Knockdown of Liver-Derived C1 Inhibitor Results in Brain Pathology in mice

Running title: C1INH, the gatekeeper of the CNS

Dorit Farfara[1], Emily Feierman[1], Allison Richards[1], Alexey S. Revenko[2], Robert A. MacLeod[2], Erin H. Norris[1] and Sidney Strickland[1]

[1]Patricia and John Rosenwald Laboratory of Neurobiology and Genetics, The Rockefeller University, New York, NY 10065, USA

[2]Department of Antisense Drug Discovery, IONIS Pharmaceuticals Inc, Carlsbad, CA, USA

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Abstract

Activation of the innate immune system, mediated by the complement and the contact systems, induces inflammation. The inhibition of these pathways by C1 inhibitor (C1INH) has been shown to decrease pro-inflammatory response and mediate vascular permeability in the periphery. However, the role of peripheral C1INH has not been associated with brain function. Using an antisense oligonucleotide (ASO) to deplete circulating liver-derived C1INH in wildtype (WT) mice, we induced the activation of the kallikrein-kinin system (KKS), which produced bradykinin in the plasma, resulting in hypotension. Interestingly, the complement system was quiescent. Depletion of liver-derived C1INH increased blood-brain barrier (BBB) permeability, increased expression of bradykinin 2 receptor, activated resident glial cells to secrete pro-inflammatory mediators such as Il-1beta, Il-6, TNF-alpha and iNOS, and induced cognitive decline. The results of this study emphasize the important role of circulating C1INH in mediating brain function through the activation of the KKS. Thus, manipulation of C1INH in neuro-vascular disorders might be therapeutically beneficial.

Significant statement

By using Antisenseoligonucleotide (ASO) techniques we knocked-down liver-derived C1INH for 12 weeks. This elimination led to the activation of the kallikrein-kinin system in the plasma to produce elevated levels of bradykinin leading to increases bradykinin 2 receptor expressions in the brain, blood-brain barrier permeability, activation of astrocytes and microglial cells towards a pro-inflammatory response, and cognitive decline. These results suggest that liver-derived C1INH must be monitored and properly mediated to maintain normal brain functions.

Introduction

The involvement of the peripheral immune system in brain pathology is now supported by mounting evidence. C1 inhibitor (C1INH) belongs to the super-family of serine protease inhibitors (serpins). C1INH inhibits the initiating proteases of the complement system (Ratnoff and Lepow, 1957, Ziccardi and Cooper, 1979) and the kallikrein system (Schapira et al., 1982; van der Graaf et al., 1983). The complement system and KKS share important features (Bossi et al., 2011), and both involve in proteolytic cascades resulting in a pro-inflammatory response (Ghebrehiwet et al., 2016).

Activation of the KKS is induced by plasma kallikrein which cleaves high molecular weight kininogen (HK) to liberate bradykinin, a 9-amino acid peptide, known to cause vasodilation, reduce blood pressure, and induce a pro-inflammatory response through activation of bradykinin receptors 1 and 2 (B1R and B2R) (Schmaier and McCrae, 2007).

The distribution of C1IHN is very similar in mouse and human tissues (Lener et al., 1998; Vinci et al., 2002). Hepatocytes are thought to be the main source for C1INH biosynthesis (Colten, 1972). In the CNS, C1INH is highly expressed in the cortex and the external capsule of the forebrain (Vinci et al., 2002). The synthesis and/or secretion of C1INH have been detected in astrocyte-derived tumor cell lines (Gasque et al., 1993), brain tissue, neuroblastoma cell lines, microglia, monocytes (Gasque et al., 1995; Gasque et al., 1996), and neurons (Walker et al., 1995).

In this study, we show the important role of balanced liver-derived C1INH in maintaining brain integrity and function. By using ASO-mediated knockdown, we eliminated liver-derived C1INH in mouse plasma, which resulted in kallikrein-kinin system (KKS) activation. KKS activation led to the production of bradykinin, which in turn led to hypotension and increased the expression of bradykinin 2 receptor (B2R) in the brain. Impaired blood-brain barrier (BBB) integrity, activated astrocytes and microglia cells to secrete pro-inflammatory mediators, and impaired cognition were the pathological result of this knockdown. These results highlights the important role of normal levels of liver-derived C1INH in maintaining proper brain function.

Results

Subcutaneous administration of C1INH ASO eliminates C1INH in the plasma and activates the KKS. In order to prevent C1INH production in the liver, we subcutaneously administered C1INH ASO or control ASO (CTRL ASO) to ten-week-old WT mice for three months. Plasma levels of C1INH were significantly downregulated in mice treated with C1INH ASO compared to CTRL ASO (Fig 1A and B and Supplementary figure 1A for C57/C3H). Similar results were obtained in non-reduced conditions (Supp Fig.1B-C). In addition, C1INH ASO treatment, targeted against liver C1INH did not eliminate the protein expression in splenocytes and macrophages measures by FACS (Supp. Fig 1D-F). These findings suggest that the reduction of C1INH in the circulation is due to the targeted treatment of C1INH in the liver which results in decreased expression of the secreted form of C1INH and that the ASO does not target other peripheral cells.

Since C1INH can also inhibit the KKS through plasma kallikrein, we analyzed the cleavage of high molecular weight kininogen (HK) after C1INH ASO administration. Using Western blot analysis, we found a 50% reduction in levels of intact HK (iHK; mHK+ΔD5) in plasma from C1INH ASO-treated mice compared to CTRL ASO-treated mice (Fig 1C and D and Supplementary figure 2A), indicative of increased HK cleavage as previously suggested (Zamolodchikov et al., 2015). Non-reduced HK levels were also significantly reduced in C1INH ASO mice (Supplementary figure 2B, C). Furthermore, levels of plasma kallikrein, which activates HK cleavage, were significantly increased in plasma from C1INH ASO-treated mice compared to controls (Fig 1E, F). However, when we evaluated the plasma after eight weeks of treatment, we found no significant differences in iHK and plasma kallikrein levels in C1INH targeted by ASO (Supplementary figure 2D). Therefore, we continued our evaluations using the chronic treatment time point (12 weeks of treatment).

We measured kallikrein activity in plasma of C1INH ASO-treated mice compared to CTRL ASO, as previously described (Zamolodchikov et al., 2015). Kallikrein activity levels were significantly increased by 48% ($p=0.0244$) compared to plasma from control treated mice (Fig 1G and H),

supporting the results of KKS activation seen in western blots.

The end product of HK cleavage is a 9-amino acid peptide called bradykinin. Bradykinin is unstable in the plasma and rapidly eliminated by degradation. Using a sensitive ELISA kit for detecting bradykinin, we found that levels of bradykinin were significantly increased in C1INH ASO-treated mice compared to CTRL ASO-treated mice (Fig 1I). These results suggest that chronic knockdown of liver-derived C1INH leads to activation of the KKS and to the generation of bradykinin.

Studies have shown that elevated levels of bradykinin correlate with low blood pressure (Guilarte et al., 2017; Sala-Cunill et al., 2015; Waeber et al., 1984). Since plasma from our C1INH ASO-treated mice showed a significant increase in bradykinin, we examined the blood pressure of these mice. We found a significant decrease in systolic, diastolic, and mean blood pressure in C1INH ASO-treated mice compared to CTRL ASO-treated mice (Fig 1J).

