Adeno-associated virus-mediated expression of β-hexosaminidase prevents neuronal loss in the Sandhoff mouse brain

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Sandhoff disease, a GM2 gangliosidosis caused by a deficiency in β-hexosaminidase, is characterized by progressive neurodegeneration. Although loss of neurons in association with lysosomal storage of glycosphingolipids occurs in patients with this disease, the molecular pathways that lead to the accompanying neurological defects are unclear. Using an authentic murine model of GM2 gangliosidosis, we examined the pattern of neuronal loss in the central nervous system and investigated the effects of gene transfer using recombinant adeno-associated viral vectors expressing β-hexosaminidase subunits (rAAV2/1-Hex). In 4-month-old Sandhoff mice with neurological deficits, cells staining positively for the apoptotic signature in the TUNEL reaction were found in the ventroposterior medial and ventroposterior lateral (VPM/VPL) nuclei of the thalamus. There was progressive loss of neuronal density in this region with age. Comparable loss of neuronal density was identified in the lateral vestibular nucleus of the brainstem and a small but statistically significant loss was present in the ventral spinal cord. Loss of neurons was not detected in other regions that were analysed. Administration of rAAV2/1-Hex into the brain of Sandhoff mice prevented the decline in neuronal density in the VPM/VPL. Preservation of neurons in the VPM/VPL was variable at the humane endpoint in treated animals, but correlated directly with increased lifespan. Loss of neurons was localized to only a few regions in the Sandhoff brain and was prevented by rAAV-mediated transfer of β-hexosaminidase gene function at considerable distances from the site of vector administration.

INTRODUCTION

The GM2 gangliosidoses, Tay-Sachs disease (1–2), Sandhoff disease (3) and GM2 activator deficiency (4) are lysosomal storage disorders with progressive widespread manifestations in the nervous system. These diseases are caused by defective catabolism of GM2 and related glyco-conjugates (5–7), principally in neurons, due to impaired function of β-hexosaminidases in the lysosome.

The murine model of Sandhoff disease Hexb knock out (KO) (8) displays a phenotype closely resembling early-onset GM2 gangliosidoses in humans. At 3 months of age, the animals develop tremor and increased limb tone, which is worse in the hind legs. These manifestations become progressively more severe until 4–5 months of age, when the animal becomes moribund and rapidly loses weight. Neurons appear distended by lysosomal storage material and signs of neuroinflammation are present (9) accompanied by an increase in terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining (9–11). In infants with GM2 gangliosidosis, whole brain weight is greatly increased for age, and post-mortem examination reveals marked loss of neurons in the cerebral cortex and cerebellum with extensive gliosis (12). TUNEL staining in the Sandhoff mouse brain has been widely interpreted as indicative of neuronal loss, although no definitive quantification has been provided.
Recombinant adeno-associated virus-mediated expression of β-hexosaminidase (rAAV2/1-Hex) is an effective treatment for murine Sandhoff disease (13). Treatment by gene transfer prevents lysosomal storage, neuroinflammation and greatly prolongs the life of the Sandhoff mouse. This improved survival exceeds that reported with other treatments such as substrate reduction therapy, bone marrow transplantation and stem cell therapy (14–16). Here, we investigated whether gene therapy would improve outcome by preventing neuronal cell loss. This led us to assess the extent of neuronal loss in this model and its contribution to the pattern and onset of disease manifestations. A combination of TUNEL staining and immunostaining for the signature neuronal antigen NeuN showed marked but highly localized loss of neurons that was prevented by rAAV-mediated functional complementation of β-hexosaminidase.

RESULTS

TUNEL staining in Sandhoff mouse brain reveals cell changes with marked regional specificity

For determination of TUNEL-staining distribution in the Sandhoff mouse brain, 4-month-old Sandhoff animals were compared with age-matched normal controls (Fig. 1). For examples of TUNEL-staining nuclei in the ventroposterior medial and lateral nuclei (VPM/VPL), see Supplementary Material, Figure S1. The brains of four Sandhoff mice and four age-matched controls were examined. TUNEL-stained cells were systematically counted throughout the brain. Structures included in the analysis were defined as the telencephalon (the rostral migratory stream was not included in the analysis); diencephalon; mesencephalon; rhombencephalon and the cerebellum (counted separately from the rhombencephalon). TUNEL-staining nuclei quantified in the telencephalon were similar between Sandhoff animals and controls. In contrast, the Sandhoff diencephalon showed an increase in TUNEL-positive nuclei compared with age-matched controls (Fig. 2, P < 0.01, Bonferroni post hoc test). TUNEL staining in the Sandhoff diencephalon was localized to the ventrobasal thalamus (VPM/VPL). Dense TUNEL staining present in the VPM/VPL was not seen in other thalamic structures such as anterior motor nuclei. No appreciable increases in TUNEL staining were noted elsewhere in the Sandhoff brain.

