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Focused ultrasound for targeted delivery of siRNA and efficient knockdown of *Htt* expression

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Abstract

RNA interference is a promising strategy for treatment of Huntington's disease (HD) as it can specifically decrease the expression of the mutant Huntingtin protein (Htt). However, siRNA does not cross the blood-brain barrier and therefore delivery to the brain is limited to direct CNS delivery. Non-invasive delivery of siRNA through the blood-brain barrier (BBB) would be a significant advantage for translating this therapy to HD patients. Focused ultrasound (FUS), combined with intravascular delivery of microbubble contrast agent, was used to locally and transiently disrupt the BBB in the right striatum of adult rats. 48 hrs following treatment with siRNA, the right (treated) and left (control) striatum was dissected and analyzed for Htt mRNA levels. We demonstrate that FUS can non-invasively deliver siRNA-Htt directly to the striatum leading to a significant reduction of Htt expression in a dose dependent manner. Furthermore, we show that reduction of Htt with siRNA-Htt was greater when the extent of BBB disruption was increased. This study demonstrates that siRNA treatment for knockdown of mutant Htt is feasible without the surgical intervention previously required for direct delivery to the brain.

Keywords

Focused ultrasound; siRNA; drug delivery; Huntington's disease; blood-brain barrier

Introduction

Huntington's disease (HD) is a progressive neurodegenerative disease caused by an expansion of a CAG repeat in the Huntingtin (Htt) gene [1]. The mutation causes a gain of function of the Htt protein resulting in neuronal dysfunction and cell death, leading to the involuntary movements, cognitive dysfunction, and behavioral changes characteristic of HD.

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Although the disease is monogenic and the etiology is known, there is no current treatment [2]. In a conditional mouse model of HD, it was observed that silencing the mutant Htt transgene using tetracycline reversed or halted the progression of the disease [3]. This research indicated that therapeutic approaches to reduce levels of Htt might be an effective strategy to treat HD [2]. Since then, it has been shown that short hairpin RNA (shRNA) delivered by viral vectors leads to a decrease in Htt protein and associated pathology [4,5]; however, shRNAs can be toxic when integrated into the genome.

A similar approach is to use small interfering RNA (siRNA) to suppress expression of the Htt gene. Conjugation of bioactive siRNAs to cholesterol is known to improve cellular uptake of siRNA *in vivo* [6,7]. Delivery of cholesterol-conjugated (cc) siRNA against Htt reversed pathology in a HD mouse model [7]. In that study, cc-siRNA-Htt was injected directly into the striatum and was found to decrease Htt protein expression, improve motor function and increase neuronal survival. In a recent study in non-human primates, unconjugated siRNA targeting Htt, administered by continuous infusion into the putamen, was effective in reducing Htt expression throughout most of the striatum [8].

siRNA has been administered into the cerebral ventricles and the brain parenchyma and was shown to be safe and effective in mice and non-human primates [9,10]. However, systemic delivery of siRNA able to pass through the blood-brain barrier (BBB) would be useful for treating HD, since invasive surgical intervention could be avoided.

In this study, we investigate the potential of MRI-guided focused ultrasound (MRIGFUS) to non-invasively deliver cc-siRNA-Htt directly to the striatum. Advances in FUS technology have been used to transiently increase the permeability of the BBB, allowing agents to cross from the blood stream to the brain [11]. FUS applies concentrated acoustic energy to a focal spot measuring a few millimeters in diameter. A microbubble contrast agent is administered systemically and when FUS is applied transcranially to the target location, the circulating microbubbles begin to oscillate. This leads to mechanical changes in the blood vessel wall and a transient increase in the permeability of the BBB [11]. Previous work has shown that transient changes in BBB permeability induced by FUS, permits entry of chemotherapeutics and therapeutic antibodies to targeted areas of the brain [12–15].