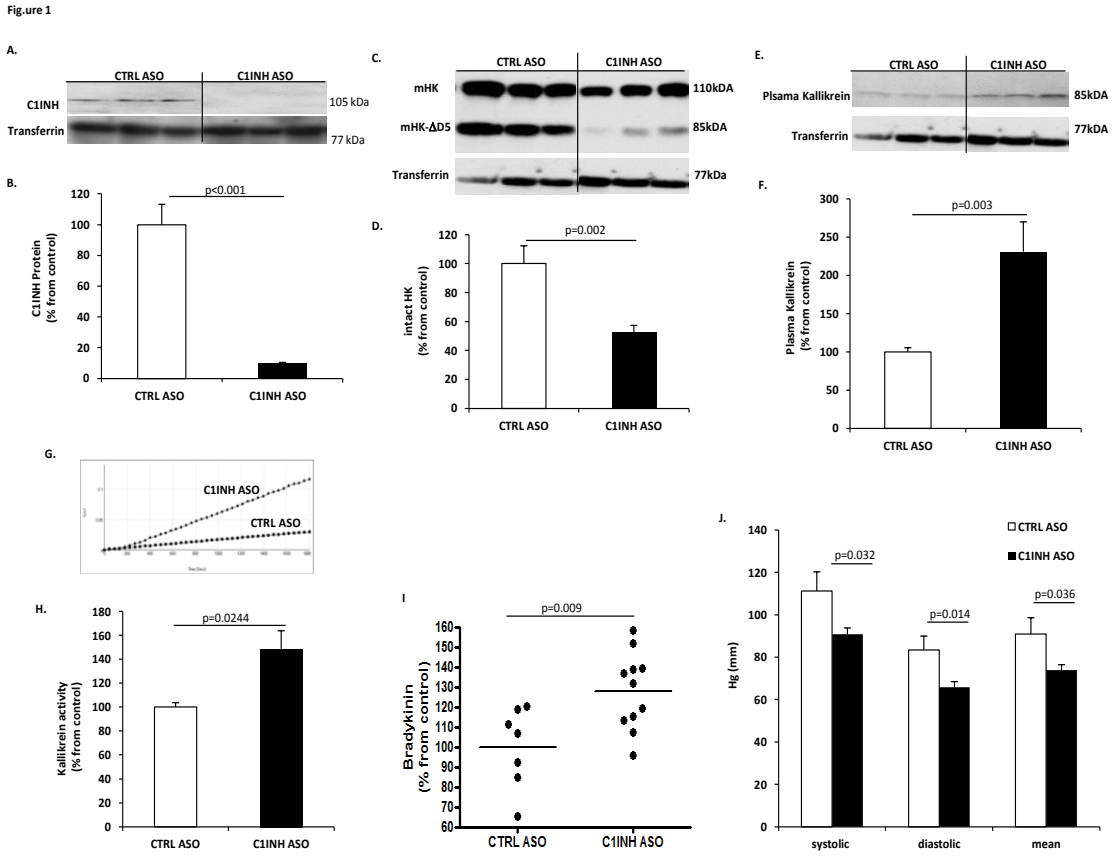

Figure 1. Elimination of C1INH in plasma of C1INH ASO treated mice and activation of the KKS. (A) Representative Western blot of plasma from mice treated with CTRL ASO vs C1INH ASO, probed with an anti-C1INH (A), HK comprised of two bands (110 kDa and 85 kDa) (C) and plasma kallikrein (E) antibodies under reduced conditions. Quantification of C1INH (B) levels show a significant reduction in C1INH ASO treated mice compared to controls

(p<0.001, n=12/group). Quantification of Western blots showed a significant decrease in the total level of intact HK (110 kDa + 85 kDa; D; p=0.002, n=9-10/group) and a significant increase in the level of plasma kallikerin (F; p=0.003, n=9-10/group) between plasma from C1INH ASO treated mice compared to CTRL ASO treated mice. Samples were normalized to transferrin (TF). (G, H) Representative kallikrein activity plot and quantification of only males treated with CTRL ASO or C1INH ASO. Plasma from C1INH ASO treated mice show a significant increase in KKS activity (p=0.0244, n=5-7/group) compared to CTRL ASO. (I) ELISA analysis indicates there was significantly more bradykinin in the plasma of C1INH ASO treated mice compared to the control group (p=0.009, n=7-11/group). (J) Blood pressure measurement was determined by tail cuff plethysmography. Systolic, diastolic, and mean blood pressure readings indicate that C1INH ASO treated mice are hypotensive in comparison to the control group (p=0.032, p=0.014, p=0.036; n=9- 11/group).

C1INH was first characterized as the major inhibitor of the complement system initiation cascade, therefore we explored if the downregulation of peripheral C1INH induced complement system protein activation. Surprisingly, we found no changes in the levels of C1qA and C1r by Western blot (Supp Fig 3A-D and Supplementary figure 3H). Furthermore, levels of peripheral C3a were not changed when depleting peripheral C1INH, examined by Western blot and ELISA (Supp Fig 3E-G). These results suggest that knockdown of endogenous peripheral C1INH does not induce the activation of the complement system in the periphery.

ASO treatments target mRNA in the liver which may cause liver dysfunction resulting in pro-inflammatory responses (Lapinski, 2001; Mehta et al., 2015), (Liu, 2009), neurological deficits, brain edema, and brain inflammation (Jiang et al., 2009; O'Beirne et al., 2006; Sawhney et al., 2016). We ruled out liver dysfunction as a possible outcome of ASO treatment effects (Butterworth, 2013) by using alanine aminotransferase (ALT) assay and ELISA for pro-inflammatory cytokines in the plasma (Supp Fig 4A-D) which showed no significant changes between the treated groups. Furthermore, we measured the spleen as another indication of liver dysfunction and found no differences in weight between the groups (Supplementary figure 4E), suggesting no toxic liver dysfunction apparent due to the ASO treatment. We also ruled out stress, as a cause for KKS activation (Couto Alves et al., 2013; Kadoch et al., 2014), measured by corticosterone (CORT) levels (Supplementary figure 4F). These low levels of CORT indicate that chronic injections did not induce a stress response which might result in activation of the KKS.

Chronic knockdown of liver-derived C1INH increased bradykinin 2 receptor (B2R) expression in the brain and BBB dysfunction. The bradykinin system is known to be involved in BBB integrity (Bartus et al., 1996), and B2R antagonists can prevent BBB permeability induced by elevated levels of bradykinin (Marcos-Contreras et al., 2016). Bradykinin was suggested to be an important pro-inflammatory inducer in the nervous system (Wahl et al., 1996). Since we detected elevated levels of plasma bradykinin in C1INH ASO-treated mice, we measured the levels of B2R in the brain. Both gene and protein expression levels of B2R were significantly increased by two-fold in C1INH ASO mice compared to controls (Fig 2A-C), suggesting an activation of the bradykinin pathway in the brain.

Next, we examined BBB integrity by immunofluorescence staining of coronal brain sections for IgG. IgG is usually not expressed in healthy brain. There was increased staining of IgG in the parenchyma, outside laminin-positive vessels, specifically in borders of the lateral ventricle and the fimbria (Fig 2D). Using Evans blue, we evaluated the BBB leakage in the brains of C1INH ASO-treated mice compared to CTRL ASO-treated mice. We found an increase in blue staining in the freshly frozen brains of C1INH ASO mice (Fig 2E-F). Moreover, Evans blue dye was more readily detected by fluorescence microscopy in the brain tissue of C1INH ASO mice (Fig 2G), specifically in the margins of the ventricles and parenchyma. When we analyzed the brains for differences in water content, to test for brain edema, we did not find any changes between groups (Supp Fig 5). These results suggest a possible breakdown of the BBB specifically in the lateral ventricle region, with increased expression of B2R.