NeuN immunostaining reveals localized reduction of neuronal density

TUNEL staining was most intense in the VPM/VPL of the Sandhoff mouse. To determine whether neuronal loss occurred in the VPM/VPL nuclei and to corroborate the signals obtained with TUNEL staining, sections were stained with NeuN to identify neurons and counted to determine relative neuronal density. Anti-NeuN staining was used as it recognizes the neuron-specific DNA-binding protein in most areas of the central nervous system (CNS). To determine whether neuronal loss is a localized phenomenon in the Sandhoff CNS, NeuN immunostained cells were counted also in other regions. These regions included the primary somatosensory and motor cortices, the lateral vestibular nucleus (LVN), the facial motor nucleus (nucleus of cranial nerve VII) and total neurons in the ventral horns of the spinal cord. Purkinje neurons of the cerebellum were stained with haematoxylin and eosin and counted. The relevance of these structures to...
Sandhoff disease and justification for inclusion in the study is set out in Table 1.

NeuN staining in the Sandhoff animal revealed progressive neuronal loss in the VPM/VPL and LVN compared with age-matched control animals (Figs 3 and 4). Neuronal loss was apparent in the VPM/VPL by 3 months of age (39% decrease, \( P < 0.01 \), Bonferroni post hoc test) and by 4 months of age, relative neuronal density had fallen to less than one half of the density in control animals (58% decrease, \( P < 0.01 \), Bonferroni post hoc test, Figs 3 and 5A). Similarly, loss of neuronal density in the LVN was apparent by 3 months of age (57% decrease, \( P < 0.01 \), Bonferroni post hoc test); however, this loss did not increase beyond 3 months (58% decrease, \( P < 0.01 \), Bonferroni post hoc test) (Figs 4 and 5b).

Neurons in the spinal cord were counted in the ventral horns—defined as all neurons ventral to the central canal. Quantification of NeuN-staining cells in sections sampled along the length of the spinal cord (on average, 18 sections were sampled per animal) showed a small but statistically significant loss (15% loss, \( P = 0.013 \), Student’s t-test) in the Sandhoff animal at 4 months of age compared with an age-matched control. In contrast, neuronal density in the primary motor cortex, the somatosensory cortex (the forelimb and hindlimb area), the facial motor nucleus and the Purkinje neurons of the cerebellum was unchanged (see Supplementary Material, Table S1 for results).

Collectively, these data show that neuronal loss in the brain and spinal cord of Sandhoff mice is confined to localized anatomical regions and is not a widespread phenomenon.

Figure 3. The Sandhoff VPM/VPL shows progressive loss in neuronal density. At 2 months of age, NeuN staining (brown DAB stain) is roughly equal between control and Sandhoff animals (A and B). By 3 months of age, overt loss in neuronal density has occurred in the VPM/VPL of the Sandhoff animal (shown by dashed line, C and D). By 4 months of age (E and F), this loss has progressed to over half the neurons present in the normal control. Scale bars = 200 \( \mu \)m.

Table 1. Regions of the CNS assessed for loss in neuronal density

<table>
<thead>
<tr>
<th>Anatomical region</th>
<th>Reason for inclusion/attributed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalamus (VPM/VPL)</td>
<td>This region showed dense TUNEL staining in the Sandhoff mouse. Proprioceptive and somatosensory information are relayed through these nuclei (30)</td>
</tr>
<tr>
<td>LVN</td>
<td>The LVN receives afferent projections from the cerebellar Purkinje neurons. This nucleus modulates antigravity muscles by projecting onto spinal interneurons (32)</td>
</tr>
<tr>
<td>Primary somatosensory cortex (layers II–IV and V and VI—forelimb and hindlimb area)</td>
<td>This somatosensory cortex receives afferent projections from the VPM/VPL and could be affected by thalamic neuronal loss</td>
</tr>
<tr>
<td>Primary motor cortex (layers II–III and V and VI)</td>
<td>Contains upper motor neurons. Loss of these neurons could explain hindlimb hypertonicity seen in Sandhoff animals (42)</td>
</tr>
<tr>
<td>Facial motor nucleus (VII)</td>
<td>Facial paralysis is not a recognized symptom in GM2 gangliosidosis patients. As such this nucleus was not suspected to play a role in disease and should not show neuronal loss</td>
</tr>
<tr>
<td>Cerebellar Purkinje neurons (third, fourth and fifth cerebellar lobules)</td>
<td>Through connections with the deep cerebellar and vestibular nuclei, Purkinje cells represent the sole efferent pathway from the cerebellum</td>
</tr>
<tr>
<td>Spinal cord (ventral horn neurons)</td>
<td>Contains interneurons and lower motor neurons that participate in the cortico-spinal tract. Loss of interneurons [seen in mouse models of spasticity (43)] could explain hindlimb hypertonicity seen in Sandhoff animals</td>
</tr>
</tbody>
</table>

