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Richard Grondin
University of Kentucky, richard.grondin@uky.edu

Pei Ge
Alnylam Pharmaceuticals

Qingmin Chen
Alnylam Pharmaceuticals

Jessica E. Sutherland
Alnylam Pharmaceuticals

Zhiming Zhang
University of Kentucky, zzhan01@uky.edu

See next page for additional authors

Authors
Richard Grondin, Pei Ge, Qingmin Chen, Jessica E. Sutherland, Zhiming Zhang, Don M. Gash, David K. Stiles, Gregory R. Stewart, Dinah W. Y. Sah, and William F. Kaemmerer

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Onset Time and Durability of Huntingtin Suppression in Rhesus Putamen After Direct Infusion of Antihuntingtin siRNA

Richard Grondin[1], Pei Ge[2], Qingmin Chen[2], Jessica E. Sutherland[2], Zhiming Zhang[1], Don M Gash[1], David K Stiles[3], Gregory R Stewart[3], Dinah WY Sah[2] and William F Kaemmerer[3]

One possible treatment for Huntington's disease involves direct infusion of a small, interfering RNA (siRNA) designed to reduce huntingtin expression into brain tissue from a chronically implanted programmable pump. Here, we studied the suppression of huntingtin mRNA achievable with short infusion times, and investigated how long suppression may persist after infusion ceases. Rhesus monkeys received 3 days of infusion of Magnevist into the putamen to confirm catheter patency and fluid distribution. After a 1-week washout period, monkeys received radiolabeled siRNA targeting huntingtin. After 1 or 3 days of siRNA delivery, monkeys were either terminated, or their pumps were shut off and they were terminated 10 or 24 days later. Results indicate that the onset of huntingtin mRNA suppression in the rhesus putamen occurs rapidly, achieving a plateau throughout the putamen within 4 days. Conversely, loss of huntingtin suppression progresses slowly, persisting an estimated 27–39 days in the putamen and surrounding white matter. These findings indicate the rapid onset and durability of siRNA-mediated target gene suppression observed in other organs also occurs in the brain, and support the use of episodic delivery of siRNA into the brain for treatment of Huntington's disease and possibly other neurodegenerative diseases.
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Introduction

Because Huntington's disease is known to be caused by expression of huntingtin (HTT) protein containing an expanded polyglutamine repeat region,[1] a logical approach to treatment of the disease is to administer a therapeutic agent designed to lower HTT expression. Ideally, the agent would be administered systemically and would reduce the expression of only the mutant allele in heterozygous individuals by targeting the expanded repeat region or a mutant-allele-associated single-nucleotide polymorphism.[2] However, since chronic reduction of the expression of wildtype HTT has been shown to be well-tolerated in the rodent and nonhuman primate brain,[3–6] another approach is to directly deliver a nonallele-specific siRNA against HTT mRNA to affected brain regions. We have previously shown that direct infusion of an siRNA that targets both rhesus and human *HTT* into the putamen of rhesus monkeys can achieve significant reduction in *HTT* mRNA over a substantial region of the primate putamen.[7] Furthermore, we have found that the volume of brain tissue reached by the infusion increases in proportion to the rate of fluid flow used for the infusion, as long as the rate is not so high as to cause loss of fluid due to backflow up the catheter path, or loss into a sulcus or ventricle. Risk of these limitations can be minimized by appropriate catheter design and careful trajectory planning.

Previous data support that chronic infusion of siRNA into a patient's brain could be accomplished using existing programmable, implantable and transdermally refillable drug pumps positioned in the abdomen, and connected with as yet investigational brain infusion catheters implanted into brain targets and anchored in place at the skull.[7] However, there are additional issues beyond fluid backflow concerning the use of a relatively high fluid flow rate to maximize brain tissue coverage. These include the possibility of eventual fluid build-up in the brain, and the inconvenience of relatively rapid emptying of the pump's reservoir necessitating frequent refilling. A way to minimize these concerns without sacrificing the goal of maximizing the volume of brain tissue treated by the siRNA may be to program the pump to deliver the fluid in a repeated pattern, delivering at a relatively high flow rate for a limited period of time, then dropping the flow rate to a minimal, basal level for an intervening period before repeating the cycle. The effectiveness of this type of therapy regimen depends upon whether the effect of the siRNA in reducing the expression of *HTT* in the treated brain tissue is durable, lasting for at least hours, if not days or weeks, following delivery of the fluid to regions of tissue. The effect of siRNA has been found to be durable in other organs and tissues of the body. For example, the durability of suppression of hepatocyte-derived transthyretin protein as measured in serum following a single intravenous dose of lipid nanoparticle encapsulated siRNA targeting transthyretin (ALN-TTR02) is at least 28–43 days in both humans and nonhuman primates.[8] However, it has not been previously known whether such findings would generalize to unencapsulated ("naked") siRNA delivered to the brain.

The aims of our study were to assess the effect of the duration of infusion of siRNA on the level of suppression of *HTT* mRNA attained in the putamen and surrounding brain regions,

[1]Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Lexington, Kentucky, USA; [2]Alnylam Pharmaceuticals, Cambridge, Massachusetts, USA; [3]Drug Delivery R&D, Neuromodulation, Medtronic Inc., Minneapolis, Minnesota, USA. Correspondence: William F Kaemmerer, Medtronic, Inc. RCE280, 7000 Central Avenue N.E., Minneapolis, MN 55432 USA. E-mail: bill.kaemmerer@medtronic.com
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and to evaluate the durability of the suppression in the tissue after delivery is stopped. We implanted drug delivery pumps and intracranial catheters into rhesus putamen and delivered a dilute solution of the magnetic resonance imaging (MRI) contrast agent gadopentetate dimeglumine (Magnevist) for a period of several days, during which time MR imaging was used to confirm catheter patency and document intraparenchymal fluid distribution. After a 1-week washout period, we delivered radiolabeled siRNA via the same systems for various periods of time, and terminated animals at various time points. The distribution of the siRNA in the brain was assessed via autoradiography and the suppression of *HTT* mRNA by the siRNA at various locations in the brain was assessed via RT-qPCR. Our data support that, as has been found in other organs and tissues, the effect of siRNA on the level of expression of the targeted gene is durable for several weeks in the brain. Furthermore, the onset of the siRNA effect is rapid, such that a cyclical delivery regimen involving an intermittently high flow rate might be used to maintain a fairly continuous therapeutic effect in targeted brain regions extending well beyond the volume reached by a lower flow rate of delivery. These findings may be applicable not only to Huntington's disease treatment, but also treatment of other neurodegenerative diseases for which the lowering of a toxic gene product in the brain may be efficacious.

Results

Fifteen adult female rhesus monkeys were implanted with intracranial catheters primed with 5 mmol/l Magnevist in phosphate-buffered saline (PBS) and connected to abdominally implanted infusion pumps. All animals were infused with Magnevist from their implanted pump at a rate of 0.3 µl per minute for 3 days. In all animals, the catheter needle tips were found to be within a 2 mm radius of the target location in the putamen based on the postoperative MRI taken immediately after catheter placement (on day –10). Also, all catheters were patent based on Magnevist visualization in the brain via the MRI taken 3 days postsurgery (on day –7). After an intervening 7-day infusion of PBS at a rate of 0.1 µl per minute (for "washout" of the Magnevist), the pump reservoir was filled (on day 0) with radiolabeled anti-*HTT* siRNA for all animals but a PBS control group ($n = 3$), and the infusion continued at a rate of 0.3 µl per minute for 2 or 4 days. The purpose of these time periods was to yield 1- and 3-day infusions of the siRNA into the brain, given that the time required for the siRNA to reach the brain from the abdominal pump, at a rate of 0.3 µl/minute, is about 24 hours. At the end of this infusion, either the monkeys were terminated, or the infusion was stopped for a period of 10 or 24 days at which point the animals were terminated (**Figure 1a**). All 15 catheters were determined to be patent at the time of the animal's necropsy based on a postmortem check of fluid delivery through the catheter from the pump.