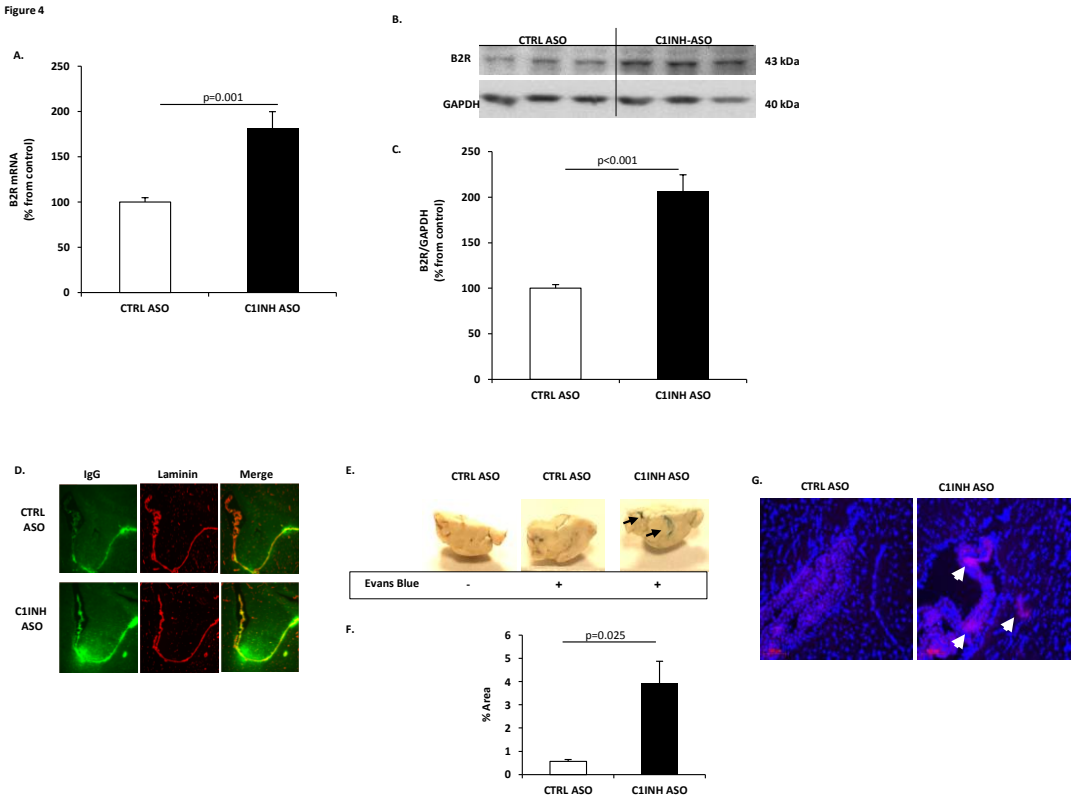

Figure 2. Increased B2R expression and BBB permeability is caused by depletion of peripheral C1INH. RTPCR of coronal brain samples showed elevated B2R gene expression in mouse brains from C1INH-ASO treated mice compared to controls (A; p=0.001, n=6-7/group). (B, C) Western blot of coronal brain homogenates were evaluated for B2R protein expression between groups. C1INH ASO group showed significantly higher levels of B2R in the brain than did the CTRL ASO group (p<0.001, n=5/group). GAPDH was used for normalization. (D) IgG immunofluorescence staining (green) at the fimbria of the hippocampus outside the vessels of C1INH ASO treated mice compared to that of CTRL ASO indicates a disruption of BBB integrity (arrows). Laminin staining (red) was used to outline blood vessels in the region (100µm scale). (E) Representative images of brain hemispheres from mice after Evans blue injection and perfusion. C1INH ASO mice administered Evans blue show a much greater amount of blue coloring in their brains than Evans blue-injected CTRL ASO mice and untreated mice. (F) Quantification of blue area from brain sectioned area in percentage using ImageJ (p=0.025, n=3) (G) Representative staining of Evans blue (red) in the brain parenchyma and the lateral ventricle. Blue signal is DAPI staining (100µm scale).

Chronic depletion of liver-derived C1INH induced activation of resident glial cell towards a pro-inflammatory response and cognitive decline. Emerging evidence suggests that peripheral inflammation can induce brain pathology leading to neuroinflammation and cognitive decline (Hu et al., 2010; Obasi et al., 2012; Simen et al., 2011). We therefore examined this phenomenon in our studies. We found increased expression of GFAP and CD11b, markers of astrocyte and microglial cells activation,

respectively, in the cortex of C1INH ASO-treated mice compared to CTRL ASO-treated mice (Fig 3). Expression levels of both genes and proteins were significantly increased in the cortex as shown by mRNA analysis (Fig 3A for GFAP; 3E for CD11b) and western blot analysis (Fig 3B&C for astrocytes; Fig 3F&G for microglia/macrophages). We have also confirmed the results using immunofluorescence staining for astrocytes and microglia (Fig 3D for astrocytes; Fig 3H for microglia/macrophages). These results suggest that the activation of resident immune cells in the brain is influenced by reduced levels of C1INH in the periphery.

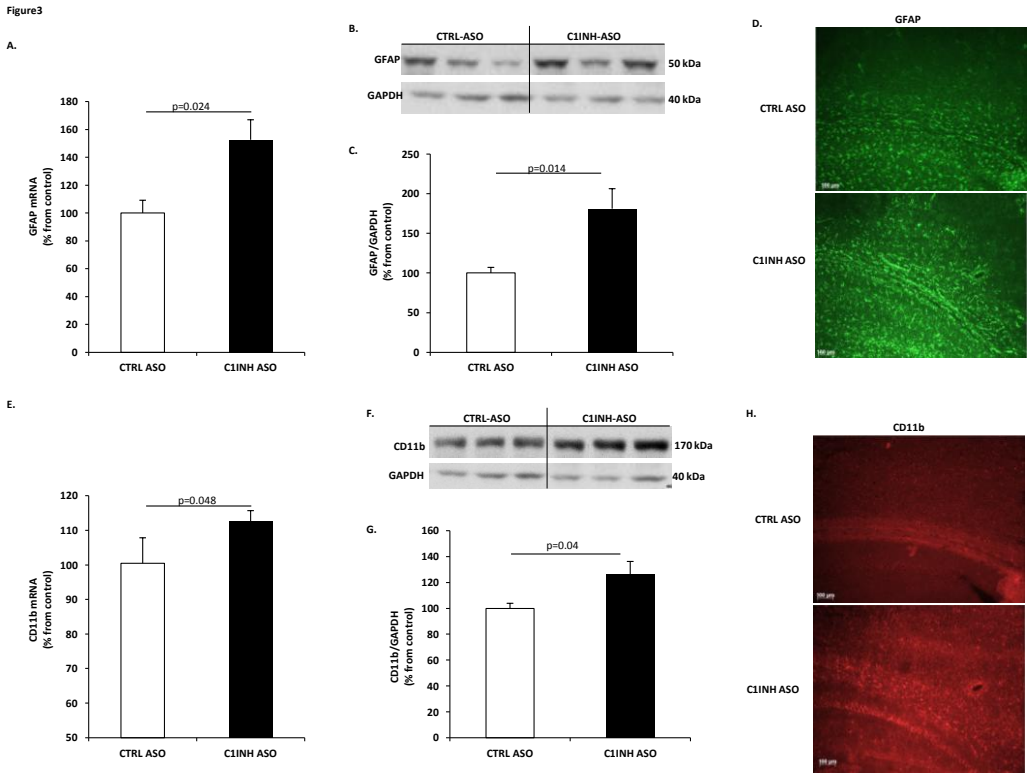

Figure 3. Glial cell activation in the cortex is induced by depletion of peripheral C1INH. (A) Gene expression of GFAP is increased (p=0.024; n=4-10/group) in the cortex of mice treated with C1INH ASO compared to control treatment. (B) Representative Western blot of GFAP in the cortex of C1INH ASO treated mice vs. CTRL ASO treated mice. Samples were normalized to GAPDH. (C) GFAP protein expression is significantly increased (p=0.014, n=5) in the cortex of C1INH ASO treated mice compared to CTRL ASO treated mice. (D) Representative immunofluorescence staining of GFAP in the cortex of C1INH ASO vs. CTRL ASO treated mice (100μm scale). (E) Gene expression of CD11b is increased (p=0.048, n=4-10/group) in cortex of C1INH ASO vs. CTRL ASO treated mice. (F) Representative Western blot of CD11b in the cortex of C1INH ASO vs. CTRL ASO treated mice. Samples were normalized to GAPDH. (G) CD11b protein expression is increased (p=0.04, n=5) in the cortex of C1INH ASO treated mice compared to CTRL ASO treated mice. (H) Representative immunofluorescence staining of CD11b in the cortex of C1INH ASO vs CTRL ASO treated mice (100μm scale).