This table shows regions of the CNS where numbers of NeuN-staining cells were counted and relative densities were determined. Reasons for inclusion and function of neuroanatomy are also described.

Gene therapy prevents neuronal loss in the Sandhoff mouse

To determine whether gene augmentation therapy could prevent neuronal loss in the Sandhoff brain, 1-month-old mice were
treated with a single striatal injection of rAAV2/1 encoding β-hexosaminidases A and B. Viruses were either therapeutic rAAV2/1-Hex or control rAAV2/1-enhanced green fluorescent protein (EGFP) (see Table 2 for a summary of animals used). Three Sandhoff and four age-matched control animals received rAAV2/1-Hex in the right striatum and vehicle contralaterally and were culled for analysis at 4 months of age. Two Sandhoff animals that were treated with rAAV2/1-EGFP were culled at 4 months of age. A further three Sandhoff animals and two control animals that received gene therapy were killed at their humane endpoint for analysis.

After intervention by rAAV2/1-Hex, mice culled for analysis at 4 months of age did not display overt disease signs, such as coarse tremor, present in untreated Sandhoff animals of the same age. At 4 months, relative neuronal density in the VPM/VPL of treated Sandhoff mice was indistinguishable from the density in treated control mice. At this time point in the untreated Sandhoff animal, over half of the neurons had died (Fig. 7A–D). Two Sandhoff animals that had received rAAV2/1-EGFP as a control vector displayed disease signs and neuronal densities in the VPM/VPL similar to that observed in untreated Sandhoff disease animals (Fig. 7D). This indicates that expression of β-hexosaminidase activity rescued neuronal viability. In contrast to the thalamus, gene therapy delivered at a single site in the striatum failed to prevent loss of neuronal density in the distant LVN at 4 months of age (Fig. 7E). In this group of animals, there was a significant difference between virus-treated control animals and untreated Sandhoff animals (P < 0.05, Bonferroni post hoc test).

Sandhoff animals treated by gene transfer maintained to the humane endpoint (n = 3, survival = 151, 226 and 258 days) showed variable neuronal loss in the VPM/VPL when compared with two normal controls that had also received therapeutic vector. Preservation of neuronal density in the VPM/VPL directly correlated with increased survival (Fig. 7F). In one animal that received little benefit from rAAV treatment (survival = 151 days), neuronal density in the VPM/VPL was similar to 4-month-old untreated Sandhoff disease [248 and 331 neurons/mm² in phosphate-buffered saline (PBS) and rAAV-treated hemispheres, respectively]. The animal that survived to an intermediate age (226 days) had rescue of neuronal loss in the VPM/VPL confined to the rAAV-treated hemisphere; the hemisphere receiving only PBS showed neuronal loss in the range of untreated mice at the humane endpoint (295 and 728 neurons/mm² in PBS- and rAAV-treated hemispheres, respectively). The longest surviving animal (258 days) had bilateral preservation of VPM/VPL neurons even though only one of the hemispheres was treated with rAAV2/1-Hex (777 and 816 neurons/mm² in PBS- and rAAV-treated hemispheres, respectively). Loss of neurons in the VPM/VPL at the humane endpoint correlated well with neuronal storage, as determined by cytoplasmic vacuolation revealed by NeuN-staining (Supplementary Material, Fig. S2). VPM neurons in hemispheres that underwent neuronal loss appeared vacuolated with neuronal storage. Where there was preservation of neurons in the VPM/VPL of treated Sandhoff mice, cytoplasmic vacuolation was absent.

Importantly, and despite bithalamic neuronal rescue by gene therapy, the animal, which showed bithalamic rescue of neurons and that had lived until 258 days of age, reached its humane endpoint and had the characteristic signs of experimental Sandhoff disease. Rapid weight loss of between 10 and 20% of presymptomatic weight that defines the humane endpoint in our experiments with this mouse strain is shown in Figure 8. The Sandhoff LVN showed little therapeutic benefit from gene transfer, although in the longest surviving mouse, the rAAV-treated side had a neuronal density which was greater than the Sandhoff disease range (Fig. 7G).