Here, we performed a proof of principle study to address whether MRIGFUS could be an effective delivery method of siRNA to the striatum in patients with HD. We demonstrate that the striatum can be accurately targeted by MRIGFUS in rats. We also show that the siRNA is biologically active *in vivo* after delivery with MRIGFUS. Administration of cc-siRNA-Htt led to a significant reduction of Htt expression in a dose dependent manner with no reduction observed using control siRNA. Furthermore, we demonstrated that Htt knockdown is correlated with the amount of contrast enhancement observed on T1 weighted (T1w) MR images. Together, these results suggest that MRIGFUS is an effective tool for non-invasive delivery of siRNA and should be investigated as a treatment of HD.

Material and Methods

siRNA preparation

All single-stranded RNAs were synthesized at Alnylam Pharmaceuticals and were characterized by ESI-MS and anion exchange HPLC. Single-stranded RNAs were synthesized as 19-mers with deoxythymidine (dT) overhangs including 2'-O-Me substitutions on a subset of internal nucleotides, and phosphorothioate (s) linkages to provide stabilization against nucleases. To generate siRNAs from RNA single strands, equimolar amounts of complementary sense and antisense strands were annealed. The sequences for siRNA-Htt [8] and siRNA-LUC [16] have been previously published.

Animals

52 Wistar rats (300–350g) were received from Charles River Laboratories (Sherbrooke QC, Canada). All experimental procedures were approved by the Sunnybrook Research Institute Animal Care and Use Committee and conformed to the guidelines set by the Animals for Research Act of Ontario and the Canadian Council on Animal Care

MRlgFUS

Animals were anesthetized with ketamine [50 mg/kg] and xylazine [10 mg/kg] and depilatory cream was used to remove hair from the head. Animals were placed supine on a custom-built FUS positioning system [17], heads were coupled to a degassed water bag and the entire system was placed in the 1.5T MR scanner (Signia, GE Healthcare, Milwaukee, WI). Baseline T1- (TE= 10 ms, TR=500 ms) and T2-weighted (TE=60 ms, TR=2000 ms) images were acquired. The T2w images, combined with a stereotaxic atlas, were used to choose a square of 4 precise target points with 1.5 mm spacing, in the right striatum (Figure 1). Animals were placed into 4 groups: 1) control rats which received intravenous saline injection only (n=6), 2) vehicle controls which received intravenous injection of cc-siRNA-LUC (n=5), 3) treatment rats which received intravenous injection of cc-siRNA-Htt [0–100mg/kg] (n=4–6 per dose) and 4) carotid rats which received intra-carotid injection of cc-siRNA-Htt [50mg/kg] (n=5). In all cases, cc-siRNA-Htt was injected through a tail vein catheter or intra-carotid catheter, followed by injection of Definity® microbubble contrast agent (0.02 ml/kg, Lantheus Medical, N. Billerica, MA) immediately prior to sonications. Sonications were completed using a 558 kHz transducer (0.3 MPa estimated *in situ* pressure, 10 ms bursts, 1 Hz pulse repetition frequency, 120 s total exposure duration). Following sonications, gadolinium contrast agent (0.2 ml/kg; Omniscan, GE Healthcare, Milwaukee, WI) was injected through the tail vein followed by a 0.5 ml saline flush. Contrast enhanced T1w images were used to confirm BBB opening with FUS. Animals were maintained under anesthetic and were sonicated a second time using the same parameters, 1 hr following the first sonication. A second injection of siRNA was administered just prior to the second sonication. Animals were sacrificed 48 hrs after the second sonication.

Branched DNA (bdNA) analysis

After sacrifice, the left and right striatum were dissected from the brain and snap frozen on dry ice. The QuantiGene assay (Affymetrix, Santa Clara, CA) was used to quantify mRNA

levels in tissue sample lysates according to the manufacturer's instructions. Htt mRNA levels were normalized to GAPDH mRNA levels and then further normalized to control animals.