Activation of glial cells can trigger inflammatory pathways (Farfara et al., 2008). In order to understand the response of resident glial cell activation, we measured levels of pro-inflammatory cytokines in the brain. We first measured gene expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, since they are the major secreted cytokines from activated glial cells. Comparing brain samples from C1INH ASO- and CTRL ASO-treated mice, RT-PCR analysis confirmed the elevation in pro-inflammatory cytokine gene expression (Fig 4A-C) and ELISA confirmed an increase in their proteins (Fig 6D-F). Furthermore, since nitric oxide is known to be secreted from activated resident immune cells during pro-inflammatory response (Farfara et al., 2008), we used immunofluorescence and Western blotting to examine changes in inducible nitric oxide synthase (iNOS). We observed an increase in iNOS expression in brains of C1INH ASO mice compared to CTRL ASO-treated mice (Fig 4G-I).

At the end of the chronic C1INH ASO treatment, we performed behavioral tests to evaluate motor and cognitive function in mice. Using the fear conditioning paradigm, C1INH ASO-treated mice showed reduced percentage of total freezing time on the test day compared to the CTRL ASO group (Fig 4J), indicative of memory impairment. We have also examined cognitive function after 8 weeks of treatment, and found no significant changes (Supp Fig 6A), suggesting the chronic, rather than short term, depletion of liver-derived C1INH induced brain dysfunction. In order to evaluate motor abilities that might be affected by the ASO treatment, we quantified the average distance traveled on the first day of the fear conditioning test prior to foot shock. No motor dysfunction was observed between the groups (Supp Fig 6B). We also performed an open field test to compare the distance which mice traveled after treatment and found no differences (Supp Fig 6C). Since there was no difference observed in motor function between the C1INH ASO and CTRL ASO groups, the reduced freezing time during fear conditioning is most likely a result of cognitive impairment due to the chronic depletion of peripheral C1INH. Although cognitive impairment is often correlated with neurodegeneration, we did not observe evidence of neuronal degeneration as measured by evaluation of

apoptosis (Supplementary figure 6D). This result suggests that the cognitive decline observed in C1INH ASO-treated mice might be due to the loss of BBB integrity and the pro-inflammatory response in the brain, resulting from peripheral activation of the KKS. In conclusion, these results suggest that brain function is mediated by peripheral C1INH.

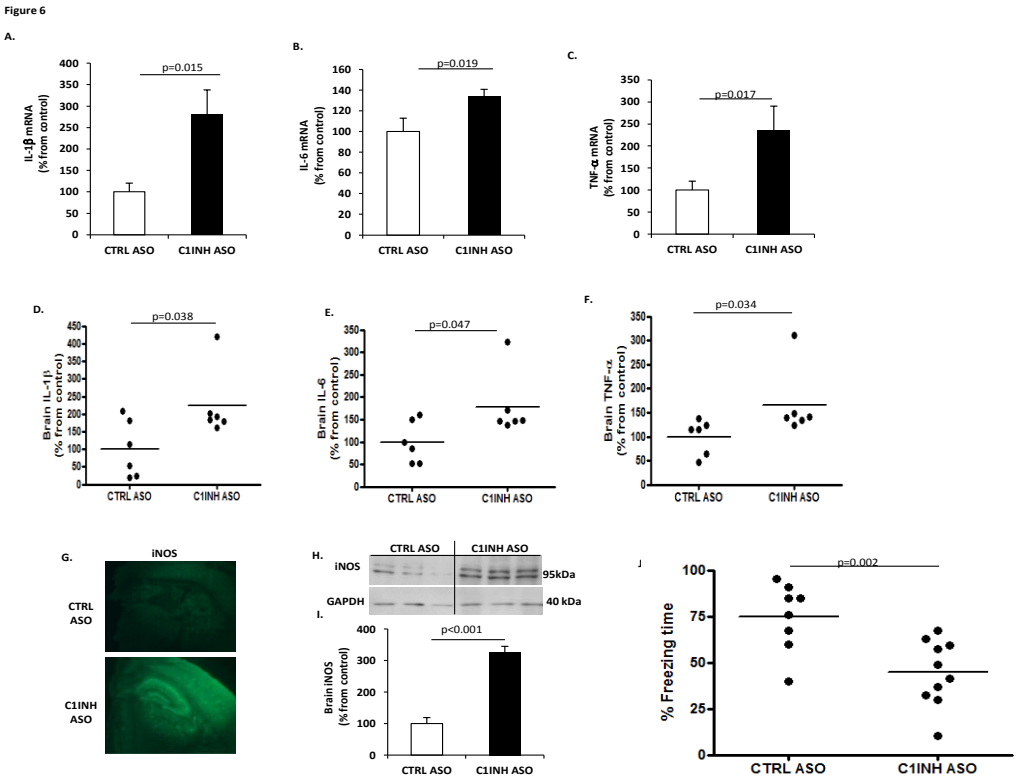

Figure 4. Increased pro-inflammatory response and cognitive decline in the CNS of mice treated with C1INH ASO. (A-C) Elevated gene expression of (A) IL-1β (p=0.015, n=5-7/group), (B) IL-6 (p=0.019, n=5- 7/group), and (C) TNF-α (p=0.017, n=5-7/group) in brains of C1INH ASO treated mice compared to CTRL ASO treated mice. (D-F) Protein expression levels of pro-inflammatory cytokines are increased in brains of C1INH ASO treated mice compared to controls: (D) Il-1β (p=0.038, n=6), (E) IL-6 (p=0.047, n=6), and (F) TNF-α (p=0.034, n=6). (G) Representative immunofluorescence staining of iNOS in the brains of C1INH ASO treated mice compared to CTRL ASO treated mice. (H) Representative Western blot of iNOS in brain homogenates of C1INH ASO treated mice compared to controls. (I) iNOS protein expression is increased (p<0.001, n=6) in C1INH ASO treated mice compared to controls. See my handwritten suggestions and notes on this Figure. (J) Fear conditioning test (day 2) demonstrates decreased freezing time in C1INH ASO treated mice compared to controls (74.80 ± 6.521 vs. 74.80 ± 6.521 N=8, p=0.022, n=8-10/group).

Discussion

Emerging evidences are raising regarding the effect the peripheral blood system on brain function. Blood proteins and activated immune cells are known to play a role in many brain pathologies. However, the exact mechanism and the routes involved are still under investigation.

In this study, we show that chronic depletion of liver-derived C1INH lead to BBB permeability, brain inflammation, and cognitive dysfunction. The mechanistic link underlying these results is through the activation of the KKS to produce bradykinin in the periphery but spares the activation of the complement system.

By using an ASO technique that targets the production of C1INH in the liver, we reduced the protein expression in the periphery. We first tested whether C1INH ASO mice demonstrated changes in the initiation factors of the complement system and found no differences in the activation state of the complement system in the plasma or the brain. However, we observed increased activation of the KKS, through the cleavage of intact HK by plasma kallikrein, resulting in a production of bradykinin. This toxic protein is known to induce peripheral vascular permeability and hypotension mediated by bradykinin 2 receptor (Han et al., 2002; Sala-Cunill et al., 2015) however, no data was presented regarding brain integrity and function.