**DISCUSSION**

Here we show that the loss of thalamic neurons in the Sandhoff mouse brain is prevented by a single administration of rAAV2/1-mediated gene therapy delivered into the right striatum (Fig. 7A–D and F). Not only was delivery of treatment to the striatum sufficient to prevent neuronal loss in a neighbouring brain structure, the thalamus, it also prevented neuronal loss in the contralateral thalamus (Fig. 7D and F). A large part of this observed correction probably resulted from secretion by transduced cells and recapture of the β-hexosaminidase corrective factor via the mannose-6-
phosphate or a related receptor-mediated uptake pathway (17).

Prevention of neuronal loss in the thalamus is congruent with previous work describing gene therapy of experimental GM2 gangliosidosis from our laboratory; the widespread distribution of \( \beta \)-hexosaminidase activity after gene therapy delivered into the striatal parenchyma is a noteworthy feature of this intervention (13).

While in Sandhoff mice culled at the humane endpoint after gene therapy, rescue of thalamic neurons was predictive of survival, this simply reflects the extent to which gene therapy was able to complement the enzyme deficiency in that individual animal. Since all animals eventually showed end-stage signs of Sandhoff disease at the end of their lives, it is clear that the clinical signs were independent of the status of the thalamus. As the three animals used in this experiment demonstrated such extreme variation in VPM/VPL neuronal density that was concomitant with end-stage Sandhoff disease (Fig. 7F), neuronal loss in the thalamus cannot contribute significantly to the development of overt motor signs of disease. This result has implications for other models of lysosomal disorders in which the role of thalamic neuronal loss in pathogenesis has been recently questioned (18).

A localized immune response to the vector or the transgene, or both, is present in these animals; cellular infiltrate and inflammatory markers are present around the injection site. Localized loss of neurons was also observed around the vector delivery site and was restricted to the striatum. However, the local inflammatory reaction is temporary and it is in agreement with published findings by others. Animals injected at 1 month of age with a dose of vector

Figure 5. Quantification of relative loss in neuronal density in the Sandhoff thalamus and brainstem. (A) Loss in neuronal density is progressive in the Sandhoff VPM/VPL (white bars) when compared with age-matched normal controls (black bars) from 2 months of age onwards. Significant \( (P < 0.01) \) neuronal loss has occurred by 3 months of age. By 4 months, less than half of neurons present in controls remain. (B) A decrease in neuronal density in the Sandhoff LVN, also seen from 3 months onwards. A mixture of Hexb \(+/-\) and Hexb \(+/-\) mice were used as normal controls to compare with Sandhoff mice. Neuronal density between Hexb \(+/-\) and Hexb \(+/-\) mice were entirely comparable. \( n = 3, 3 \) and 4 Sandhoff and control mice at 2, 3 and 4 months of age, respectively. \( * P < 0.01, \) Bonferroni post hoc test.

Table 2. Animals used to determine whether gene therapy can prevent neuronal loss

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>n</th>
<th>Age at which mice were culled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandhoff</td>
<td>rAAV2/1-Hex</td>
<td>3</td>
<td>4 months</td>
</tr>
<tr>
<td>Normal controls</td>
<td>rAAV2/1-Hex</td>
<td>4</td>
<td>4 months</td>
</tr>
<tr>
<td>Sandhoff</td>
<td>rAAV2/1-EGFP</td>
<td>2</td>
<td>4 months</td>
</tr>
<tr>
<td>Normal controls</td>
<td>rAAV2/1-Hex</td>
<td>2</td>
<td>Humane endpoint controls</td>
</tr>
<tr>
<td>Sandhoff</td>
<td>rAAV2/1-Hex</td>
<td>3</td>
<td>Humane endpoint (151, 226 and 258 days—male, female and male, respectively)</td>
</tr>
</tbody>
</table>

All animals were injected with rAAV2/1 at 1 month of age.