Distribution of infusates

To determine whether an infusion of Magnevist would have utility in assessing the extent of siRNA distribution achieved in an individual's brain, the volume of distribution of Magnevist was determined from the T1-weighted MRI images taken at the completion of the 3 days infusion period and compared to the distribution of siRNA assessed later by autoradiography (**Figure 1b**,**c**). The volume of Magnevist distribution was obtained by thresholding the gray scale MRI image using a voxel value that was 10% of the maximum T1 intensity obtained from the Magnevist signal at the catheter tip in the putamen. Since the absolute concentration of Magnevist did not vary at this point, it provided a reference to standardize the threshold from scan to scan. The volume of siRNA delivery was quantified by identifying the regions of autoradiography sections with a signal corresponding to 0.65 mg or more of siRNA per gram of tissue, which is the concentration of siRNA previously found to correspond to approximately 45% suppression of *HTT* mRNA.[7] We refer to this as the "volume of ≥45% suppression." Only the animals terminated immediately after 2 days of infusion (group 2, 1 day of siRNA delivery to the brain) or 4 days of infusion (group 3, 3 days of siRNA delivery to the brain) had sufficient radioactive material in the brain tissue to visualize intraparenchymal distribution of siRNA by autoradiography. All other groups (with longer survival intervals postinfusion) had negligible signal. The volume of ≥45% suppression (mean ± SE) obtained in the monkeys at time points 1 and 3 was 584.8 ± 45.6 and 674.5 ± 58.4 mm^3, respectively, a difference that is not statistically significant ($t = 1.21$, df = 4, $P = 0.29$). The volume of Magnevist distribution in these groups was 1,262.8 ± 307.9 and 1,747.8 ± 641.8 mm^3, respectively ($t = 0.68$, df = 4, $P = 0.533$). For every animal, the volume of Magnevist distribution exceeded the volume of ≥45% suppression, but the difference between the volume of Magnevist distribution and the volume of ≥45% suppression was highly variable from animal to animal (**Figure 2**) and was not significant (875.7 ± 350.6, $t = 2.5$, df = 5, $P = 0.055$). The correlation between the volumes was $r = –0.32$ ($P = 0.54$). Thus, a quantitative relationship between the volume of Magnevist distribution and the subsequent siRNA volume of ≥45% suppression could not be defined using the observations from this small number of animals.

Onset of suppression

Using HTT suppression measurements normalized to the corresponding PBS group averages (on a punch location-by-punch-location basis), a mixed-model analysis of variance (ANOVA) of *HTT* suppression with punch location as the within-monkey factor and membership in groups receiving siRNA for zero (PBS group), 1, or 3 days as the between monkey factor was performed. Two outlier observations (with standardized residuals more than 5.0 standard deviations from their respective group mean) were omitted from the analysis: one punch from one monkey in the PBS group (calculated percent suppression = –96.13%, *i.e.*, an apparent increase in *HTT* expression) and one punch from one monkey in the siRNA1+3 group (calculated percent suppression = –52.20%, also indicating an apparent increase in *HTT* expression). The ANOVA resulted in a significant difference among punch locations ($F = 2.12$, df = 68, 359, $P < 0.001$) and a significant interaction effect of punches by treatment group ($F = 1.35$, df = 136, 359, $P = 0.015$), indicating there is evidence for true differences in the level of *HTT* mRNA suppression at various anatomical sites depending upon the treatment administered, as expected. To further characterize the relationship between level of *HTT* mRNA suppression, location in the brain, and treatment group,

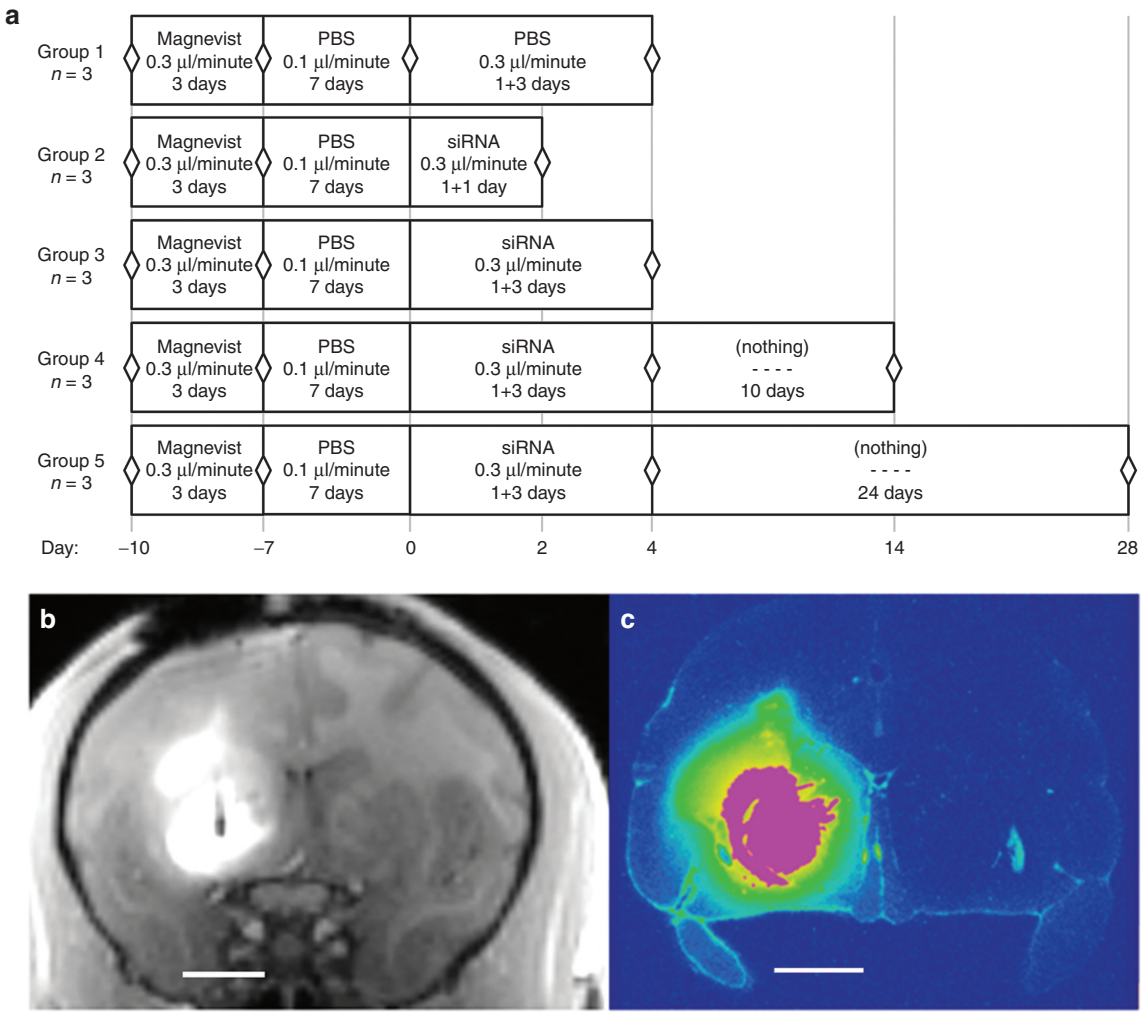

Figure 1 Study design and distribution of infusates. (**a**) Diagram of the experimental groups and time course of events in the study. Diamonds indicate MRIs taken after implantation of the delivery hardware and at later points in the study. Groups 4 and 5 had their pumps completely turned off after 4 days of infusion. (**b**) Distribution of Magnevist after 3 days of delivery to the putamen at a rate of 0.3 µl per minute. (**c**) siRNA distribution in the same monkey after 3 days of delivery to the putamen at a rate of 0.3 µl per minute, visualized by autoradiography. The autoradiography is color-coded for siRNA concentrations. Magenta represents a potentially "efficacious" level of siRNA (≥ 0.65 mg of siRNA per gram of tissue). At this coronal plane, there was near complete coverage of the putamen with some spread to adjacent structures, in particular some tracking of infusate dorsally into cortex. Scale bars = 1 cm.

the punches were categorized according to whether they were within or outside the putamen (the site of the infusion), and a mixed-model ANOVA was performed with membership in groups receiving siRNA for zero (PBS group), 1, or 3 days as the between-monkey factor, and punch category (within or outside the putamen) as a within-monkey factor, with punches nested within punch category. The ANOVA resulted in a significant interaction between punch category and treatment group ($F = 14.99$, df = 2, 493, $P < 0.001$). This interaction is portrayed in the graph in **Figure 3**, which shows no differences among the monkeys receiving PBS or siRNA in HTT mRNA suppression in locations outside the putamen, but a difference among the monkeys receiving PBS or siRNA for differing lengths of time in HTT mRNA suppression in locations within the putamen.

Separately analyzing the punches taken from other anatomical regions in the brain (caudate, cerebral cortex, and white matter structures surrounding the basal ganglia) did not show any significant differences in *HTT* mRNA suppression across treatment groups nor any treatment group by punch location interaction in regions beyond the putamen (data not shown).

To more fully characterize and mathematically model the onset of suppression resulting from siRNA infusion into the rhesus putamen, a meta-analysis was performed using the data from groups 2 and 3 from the current study (siRNA1+1 and siRNA 1+3, respectively) and *HTT* suppression data from the same tissue punch locations from five additional monkeys used in a prior study.[7] These five monkeys received the same siRNA at the same concentration and infusion rate, and via the same infusion system implanted by the same surgical team as the current study, for 7 days prior to termination. As with the monkeys in the present study, it took 24 hours for the siRNA to reach the brain from the pump reservoir in the abdomen, so the data from this latter group of five monkeys

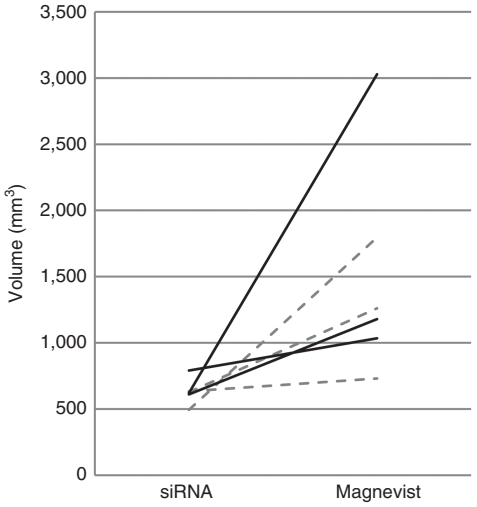

Figure 2 Volumes of Magnevist and siRNA distributions by monkey. Data plotted are the volume of siRNA at a concentration ≥ 0.65 mg of siRNA per gram of tissue and the volume of Magnevist distribution. Lines connect values from the same monkey (gray dotted lines, monkeys receiving siRNA in putamen for 1 day; solid black lines, monkeys receiving siRNA in putamen for 3 days).