ASOs that target the liver may produce liver disorders which can correlate with an enlarged spleen, elevated pro-inflammatory cytokines(Reid, 1986), and induce neurological deficits and brain inflammation (Jiang et al., 2009; O'Beirne et al., 2006; Sawhney et al., 2016). We analyzed liver dysfunction and secretion of pro-inflammatory mediators in the plasma and found no liver stress measured by ALT or pro-inflammatory response such as IL-6, IL-1β, and TNF-α. Spleen weight was also unchanged between the treated groups, suggesting no toxic liver dysfunction apparent due to the ASO treatment. Altogether, these results indicate that the activation of the KKS in the periphery is not associated with the chronic treatment of the ASO but by the reduction of liver-derived C1INH alone. In the brain, we observed leakage of the BBB, pro-inflammatory response and cognitive decline which

are not a result of liver dysfunction due to the chronic C1INH ASO treatment, but because of decreased C1INH levels in the plasma.

Our mouse model partially mimics the clinical pathology of Hereditary angioedema (HAE) type-1, a condition caused by mutations in the SERPING1 gene leading to depletion of C1INH production in humans. HAE type-1 and acquired angioedema (AAE) type-1 are diseases characterized by recurrent episodes of severe, localized inflammation and increased vascular permeability affecting soft tissues, including the gastrointestinal tract, upper airways, and the mucosa (Agostoni and Cicardi, 1992; Carugati et al., 2001). The acute attacks in HAE patients are thought to be mediated by the activation of the KKS and the complement system (Gao et al., 2013). Plasma from C1INH-deficient patients contains decreased levels of intact HK (Schapira et al., 1983). During inflammatory attacks, HK is cleaved (Berrettini et al., 1986) and bradykinin is released (Nussberger et al., 1998). Activation of the contact system was shown to mediate vascular permeability via B2R n in the C1INH-/- model (Han et al., 2002). Today, HAE patients are treated during attacks with either recombinant C1INH or kallikrein inhibitors, which halt the production of bradykinin (Cugno et al., 2009). Moreover, prophylactic treatment with C1INH was shown to reduce the frequency of acute attacks (Ma et al., 2011). Previously, the term angioneurotic edema was used to characterize angioedema because of the psychological stress involved with this disorder (C.H Douglas, 1957) suggesting an involvement of stress hormones. Physiological stress is considered a trigger for acute attacks in the cases of HAE (Milder et al., 2007). To understand if the chronic treatment induced a stress response and thus contribute to the activation of the KKS, we tested corticosteroid levels in the plasma of ASO-treated mice compared with that of restrained mice, which demonstrate high levels of plasma corticosteroid. ASO-treated mice did not show elevated levels of plasma corticosteroid, suggesting that the cause of KKS activation and bradykinin overproduction was due to the depletion of the C1INH itself, indicating an important role of controlled, balanced levels of C1INH in the plasma. Depletion of peripheral C1INH led to low blood pressure likely due to the high levels of bradykinin detected in the plasma, which causes blood vessels to dilate. Thus, we concluded KKS

activation upon C1INH ASO treatment was not driven by inflammation in the periphery and was specific to C1INH depletion.

C1INH was first suggested to be involved in brain injury in 1983 (Spiteri et al., 1983). Twelve years later, C1INH was shown to be synthesized in the brain and was correlated with Alzheimer's disease (AD) pathology (Walker et al., 1995). Our group has also shown a decreased expression of C1INH in AD plasma compared to non-demented controls (Zamolodchikov et al., 2016). C1INH, as a modulator of inflammation, was increased in the brains of C1INH ASO treated mice as a result of the pro-inflammatory response and the permeable BBB affected by the down-regulated expression of the liver-derived C1INH expression.

The therapeutic roles of C1INH have been observed in several animal models of peripheral inflammatory disease including sepsis (Croner et al., 2004; Giebler et al., 1999; Guerrero et al., 1993; Jansen et al., 1998; Liu et al., 2007; Scherer et al., 1996), xenograft transplant rejection (Fiane et al., 1999; Hecker et al., 2002a; Hecker et al., 2002b), myocardium ischemia-reperfusion injury(Akita et al., 2003; Horstick, 2002; Horstick et al., 2001a; Nielsen et al., 2002), and hemorrhagic shock (Horstick et al., 2001b). Beyond clinical treatment with recombinant C1INH for HAE, clinical treatment with C1INH in humans revealed therapeutic effects in coronary and myocardial infarction (Bauernschmitt et al.,1998; de Zwaan et al., 2002), septic shock (Hack et al., 1993), vascular leak syndrome (Hack et al., 1993), and other acute inflammatory diseases (Niederau et al., 1995; Ogilvie et al., 1994; Tassani et al., 2001). Moreover, mice infected with *Streptococcus pneumonia* and treated with C1INH showed an improvement in clinical signs of bacterial clearance in the cerebrospinal fluid (CSF) and blood, decreased leukocyte infiltration to the CSF, meningitis, and reduced Il-6 levels (Zwijnenburg et al., 2007). Furthermore, administration of C1INH was shown to have neuroprotective roles in acute neurodegenerative diseases such as stroke and traumatic brain injury (Albert-Weissenberger et al., 2014; De Simoni et al., 2003; Heydenreich et al., 2012) by reducing the infarct volume and the neuronal damage in the temporal cortex, striatum, hippocampus, and thalamus (De Simoni et al., 2003).

Our data suggest that chronic depletion of C1INH can cause serious brain pathologies via the activation of the KKS alone, and treatment with recombinant C1INH, KKS inhibitors, or B2R antagonists might be considered a necessary prophylactic treatment for down-regulated C1INH brain derived-disorders. Furthermore, manipulation of C1INH levels in the periphery might be used to intentionally open the BBB for drug administration. It will also be interesting to determine the role of peripheral C1INH protein in acute and chronic neurodegenerative diseases such as stroke and Alzheimer's disease and evaluate the therapeutic potential of recombinant C1INH, KKS inhibitors, or B2R antagonists in neuro-vascular disorders.

Materials and Methods

Animals. All animal experiments were conducted in accordance with the guidelines of the US NIH Guide for the Care and Use of Laboratory Animals and with approval from the Animal Care and Use Committee of The Rockefeller University. C57/Bl6J mice (males and females) were used for all experiments. A second WT model, C57/C3H, was also used. C1INH ASO (murine sequence)(Bhattacharjee et al., 2013) and control ASO (CTRL ASO, no homologies to the mouse genome) were provided by Ionis Pharmaceuticals.

ASO preparation and treatment. ASOs were dissolved in saline and injected subcutaneously at 150 mg/kg/week for two weeks and then 50 mg/kg/week for 10 weeks. Ten-week-old WT mice were treated with C1INH ASO or CTRL ASO for 12 weeks (n=6-14 mice/group).

Plasma processing. Plasma was collected the day before the treatment started, after two weeks of treatment with 150mg/kg/week (sub-mandibularly), and the day of sacrifice (12 weeks, cardiac puncture). Animals were anesthetized at the end of the treatment using regulated CO_2. Mice were then transcardially perfused with saline. Upon collection, blood was immediately processed in EDTA-containing tubes (BD Microtainer). Heparin was avoided as it has been shown to bind to C1INH (Rossi et al., 2010). Blood was centrifuged at 1300 rpm for 15 min at room temperature (RT). The upper phase of the supernatant was transferred to a

second tube containing 0.5 M EDTA PH8 and centrifuged again. The plasma was aliquoted and immediately frozen and stored at -80 ^{0}C until analysis. During the lifespan of the mice, blood was taken submandibularly. At the end of the experiment, blood was taken from the heart.