phosphate or a related receptor-mediated uptake pathway (17).
Figure 7. rAAV2/1-Hex-mediated gene therapy prevents neuronal loss in the Sandhoff VPM/VPL. (A) Marked neuronal loss (shown by DAB-stained NeuN immunoreactivity) in the untreated Sandhoff VPM/VPL at 4 months of age (arrows). In contrast, rAAV2/1-Hex-treated Sandhoff mice do not show neuronal loss at 4 months of age (B). In fact they are indistinguishable from rAAV2/1-Hex-treated controls (C). (D) rAAV2/1-Hex-treated 4-month-old Sandhoff animals (n = 3, white bars) had VPM/VPL neuronal densities similar to treated control animals (n = 4, black bars). This was true for both the rAAV2/1-Hex-treated hemisphere and the contralateral control PBS-treated hemisphere. Two Sandhoff animals were injected with EGFP-expressing rAAV2/1. These animals exhibited disease range neuronal loss in the VPM/VPL at 4 months of age (green diamonds). (E) The effect of gene therapy on neuronal loss in the LVN. In this analysis, virus-treated control animals are significantly different from untreated Sandhoff animals (P < 0.05); however, virus-treated Sandhoff animals are not statistically different from untreated Sandhoff animals. In (F) and (G), individual animals taken to their humane endpoints are represented for both PBS (black bars) and rAAV2/1-Hex (white bars) treated hemispheres. Survival in days is indicated on the x-axis. In the VPM/VPL (F), treated animals exhibited no prevention of neuronal loss (survival time = 151 days), unilateral rescue from neuronal loss (survival time = 226 days) or bilateral rescue from neuronal loss (survival time = 258 days). (G) Gene therapy administered in the striatum did not rescue neurons in the LVN at the humane endpoint, apart from in the longest surviving Sandhoff animal. Scale bars = 200 μm (A–C). Bars = mean ± SEM (D–G) or singular data points (F and G). The red horizontal bar represents the 4-month-old untreated Sandhoff disease minima and maxima (D–G). A mixture of Hexb +/+ and Hexb +/− mice were used as treated normal controls to compare with Sandhoff and treated Sandhoff mice. Neuronal density between Hexb +/+ and Hexb +/− mice were entirely comparable.
animals. Figure 8. Weights of treated Sandhoff animals at the humane endpoint. This figure shows weights recorded for rAAV2/1-Hex-treated Sandhoff animals that were allowed to survive to their humane endpoint. Survival time for rAAV2/1-Hex-treated Sandhoff animals is shown in the figure legend. Compared to an rAAV2/1-Hex-treated control mouse that increased in weight at a stable rate, treated Sandhoff animals eventually displayed acute weight loss which signalled their humane endpoint. Data plots represent individual animals.

higher than that used in the present study and culled at 2 years of age did not show this localized inflammatory reaction (1 out of 29 animals that received rAAV2/1-Hex at 1 month of age and were sacrificed at 2 years of age showed cellular infiltrate around vessels at an injection site, unpublished observations). In spite of these findings, we contend that the immune response is unlikely to have attenuated the therapeutic rescue of Sandhoff disease here demonstrated: robust expression of corrective enzyme is maintained in the brain for at least 2 years after treatment with rAAV2/1-Hex (13 and unpublished observations). Partial rescue of Sandhoff disease more likely results from incomplete distribution of the vector and enzyme in the nervous system. Even in the longest surviving animal in the present study, neuronal storage was present in the brainstem at the humane endpoint (Supplementary Material, Fig. S2B(ix)).

With combined TUNEL (Figs 1 and 2) and NeuN staining (Figs 3–5), we show that the neuronal loss in the Sandhoff mouse brain is localized. Intense TUNEL staining was observed in defined thalamic nuclei and this contrasted with sparse staining elsewhere in the Sandhoff brain. Within the thalamus, TUNEL staining was localized almost entirely to the somatosensory VPM and VPL nuclei. Dense TUNEL staining was not observed in the LVN, where neuronal loss was also observed. This may be because TUNEL staining was only performed on 4-month-old animals and by that stage neuronal loss had apparently stopped. A factor that could bias interpretation of TUNEL staining is how disease influences the rate of clearance of apoptotic bodies. Under conditions of adrenalectomy and corticosterone supplementation in the rodent brain, initiation of neuronal apoptosis to clearance of debris visible at the light microscopic level takes \( \sim 72 \) h (19). Based on this study, a TUNEL-labelled apoptotic body may be present in the brain for 2–3 days before its components are phagocytosed. However, in some diseases, the time taken to clear apoptotic neurons may vary. Accumulation of late apoptotic bodies occurs in a Drosophila model of mucolipidosis type IV (a lysosomal disorder) as a result of impaired clearance (20). Impaired clearance of dead cells in the brain could exacerbate neuroinflammation and lead to further cell death (9). In contrast, clearance of apoptotic bodies can occur more rapidly than normal. For example, a decrease in progranulin expression can accelerate apoptotic clearance; a mechanism thought to be important in frontotemporal lobar degeneration (21). We identified very few pyknotic bodies (late stage apoptotic morphology, Supplementary Material, Fig. S1) staining for TUNEL. This suggests that clearance remains active in the Sandhoff mouse. However, if Sandhoff disease were to accelerate phagocytosis of apoptotic particles, the TUNEL staining presented here may lead to underestimates of the number and distribution of dying cells.