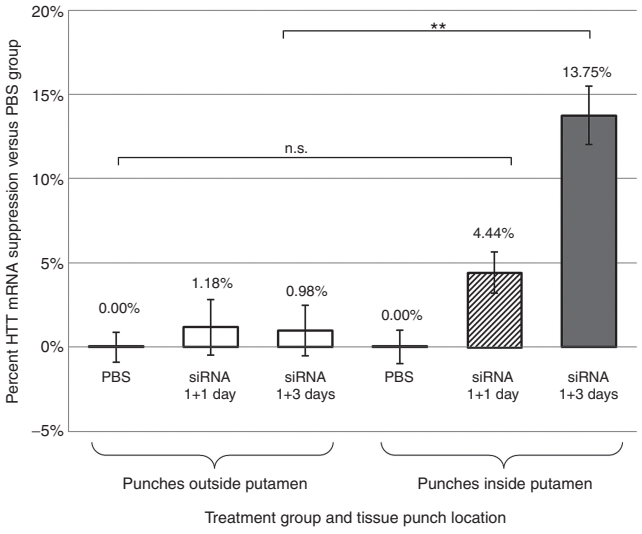

Figure 3 Suppression of HTT mRNA in the putamen. Average percent suppression of HTT mRNA in tissue punches from outside the putamen or from within the putamen, by treatment group, after subtracting the average percent suppression at the corresponding tissue punch locations in phosphate-buffered saline Group 1. Treatment and punch location combinations that are significantly different by *post-hoc* analysis (Tukey method[15]): n.s. = not significantly different; $^{**}P < 0.01$. HTT, huntingtin.

("Group 6") serves as an additional time point at day 6 in the modeling of suppression onset. The assay of the level of suppression of *HTT* mRNA in the tissue punches from the Group 6 monkeys was conducted using the same cDNA standard to define the level of HTT mRNA in rhesus putamen considered "zero percent suppression" as was used in the current study (see Materials and Methods.) Therefore, the percent suppression information is on the same measurement scale across studies, such that a meta-analysis is meaningful.

In this meta-analysis, using just the tissue punches from the locations inside the putamen, a mixed-model ANOVA of *HTT* suppression (normalized to corresponding PBS group averages) with punch location as a within-monkey factor and membership in groups as the between-monkey factor was performed. The groups included were Groups 1, 2, and 3 from the current study (c.f. **Figure 1a**), and Group 6 from the prior study, with the latter three groups receiving siRNA infusion into the putamen for 1, 3, or 6 days, respectively. The ANOVA resulted in significant differences among groups ($F = 30.91$, df = 3, 10, $P < 0.001$), and among the various punches ($F = 2.41$, df = 39, 334, $P < 0.001$). One-sample *T*-tests of the average suppression across all putamenal punches in Group 2, Group 3, and Group 6 (against the null hypothesis of zero suppression) showed that suppression (relative to PBS) was significantly greater than zero after after 3 days (mean difference = 13.47%, SD = 7.85%, $T = 2.97$, df = 2, $P < 0.05$, one-tailed) and significantly greater than zero after 6 days of siRNA exposure (mean = 38.64%, SD = 4.41%, $T = 19.75$, df = 4, $P < 0.001$).

Continuing with the meta-analysis, an ANOVA of the overall average HTT suppression in each monkey's putamen derived from just those punches in the putamen that were 4 mm away from the infusion site (the "B" series of 26 punches, see **Supplementary Materials**) showed a significant difference among groups ($F = 36.08$, df = 3, $P = 0.001$). Suppression averaged 4.1% above the corresponding PBS group values after 1 day and 18.4% after 3 days. This gain of 14.3 percentage points in average *HTT* suppression in Group 3 (siRNA1+3) compared to Group 2 (siRNA1+1) is statistically significant ($P < 0.05$ by the Tukey *post-hoc* test of pairwise comparisons). These data indicate that continuing to expose the putamen to siRNA for additional days after one day of siRNA exposure is effectual and perhaps necessary for therapeutic benefit.

A main objective of the current study was to characterize whether the onset of suppression is linear or curvilinear, the shape of any curvilinear relationship, and the likely timing of the asymptote of suppression levels, and thus the time point beyond which continuous exposure of the brain tissue to the siRNA could be suspended for awhile without sacrificing the potential for even greater suppression. For this purpose, the analysis plan was to identify the best-fitting linear regression line and the best fitting-fitting parameter values for two curvilinear functions, each computed on a punch-location-by-punch-location basis, in a meta-analysis using Groups 2, 3, and 6, mathematically fixing the percent suppression at time zero to zero. **Figure 4** illustrates the shape of the equations. Using the difference in percent suppression above the PBS group average at the corresponding punch location in the brain as the measure, this analysis yielded no consistent result, and no consensual estimate regarding the time of siRNA exposure sufficient to reach an asymptote. Rather, in 11 of 40 punch locations, the best-fitting relationship between time and suppression was concave down, in 4 punch locations (not anatomically contiguous) the best-fitting

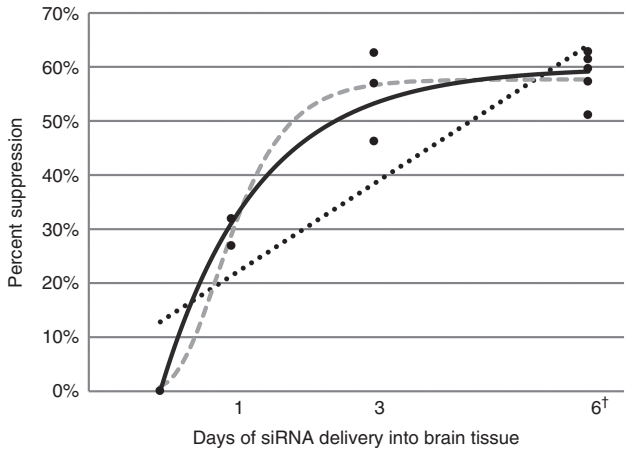

Figure 4 Illustration of alternative curve fits for time course of suppression onset. Linear, sigmoidal, and concave down curve fit to onset of suppression of huntingtin measured in RNA from punch location B02 from the putamen of monkeys after 1, 3, and 6 days of siRNA delivery (group 2, group 3, and group 6, respectively). Dotted line: best linear fit, Supp = 12.84 + (8.53 * day); gray dashed curve: best sigmoidal fit, Supp = 57.69 * exp(–exp(1.45 – 1.83 * day)); solid black curve = best concave down fit, Supp = 59.88 * (1 – exp(–0.73*day)). The curve fits for each punch location in the putamen are provided in the **supplemental materials**. †Group 6 is the group from a prior study used for this meta-analysis of suppression onset.

relationship was sigmoidal, in 11 locations it was linear, and in 14 locations it followed an exponential growth curve, implying no limit to the amount of suppression attainable (or at least that in a variety of non-contiguous punch locations scattered through the putamen the asymptote was not yet attained after 6 days of exposure).

Because of the possibility that these complex and uninformative results were due to the analysis of the difference in suppression above the corresponding PBS averages, possibly exacerbating variability in the data, the analysis was performed directly on the percent suppression over the zero point provided by the common cDNA standard (see Methods). This analysis yielded consistent, consensus results across punch locations. In 37 of 41 punch locations, the equation type that best fits the data is curvilinear, concave down (see **Supplementary Table S1**), indicating there is minimal lag in the initiation of the suppression (else, a sigmoidal curve would fit better) and an asymptote to the maximum suppression that can be attained occurs within a 6-day period. On average, the curve fits across the 41 punch locations in the putamen indicate that 97% of the maximum attainable suppression of *HTT* is reached within 3.23±0.25 days (mean ± SE) of infusion initiation. There is no significant relationship between the distance of the punch from the infusion site and the predicted number of days to reach 97% of the maximum attainable suppression at that site (correlation = –0.28, $P = 0.116$, $n = 33$).

A visualization of how the level of suppression of *HTT* mRNA in the various punch locations in putamen of the monkeys varied with differing days of delivery of the siRNA is provided in **Figure 5**. **Supplementary Materials** present the data (means ± SE) in tabular form in **Supplementary Table S2**, with larger schematic images showing the locations of the tissue punches in the monkey brain provided in **Supplementary Figure S1**.

