Targeted gene transfer into ependymal cells through intraventricular injection of AAV1 vector and long-term enzyme replacement via the CSF

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Enzyme replacement via the cerebrospinal fluid (CSF) has been shown to ameliorate neurological symptoms in model animals with neuropathic metabolic disorders. Gene therapy via the CSF offers a means to achieve a long-term sustainable supply of therapeutic proteins within the central nervous system (CNS) by setting up a continuous source of transgenic products. In the present study, a serotype 1 adeno-associated virus (AAV1) vector was injected into a lateral cerebral ventricle in adult mice to transduce the gene encoding human lysosomal enzyme arylsulfatase A (hASA) into the cells of the CNS. Widespread transduction and stable expression of hASA in the choroid plexus and ependymal cells was observed throughout the ventricles for more than 1 year after vector injection. Although humoral immunity to hASA developed after 6 weeks, which diminished the hASA levels detected in CSF from AAV1-injected mice, hASA levels in CSF were maintained for at least 12 weeks when the mice were tolerized to hASA prior of vector injection. Our results suggest that the cells lining the ventricles could potentially serve as a biological reservoir for long-term continuous secretion of lysosomal enzymes into the CSF following intracerebroventricular injection of an AAV1 vector.

Lysosomal storage disease (LSD) is a diverse group of genetic disorders characterized by an inherited deficiency in specific lysosomal enzymes and a consequent accumulation of undigested substances within lysosomes. Some LSDs have been successfully treated using systemic enzyme replacement therapy (ERT). With that therapy, intravenously delivered lysosomal enzymes are taken up by the target cells via the mannose-6-phosphate receptor-mediated pathway and cross-correct the enzyme deficiency. However, the clinical efficacy of ERT for LSD with neurological symptoms, such as type 3 Gaucher disease and metachromatic leukodystrophy (MLD), is very limited, as lysosomal enzymes cannot cross the blood-brain barrier (BBB). Thus, alternative drug delivery strategies that circumvent the BBB will be required to treat the central nervous system (CNS) manifestations of LSD.

One possible approach to delivering therapeutic proteins to the CNS is direct injection of a viral vector into the brain parenchyma. We previously showed that in MLD model mice lacking the lysosomal enzyme arylsulfatase A (ASA), a single injection of serotype 1 adeno-associated virus (AAV1) vector encoding human ASA (hASA) into the hippocampus leads to widely distributed expression of hASA protein and a subsequent reduction in sulfatide levels throughout the brain. To apply this approach to large animals, including humans, multiple vector injections with invasive surgical trepanation of the skull is required because the volume of the adult human brain is supposed to be around 3,000 times greater than that of the adult mouse. So far, clinical trials for AAV-mediated treatment of Canavan’s disease, Batten’s disease (ClinicalTrials.gov identifier: NCT00151216 and NCT01161576) and Sanfilippo A syndrome (ClinicalTrials.gov identifier: NCT01474343) have been performed or are ongoing. In these studies, the vector is administered through multiple surgical burr holes, but the efficacy of these treatments has been somewhat equivocal.

Another approach is enzyme replacement via the cerebrospinal fluid (CSF), which enhances the enzyme’s distribution within the CNS. Repeated or continuous infusion of recombinant protein through intrathecal or intraventricular delivery has been shown to improve neurological symptoms in model animals with neuropathic LSDs. However, repeated lumbar puncture and intrathecal catheter insertion, both of which are considered
minimally invasive, would likely become unacceptably invasive and expensive for patients who had to continue the regular administration of enzymes over their entire lifespan. In such cases, brain-directed gene therapy could help to reduce the burden on patients by setting up transduced cells within their CNS to continuously secrete the therapeutic enzymes into the CSF for sustained periods. Our aim in the present study is to assess the feasibility of AAV1-mediated enzyme replacement within the CNS via the CSF.

