Contribution of brain inflammation to neuronal cell death in neuronopathic forms of Gaucher’s disease

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Gaucher’s disease, the most common lysosomal storage disorder, is caused by the defective activity of glucocerebrosidase, the lysosomal hydrolase that degrades glucosylceramide. The neuronopathic forms of Gaucher’s disease are characterized by severe neuronal loss, astrocytosis and microglial proliferation, but the cellular and molecular pathways causing these changes are not known. In the current study, we delineate the role of neuroinflammation in the pathogenesis of neuronopathic Gaucher’s disease and show significant changes in levels of inflammatory mediators in the brain of a neuronopathic Gaucher’s disease mouse model. Levels of messenger RNA expression of interleukin -1β, tumour necrosis factor-α, tumour necrosis factor-α receptor, macrophage colony-stimulating factor and transforming growth factor-β were elevated by up to ~30-fold, with the time-course of the increase correlating with the progression of disease severity. The most significant elevation was detected for the chemokines CCL2, CCL3 and CCL5. Blood–brain barrier disruption was also evident in mice with neuronopathic Gaucher’s disease. Finally, extensive elevation of nitrotyrosine, a hallmark of peroxynitrite (ONOO−) formation, was observed, consistent with oxidative damage caused by macrophage/microglia activation. Together, our results suggest a cytotoxic role for activated microglia in neuronopathic Gaucher’s disease. We suggest that once a critical threshold of glucosylceramide storage is reached in neurons, a signalling cascade is triggered that activates microglia, which in turn releases inflammatory cytokines that amplify the inflammatory response, contributing to neuronal death.

Keywords: Gaucher’s disease; neuroinflammation; lysosomal storage disorders

Abbreviations: Gd-DTPA = gadolinium diethylenetriaminopentaacetic acid; HPRT = hypoxanthine phosphoribosyltransferase 1; IL = interleukin; PBS = phosphate-buffered saline; TNF-α = tumour necrosis factor-α

Introduction

Gaucher’s disease is an inherited metabolic disorder caused by mutations in the gene encoding glucocerebrosidase. Impairment of glucocerebrosidase activity leads to accumulation of the sphingolipid, glucosylceramide, which via largely unknown mechanisms, results in disease pathology (Wong et al., 2004; Vitner et al., 2010b; Gupta et al., 2011). The neuronopathic forms of Gaucher’s disease (types 2 and 3), which comprise ~6% of patients with Gaucher’s disease, are characterized by neuronal loss, astrocytosis and microgliosis (Wong et al., 2004; Farfel-Becker et al., 2011b). Little is known about the molecular events leading to neuronal death, and at present there is no treatment available for neuronopathic Gaucher’s disease. Thus, understanding the biochemical and cellular pathways that cause neuronopathic Gaucher’s disease may potentially offer new therapeutic approaches for disease management.
and could also shed light on the mechanisms at play in other lysosomal storage diseases, of which Gaucher’s disease is the most common. Moreover, a link has recently been suggested between Gaucher’s disease and Parkinson’s disease (Sidransky, 2004; Yap et al., 2011), although this link may be more widely spread among the lysosomal storage disorders than originally thought (Shachar et al., 2011).

We recently demonstrated elevation of cathepsin D in microglia in a mouse model of neuronopathic Gaucher’s disease (Vitner et al., 2010a), which suggested that reactive microglia may play a role in neuronal degeneration. This assumption was further supported by findings showing that microglial activation and astrogliosis are spatially and temporally correlated with selective neuronal loss (Farfel-Becker et al., 2011b). Activated microglia release a variety of soluble proinflammatory and potentially cytotoxic factors [such as superoxide, nitric oxide and tumour necrosis factor-α (TNF-α)] (Block and Hong, 2005). However, activated microglia can also enhance neuronal survival through release of trophic and anti-inflammatory factors (Block and Hong, 2005), with the nature of the released factors determining the outcome of the inflammatory reaction.

Brain inflammation has been implicated in a wide range of neurodegenerative disorders such as Alzheimer’s and Parkinson’s diseases (Barnum and Tansey, 2010; Lee et al., 2010) and also in a number of lysosomal storage disorders such as the GM1 and GM2 gangliosidoses (Wada et al., 2000; Jeyakumar et al., 2003), mucopolysaccharidoses types I and IIIB (Ohmi et al., 2003; Ausseil et al., 2008) and Niemann–Pick type C (German et al., 2002; Smith et al., 2009). In contrast, most of the studies examining inflammation in Gaucher’s disease have focused on type 1, which lacks a severe neuronal phenotype. Thus, glucosylceramide storage in macrophages (the main cell type affected in type 1 Gaucher’s disease) leads to macrophage activation and release of multiple cytokines (Imoudiak and Futerman, 2005). Levels of interleukin (IL)-1β, IL-1 receptor antagonist, IL-6, TNF-α and soluble IL-2 receptor (sIL-2R) are all elevated in the serum of patients with Gaucher’s disease (Barak et al., 1999), as are CD14 and macrophage colony-stimulating factor (Hollak et al., 1997). In the foetal brain of the Gba mouse (a complete glucocerebrosidase knockout; Tybulewicz et al., 1992), levels of IL-1β, IL-6 and TNF-α were elevated, as were reactive oxidative species (Hong et al., 2006), but since these mice die at birth, these data cannot be extrapolated to changes that may occur in the adult mouse brain (Willemsen et al., 1995). One study has demonstrated elevated messenger RNA levels of TNF-α and IL-6 messenger RNA in the midbrain of a Gaucher’s disease-like mouse model (V934L/V934L saposin C −/−) (Sun et al., 2010, 2011), but the relevance of this mouse to neuronopathic Gaucher’s disease pathology is currently unclear (Farfel-Becker et al., 2011a).