Duration of suppression

Another main objective of the current study was to determine how long delivery of siRNA to putamenal tissue could be suspended before suppression of *HTT* would be substantially lost. To investigate the duration of suppression of *HTT* by the siRNA, we again used HTT suppression measurements normalized to the corresponding PBS group (Group 1) averages on a punch location-by-punch-location basis, and performed a mixed-model ANOVA of *HTT* suppression with punch location as the within-monkey factor and membership in the group terminated at 0, 10, or 24 days following cessation of the siRNA delivery as the between-monkey factor. This ANOVA resulted in a significant difference among groups ($F = 12.03$, df = 2, 6, $P = 0.008$), among punch locations ($F = 2.75$, df = 69, 355, $P < 0.001$) and interaction between punches and group ($F = 1.33$, df = 138, 355, $P = 0.018$).

Restricting the analysis to punches located within the putamen produced the following ANOVA results: there are significant differences among the experimental groups ($F = 10.67$, df = 2, 6, $P = 0.010$) and among the various punches ($F = 2.29$, df = 40, 203, $P < 0.001$). Furthermore, there is no evidence that the differences among experimental group varied by punch location (interaction of group × punch, $F = 0.79$, df = 80, 203, $P = 0.891$). That is, the time course of the suppression was similar regardless of the particular location of the punch within the putamen. Planned follow-up questions regarding the durability of suppression were addressed by *T*-tests at the various time points, separately, as shown in **Figure 6a**. **Figure 6a** also shows that when the percent suppression is normalized by subtracting the averages of the PBS group (Group 1), none of the other groups' averages are significantly different from zero, and the average of Group 5 (24 days after cessation of siRNA delivery) trends below zero ($P = 0.079$, two-tailed) which would be indicative of a "rebound" effect with even greater *HTT* expression (above baseline) after the siRNA treatment is stopped. However, there is no evidence of such a rebound effect in the *HTT* suppression values using zero point defined by the common cDNA standard; the apparent increase in HTT expression is an artifact of the mathematical subtraction of the PBS group values.

To determine whether the decline in suppression with time is linear or curvilinear, the best-fitting linear regression line and the best-fitting parameter values for two curvilinear functions (concave up and concave down) were computed on a punch-location-by-punch-location basis. **Figure 6b** illustrates the shape of these three equations. Using the difference in percent suppression above the PBS group average at the corresponding punch location in the brain as the measure, this analysis yielded inconsistent and uninformative results. In 19 of 38 punch locations, the best-fitting relationship between time and suppression was linear, in 16 of 38 punch locations the best-fitting relationship was concave down, in 1 the best-fitting relationship was concave up, and in 2, the best-fitting relationship was none of these, but rather a paradoxical quadratic

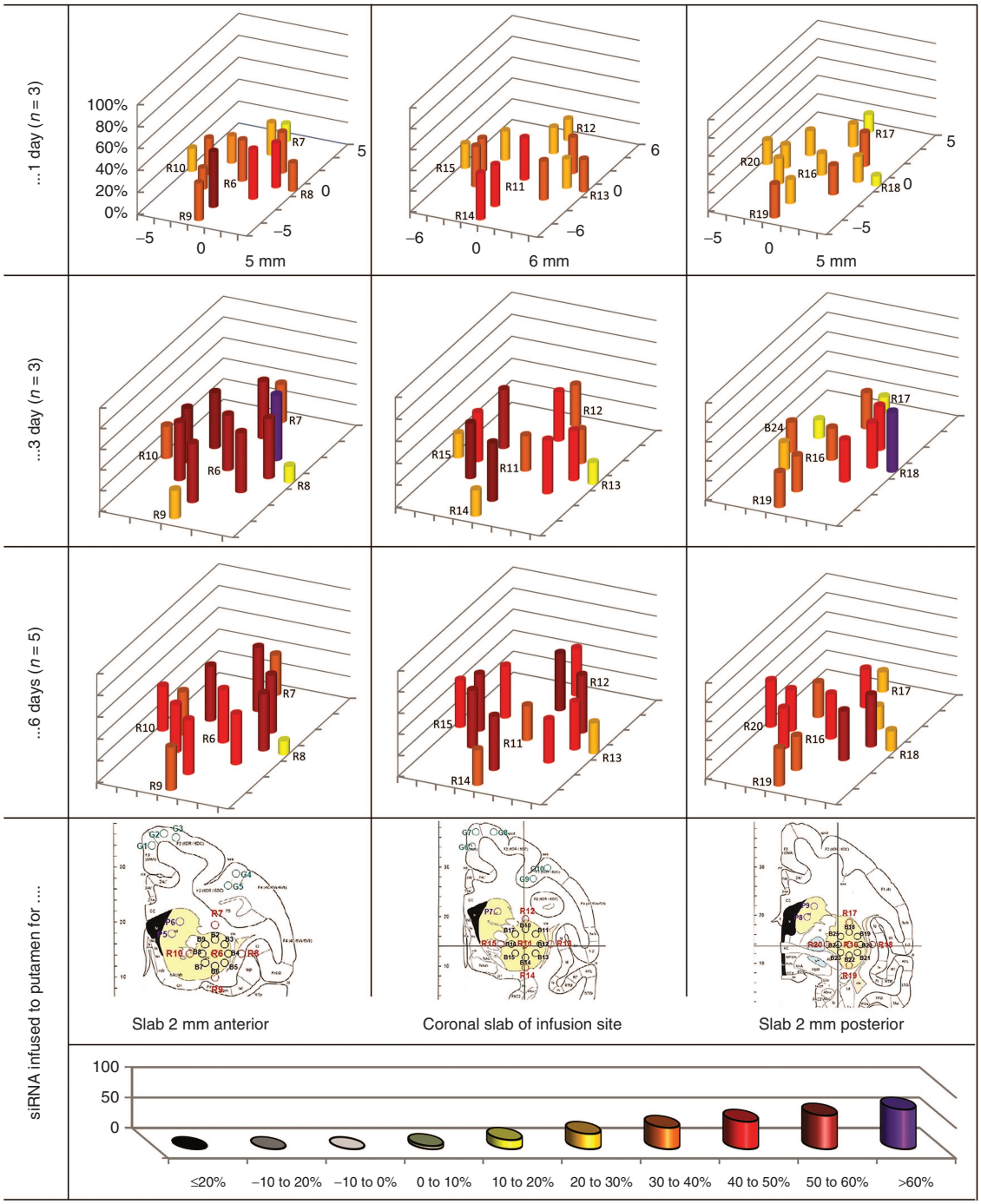

Figure 5 Suppression onset in the putamen. The average suppression of *HTT* mRNA measured in punches taken from the putamen in the coronal slab containing the infusion site and the 2-mm slabs anterior and posterior to that slab is shown for each of three time points.

(inverted U) relationship, with greater suppression in the group assayed 10 days after siRNA delivery cessation than in the groups assayed at 0 or 24 days after siRNA delivery cessation. Estimates across punch locations of the number of days from the cessation of siRNA administration to the point in time when the level of suppression would be back to zero could not be calculated using either the linear or concave down model (leading to a nonsensical –43.3 days in the linear case, and a negative logarithm in the concave down case).

Therefore, as with the onset characterization, the analysis to characterize duration of suppression was performed directly on the percent suppression over the zero point provided by the common cDNA standard. This analysis yielded consistent results. Regression analyses yielded a statistically significant coefficient for the linear term in the majority of punches (24 of the 38). A concave up (exponential decay) model is the best fit to the data in only 8 out of 38 punch locations (see **Supplementary Table S3**) and only 3 punch