In our hands, TUNEL staining also proved to be variable, depending on how the tissue was processed. Dense TUNEL staining was only observed in 4-month-old Sandhoff mouse brains when the tissue was embedded in paraffin before processing. Staining was also affected by length of exposure to paraformaldehyde so that postfixation of no more than 2 h was optimal. It was notable that the TUNEL signal was never intense when examined in tissue snap-frozen when fresh, cryosectioned and then fixed in PBS containing 4% paraformaldehyde immediately before staining. Given the difficulties associated with TUNEL staining, further analysis of cell loss in the Sandhoff mouse was performed with NeuN staining for neurons, which proved to be very robust.

The significance of the small loss of neurons seen in the ventral spinal cord is unclear and requires further study to delineate what type of spinal neurons have been lost in the 4-month-old Sandhoff mouse. In contrast, primary motor cortex, somatosensory cortex, facial motor nucleus and cerebellar Purkinje neurons showed no significant loss of neurons. Localized loss of neurons in the Sandhoff mouse brain was accompanied by co-varying GFAP staining (Fig. 6). As expected, neuroinflammation shown by GFAP staining appeared high in regions of marked neuronal death. Thus, reduced neuronal density is a localized finding in the murine Sandhoff brain. Accumulation of glycolipids in the Sandhoff brain shows regional differences, for example, storage of glyco-conjugates revealed by periodic acid-Schiff staining in the Sandhoff cerebral cortex is prominent in layers V and VIb, but not in layers II/III, IV and VIa. Additionally, pyramidal neurons in the CA3 field of the hippocampus appear distended, but pyramidal neurons in the CA1 field of Ammon’s horn do not. In spite of this, regional variations in storage material do not account for the pattern of neuronal loss observed: neurons of the VPM/VPL stain very strongly for storage material (Supplementary Material, Fig. S1), and this region shows marked neuronal loss. On the other hand, large motor neurons in the facial nucleus of the brainstem also contain substantial amounts of storage material but do not show decreased numbers (Supplementary Material, Table S1); neither does the facial motor nucleus show the same density of GFAP staining as the VPM/VPL (Fig. 6).

Selective neuronal loss that does not correlate with the distribution of storage material has been recently noted in ovine neuronal ceroid lipofuscinosis (Batten’s disease) (22). The authors of this study interpreted the observation as evidence that lysosomal storage body accumulation is not central to pathogenesis. We contend it is equally probable that neuronal pathogenesis. We contend it is equally probable that neuronal...
lyosomal storage may indeed contribute to pathological processes that ultimately cause loss of neurons with varying degrees of susceptibility. We hypothesize that the loss of local populations or networks of neurons may result from variation in individual susceptibility to disturbances of specialized functions or cellular structures. In essence, regionalized neuronal death could result from a selectively lowered threshold to the toxic effects of lysosomal storage. Preferential injury as a result of particular physiological stress may also explain the apparent selective vulnerability of specific neurons in other neurological diseases. In amyotrophic lateral sclerosis, selective loss of motor neurons has been ascribed to reduce buffering of cytosolic calcium ions (23). Under physiological conditions, weak calcium buffering allows rapid calcium signalling at low energy cost—however, this phenomenon leaves the motor neuron susceptible to pathological calcium fluxes (23). In a similar manner, preferential loss of striatal neurons in Huntington’s disease may be explained by enhanced mitochondrial sensitivity to increased intracellular calcium concentrations (24).

Neuronal loss in the VPM/VPL nuclei of the thalamus is not restricted to Sandhoff mice. Neuronal loss in thalamic nuclei also occurs in the Niemann-Pick type C1 (25), mouse models of infantile neuronal ceroid lipofuscinosis (26) and in models of neuronopathic Gaucher disease (27,28). Collectively, these findings suggest that thalamic relay nuclei are especially vulnerable to the pathological effects of diverse lysosomal disorder. In the Sandhoff disease brain, it is unknown as to whether the cell death is induced by factors intrinsic or extrinsic to the neuron. Studies in other lysosomal diseases suggest that the cell loss is neuron intrinsic and not solely triggered by external factors, such as those elaborated by abnormally behaving microglia. A neuronopathic model of Gaucher disease in which lysosomal glucocerebrosidase is deficient only in neurons and macroglia shows clear loss of neurons (27). Purkinje cell loss in Niemann-Pick type C1 also appears to be caused by intrinsic biochemical changes (29).