Splenocytes and peritoneal macrophages. Spleens and peritoneal macrophages were taken from CTRL ASO and C1INH ASO-treated mice, and single-cell suspensions were prepared (Farfara et al., 2011; Weiss et al., 2011). CD4+ cells and CD8+ cells were positively stained (BD Bioscience) along with C1INH-conjugated FITC (biorbyt) for FACS analysis.

Chronic restraint stress. Chronic restraint stress (CRS) was induced(Wood et al., 2008) with slight modifications. Mice were immobilized for 6 hours using a disposable netting. Blood was collected following the restraint period, and plasma was prepared. Levels of plasma corticosterone were determined using Corticosterone Enzyme Immunoassay Kit (K014-H1, Arbor Assays).

LPS treatment. Mice were injected intraperitoneally (i.p.) with 100 μl of 1 mg/ml lipopolysaccharide (LPS; L-2630 Sigma-Aldrich). Blood was collected and plasma prepared 12 hours after LPS injection. This source was used as a positive control during all ELISA examinations (see below).

Alanine aminotransferase (ALT) activity assay. Liver enzyme function was examined using the ALT Activity Assay (MAK052, Sigma-Aldrich). As a positive control group for this assay, we used lipopolysaccharide (LPS) to induce high levels of secreted ALT levels in the plasma (Baveja et al., 2002).

Blood pressure (Gonsette, 1978). Blood pressure was measured at the end of ASO treatment using tail-cuff plethysmography (Kent Scientific). An average of 3 readings was obtained for each animal during measurement.

Evans blue and brain edema (Lim et al., 1984). Twelve hours before the sacrifice, 2% Evans blue in saline was injected into mice intraperitoneally. After perfusion with saline, one hemisphere was taken to assess percent of H_2O volume using the wet/dry procedure(Hellal et al., 2004). Hemispheres were immediately weighed to obtain wet weight (WW) and heated to 100

^{0}C for 24h. Then samples were weighed again to obtain the dry weight (DW). Brain water content was calculated as %H_2O = (WW-DW) X 100/WW. The other brain hemisphere was collected and sectioned. Loss of BBB integrity was revealed by visualizing Evans blue under the fluorescence microscope.

Immunohistochemistry (Avrahami et al., 2013; Farfara et al., 2015). The primary antibodies used were: anti-GFAP (DAKO Z0334), anti-CD11b (Abcam ab-8878), IgG (Thermo scientific), anti-laminin (Fisher scientific RT-795-PO), and anti-iNOS (Abcam ab-129372). For secondary antibodies, we used IgG anti- mouse, rat, or rabbit, depending on the host of the first antibody (Thermo Scientific). Vectasheild-DAPI (Vector Labs) was used to seal the slides. Brain sections were visualized using Zeiss Axiovert200. Images were analyzed using ImageJ software.

Tunnel assay (Yao et al., 2014). Cell death by apoptosis was examined using the In Situ Cell Death Detection Kit, TMR red (Roche).

Western blot analysis (Chen et al., 2017)**.** Antibodies used for plasma samples: anti-HK light chain (R&D MAB22061); anti-FXII (HTI PAHFFXII-s); anti-plasma kallikrein (R&D AF2498); anti-transferrin (Abcam ab82411). Antibodies used for brain samples: anti-GFAP (DAKO Z0334); anti-CD11b (Abcam ab-133357); anti-B2R (LSBio LS-C405461); anti-GAPDH (Abcam ab9484 or Proteintech 60004-1-Ig). Protein levels were quantified using NIH Image J densitometry. Western blot results for plasma samples were normalized to transferrin, and brain protein extracts were normalized to GAPDH or Actin.

ELISA (Farfara et al., 2011). ELISA was used to determine level of C3a (Molecular Innovations) and bradykinin (ENZO) in plasma and brain homogenate. The samples were normalized to 100% from CTRL-ASO to pool results from three different tests. For detection of pro-inflammatory cytokines, we used Mouse DuoSet ELISA kits for IL-1b, IL-6, and TNF-α (R&D). For a positive control in these assays, we used plasma from an LPS-injected mouse (Qadri et al., 2016).

Gene expression (Farfara et al., 2011). RNA was extracted from fresh cortex or frozen whole brain using RNeasy Lipid Tissue mini kit (74804, Qiagen). RNA was converted to cDNA using High Capacity cDNA Reverse

Transcription Kit (4368814, Life technologies). Gene expression levels were amplified using Taqman enzyme (Applied Biosystems, 4370048) and primers (IL1-β Mm00434228_m1, IL-6 Mm00446190_m1, TNF-α Mm00443258_m1, GFAP Mm01253033_m1, CD11b Mm00434455_m1) and normalized to endogenous mouse GAPDH (Mm99999915_g1). ΔΔCT was quantified and compared between samples.

Kallikrein activity assay (Zamolodchikov et al., 2015). Plasma was evaluated for KKS activity using the chromogenic substrate S-2303 (Diapharma) or Pefachrome PK8092 (Pentapharm).

Behavioral analysis (Farfara et al., 2015). All behavioral experiments were performed and analyzed with a researcher blinded to genotype and treatment. Fear conditioning was performed as previously described(Farfara et al., 2015) with slight changes. Two foot shocks were given (0.7 milliamp, 0.5 sec), one after the first three minutes and the second at the end of the five minutes of the first day. Open field test was done as previously described (Gould, 2009).

Statistical analysis. All statistical analyses as indicated in the figure legends were determined by two-tailed t-test. One-tailed t-test was performed when the results confirmed the hypothesis. All numerical values presented in graphs are mean ±SEM.

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Authorship:

Contributions: D.F. designed the study, performed experiments, analyzed data, and wrote the manuscript; E.F. and A.R. performed experiments and provided technical assistance; E.H.N and S.S participated in data analysis and manuscript preparation. Conflict-of-interest disclosure: Alexey S. Revenko and A. Robert MacLeod are employees and stockholders of Ionis Pharmaceuticals. The authors declare no additional competing financial interests.