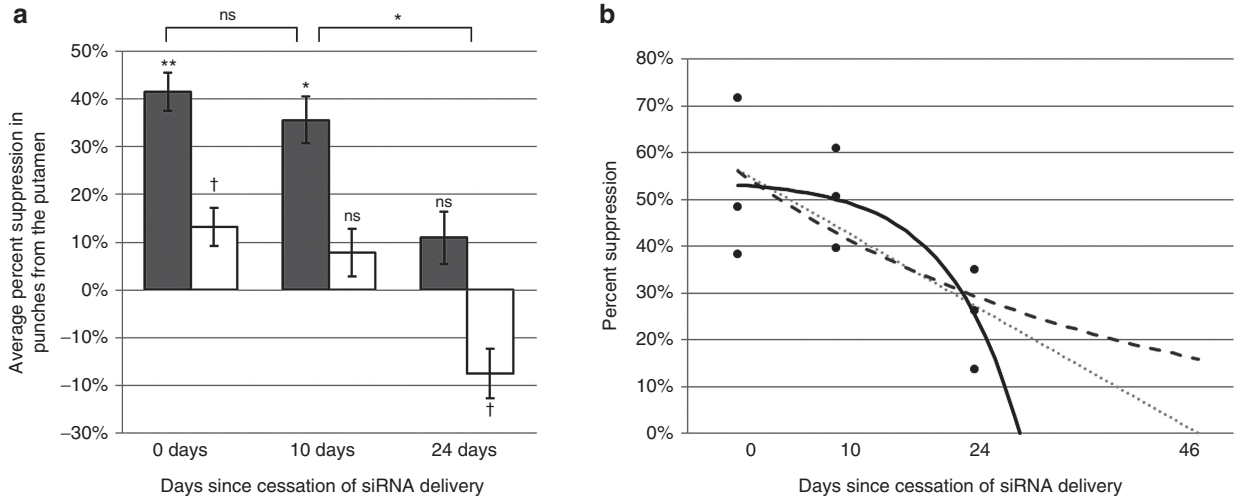

Figure 6 Suppression durability in the putamen. (**a**) Mean and standard error of suppression averaged over all tissue punches from the putamen in monkeys terminated 0, 10 or 24 days after siRNA delivery cessation (groups 3, 4, and 5, respectively). Gray-filled bars are the suppression values based on the zero point provided by the common cDNA standard; white bars are the suppression values after subtracting the corresponding phosphate-buffered saline Group1 average from the same tisue punch locations. $^{**}P < 0.01$ versus zero, $^{*}P < 0.05$ (Tukey *post-hoc* test), $^{\dagger}P < 0.10$ versus zero, two-tailed; ns = not significant. (**b**) Linear, concave up, and concave down curve fit to the decline of suppression measured in RNA from punch location B14 from the putamen of monkeys at 0, 10, and 24 days after cessation of siRNA delivery using the suppression values based on the zero point provided by the common cDNA standard. Dotted line: best linear fit, Supp = 56.34 + (–1.21 * day); gray-dashed curve: best concave up fit, Supp = 56.13 * exp(–0.027 * day); solid black curve: best concave down fit, Supp = 54.10 – (exp(0.14 * day)). The curve fits for each punch location in the putamen are provided in the **supplemental materials**.

locations yielded a statistically significant coefficient for any curvilinear term (concave up or down). Thus, the most parsimonious model for the decay of *HTT* mRNA suppression based on these data is a linear function of time, declining at the rate of about 1.4 percentage points per day. The average estimate (across punches) for the number of days from the cessation of siRNA administration to the point in time when the level of suppression would be back to zero using this linear model is 39.3 days (95% confidence interval = 31.4–47.1 days). A more conservative estimate using a concave down model is 27.3 days (95% confidence interval = 25.4–29.1 days).

A visualization of how the level of suppression of *HTT* mRNA in the various punch locations in putamen of the monkeys varied with time since the cessation of the siRNA delivery is provided in **Figure 7**. The same data (means ± SE) are presented in tabular form in **Supplementary Table S4**.

Discussion

We believe the data presented here are the first of their kind for siRNA administered to the nonhuman primate brain. Specifically, the data provide information about the time course of the onset and durability of siRNA-mediated suppression of a target gene such as *HTT* in the putamen. The results suggest that siRNA might be effectively delivered to a human patient's putamen via an episodic dosing paradigm as an alternative to continuous infusion.

Comparison of Magnevist and the siRNA infusion in this study showed that whether the siRNA had been delivered for 1 or 3 days, the Magnevist distribution was greater than the siRNA distribution (volume of siRNA tissue concentration previously found[7] to be equivalent to ≥45% suppression) that was later attained in the same animal. The Magnevist and siRNA distributions bore enough similarity that it may be reasonable to use Magnevist in the clinical setting as a surrogate for siRNA in terms of visualizing the *directions* of the anatomical spread of the siRNA, in addition to confirmation of catheter patency, system integrity, and fluid delivery into tissue. It is also possible that an MRI of Magnevist distribution taken sooner after infusion initiation would not yield as large and variable an overestimate of the siRNA distribution as was found here. However, pending further research, this could not be used clinically for an estimate of the extent of siRNA spread and the resulting extent of *HTT* suppression in a patient.

The three monkeys receiving PBS (Group 1, 3 days of Magnevist, followed by 7 days of PBS delivered at 0.1 ul/minute for Magnevist washout, followed by PBS at 0.3 µl/minute for 4 days) showed an unexpectedly low level of *HTT* mRNA normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression and compared to the zero point provided by the common cDNA standard (see Methods). This resulted in apparent *HTT* suppression by PBS alone. The reason for this result is unclear, and it is inconsistent with several previous studies of PBS versus ALN-HTT01 delivery to the rhesus putamen by this delivery system, including the results from five monkeys receiving PBS only in a prior study,[7] for which the average HTT mRNA suppression in the putamen, measured by the same technique and against the same standard used here, was 2.98 ± 4.47% (mean ± SD, $n = 5$). In any case, a significantly greater level of *HTT* suppression in the putamen was seen in the monkeys receiving the siRNA for 3 days, compared to this contemporaneous PBS group. However, for purposes of the meta-analysis to characterize the relationship between time and onset of HTT suppression,

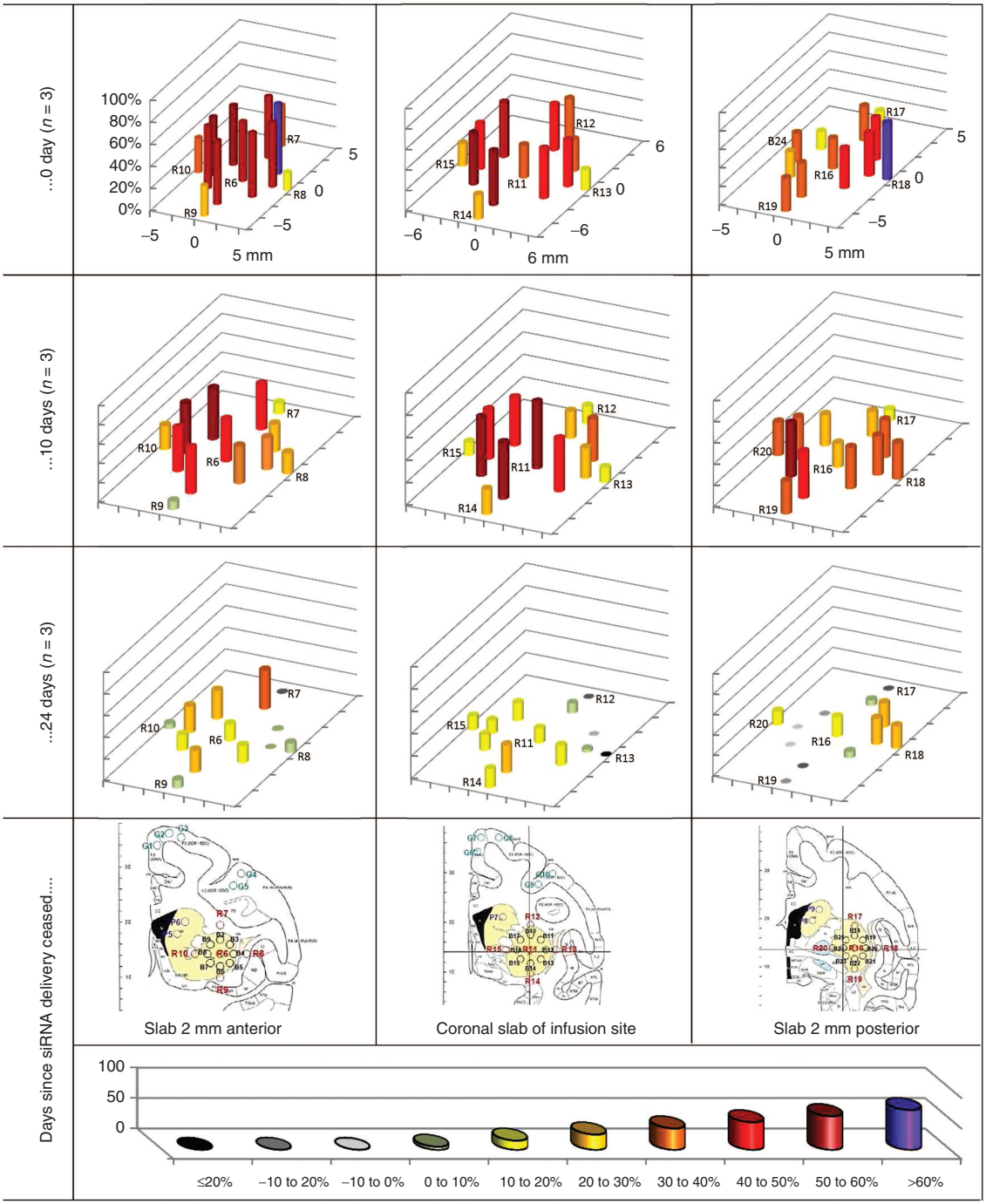

Figure 7 Suppression durability in the putamen. The average suppression of huntingtin mRNA measured in punches taken from the putamen in the coronal slab containing the infusion site, and the 2-mm slabs anterior and posterior to that slab is shown for each of three time points.

the unexpected low level of HTT in Group 1 made it impossible to identify a consistent relationship between time and HTT suppression if the data were analyzed as the difference in suppression over the PBS group. In contrast, direct analysis of the level of suppression against the zero point provided by the common cDNA standard yielded a consistent relationship and plausible estimate of the time to maximal suppression. In this meta-analysis of the data on a punch-by-punch basis (41 repetitions of the same analysis), 88% of the punch locations yielded a best-fitting mathematical equation of the concave down form, modeling the increase in suppression over time as reaching 97% of its asymptote within 3–4 days (3.22 ± 0.25 days, means ± SE; **Supplementary Table S1**). A risk of this analysis is that the suppression assessed may be a mixture of true siRNA effects (*i.e.*, RNA interference) plus other effects such as the effect of fluid infusion into the

putamen. However, to the extent that these other effects on *HTT* expression are reproducible, they may be part of the overall effect of the therapy in its clinical application.