**Results**

**Injection of AAV1 vectors into the CSF leads to widespread transduction of ventricular ependymal cells.** To evaluate the feasibility of AAV1-mediated enzyme replacement via the CSF, we first examined the transgene distribution in mouse brain following intracerebroventricular injection of AAV1 bicistronic vectors encoding green fluorescence protein (GFP) and hASA. Immunohistochemical analysis showed that 3 weeks after a single injection of the AAV1 vector into the CSF, GFP expression was broadly distributed in the choroid plexus and ependymal cells throughout the cerebral ventricles (Figure 1). Coronal brain sections revealed the widespread diffusion of AAV1 vectors from the injection site to the contralateral, anterior lateral (Figure 1a, 1b) and third (Figure 1c, 1d) ventricles, as well as fourth ventricles via the cerebral aqueduct (Figure 1e, 1f). GFP expression was mainly confined to the choroid plexus and ependymal cells, with little or no detection in the brain parenchyma or spinal cord. The transduction of ependymal cells was confirmed using an anti-vimentin antibody as a marker of the ependymal cell layer (Figure 2a). Moreover, co-immunostaining of GFP and hASA validated the concurrent expression of these two proteins in same ependymal cells (Figure 2a, 2b). On the other hand, the distribution of these proteins within the cells differed considerably. Whereas GFP was uniformly distributed throughout each cell, the staining pattern of hASA showed a granular distribution surrounding the nucleus (Figure 2b), which suggests transport of the human lysosomal protein into the murine lysosomes. Double

Figure 1 | Transgene expression in coronal sections of mouse brain 3 weeks after injection of bicistronic AAV1 vector into the right lateral ventricle. The injection needle track is indicated with an arrowhead in b. (a–c) Low magnification photomicrographs showing GFP expression in the ependymal cells throughout the right and left lateral ventricles (LV) as well as the dorsal (D3V) and main (3V) parts of the third ventricles. Distances from bregma are 0.08 mm anterior (a), 0.40 mm posterior (b) and 2.32 mm posterior (c), respectively. (d–f) Higher magnification micrographs showing GFP expression in the choroid plexus (arrowhead in d) and the ependymal cells lining the D3V (d), cerebral aqueduct (e) and fourth ventricle (4V) (f). Cell nuclei are stained with DAPI (blue) and neurons in the granular layer (GL) in the cerebellum are labeled with anti-NeuN antibody (red). Distances from bregma are 0.88 (d), 3.28 (e) and 6.16 (f) mm posterior, respectively.

Figure 2 | Concurrent expression and distribution of transduced GFP and hASA within ependymal cells. (a) Co-immunostaining showing that GFP-positive cells (green) are in the ependymal layer and are also stained with anti-vimentin (blue), and that hASA (red) is concurrently expressed in the GFP-positive cells. (b) Higher magnification micrographs showing that GFP is relatively uniformly distributed within ependymal cells, while hASA exhibits more granular localization surrounding the DAPI-stained (blue) cell nuclei. (c) LAMP-2-positivity (blue) confirmed that the transduced hASA is mainly localized in the lysosomes.
the same time as CSF collection were diluted serially, incubated with
measures to determine the time-dependent development of an anti-hASA anti-
despite robust expression of the protein, we estimated the effect of
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tions confirm that long-term stable expression of the hASA trans-
seen more than a year post-injection (Figure 4i, 4l). These observa-
at 12 weeks after AAV1 injection (Figure 4h, 4k), and could still be
however, substantial expression of hASA was observed in the brain 