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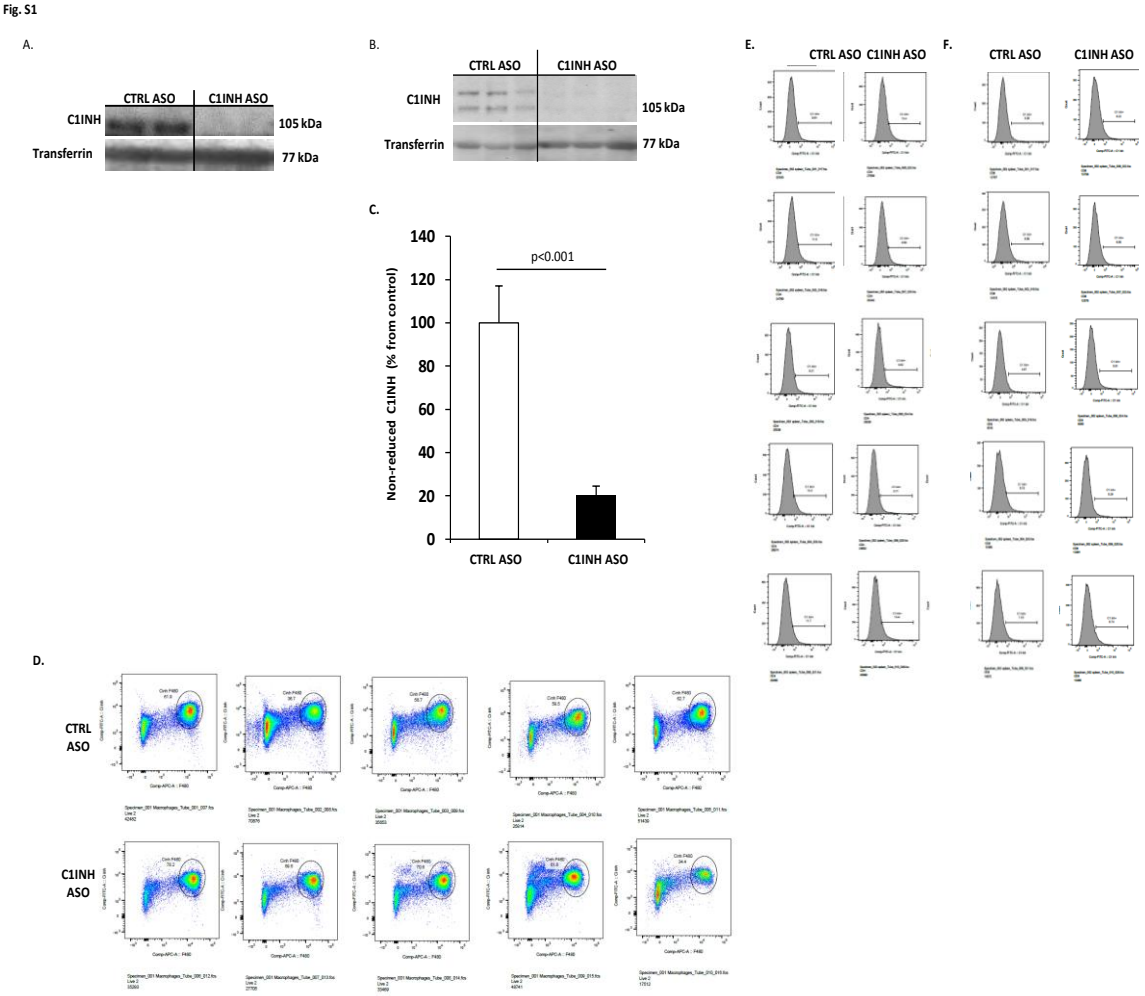
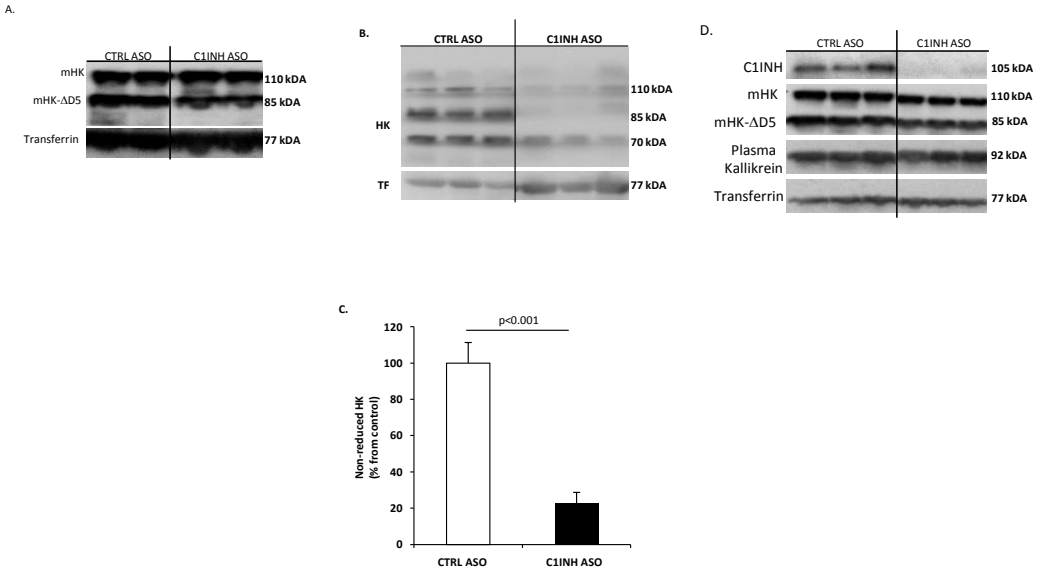
Supplementry Figures:



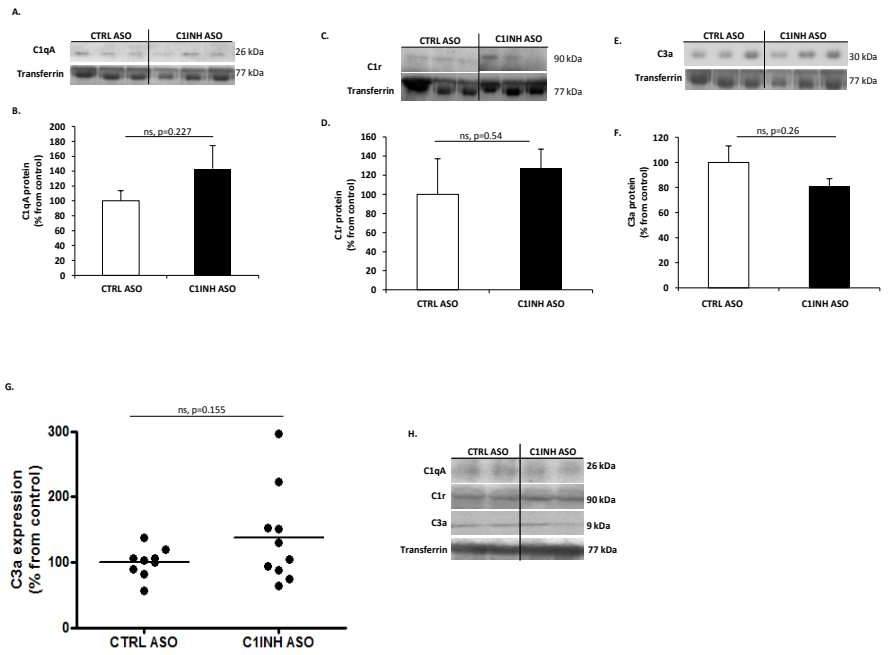
Supplementary Figure 1. Down regulation of liver-derived C1INH in C57/C3H and no observed changes in C1INH expression in macrophages or splenocytes nor activated the complement system. Plasma from C57/C3H mouse model was used to confirm the results seen in C57/Bl6J. (A) Representative Western blots of C1INH depletion from CTRL ASO compared to WT treated with C1INH ASO (B) Representative Western blots of non-reduced C1INH and (C) Quantification analysis of C1INH expression shows an 80% reduction ($p<0.001$, $n=6$). (D) FACS analysis of C1INH (FITC) from IP macrophages (F4/80+ APC) and (E) CD4+ cells and (F) CD8+ cells from spleen. (G) Representative Western blots of C1qA, C1r and C3a in C57/C3H mouse model. Transferrin was used as loading control.

