

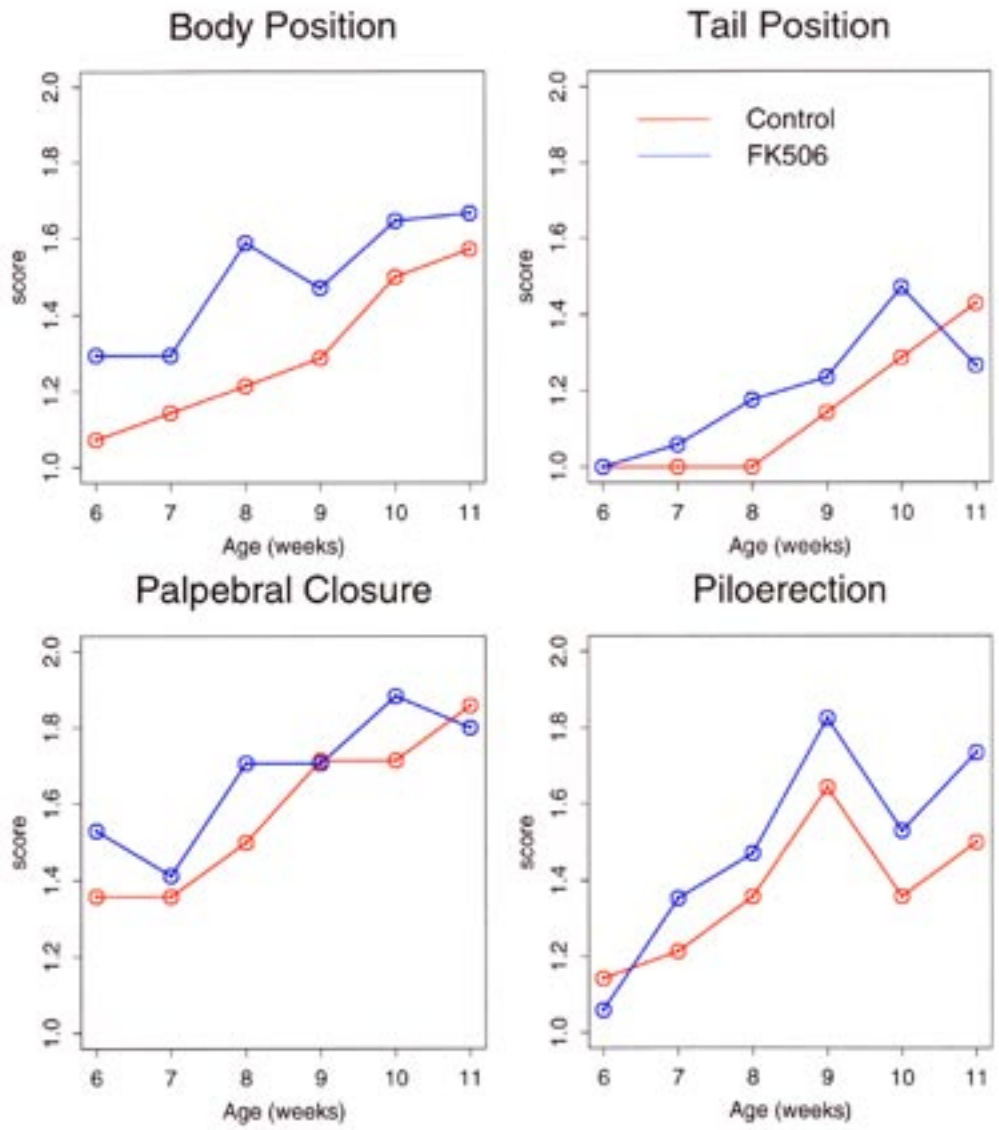


**Figure 5. No improvement in physical phenotype assessment scores with FK506 treatment.** Graphs of mean data shown in panels ($n$ = 15–19). Scores for individual components and $P$ values are shown in Table 2. The cumulative $z$ score for all six measures showed a trend toward better scores in control R6/2 mice compared to FK506-treated mice ($P < 0.08$). $z$ scores were based on 7–11-week-old mice as described in text.