increase gradually over the period from 1 to 6 weeks after injection 
while hASA expression in the AAV1-injected brains appeared to
jected mice showed no hASA immunoreactivity (Figure 4a, 4d),
Consistent with the results of the ELISA, brain sections from unin-
we next assessed the secretion of hASA from 
the transduced cells into the CSF of mice. CSF samples collected from 
the cisterna magna of both AAV1-injected and uninjected mice were 
assayed for their hASA content using an enzyme-linked immunoabsorbent assay (ELISA, Figure 3). This confirmed the 
absence of hASA in CSF from uninjected control mice (n = 5). By 
contrast, in the AAV1-injected mice the average hASA levels in the 
CSF increased gradually during the period from 1 to 6 weeks after injection (0.15 ± 0.15 ng/ml at 1 week (n = 5), 1.59 ± 0.61 ng/ml at 
3 weeks (n = 8) and 2.36 ± 1.42 ng/ml at 6 weeks (n = 6)), but levels 
had returned nearly to zero by 12 weeks after injection (0.13 ± 0.06 ng/ml (n = 5)), indicating that a net peak of the expression of 
hASA in the CSF has been between 3 and 12 weeks after viral 
injection. Using a nonparametric rank test because of the zero 
concentrations in 2 of the 6 samples, a significant difference 
between AAV1-injected mice and their uninjected counterparts 
was detected 3 weeks after injection, but not 6 weeks after. In 
addition, no hASA was detected in the plasma samples collected at 
same time as the CSF in any of the mice used in this study (data not 
shown).

To determine why hASA levels in CSF declined within 12 weeks 
after AAV1 injection, we used immunohistochemical analysis to 
examine the time course of hASA expression in the brain. Consistent with the results of the ELISA, brain sections from unin-
jected mice showed no hASA immunoreactivity (Figure 4a, 4d), 
while hASA expression in the AAV1-injected brains appeared to 
increase gradually over the period from 1 to 6 weeks after injection 
(Figure 4b, 4c, 4e–g, 4j). In sharp contrast to the levels in CSF, 
however, substantial expression of hASA was observed in the brain 
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to clarify the reason why hASA levels in CSF were suppressed 

despite robust expression of the protein, we estimated the effect of 
humoral immunity by using an immunoprecipitation method to 
to measure the time-dependent development of an anti-hASA anti-
body. Blood plasma samples collected from AAV1-injected mice at 
the same time as CSF collection were diluted serially, incubated with

long-term replacement of hASA in murine CSF is achieved in 
immunotolerized animals. To further characterize the effect of 
antibody development on hASA levels in CSF, we induced C57BL/ 6 mice to develop tolerance to hASA (Figure 6a). When neonatal 
mice were intraperitoneally administered 10 μg of hASA 30–40 h 
and then 5 days after birth, subsequent immunoprecipitation 
analysis of plasma samples showed suppression of antibody 
development (Figure 6b, 6c), as comparing to untreated mice 
(Figure 5). Although the immunotolerization protocol did not 
produce complete tolerance in all treated animals, significant 
antibody development was suppressed during the 12 weeks after 
AAV1 injection (Figure 6c).

Using the tolerated mice, we were able to achieve long-term hASA 
replacement via the CSF (Figure 6d). We confirmed that whereas 
hASA was not detected in CSF from untreated tolerated mice (n = 4), 
substantial levels were present in CSF from AAV1-injected tolerated 
mice, with significant increases detected at 6 and 12 weeks (0.43 ± 0.27 ng/ml at 3 weeks (n = 3), 2.13 ± 0.39 ng/ml at 6 weeks (n = 4) 
and 2.37 ± 1.27 ng/ml at 12 weeks (n = 5)). At 12 weeks, moreover, 
hASA levels were significantly higher in CSF from tolerated mice 
than from non-tolerized mice (compare Figure 6d and 3; P < 0.01, 
Mann-Whitney test). These results suggest that AAV1-mediated 
long-term enzyme replacement via the CSF is feasible under condi-
tions in which humoral immunity does not neutralize the enzyme 
secreted from the transduced cells. The involvement of an antibody 
in the inhibition of hASA in CSF was also apparent when the hASA 
concentration in CSF was plotted against the anti-hASA antibody 
titer in the plasma of AAV1-injected mice (Figure 7).