Based on the meta-analysis, the median value of the asymptotic level of suppression calculated across all tissue punches in the putamen was 48.4% (**Supplementary Table S1**). In animal models of Huntington's disease, reduction of mutant *HTT* expression by ~45% to ~75% has been sufficient to produce phenotypic improvement in behavior as well as neuropathology.[3,4,9–12] For example, Harper *et al.*[9] found that reduction of the mutant *HTT* transcript in HD-N171-82Q mice by 51–55% led to significant improvements in gait deficits and rotarod performance. Kordasiewicz *et al.*[10] report that an antisense oligonucleotide that reduced the production of human mutant exon1 mRNA in the R6/2 mouse brain by 43 ± 5% was sufficient to block further brain weight loss and significantly extend the lifespan of these mice. In the primate study reported by Kordasiewicz *et al.*,[10] 4 mg of antisense oligonucleotide was delivered to the cerebrospinal fluid of rhesus monkeys per day for 21 days, with the result that *HTT* mRNA was suppressed by 53% (*i.e.*, to 47% of control levels) in the frontal cortex and by about 25% in the caudate nucleus, though the latter level was not statistically significant. Of course, whether this amount of suppression in treated tissue will be sufficient to achieve a therapeutic effect in patients affected by the human disease will only be determined by clinical trials. It appears likely that approaches to *HTT*-lowering might advance to first-in-human trials on the assumption that about 25–55% suppression of *HTT* mRNA and protein in the brain will be sufficient to have a therapeutic effect on brain tissue, with the hope that sufficient key regions of tissue can be treated to produce a beneficial outcome for the patient.

It is notable that 3 days versus 1 day of siRNA delivery to the brain resulted in a greater *level* of suppression of *HTT* in the treated tissue, but not in a greater *volume* of the putamen treated (see left endpoints of lines in **Figure 2**). This suggests that the *volume* of tissue treated may be a function of the rate of delivery of fluid to the brain from the infusion system rather than the number of days of delivery *per se*. Clinically, this could be controlled by how the pump is programmed.

A limitation of this study is that the time course of *HTT* suppression was measured across animals, rather than within the same animals over time. It is assumed but cannot be guaranteed that this time course reflects what will occur in an individual's brain over time. In addition, the level of suppression was measured in tissue punches, and not individual cells; it is likely that the level of suppression attained in individual cells or cell types is variable. Further research would be needed to characterize siRNA uptake and *HTT* suppression in various cell types, and the amount of mutant *HTT* suppression that is adequate for rescue of function in various cell types. Nevertheless, based on the tissue punches taken from the putamen of the animals, not only from the slab containing the infusion site, but also from multiple slabs anterior and posterior to the infusion, the study showed that the onset of *HTT* mRNA suppression resulting from the infused siRNA was fairly rapid.

Monkeys terminated at 10 or 24 days after cessation of siRNA delivery had insufficient autoradiography signals to allow for distribution analysis, suggesting that after delivery of siRNA is stopped, washout of siRNA to well below 0.65 mg per gram of tissue occurs in less than 10 days. However, significant suppression of *HTT* in the treated region of the brain persists at this time point. Based on tissue punches from throughout the putamen, the decline in the percent suppression of *HTT* upon cessation of delivery of the siRNA is slow, and most parsimoniously described as a linear function of time, taking about 33 days for the suppression to return to zero. An alternative, concave down curvilinear model for the decline in percent suppression of *HTT* also fits the data obtained in this study, but with less statistical confidence. This model predicts it would take about 25.9–26.8 days for suppression to return to zero upon cessation of siRNA delivery. Because it is much more common for biological processes to be nonlinear rather than linear in nature, it is tempting to assume that the nonlinear model is correct. A mechanistic explanation for what is happening at the molecular level may be that the siRNA molecules, once inside the cell (and associated with the RISC complex) undergo a time-dependent wearing out, such that the probability that any given molecule of siRNA will cease to suppress *HTT* mRNA is a steadily increasing function of how long that particular molecule of siRNA has been inside the cell. Such a time-dependent loss-of-function can yield a curvilinear decline in suppression overall for the population of molecules in a cell (**Supplementary Figure S2**). Given the limited number of time points in this study, we cannot distinguish between a linear and concave-down model for the decline. Pending further studies providing more time points, the most parsimonious course of action is to assume the linear model is the best description of the time course of the decline in siRNA-mediated suppression of *HTT* mRNA in the brain. It is notable, however, that the results of this study rule-out a concave-up, exponential decay model for the decline in suppression of *HTT* by the siRNA. Mechanistically, this means we can rule-out that the decline in suppression by the siRNA is governed by the physical washout of the siRNA from the brain.

This study supports the conclusion that 3–4 days of siRNA infusion into the brain is sufficient time to achieve the maximum suppression of *HTT* mRNA attainable by this siRNA. Computational modeling studies are underway to estimate the flow rate for the infusion that may yield optimal coverage of the HD patient's brain tissue; ideally, the maximum flow rate used in human patients can be higher than that used in the rhesus monkey. After 4 days, the flow rate would automatically be reduced to the minimum programmable rate of the infusion pump, and continue at this rate for 10–24 days. Then the process would repeat, resulting in a recurring cycle of 14 or 28 days in length. This episodic delivery pattern incorporating a high flow period may produce a therapeutic effect over a broader volume of brain tissue than produced by a constant infusion at a lower rate, while offering the advantages of slower depletion of siRNA from the drug pump reservoir, and avoidance of any potential problems that might arise from continuously delivering exogenous fluid into the brain at a relatively high rate.

In the report cited earlier, Kordasiewicz *et al.*[10] found evidence in mouse models of Huntington's disease that the beneficial effects of *HTT*-lowering on the animal's phenotype

out-lasted not only the delivery of the oligonucleotide and its presence in the brain, but even the time period for which *HTT* protein expression was reduced. This finding has led to the hypothesis that a "holiday" from the mutant *HTT* protein's effects on a neuron may be sufficient for a neuron to recover some of what has been, so far, its life-long ability to cope with mutant *HTT*'s toxic effects.[13] If so, periodic delivery of a *HTT*-lowering agent may have beneficial effects that are more stable than might otherwise be predicted from the direct effects on the agent on gene expression, such that the exact amount of time between doses may not be highly critical, at least in the case of Huntington's disease.

To the extent that both the distribution and uptake of the siRNA used in this study are representative of the distribution and uptake into brain tissue of siRNA that might be used against other disease targets, these results suggest that episodic delivery of siRNA may be useful in a many future therapies for neurological disorders.

Materials and methods

Brain infusions. Fifteen adult female rhesus *(Macaca mulatta*) monkeys were obtained from a commercial supplier (Covance, Alice, TX) and equally divided into five test groups. All animals were implanted with the intraparenchymal brain infusion hardware consisting of a Medtronic SynchroMed II infusion pump (model 8637-20, Medtronic, Minneapolis, MN), and investigational catheter system consisting of a sutureless pump catheter, cranial anchor and cranial catheter (Medtronic). All animal procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee and conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Surgical methods. For each animal, a series of presurgical T1-weighted and T2-weighted MR images was obtained prior to the surgery for screening and surgical planning. The preoperative MRI was read to confirm that there were no brain abnormalities, and to plan the stereotactic coordinates for the catheter implantation. On the day of surgery, an MRI was obtained postoperatively to verify that the infusion point (catheter needle tip) was within a 2 mm radius of the intended surgical target: the approximate center of the putamen at the rostrocaudal and lateral coordinates and 3 mm above the ventral border of the putamen at the vertical coordinate. A second postoperative MRI was conducted after 3 days of infusion of Magnevist (5 mmol/l, Bayer HealthCare Pharmaceuticals, Wayne, NJ) at a rate of 0.3 µl/minute to confirm catheter patency and to determine infusate distribution. All groups had a final MRI taken on the day of termination.