HD are screened in mouse models, it seems reasonable to focus on end points that delay onset of debilitating symptoms and specifically avoid compounds that prolong the highly symptomatic phase of disease.

The number of drugs that show efficacy in invertebrate or in vitro HD assays is increasing rapidly. As these compounds move into murine efficacy studies, it is imperative to develop study end points that can be measured rapidly and reproducibly. For this reason, we focused on developing a rapid neurobehavioral assessment. Except for the rotarod test and clasping assessment, the neurobehavioral assessment described in this report can be completed in 2 min per mouse, and the results are reproducible between multiple investigators.

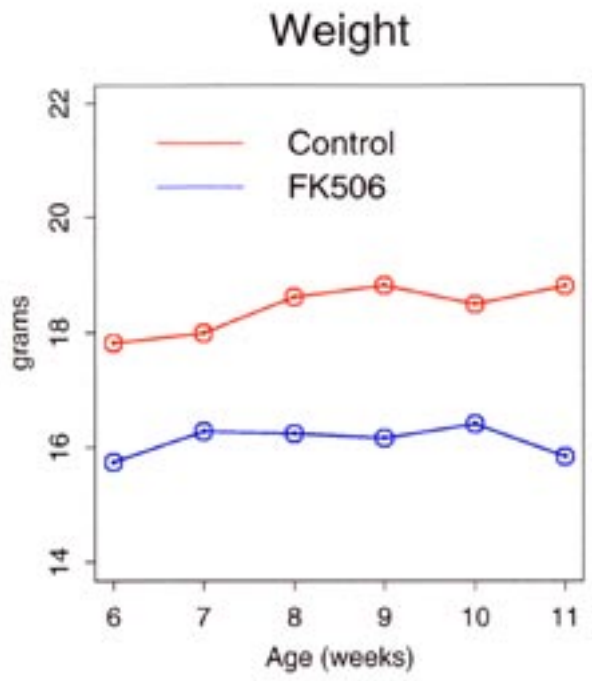


**Figure 6. Weight measurements for FK506-treated and control R6/2 mice.**

The difference in mean neurobehavioral assessment composite scores between R6/2 and wild-type mice provides a scale to interpret the potential therapeutic impact of new drugs. Given the distinction seen between R6/2 and wild-type mice, it is feasible to conduct studies to detect an improvement equal to a small fraction (25% or 33%) of this difference in the composite neurobehavioral assessment score. Such a study could have as few as 15 mice per arm, yet achieve 90% power for a 25% improvement in the neurobehavioral assessment assuming the variance seen in R6/2 mice scores is similar to this study.

Development of the neurobehavioral assessment was motivated by a pilot study of FK506 treatment in R6/2 mice. In the pilot study, we noted that FK506-treated mice behaved like wild-type mice even as control R6/2 mice became debilitated. Following development of the neurobehavioral assessment, we conducted a full-scale FK506 study in which the neurobehavioral assessment was applied prospectively by investigators that were blinded to drug treatment. The assessment was based on a comprehensive phenotype assessment tool (SHIRPA) that is widely accepted but unsuitable for drug screening and in previous assessments of motor deficits in R6/2 mice (15,17,18). In this study, FK506 reduced the percentage of R6/2 mice that exhibited neurologic deficits in the early symptomatic phase, but did not prolong the highly symptomatic phase.