Results

MRlgFUS disruption of the BBB effectively delivers cc-siRNA-Htt to the striatum

T2w images, combined with a rat stereotaxic atlas, were used to precisely target a 1 mm square region in the striatum of the right hemisphere (Figure 1A,B). siRNA was injected through the tail vein immediately prior to sonication. Following FUS, BBB opening was confirmed by injecting MR contrast agent through the tail vein and acquiring contrast-enhanced T1w images (Figure 1C). The post-sonication T1w images demonstrate that the BBB was disrupted in the targeted striatum confirming that the targeting was accurate and that siRNA was delivered to the intended region.

Cholesterol conjugation has been shown to improve cellular uptake of siRNA in hepatocytes [6] and neurons [7]. Therefore, we synthesized cholesterol conjugated (cc) siRNA to silence Htt (cc-siRNA-Htt) and luciferase (cc-siRNA-LUC), the latter to be used as a control. cc-siRNA was delivered to the right striatum brain through 2 separate sonications. bDNA analysis, performed 48 hrs later, showed that cc-siRNA-Htt significantly decreased Htt bDNA (* $p < 0.05$; Figure 2) whereas the cc-siRNA-LUC had no effect on Htt bDNA levels. These results confirm the specificity of cc-siRNA-Htt to the Htt mRNA. Furthermore these results clearly demonstrate that MRlgFUS is an effective method for delivery of bioactive siRNA to the striatum.

cc-siRNA-Htt decreases Htt bDNA in a dose dependent manner

We delivered various doses of siRNA to determine the achievable level of Htt reduction *in vivo*. Immediately prior to BBB disruption, various concentrations of cc-siRNA-Htt (5, 12.5, 25, 50, and 100 mg/kg) were administered through the tail vein and the animals were survived for 48 hrs. bDNA analysis showed a dose dependent correlation between decreases in Htt bDNA and the concentration of cc-siRNA-Htt at doses of 5, 12.5 and 25 mg/kg (Figure 3). At 25 mg/kg, levels of Htt were decreased by 32% (* $p < 0.05$). When larger doses of siRNA (50 and 100 mg/kg) were administered, the decrease in Htt levels was not significantly different from 25 mg/kg (Figure 3) suggesting that the maximal reduction of Htt using this system is ~35%. However, it has been suggested that a modest reduction in Htt function can lead to a substantial improvement in disease symptoms [18].

Improved BBB opening is correlated to improved effect of cc-siRNA-Htt in vivo

We examined whether we could increase the amount of cc-siRNA-Htt entering the brain on the first pass by administering the siRNA through the carotid artery. In these animals, siRNA was administered in the carotid simultaneous to the injection of microbubbles through the tail vein. Our results showed that carotid delivery did not increase Htt knockdown compared to delivery of siRNA in the tail vein (data not shown).

We next compared the amount of Htt knockdown to the extent of BBB opening as determined by measuring the intensity of the enhancement in the right (sonicated) striatum and the left (non-sonicated) striatum in post-treatment MR images. The relative enhancement ranged from 12–58% (Table 1). We plotted the % enhancement against the amount of Htt mRNA as expressed as a percentage of the control and found that enhancement and Htt knockdown were positively correlated. The extent of Htt knockdown was found to be positively correlated to the extent of enhancement ($r^2=0.51$; Figure 4A). When the data was further separated into groups with <20% enhancement and >20% enhancement, the positive relationship between enhancement and Htt knockdown was confirmed (Figure 4B).

Discussion

Reducing the level of the mutant Htt in patients with HD represents the ultimate and most direct approach to treatment. Delivery of siRNA is a plausible option for reducing Htt expression and studies using siRNA in animal models of HD have shown improvements in disease outcome [4,7]. One major concern for this treatment has been the delivery of siRNA to the brain without the use of toxic transfection agents or surgery [18]. In this study, we demonstrate that MRIGFUS can non-invasively and effectively deliver siRNA to the striatum leading to a decrease in Htt gene expression.