Briefly, the implant procedure was as follows. After induction of anesthesia, the animal was positioned in an MRI-compatible stereotaxic apparatus and the surgical sites prepped using aseptic techniques. Then, a burr hole was drilled through the skull under sterile field conditions at a location derived from the preoperative MRI used to plan the catheter trajectory. Using a micro manipulator, a cannula containing an obturator was lowered into the brain at a rate of 1 mm per minute to a depth of 7 mm above the target. Next, the obturator was removed from the cannula and a needle-tipped intraparenchymal infusion catheter (investigational device, Medtronic) containing a hollow stylet was lowered until its needle tip was at a depth corresponding to the planned target. Throughout this procedure, sterile saline was infused through the hollow stylet and catheter at a rate of 1 µl/minute to maintain catheter patency. The catheter was secured at the skull using a cranial anchor (investigational device, Medtronic) designed to prevent mechanical forces from the extra-cranial catheter tubing that connects to the abdominally implanted pump from being transmitted to the intracranial portion of the catheter. An infusion pump (SynchroMed II Model 8637-20, Medtronic) was filled with 5 mmol/l Magnevist, then implanted into a subcutaneous pocket in the animal's abdomen. A catheter tunneled subcutaneously from the pump's outlet port to the site of the cranial anchor was also filled with 5 mmol/l Magnevist and connected to the inlet port of the cranial anchor. The pump was programmed to deliver a single 100 µl bolus of fluid at a rate of 10 µl/minute for 10 minutes to ensure catheter patency, then the flow rate was reduced to a basal rate of 0.3 µl/minute for 3 days of Magnevist delivery. The animal was taken from surgery to MRI immediately postoperatively to confirm correct catheter placement and patency.

siRNA development. An siRNA molecule (Alnylam Pharmaceuticals, Cambridge, MA) that targets a region of *HTT* mRNA that is 100% homologous between rhesus and human was developed by first identifying potential target sequences and screening them by Alnylam-proprietary criteria for likely activity and specificity. Forty-two siRNA candidates were then screened *in vitro* in HeLa cells for activity against human *HTT*. Top candidates were further assessed for potency in Neuroscreen-1 and U87MG cell lines, and empirically tested in Cos7 cells to rule out inhibition of 17 possible off-target genes identified based on partial sequence match to the siRNA sequence. The lead candidate (ALN-HTT01, see Stiles *et al.*[7] for sequence) inhibits *HTT in vitro* with an IC_{50} of 56 pM, but does not attain 50% inhibition of any of the 17 off-target candidates when applied at 200 nmol/l (see **Supplementary Materials**). ALN-HTT01 was also tested for stability in cerebrospinal fluid (CSF) collected from individuals with normal *HTT* CAG repeat lengths and from both presymptomatic and symptomatic HD mutant gene carriers and patients. Greater than 80% recovery of the full-length antisense strand was obtained after 8 hours incubation in CSF at 37 °C independent of the clinical stage of the disease of the CSF donor (data not shown).

The siRNA used for this study is a radiolabeled version of ALN-HTT01. It was synthesized and purified according to standard procedures, but with the following modifications for radiolabeling. Specifically, a deoxythymidine phosphoramidite building block ^{14}C-labeled at C-4 was synthesized and coupled to a solid-support-bound, protected oligonucleotide precursor, followed by deprotection and anion-exchange HPLC purification of the radiolabeled antisense strand (AM Chemicals, LLC, Oceanside, CA). The radiolabeled antisense strand was then annealed to the unlabeled sense strand, and diluted in PBS. The *HTT* mRNA suppression activity of the radiolabeled siRNA was confirmed by an *in vitro* dose–response assay in HeLa cells to be essentially identical to

that of ALN-HTT01. The final specific activity of this ^{14}C-siHtt was 2.0 µCi/mg, and osmolarity and endotoxin levels were confirmed to be within acceptable levels.

siRNA delivery. Monkeys were infused with test articles (PBS, or 12 mg/ml siRNA in PBS) by transcutaneously refilling the abdominally implanted pump with the appropriate article on the planned days per the experimental design outlined in **Figure 1a**. The refill procedures were performed in the animals under anesthesia using aseptic techniques. First, the reservoir refill port was located by palpating the skin over the pump. Then, a sterile 22-gauge noncoring needle was inserted through the skin and the silicone septum of the port until the needle contacted the pump's internal reservoir needle stop. The existing contents of the reservoir were aspirated and discarded, then the reservoir was rinsed twice with 5 ml of test article. Upon final aspiration of the rinse volume, the reservoir was filled with 20 ml of test article and the refill needle was removed.

Tissue harvesting and processing. Animals were euthanized by transcardial perfusion of ice-cold saline under general anesthesia per University of Kentucky standard operating procedures. The unfixed brain was removed from the skull and cut into seven 2-mm thick coronal sections, with one section at the infusion site, and three additional sections anterior, and three posterior to the infusion site. The tissue was frozen, stored at –70 °C, and sent on dry ice to a contract laboratory (QPS LLC, Newark, Delaware) for processing, including sectioning for quantitative autoradiography and subsequent tissue punching. Briefly, the 2-mm thick brain slabs were secured with carboxymethylcellulose to a microtome plate, and sectioned just enough to produce a coronal section spanning the entire brain. Then, the next two 40-µm-thick sections were removed and used for autoradiography. Punches were then harvested from the remaining tissue at prespecified locations by pressing a Harris tissue punch (Harris Uni-Core 1.25 mm, American MasterTech, Lodi, CA) into the tissue to collect a tissue "core." The resulting tissue samples were placed in 1 ml of Trizol reagent (Life Technologies, Grand Island NY), and stored at –80 °C until later analyzed by RT-qPCR for *HTT* and GAPDH mRNA levels.

Autoradiography. The tissue sections for quantitative autoradiography were covered with plastic wrap and exposed to phosphorimaging screens. Carbon 14 radiolabeled glucose-spiked blood calibration standards were coexposed with all sections and the images were used for calibrating the image analysis software. The exposed screens were scanned using a Molecular Dynamics Typhoon 9410 Phosphor Imager (GE Healthcare Life Sciences, Pittsburgh, PA) and image calibration and concentration profile data were acquired as millicandellas per square millimeter. The calibrated standard curve enabled the conversion of the autoradiographic signal to units of microcuries per gram of tissue (µCi/g). The images were stacked and aligned in Amira software (version 5.4.5, FEI Visualization Sciences Group, Burlington, MA) in a fashion resembling an MRI image stack. Using this stack, the approximate "volume of ≥45% suppression" was calculated for each monkey by identifying the area within each slice where the tissue concentration was greater than 0.65 mg of siRNA per gram of tissue. This tissue concentration has been previously determined[7] to correspond to 45% suppression of *HTT* mRNA, used as a target value for potential clinical effect. The labeled area in each slice was multiplied by 2 mm (the distance between successive slices) to convert area to volume. The total volume of ≥45% suppression for each monkey is the sum of the individual slice volumes.

RNA isolation. Tissue punch samples were homogenized in a TissueLyser (Qiagen, Valencia, CA) then total RNA was isolated from each sample using the RNeasy Lipid Tissue Kit (Qiagen) according to the manufacturer's protocol. The optional on-column DNase I digestion step of the manufacturer's protocol was performed to remove any genomic DNA contamination. RNA was eluted twice in 40 µl of nuclease-free water (final elution volume of 80 µl) to maximize the total RNA yield. Following elution, a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) was used to determine the RNA concentration and to measure the ratio of absorbance at wavelengths of 260 and 280 nm (A260/A280 ratio), a standard means of assessing RNA sample purity. The integrity and quality of the RNA was further characterized using an Agilent (Palo Alto, CA) 2100 Bioanalyzer. Analysis was performed using the Agilent RNA Pico 6000 kit. The RNA integrity number and associated electropherogram for each sample was used to assess RNA integrity and quality. To be included in the final analysis, the RNA sample from a given tissue punch was required to have an RNA integrity number (indicator of lack of degradation) equal to 6.0 or greater, and an A260/A280 ratio (indication of purity) equal to 1.6 or greater. Out of 1,049 tissue punches, 5 were eliminated due to failure to meet the integrity criterion, and 21 more were eliminated due to failure to meet the purity criterion.

Measurement of Huntingtin mRNA suppression. For cDNA synthesis, 200 ng of total RNA from a given tissue punch was used in a reverse transcription reaction (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA) following the manufacturer's protocol. For the measurement of *HTT* mRNA, a custom TaqMan assay specific for rhesus monkey *HTT* consisting of a forward primer (ggcagcttcggagtgaca), reverse primer (tgctgtgtatgatgtaacgtc agtt) and monkey *HTT* Taqman probe (cagcttgtccaggtttat) was used. An assay specific for rhesus monkey GAPDH (Assay ID Rh02621745_g1) was validated by and purchased from Applied Biosystems and served as the endogenous control in the experiments performed.