In light of the findings reported here, we contend that further understanding of the pathogenesis of GM2 gangliosidosis, one of the most disabling of the neurodegenerative disorders caused by lysosomal defects, will require efforts to reconcile the signs of Sandhoff disease with defined neuropathological lesions. The localized loss of neurons reported here can inform us about clinical disease experienced by the Sandhoff mouse model. As gross loss of neurons was seen in the Sandhoff VPM/VPL somatosensory relay nuclei, these findings suggest that thalamic relay nuclei are especially vulnerable to the pathological effects of diverse lysosomal disorder. In the Sandhoff disease brain, it is unknown as to whether the cell death is induced by factors intrinsic or extrinsic to the neuron. Studies in other lysosomal diseases suggest that the cell loss is neuron intrinsic and not solely triggered by external factors, such as those elaborated by abnormally behaving microglia. A neuronopathic model of Gaucher disease in which lysosomal glucocerebrosidase is deficient only in neurons and macroglia shows clear loss of neurons (27). Purkinje cell loss in Niemann-Pick type C1 also appears to be caused by intrinsic biochemical changes (29).

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In light of the findings reported here, we contend that further understanding of the pathogenesis of GM2 gangliosidosis, one of the most disabling of the neurodegenerative disorders caused by lysosomal defects, will require efforts to reconcile the signs of Sandhoff disease with defined neuropathological lesions. The localized loss of neurons reported here can inform us about clinical disease experienced by the Sandhoff mouse model. As gross loss of neurons was seen in the Sandhoff VPM/VPL somatosensory relay nuclei, these animals are likely to have somatosensory impairment with reduced sensation to touch, nociception and proprioception (30). Progressive loss of somatosensory function in the Sandhoff mouse has been shown at 2 and 3 months of age (31). In this study, gene therapy directed towards dorsal root ganglion neurons in Sandhoff mice improved conduction velocity and action potential of these neurons. However, sensory deficit persisted and progressed at 3 months of age. Neuronal loss shown in the VPM/VPL in the current study would explain a large part of the persisting sensory loss shown in the study by Terashima et al. (31); the VPM/VPL represents the relay link between peripheral somatosensation and processing in the cerebral cortex. Loss of neurons in the LVN would be expected to impair cerebellar modulation of equilibratory reflexes via a pathway that links cerebellar Purkinje neurons to spinal interneurons (32). This could impair the righting reflex; however, the overt ataxia and tremor are inconsistent with this specific finding and are a strong indication of additional cerebellar disease.

In the acute murine model, movement-induced tremor is prominent: however, overt neuronal loss is not observed in the cerebellum. Similarly, there is increased hindlimb tone, but little or no evidence of neuronal loss from the motor cortex. We propose that the origin of these signs of disease will be found in other aspects of Sandhoff disease neuropathology. Axonal dystrophy is a prevalent histological feature in lysosomal disorders with a neurological component and in some instances this occurs in regions that might account for the neurological manifestations (33). One cellular population that is very susceptible to this pathological feature is Purkinje neurons (33), which could account for tremor seen in lysosomal disorder models. Although there appear to be few indications as to how axonal dystrophy affects transmission of action potentials, the strong correlation between the appearance of axonal dystrophy and the development of neurological manifestation appears to merit more study (34). Disrupted myelin structure and disturbance in myelin lipid biochemistry have been reported in the Sandhoff mouse with hypomyelination of the spinal cord (35) and optic nerve (36). Moreover, major disruption of myelin-associated lipids has been documented in Sandhoff mice, cats and humans (37). Thus disrupted transmission of action potentials due to compromised integrity of the myelin sheath may be an additional factor contributing to the progressive and severe neurological manifestations of Sandhoff disease.

It is unclear as to why overt loss of neurons reported in the human cerebral cortex and cerebellum is not seen in the mouse. This may relate to differences in the composition of brain lipids and complement of sphingolipid-degrading enzymes between humans and rodents. A sialidase capable of degrading GM2 ganglioside to GA2 (38) provides a default degradation pathway in the rodent; as a result, GA2 constitutes a larger proportion of the glycolipids in the brain of the Sandhoff mouse compared with the human brain from Sandhoff disease patients (37). An increased proportion of GM2 in human Sandhoff neurons could explain increased loss of neurons compared with the mouse if it is more toxic than GA2. The sialic acid moiety present on GM2 is responsible for inhibition of sarco-endoplasmic reticulum Ca$^{2+}$ ATPase (39)—the resulting increase in intracytoplasmic calcium and depletion of endoplasmic reticulum calcium stores are potentially damaging to neurons (40). Alternatively, neuronal death in additional CNS structures of the human Sandhoff or Tay-Sachs brain could simply reflect the effects of better medical intervention and the presence of more protracted disease in the brains obtained at death from human patients.