We demonstrate a significant decrease in Htt expression following delivery of cc-siRNA-Htt to the striatum with MRIGFUS. We observed a 32% decrease in Htt mRNA which is similar to decreases observed in other studies [5]. It is typical for siRNA to only partially reduce the expression of endogenous neural targets, sometimes by as little as 10–20%. However, modest reductions in Htt mRNA have been shown to provide therapeutic benefit in animal models of Huntington's disease. From RNAi studies reported to-date using direct viral delivery of shRNA to the CNS in animal models of Huntington's disease, Htt mRNA suppression of approximately 45% or greater has resulted in meaningful normalization of neuropathology and behavior [4]. These details have been added to the discussion.

We did not observe a dose-dependent relationship between cc-siRNA-Htt concentration and reduction in Htt DNA beyond 25 mg/kg. This may be attributable to two different mechanisms. First, the nature of BBB disruption with MRIGFUS is transient [11,20]; thus, it is possible that the BBB repairs itself quickly, before enough siRNA can enter the brain. Our results showed that the extent of Htt knockdown was related to the amount of BBB disruption, suggesting that significant BBB disruption is required for cc-siRNA to enter the striatum. However, in similar studies, increased BBB permeability persisted for 6–24 hours [20, 21] and the half-life of cc-siRNA in circulation is expanded to ~95 min [6] suggesting adequate time for the siRNA to cross into the brain. Second, it is possible that this limitation is due to the molecular target and the ability to silence it completely. Regardless of the mechanism, the apparent limitation of a ~30% reduction of Htt may actually be ideal for treatment in the clinical setting. The optimal siRNA for treatment of HD would specifically target the mutant form of Htt but this has proved to be challenging. Recent results suggest that specificity for mutant Htt over wild-type Htt can be achieved *in vitro* [22,23], though with relatively high (nanomolar) concentrations, in contrast to the picomolar potencies

typically achieved with siRNAs *in vitro*. For this reason, our group and others have generated siRNA which result in decreased expression of both the mutant and the wildtype forms of Htt. The suppression of mutant Htt would have to be finely balanced with the suppression of wildtype Htt which is known to be important for neuronal development and survival [24,25]. Based on previous literature, we hypothesize that a 30% reduction in wildtype Htt as shown in our experiments would be well tolerated in the long term [26,27]; however, further investigations would be necessary to ensure the treatment would be suitable for clinical application.

Our evidence suggests that uptake of siRNA in the striatum was not correlated to the route of delivery. Contrary to our initial belief that more siRNA would be available to move through the BBB on its first pass through the vasculature and when the paracellular spaces are greatest, delivery of siRNA through the carotid artery did not lead to significantly more Htt knockdown than siRNA delivered through the tail vein. However, we did find a correlation between the amount of contrast enhancement on post-treatment images and Htt knockdown suggesting that the success of the siRNA was directly related to how much BBB disruption was achieved. Several routes of passage through the BBB have been described [28,29]. It is likely that the cc-siRNA moves paracellularly, through the widened tight junctions, into the brain, however, passage via transcellular mechanisms which peak approximately 1 hr after sonication [30], is also possible due to the extended half-life of the cc-siRNA [6].

There are clear advantages to using MRIgFUS for delivery of siRNAs to the brains of HD patients. First, a major limitation in HD research thus far has been adequate distribution of the compound to brain areas in sufficient quantities to observe a meaningful effect [31]. Using MRIgFUS, we have demonstrated that we can deliver siRNA to the small region of the striatum. Our mRNA analysis clearly demonstrates that sufficient quantities of siRNA can be delivered to the brain and that FUS itself does not impact the function of the siRNA. In future, combination of the cc-siRNA-Htt with the microbubbles may improve drug availability in the tissue similar to previous studies [34, 35]. BBB disruption with FUS has repeatedly been shown to occur with no or only minor red blood cell extravasation [34,35] and without impacting the efficacy of the delivered drug [15, 36]. Secondly, the effects of siRNA *in vivo* can persist for as long as 2–4 weeks [18]; however, treatment for HD will require intervention over many years. MRIgFUS provides a unique treatment option in this regard as the therapy is non-invasive and can be repeated safely multiple times [37].