Discussion

Ependymal cells have been the preferred target in several gene ther-
apy studies because they are susceptible to a variety of viral vectors 
and could potentially serve as a reservoir of therapeutic protein that is 
continuously released into the CSF circulating throughout the 
brain12–18. Protein secretion from ependymal cells could be especially 
useful for treatment of LSDs with CNS manifestations, as lysosomal 
enzymes released into the CSF would be taken up by CNS cells 
through mannose-6-phosphate receptor-mediated endocytosis. In 
the present study, we observed that a single intracerebroventricular 
injection of AAV1 vector induces transgene expression in ependymal 
cells that persists for more than a year, and that human lysosomal 
enzymes will continue to be present in the murine CSF in the absence 
of antibody against the transgene product.

At the microscopic level, intracerebroventricular injection of 
AAV1 vector showed exclusive transduction of ependymal cells lin-
ing the ventricles and the choroid plexus forming the blood-CSF 
barrier (Figure 1 and 4). This is similar to the transduction pattern 
seen after intracerebroventricular injection of other serotypes of 
AAV19,19 or adenoviral vector12–14. Although AAV1 vector has been 
reported to achieve broader neuronal transduction if they were 
injected into the lateral ventricles of neonatal mouse22, matured 
ependymal cells would prevent their transfer from the CSF to the 
brain parenchyma in adult mice because the maturation of ependy-
mal cells occurs during the first postnatal week21. In earlier studies, it 
was reported that some ependymal cells behave as stem cells, even in 
the adult mouse22, and that ependymal cells turn approximately 
every 130 days23. In a more recent study, however, ependymal cells 
were reportedly quiescent under normal conditions (i.e., in the 

taining of hASA in the lysosomal marker LAMP-2 confirmed the 
localization of hASA in the lysosomes (Figure 2c).

Secretion of hASA into the CSF and the inhibitory effect of 
humoral immunity. We next assessed the secretion of hASA from 
the transduced cells into the CSF of mice. CSF samples collected from 
the cisterna magna of both AAV1-injected and uninjected mice were 
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To determine why hASA levels in CSF declined within 12 weeks 
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body. Blood plasma samples collected from AAV1-injected mice at 
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Figure 3 | Time-dependent changes in hASA levels in CSF following 
AAV1 injection. Each CSF sample was collected from a different mouse at 
the indicated times. Error bars indicate SEM values. The asterisk indicates a 
significant difference from uninjected mice (*P < 0.05, Mann-Whitney 
test).

hASA and immunoprecipitated to determine the time-dependent 
variation in the antibody titer to hASA. Both the averaged 
(figure 5a) and individual (figure 5b) titers showed a significant 
increase between 3 and 12 weeks after AAV1 injection. From this 
finding, we would expect a negative correlation between measured 
concentrations of hASA in CSF and the anti-hASA antibody titers in 
the AAV1-injected mice.
absence of brain injury) by virtue of Notch signal activation. In fact, intracerebroventricular injection of a helper-dependent adenoviral vector, which does not induce cellular immunity, led to expression of the transgene for up to 1 year in ependymal cells expressing Notch1. Similarly, we observed stable expression of human lysosomal enzymes in murine ependymal cells over more than 1 year, which suggests the AAV1 vector has a high affinity for quiescent ependymal cells and is indicative of the suitability of the method used in the present study to set up a continuous source of lysosomal enzymes to be released into the CSF.

To estimate the amount of human enzyme secreted into the CSF of mice, we used a glass capillary tube to collect CSF samples from the cisterna magna as described previously and measured the hASA concentration using a traditional ELISA. Although we visually confirmed that there was no contamination of the collected CSF by blood, we would expect small amounts of blood to have little or no effect on the estimated concentrations of hASA in CSF, as hASA was not detected in any blood samples collected simultaneously with the CSF (data not shown). Considering that CSF ultimately drains into the bloodstream and lymphatics, it is somewhat surprising that we did not detect hASA in blood samples from the tolerized mice, in which anti-hASA antibody titers were suppressed. We suggest that the hASA concentration in the blood plasma was diluted to a level below the detection limit of our assay due to the difference between the total volumes of CSF (approximately 40 μl) and plasma (at least more than 1.2 ml). We also did not detect cells expressing hASA in the livers of the AAV1-injected mice (data not shown). Most likely, therefore, an anti-hASA antibody developed in the blood in response to hASA draining from the brain, not in response to hASA secreted from infected cells outside the CNS. Although we could not directly