FK506 was chosen for this study because it is neuroprotective and also supports neuroregeneration. Neuroprotection has been observed in cultured neuronal cells exposed to excitotoxic or oxidative stress, mice lesioned with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine, or rats exposed to forebrain ischemia (19–28). Neurotrophic and regenerative effects have been reported in primary neuronal cultures, PC12 cells, surgical lesion models, and crush injury models. FK506 likely protects neurons by binding to FK binding proteins (FKBPs), also known as immunophilins. The most widely studied FKBP is FKBP12, which is complexed with inositol triphosphate (IP3R) and ryanodine receptors (RyR) in the endoplasmic reticulum (ER) membrane and with calcineurin in adjacent cytoplasm. FK506 regulates protein kinase C-mediated phosphorylation of IP3R and inhibits calcineurin-mediated dephosphorylation, and hence activation, of nitric oxide synthetase and the pro-apoptotic molecule BAD. Thus, FK506 has been postulated to be neuroprotective by regulating calcium across the ER membrane, preventing oxygen free radical formation, and inhibiting pro-apoptotic molecules. The role of FKBP12 in neuroprotection has been questioned, however. Under certain conditions, neuroimmunophilin ligands that do not bind FKBP12 protect neurons, and in other cases, neuroprotection or regeneration are observed in FKBP12-null mice (26,27,29). Despite these findings in other paradigms, further evaluation of FKBP12 in polyglutamine disease may be warranted, in part because mRNAs that encode IP3R, RyR, calcineurin, and protein kinase C are altered in R6/2 mice compared to controls (30–32).

At least eight other FKBPs have been identified (33). FKBPs are highly diverse proteins that share as common features rotamase activity and tetratricopeptide repeat domains that facilitate protein-protein interactions. Of these, FKBP52 may also be of interest in polyglutamine protein diseases because it interacts through HSP90 with the glu-

cocorticoid receptor complex. Ligand-activated glucocorticoid receptors reduce polyglutamine aggregate formation in a cell culture model, and heat shock proteins (HSPs) modulate polyglutamine-mediated toxicity in several model systems. In addition, blocking antibodies directed against FKBP52 abrogated neurotrophic effects of FK506 (29). Other FKBPs of interest include FKBP38 and FKBP65 which, like FKBP12, are enriched in the central nervous system.

It is formally possible that FK506 enhanced neurobehavioral outcomes through a mechanism that is independent of FKBPs. FK506 caused weight reduction of approximately 10% in both R6/2 and wild-type mice. It has previously been reported that calorie restriction leads to improved motor performance and survival for HD-N171-82Q HD mice (34). We do not believe that FK506 is acting through this mechanism because dietary restriction in HD-N171-82Q mice led to weight gain rather than loss. Furthermore, we have treated R6/2 mice with several other compounds (e.g., all-*trans* retinoic acid) that caused 10% weight reduction with no improvement in motor performance or survival (not shown). Future studies with other immunophilins will provide further clarification of the mechanism of action.

Second-generation immunophilin ligands are neuroprotective and also lack immunosuppressive properties that would likely preclude clinical application of FK506 or cyclosporin for patients with neurodegenerative disease (35,36). Nonimmunosuppressive immunophilin ligands protect neurons in many, but not all, assays and are neurotrophic in others (22,24–27,37–40). These compounds show efficacy in models of Parkinson's disease and are now in human clinical trials (41–43).

Delaying onset of symptoms remains the primary goal for pharmacologic management of HD. The neurobehavioral assessment reported here provides a rapid, reproducible method for identifying drugs that specifically delay symptom onset. If survival had been used as the single primary end point, the potential efficacy of immunophilin compounds would have been missed. The neuroimmunophilin FK506 delays symptom onset in R6/2 mice but does not prolong the highly symptomatic phase of disease. Evaluation of nonimmunosuppressive immunophilins in R6/2 and full-length models of HD seems warranted at this point.

## METHODS

### Animals

The mice were hemizygous R6/2 females bred and reared in our colony. Original mice were ordered from Jackson Laboratory [(Bar Harbor, ME; code B6CBA-TgN (HDexon1) 62 (males) and C57BL/6 x CBA (females)]. Transgenic animals were identified prior to weaning by PCR of toe snip DNA, and CAG repeat size was determined. Genotyping was performed by PCR according to the Jackson Laboratory protocol. Control mice were female wild-type littermates. Mice were weaned into treatment groups at 3 weeks of age. Three mice were housed per cage with a random mix of wild-type and R6/2 mice. Animals were given free access to water and powered food (#5053 Irradiated PicoLab Rodent® Diet; LabDiet, Richmond, IN, USA).