In summary, marked neuronal loss in the Sandhoff mouse brain localized to specific thalamic relay nuclei can be prevented by rAAV-mediated gene therapy. However, loss of thalamic neurons does not materially contribute to the principal manifestations of the acute neurological disease reproducibly exhibited in the Sandhoff mouse strain.
MATERIALS AND METHODS

Animals

All animal procedures were conducted following guidelines of the Animals (Scientific Procedures) Act, 1986. Hexb KO mice were generated by disruption of exon 13 of the Hexb gene (8). Hexb +/− mice were used as normal controls in this study. Where a mixture of Hexb+/− and Hexb +/- mice were used as controls, a note has been made in the corresponding figure legend. Mice had access to food and water ad libitum. All mice in the study received transgel (Charles River Laboratories) as a dietary supplement. Sandhoff mice (Hexb −/−) allowed to live to the humane endpoint were sacrificed when they had lost between 10 and 20% of their presymptomatic body weight.

rAAV administration

Vector and construct production has been previously described (13). A single co-injection of rAAV2/1 as a 3 μl mixture of HEXA cDNA expressing viruses (9.9 × 10⁹ genome copies) and HEXB cDNA expressing viruses (1.4 × 10¹⁰ genome copies) with 0.8 μl of 20% mannitol was administered to the right striatum at anterior-posterior 0.1 mm, medial-lateral 2.0 mm and dorsal-ventral 3.0 mm from bregma. Alternatively, EGFP-expressing viruses (1.1 × 10¹¹ genome copies) were injected as a control. In the same treated animals, PBS in the presence of mannitol was injected into the contralateral striatum as an internal control. All animals were injected at 1 month of age.

Tissue processing

Mice were terminally anaesthetised by intraperitoneal injection of sodium pentobarbital and transcardially perfused with PBS followed by PBS containing 4% paraformaldehyde. The brain and spinal cord were immediately dissected out and post-fixed for 2 h in the same fixative. Brains were then washed in PBS and paraffin embedded. The brain and spinal cord were sectioned at 10 μm thickness and mounted onto Superfrost Plus microscope slides (631-0108, VWR International).

TUNEL staining

TUNEL staining was performed using the ApopTag TUNEL staining kit (S7100, Millipore). Briefly, sections (10 μm thick) were deparaffinised in xylene, rehydrated through a graded ethanol series and were treated with PBS containing proteinase K (20 μg/ml) for 15 min before continuing with the TUNEL procedure following manufacturer’s recommendations. TUNEL staining was visualized by horse radish peroxidase (HRP)–diaminobenzidine (DAB) staining. Tissue sections were counterstained for cell nuclei with methyl green.

Immunostaining

For immunohistochemistry on paraffin-embedded sections, brain sections were deparaffinized and endogenous peroxidase activity was quenched in PBS containing 3% H₂O₂ for 5 min. Tissue was antigen-retrieved by incubating slides at 95–100°C for 10 min in 10 mm trisodium citrate, pH 6.0 and then left to cool at room temperature for 20 min. Sections were blocked in PBS containing 2% bovine serum albumin (BSA) for 20 min. All antibody incubations with paraffin-embedded sections were performed in PBS containing 2% BSA. For determination of relative neuronal densities, slides were incubated with mouse anti-neuronal nuclear antigen (anti-NeuN, 1:100, MAB377, Millipore) and revealed with goat anti-mouse Ig-HRP (1:100, P0447, Dako) and DAB staining. Staining for GFAP was performed on paraffin-embedded sections as above with goat anti-GFAP (1:50, sc-6171, Santa Cruz Biotechnology) and rabbit anti-goat Ig-HRP (1:100, P0449, Dako).

Tissue sampling and counting

For analysis of TUNEL staining, serial brain sections were sampled every 240 μm (41) and TUNEL-positive nuclei were counted. Determination of relative neuronal density in defined brain structures was achieved by sampling three NeuN-stained sections bilaterally at 100 μm intervals. Area of the structure and number of neurons present on a section were determined with ImageJ software (v1.41, NIH). All statistical analysis was performed using Graphpad Prism (v5.02) software.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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