Figure 4 | Time-dependent changes in expression levels of transduced hASA in the brain. Expression patterns of AAV1-transduced hASA (red) in the LV (a–c and g–i) and 4V (d–f and e–l) are shown at the indicated pre- and post-injection times. The injection needle tracks are indicated by arrowheads in (b), (c), (g) and (i). Cell nuclei are stained with DAPI (blue). Immunohistochemical staining showed no hASA signal anywhere in the brain of an un.injected control mouse (a, d), though a weak false-positive signal was detected in the choroid plexus (inset in (d)). One week after injection, overt signals could be seen in the cortical cells surrounding the needle track (arrowhead in (b)) and in the choroid plexus (inset in (e)), but there were very few hASA-expressing ventricular ependymal cells (b, e). Three weeks after injection, hASA-positive ependymal cells were sparsely distributed throughout the ventricles (c, f), and the choroid plexus (arrowheads in (f)) was densely stained. Six weeks after injection, stable expression of hASA could be seen in both ventricular ependymal cells (g–l) and choroid plexus (arrowhead in (j) and insets in (k) and (l)), and that expression persisted for over 1 year. BS, brain stem; Cb, cerebellum; CP, choroid plexus; Ctx, cortex; St, striatum.
determine antibody titers in CSF due to the small volume of the CSF samples and the very low concentration of the anti-hASA IgG, the time course of antibody development in the CSF would be coincident with that in the plasma, since IgG present in the bloodstream can rapidly cross the BBB via receptor-mediated transcytotic pathways. Evidence from several animal LSD models with CNS manifestations suggests treatments that raise levels of lysosomal enzymes in CSF have a potential for clinical use. For example, intracerebroventricular administration of hASA to MLD model mice using osmotic minipumps improved motor function and reduced sulfatide accumulation in the CNS, but did not elicit detectable anti-hASA antibodies during the 4 weeks of treatment. In our study, however, significant development of anti-hASA antibodies was observed weeks after AAV injection and likely had a negative relationship with the measured level of hASA in the CSF (Figure 7). That hASA levels could not be sustained in the long term in the CSF of immunocompetent wild-type mice (Figure 3) indicates development of humoral immunity will be a key obstacle to the widespread use of the method.

Figure 5 | Development of anti-hASA antibody titers in plasma from AAV1-injected mice. (a) Aliquots (2 μl) of plasma collected weeks (circles, n = 8), 6 weeks (squares, n = 6) and 12 weeks (diamonds, n = 5) after AAV1 injection were serially diluted two-fold and added to a fixed amount of hASA to form IgG-hASA complexes. The immune complexes were then precipitated, and the residual hASA concentration in the supernatant was measured to evaluate the plasma’s ability to capture hASA. Data points represent average values for each group, and the error bars indicate SEM values. (b) The antibody titers in individuals (closed circles) were determined from the half-maximal value of each individual dilution curve. The dotted horizontal line indicates the antibody titer detection limit, and the data under this line (open circles) were excluded from the calculation to find the average. The short solid horizontal lines represent the average values of the detectable titers in the two groups (3 and 6 weeks after injection). During the periods spanning from 3 to 6 weeks and from 6 to 12 weeks after injection, antibody titers in each group increased significantly (*P < 0.05; **P < 0.01, Mann-Whitney test).

Figure 6 | Long-term enzyme replacement via the CSF in mice tolerized to hASA. (a) Illustration of the experimental timeline. To induce tolerance to hASA, neonatal C57BL/6 mice were intraperitoneally injected with μg of hASA 30–40 h and 5 days after birth. (b) Two microliters of plasma were then taken from the immunotolerized mice weeks (circles, n = 3), 6 weeks (squares, n = 4) and 12 weeks (diamonds, n = 5) after hASA injection. All other details are the same as in Figure 5. (c) The antibody titer in the individuals. Details are same as in Figure 5. There were no significant differences among the three groups (Kruskal-Wallis test: H = 3.20, P = 0.20). (d) Time-dependent changes in hASA levels in CSF. Asterisks indicate significant differences from uninjected mice (*P < 0.05, Mann-Whitney test).
whether the method used here in mice is able to provide the same results in larger animals with larger brains, where diffusion distances and the CSF volume to be loaded with therapeutic proteins are much greater.