### Drug

FK506 [4 mg/kg Prograf® (tacrolimus) capsules, 1 mg; Fujisawa Healthcare, Deerfield, IL] was mixed in the powdered food of treated mice. Residual food was weighed to ensure that the expected amount was consumed. FK506 levels were measured in serum of 6 mice treated with 10 mg/kg FK506 and 6 mice treated with 4 mg/kg FK506 at the University of Washington Clinical Laboratory.

### Neurobehavioral Assessment

For open field analyses, mice were evaluated weekly for 2 min in an empty sterile home cage.

**Tremor.** Mice were evaluated for presence or absence of tremors. None or mild were scored as 1 (normal) and marked was scored as 2 (abnormal).

**Grooming.** During a 2-min observation, occasional, slow, or intermittent grooming is normal mouse activity. Absence of grooming or, as in the case of R6/2 mice, excessive and repetitive grooming is abnormal. Mice were given a score of 1 for slow or occasional (normal) and 2 for either none or excessive (abnormal).

**Spontaneous activity.** Mice were evaluated for their activity level while exploring the cage for the 2-min period. The cage floor was divided into quadrants and their exploratory movements were scored. Mice were scored 1 for moderate, covering all quadrants (normal), 2 for slow, covering 1 to

3 quadrants (still within normal behavior range), and 3 for either none or darting (abnormal).

**Locomotor activity.** The investigator counted the number of times the mice touched the sides of the cage over the 2-min period. The mouse had to put at least one paw on the side and place its paws back on the floor before another touch was counted. The actual number of touches was recorded.

**Rotarod.** Rotarod ability was assessed on an accelerating Rota-Rod (Model 7650; Ugo Basile, Comerio, Italy). The mice had a training run and then were tested weekly from 6 to 11 weeks of age. The rotarod accelerated from 3 to 30 rpms over a 5-min period. Latency to fall was recorded in rpms and seconds.

**Clasping.** Mice were suspended by the tail for 1 min. In this position, wild-type mice perform a swimming motion with rapidly moving paws and an arched back. Symptomatic R6/2 mice often progress from forepaw tucking to hind and forepaw clasping with a quick release, to a complete clasp where they remain in a ball. The mice were given a score of 1 for swimming motion or forepaw tuck (normal), or 2 for clasp and release or clasp and hold (abnormal).

### Physical Phenotype Assessment

This assessment was conducted simultaneously with the neurobehavioral assessment in the sterile home cage for the 2-min time period.

**Palpebral closure.** The eyes of R6/2 mice often appear different than wild-type mice. They can appear flattened and squinty with swollen lids. A score of 1 was assigned for wide open (normal), or 2 for flattened, swollen lids, or squinty (abnormal).

**Piloerection.** Fur condition was evaluated with a score of 1 having no piloerection, namely a smooth coat (normal), or 2 having erected fur and a scruffy coat (abnormal).

**Body position.** The mice were evaluated for their body position as they moved about the cage. A score of 1 was given for an elongated posture (normal), or 2 for a hunched or rounded posture (abnormal).

**Tail position.** The tail position was observed as the mice were moving. A score of 1 was given for a horizontally extended tail (normal), or 2 for a dragging or Straub tail (abnormal).

### Statistical Considerations

The standardized *z* score for each of the component outcomes was calculated. The composite score is the total of the six scores (neurobehavioral assessment) and four scores (physical phenotype assessment) where each component is standardized using the standard deviation of the control group. A one-sided test was chosen because the primary interest is only to determine if the experimental regimen improves outcome relative to vehicle. The appropriate decisions are either (*i*) to pursue the class of experimental compounds or (*ii*) not to pursue the class of experimental compounds. In contrast, a two-sided test would be appropriate in most comparisons between two experimental regimens. The repeated measurements over multiple weeks were analyzed using a mixed effects linear model (44). Power and sample size calculations incorporated the repeated measures aspect of the design.

Survival curves for each treatment group were estimated using the Kaplan-Meier method (45). Differences in the survival time distributions between treatment arms were tested using a generalized Wilcoxan test for censored data (46). Survival times were considered censored at the time of any sacrifice.

Statistical analyses were performed in the S-PLUS statistical package (Insightful, Seattle, WA).

## ACKNOWLEDGMENTS

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