Systemic administration of unmodified siRNA has been shown to stimulate the innate immune system leading to cytokine production [38]. The extent to which immunological pathways are activated is dependent upon the sequence, structure and quality of the siRNA. The 2'-O-methyl modifications, as used in the siRNA here, have been demonstrated to eliminate immune stimulation of siRNA's as a class, without compromising silencing activity [18]. Using siRNA with similar chemical modifications, DiFiglia and colleagues [7] evaluated reactive microglia and astrocytes and determined that there was no measurable inflammatory response following injection of siRNA into the striatum. Future studies will focus on a comprehensive analysis of CNS immunostimulation after siRNA administration using MRIgFUS. Nonetheless, the current study suggests that siRNA administration using

MRIgFUS represents a promising therapeutic approach that warrants further investigation for the treatment of CNS disorders such as HD.

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Figure 1. MRIgFUS disrupts the BBB in the right striatum

The right striatum (red x) was targeted by registering stereotaxic atlas images (A) with T2-weighted images of the rat brain (B). BBB opening was confirmed using contrast-enhanced T1-weighted images (C). Hyperintense regions on the images are indicative of the presence of contrast agent in the right striatum following effective BBB disruption.

Figure 2. cc-siRNA-Htt causes Htt knockdown *in vivo*.

Cholesterol conjugated (cc)-siRNA was generated to silence Htt (cc-siRNA-Htt) and luciferase (cc-siRNA-Luc) as a control. bDNA analysis, performed 48 hrs later, showed that cc-Htt significantly decreased Htt bDNA (* $p < 0.05$) whereas the cc-Luc had no effect on Htt levels *in vivo*. Data are representative of $n=5$ animals per group.

Figure 3. cc-siRNA-Htt decreases Htt in a dose dependent manner

Various doses (5, 12.5, 25, 50, and 100 mg/kg) of cc-siRNA-Htt were administered through the tail vein and the animals were survived for 48 hrs. Analysis showed a dose dependent correlation between decreases in Htt and the concentration of cc-siRNA-Htt at doses of 5, 12.5 and 25 mg/kg. At 25 mg/kg, levels of Htt were decreased by 32% (* $p < 0.05$). When larger doses of siRNA (50 and 100mg/kg) were administered, the decrease in Htt levels was not significantly different from 25 mg/kg. Data are representative of $n=4-6$ animals per group.

Figure 4. cc-siRNA-Htt effectiveness is correlated with enhancement levels

A) The % enhancement as measured in contrast enhanced T1-weight images was plotted against the amount of Htt (expressed as a % of the control). A positive correlation was observed ($r^2=0.51$) indicating that greater Htt knockdown is found in animals in which greater levels of enhancement was observed. **B)** The data was binned into groups with <20% enhancement and >20% enhancement. At all doses of cc-siRNA-Htt, more knockdown was observed when the enhancement was greater than 20%.

Table 1
Extent of BBB opening measured by MRI contrast enhancement

The extent of BBB opening was determined by measuring the intensity of the enhancement in the right (sonicated) striatum and the left (non-sonicated) striatum in post-treatment MR images. The relative enhancement ranged from 12–58%. The values are representative of n=6 animals per group.

Drug (mg/kg)	Relative Enhancement	% Htt mRNA
5	19.0±3.6	102.0±4.9
12.5	29.1±14.0	91.8±12.9
25	33.3±4.5	68.8±4.9
50	29.4±13.6	68.4±6.2
100	37.3±7.5	67.8±14.5