Fig S2



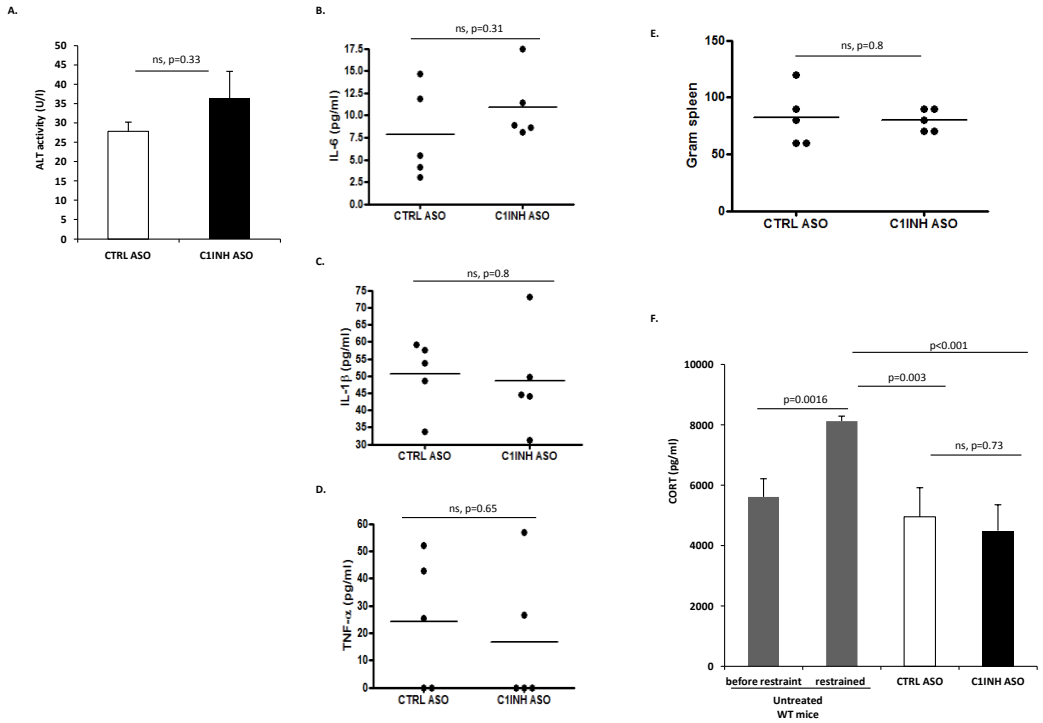
Supplementary Figure 2. Activation of the KKS in C57/C3H but no activation seen after eight weeks of treatment. (A) Representative Western blots of iHK (mHK+ΔD5) from C57/C3H mouse model treated with C1INH ASO compared to CTRL ASO shows decreased levels of iHK. (B) Non-reduced expression of iHK from plasma of CTLR ASO compared to C1INH ASO. (C) Quantification analysis of non-reduced iHK shows a decreased expression of 78% in treated C1INH ASO mice compared CTRL ASO ($p<0.001$, n=6). (D) Representative western blot of plasma from CTRL ASO compared to C1INH ASO treated for eight weeks show down regulation of C1INH expression but no differences in iHK and plasma kallikrein. Transferrin was used as loading control.

Fig S3



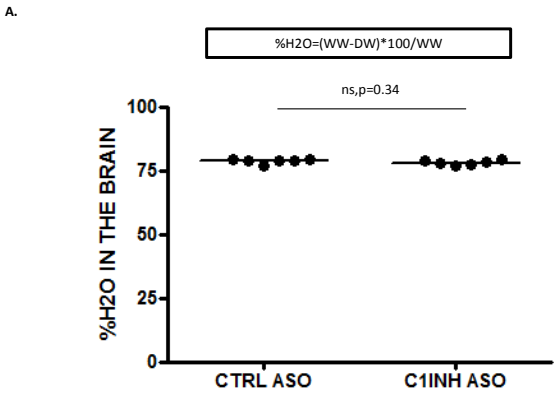
Supplementary Figure 3. Knockdown of C1INH does not induce complement activation. Representative Western blots of plasma from ASO-treated mice probed with antibodies against C1qA (A), C1r (C) and C3a (D). Samples were normalized to transferrin (TF). Quantification of Western blots shows that levels of C1qA (B), C1r (D) and C3a (E) were not significantly changed upon depletion of plasma C1INH ($p=0.227$, $p=0.54$ and $p=0.26$; n=6/group). (G) ELISA analysis of plasma demonstrates no significant change in C3a levels between C1INH ASO treated mice and CTRL ASO treated mice ($p=0.155$; n=9-10/group). (H) Representative Western blots of plasma from ASO-treated mice probed with antibodies against C1qA, C1r, and C3a from C57/C3H mice.

Fig. S4

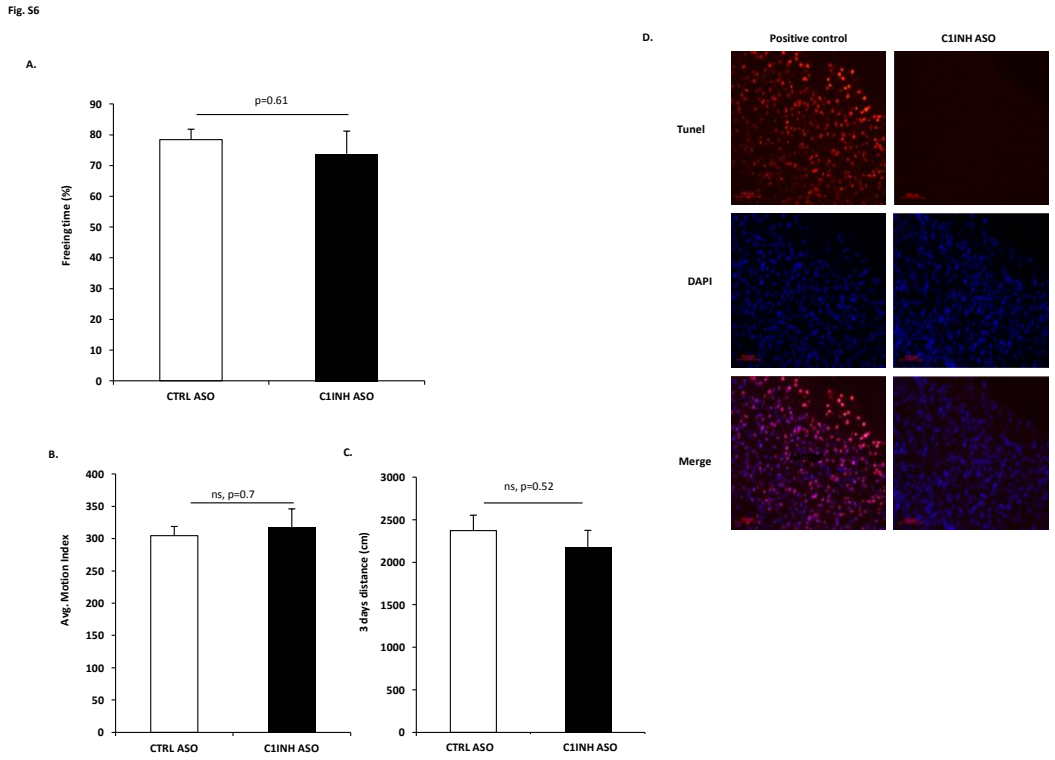


Supplementary Figure 4. No toxicity induced by C1INH ASO treatment. (A) Activity level of liver function measured by alanine transferase (ALT) assay was examined in mouse plasma. ALT activity did not change between C1INH ASO treated mice and CTRL ASO mice (p=0.33; n=4-5/group). (B-D) Levels of inflammatory cytokines did not change between C1INH ASO mice and CTRL ASO mice: IL-6 (p=0.31, n=5), IL-1β (p=0.8, n=5), and TNF-α (n=0.65, n=5). (E) Spleen weight after treatments show non differences between the groups. (F) No differences in spleen weight between the treated groups (82.00 ± 11.14, 80.00 ± 4.472, p=0.8718). (H) Corticosterone (CORT) levels did not change in plasma of C1INH ASO treated mice compared to CTRL ASO treated and mice that underwent acute restraint (p<0.001, n=5-7/group).

Fig. S5



Supplementary Figure 5. Measurement of brain edema. Brains of C1INH ASO treated mice were dried and measured for water content percentage. No differences were found between the treated groups in water content, indicating no brain edema (p=0.34, n=6/group).



Supplementary Figure 6. Cognitive dysfunction, motor function and apoptosis were not impaired after chronic elimination of liver-derived C1INH. (A) Percentage of freezing time on day 2 of the FCT showed no differences between treated groups after 8 weeks of treatment (P=0.61, n=6/group). (B) Calculation of the average motion index in the first day of the fear conditioning test prior to foot shock, which demonstrated no differences between the groups (p=0.7, n=6/group). (C) In an open field test, average of distance traveled was calculated over three days of testing. No differences were found between the groups (p=0.52, n=3-6/group). (D) Representative image of for neurodegeneration using TUNEL staining for apoptosis. No positive staining for apoptosis was determined.