Methods

AAV vector preparation. The AAV vector plasmid used here (pAAV.CAhASABE) was described as previously36. To generate the AAV1 vector, an adenovirus-free triple transfection method was performed as described previously32,33. To remove residual hASA proteins from the obtained AAV1 vector solution prior to animal injection, size exclusion chromatography was performed using an AKTA Explorer 100 HPLC system (GE Healthcare Japan, Tokyo, Japan) as described previously33,34. The absence of detectable hASA contamination in the AAV1 vector solution was then confirmed using an ELISA (see below). The AAV1 vector was determined by quantitative real-time polymerase chain reaction using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) after purification through phenol-chloroform extraction34.

Animals and injections. All animal experiments were approved by the Ethics Committee of Nippon Medical School and carried out according to the Institutional guidelines for animal care (Nippon Medical School, Tokyo, Japan). Eight- to 12-week-old C57BL/6 mice (Saitama Experimental Animal Supply Co., Saitama, Japan) were used in this study. All mice used here were healthy wild-type mice. To reduce the effect of antibody development against hASA in the AAV1 injected animals, C57BL/6 mice were immunotolerized to hASA using a published protocol in which neonatal mice were intraperitoneally injected with 10 μg of enzyme protein 30–40 h and 5 days after birth to induce tolerance36. To administer the vector, the mice were initially anesthetized with sodium pentobarbital (40 mg/kg) and then mounted on a stereotactic frame. The skin over the skull was incised and a small hole was made in the skull above the target using a microdrill. The injection site was the right lateral cerebral ventricles and the subarachnoid space but also the perivascular space and the brain parenchyma36. In addition, another immune problem which was not appeared in the rodent study will be faced with regard to the clinical trials using AAV1 vector. As a majority of healthy human population has been reported to be seropositive for neutralizing antibodies (NABs) to AAV136, it seems that AAV1 would be neutralized immediately in the majority of human patients. However, recent study has reported that intra-CSF delivered AAV vector attained the gene transfer to the canine CNS even in the presence of NABs in the serum of treating animals because the NABs titers in the CSF was much lower than that in the serum and thus did not completely block AAV vector transduction in the brain36. Considering this report, intracerebroventricular administration of AAV1 vector could have benefit to circumvent not only the BBB but also the immune response against the AAV capsid.

We previously reported that inrachippcampal injection of an AAV1 vector encoding hASA reduced sulfatide levels throughout the entire brain and improved motor function in MLD model mice6. We are now investigating using this MLD model mice if there is any beneficial metabolic effect by our intracerebroventricular injection protocol with AAV1 vector. Although we cannot compare the CSF level of hASA which was achieved in this study with that of murine ASA in the normal mice because of a lack of the information about the murine ASA level in the normal mice, our next therapeutic study will reveal whether the CSF level of hASA that will be achieved by our protocol is enough to improve the MLD symptoms or not. Furthermore, in larger animals, gene therapy via the ependymal route would be a more effective means of delivering lysosomal enzymes to the entire brain, as CSF flows not only through the cerebral ventricles and the subarachnoid space but also the perivascular space and the brain parenchyma36. In future studies, we will test.
The titer of anti-hASA antibody developed in AAV1-injected mice was determined by measuring the capacity of a twofold dilution series of 2-μl aliquots of plasma to immunoprecipitate hASA, as described previously.41 The value of the titer described by measuring the capacity of a twofold dilution series of 2-

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Author contributions

Y.Y. wrote the main manuscript text and prepared all figures; Y.H. prepared the AAV1 vectors; K.M. supervised the project; T.S. designed research and had primary responsibility for final content.

Additional information

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