All cDNA samples were run in triplicate reactions for both *HTT* and GAPDH. Both "no reverse transcriptase" and "no cDNA template" controls were assayed in parallel to the monkey tissue samples to insure that there had been no DNA contamination of either the cDNA synthesis or the amplification steps. To allow meaningful comparison of PCR results across sample plates and runs, a cDNA standard was included on all plates. This standard was prepared by pooling total RNA isolated from three tissue punches harvested from the putamen of a surgically naive rhesus monkey, then converting that RNA to cDNA. Runs were analyzed setting the PCR cycle threshold values to a constant 0.55 for the target (*HTT*) and a constant 0.53 for GAPDH across all plates and runs.

For a given tissue punch to be included in the final data analysis, the PCR results for three replicate reactions for its RNA

sample were required to be internally consistent. Specifically, the *HTT* cycle thresholds for the replicates were required to have a standard deviation equal to or less than 0.1, and coefficient of variance (CV, standard deviation divided by the mean) equal to or less than 1 percent. Similarly, the standard deviation and CV for GAPDH cycle thresholds were required to meet the same criteria. In addition, the cycle threshold for the GAPDH analysis was required to be no more than 10% higher than the average cycle threshold for GAPDH for all samples on the same analysis plate. Failure to meet this requirement would indicate that an excessively low amount of cDNA was present for the reaction to yield a reliable result; a similar criterion did not apply to the cycle threshold for *HTT*, since a low amount of *HTT* could be a valid indicator of a high degree of *HTT* suppression. Failure to meet these acceptance criteria resulted in elimination of 53 (out of 1,049 total) tissue punches from the final analysis. Three more punches were eliminated because a determination of cycle thresholds for either *HTT* or GAPDH was not possible, and two more were eliminated because the resulting calculation of percent *HTT* suppression yielded implausible results (–720.4 and –2,197.9% suppression, respectively). The rate of tissue punch acceptance into the final analysis was comparable across the experimental groups: 197/211 (93.4%), 192/205 (93.7%), 194/212 (91.5%), 196/215 (91.2%), and 186/206 (90.3%) for groups 1 through 5, respectively (chi-square test for differences = 2.39, $P = 0.66$).

To control for any difference in cDNA loading, all *HTT* expression data were normalized to the endogenous control specific for rhesus monkey GAPDH. To control for any reaction efficiency differences across assay plates or runs, the normalized HTT expression was also adjusted by subtracting out the delta Ct obtained for the cDNA standard that was included in triplicate on every plate. Note that either order for these two steps is equivalent (algebraically identical). The steps of the $\Delta\Delta C_t$ method of relative quantification[14] are as follows.

ΔC_t = (average *HTT* C_t – average GAPDH C_t)
$\Delta\Delta C_t$ = average ΔC_t – (average ΔC_t of the replicates of the cDNA standard)
2 exp(– ($\Delta\Delta C_t$)) = normalized *HTT* amount standardized to the common plate-to-plate sample.
% Suppression = (1 – (2 exp(– ($\Delta\Delta C_t$)))) × 100

Finally, for ANOVAs and other significance tests of group differences, the percent suppression of *HTT* was further normalized by subtracting the average percent suppression for the corresponding PBS control group (group 1 in **Figure 1**), or the contemporaneous PBS group for the monkeys receiving siRNA for 1+6 days (in the meta-analysis of suppression onset). The computation of these averages and their subtraction from the percent suppression was done on a punch location-by-punch location basis. However, for the mathematical modeling of onset time and the asymptote of percent suppression attainable with this siRNA, the percent suppression was used directly, rather than the difference obtained after subtracting the average of the corresponding PBS group. The reason for this is described in the results section, and commented upon in the discussion section.

Measurements of *HTT* suppression from tissue punches spanning 71 anatomical locations were available for analysis.

Modeling of the onset of HTT suppression. The time required for the siRNA to reach the brain from the SynchroMed II pump reservoir when delivered at a rate of 0.3 µl/minute following prior infusion of PBS into the brain is 23.29 hours, given a typical pump catheter and cranial catheter length of 570 and 30 mm, respectively, resulting in 419.2 µl of "dead space" in the system. Therefore, the groups in the study that received siRNA for 2, 4, (or 7) days prior to termination were deemed to be time points 1, 3, (and 6) days, respectively, for analysis (or meta-analysis) of the onset of suppression upon siRNA delivery to the brain tissue. For purposes of mathematical modeling, the level of suppression at time point zero was considered to be zero percent, by definition. The overall approach used for the statistical analysis was first to conduct a mixed-model analysis of variance (ANOVA) of the PBS-normalized data using experiment group (time point) as a between-monkey factor, and punch location as a within-monkey factor, because each monkey contributed suppression data from multiple anatomical sites. The purpose of this analysis was to verify that some true differences in *HTT* suppression among groups and/or punch locations are present in the data set, justifying proceeding to subanalyses for specific anatomical locations and groups. Next, data from each anatomical region of the brain (putamen, caudate, cerebral cortex, and white matter structures) were analyzed separately by ANOVA. Finally, only for those regions of the brain in which statistically significant differences in *HTT* suppression among groups were found, one or two group planned t-tests were used to answer the following questions regarding the onset of *HTT* suppression: (i) does suppression differ from zero after 1 day of siRNA exposure? (ii) does suppression differ from zero after 3 days of siRNA exposure? (iii) is there greater suppression after 3 days than after 1 day of siRNA exposure?, and (iv) is there greater suppression after 6 days than after 3 days of exposure?

A further meta-analysis was performed (on the percent suppression data without subtracting the PBS average) to determine whether the onset of suppression in the treated monkeys as a function of time is linear or curvilinear, and if curvilinear, whether the best fitting curve is one that is sigmoidal, or concave down. The equations chosen for this purpose were as follows:

Linear:	$\%\text{Suppression} = A + (B * \text{day})$
Sigmoidal:	$\%\text{Suppression} = A * e^{-e(B-C * \text{day})}$
Concave down:	$\%\text{Suppression} = A * (1-e^{-B * \text{day}})$

where day equals 1, 3, or 6. The analyses were performed using Minitab 16 (Microsoft, Redmond, WA) which solves for parameters A, B, and C in the respective equations by finding the set of values that minimizes the sum of squared deviations of the observed data from the values predicted by the equation. The sum of squared deviations for the best linear, sigmoidal and concave down curve fit was used to compare the goodness of fit of the data to the equations.

Modeling of the duration of HTT suppression. The analysis of the time course of the duration of *HTT* suppression, and the nature of the rate of decline in suppression over time, involved comparison of *HTT* suppression across three groups of monkeys

all from the present study. The first group was the group (Group 3, $n = 3$) that received siRNA for 4 days prior to termination (also used as time point 3 in the onset analysis, based on the time required for the siRNA to reach the brain from the pump, as described above). The second and third groups were those that received siRNA for 4 days, at which point their pumps were completely turned off, and who then were terminated 10 days (Group 4, $n = 3$) or 24 days (Group 5, $n = 3$) later. For mathematical modeling of the decline in suppression, these were considered to be time points 0, 10, and 24 days, respectively.

As with the analysis of onset, the overall approach was to first conduct a mixed-model ANOVA using experiment group (time point) as a between-monkey factor and punch location as a within-monkey factor to verify that some true differences in *HTT* suppression among groups and/or punch locations were present in the data set, justifying proceeding to subanalyses for specific anatomical locations and groups. Next, data from each anatomical region of the brain (putamen, caudate, cerebral cortex, and white matter structures) were analyzed separately by ANOVA. Finally, only for those regions of the brain in which statistically significant differences in *HTT* suppression among groups were found, one or two group planned *t*-tests were used to answer the following questions regarding the durability of *HTT* suppression: (i) does suppression differ from zero after three days of siRNA exposure (the starting point of the durability analysis)? (ii) does suppression still differ from zero 10 days after cessation of siRNA delivery? (iii) does suppression still differ from zero 24 days after cessation of siRNA delivery? (iv) is the decline in the amount of suppression with time linear or curvilinear, and if curvilinear, is the best-fitting curve one that is concave down, or concave up? The equations for these curve fits were as follows:

Linear:	$\%\text{Suppression} = A + (B * \text{day})$
Concave up (exponential decay):	$\%\text{Suppression} = A * e^{(-B * \text{day})}$
Concave down:	$\%\text{Suppression} = A - e^{(-B * \text{day})}$

where day equals 0, 10, or 24. *A* and *B* are parameters found by Minitab by solving for the set of values that most minimizes the sum of squared deviations of the observed data from the values predicted by the equation. The sum of squared deviations for the best linear, concave up (exponential decay) and concave down functions was used to compare the goodness of fit of the data to these functions.

Supplementary material

Figure S1. Diagrams of tissue punch locations.
Figure S2. Mathematically defined curves illustrating different hypothetical models of siRNA efficacy decline.
Table S1. Suppression onset in the putamen – curve fits to data by punch location.
Table S2. Suppression onset in the putamen. Percent suppression of huntingtin mRNA, means and standard errors by punch locations and groups.
Table S3. Suppression durability in the putamen – curve fits to data by punch location.
Table S4. Suppression durability in the putamen: Percent suppression of huntingtin mRNA, means and standard errors by punch locations and groups.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)