We now use a recently available neuronopathic Gaucher’s disease mouse, the Gba flox/flox;nestin-Cre mouse (hereafter referred to as the Gba −/− mouse) (Enquist et al., 2007) to attempt to delineate the role of brain inflammation in neuronopathic Gaucher’s disease pathogenesis. This mouse was designed by flanking the gba gene with two loxP sites, enabling cleavage of a segment of the gene by Cre recombinase, resulting in a null allele (Farfel-Becker et al., 2011a). Under the nestin promotor, Cre recombinase expression is limited to neural stem cells and their progeny, namely neurons, astrocytes and oligodendrocytes, leading to a specific deficiency of glucocerebrosidase in these cells. Microglia, which are mononuclear phagocytes derived from bone marrow precursor cells, are not affected by nestin-Cre (Yamaguchi et al., 2010) and do not display a lipid-engorged phenotype (Enquist et al., 2007; Yamaguchi et al., 2010). Using these mice, we are now able to rule out directly the involvement of defective microglia in the initiation of the inflammatory response.

**Materials and methods**

**Animals**

Gba flox/flox;nestin-Cre were used as a model of neuronopathic Gaucher’s disease (Enquist et al., 2007). Gba flox/flox mice were crossed with Gba flox/+;nestin-Cre to generate Gba flox/flox;nestin-Cre mice (referred to as Gba −/− mice) and Gba flox/+;nestin-Cre mice (referred to as Gba +/− mice), which served as healthy controls. Since no phenotypic difference was observed between males and females (n = 15 for each gender; Farfel-Becker et al., 2011b, and data not shown), both genders were used in this study. Genotyping was performed by polymerase chain reaction using genomic DNA extracted from mouse tails or embryonic brains (Farfel-Becker et al., 2009). The colony was maintained in the experimental animal centre of the Weizmann Institute of Science. Brains from K14-inl mice (Enquist et al., 2007), a mouse model with a strong reduction in glucocerebrosidase activity in all tissues except the skin, were kindly provided by Drs Simon Waddington and Ahad Rahim (University College London).

**RNA extraction and quantitative polymerase chain reaction**

Thalamic, brainstem and cerebellar tissues were removed from the brain, and cerebral tissue (which included the hippocampus, striatum and the cortical mantle, and is referred to as ‘grey matter’) was used for protein or RNA extraction. Total RNA was isolated using the RNAeasy mini kit (Qiagen) according to the manufacturer’s instructions, which included a DNase step and addition of β-mercaptoethanol. Complementary DNA synthesis was performed using the Reverse-iT™ first-strand synthesis kit (Thermo Scientific) using random decamers. Complementary DNA products were stored at −20°C. Quantitative polymerase chain reaction was performed using the SYBR® Green PCR Master Mix (Finzyme) and an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with complementary DNA (equivalent to 5 ng of total RNA). The primer concentration was 13 nM in a reaction volume of 20 μL. Each reaction was performed in duplicate. The thermal cycling parameters were as follows: step 1, 95°C for 10 min; step 2, 95°C for 15 s; step 3, 60°C for 30 s, 68°C for 30 s. Step 2 was repeated for 40 cycles and was followed by a dissociation step. Fold-change in messenger RNA was calculated using the comparative cycle threshold method using hypoxanthine phosphoribosyltransferase 1 (HPRT) or TATA box binding protein for normalization. P-values were calculated using a one-tailed, two-independent samples Student’s t-test. A P < 0.05 was considered statistically significant.
Table 1 Primers used for polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<tbody>
<tr>
<td>TBP</td>
<td>F: 5'-TGCTGGTTGGATTGGTTG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTGCGTTGGTGGAGGAAGT-3'</td>
</tr>
<tr>
<td>HPRT</td>
<td>F: 5'-CCGCAAATTGATGATCC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGTTAGCTGTTGGTGTAACG-3'</td>
</tr>
<tr>
<td>MCF-1</td>
<td>F: 5'-CATCGGAGTGGAGGAAACAG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GAAGCGAGCGAGGGAAGAC-3'</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>F: 5'-CGAGTAACTGCTATTGACCA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ACTGACAGCATCTTGTACCT-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: 5'-CTATGCGGAGGCAACACAC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCATGCGGAGCAAGGACG-3'</td>
</tr>
<tr>
<td>TNFR-1</td>
<td>F: 5'-GCCCACACCTTCCGCTTCAAC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GTCCAGGACCTTGCGGTTGGG-3'</td>
</tr>
<tr>
<td>CCL2/MCP-1</td>
<td>F: 5'-TCGATGCTGTCTAGCTTACCA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GACACAGACCTCTTCTTACCT-3'</td>
</tr>
<tr>
<td>CCL3/RANTES</td>
<td>F: 5'-GGCCACACCTTGGCCCTCCT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TGGTATTTGCGCTCCGCCG-3'</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>F: 5'-CGAGAGCTTGGTACTGCTAC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAGAGCGGAGCAGACAAAG-3'</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>F: 5'-ACAAAGAATGGTCTGCAATCG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCCGTTGATTGCTGCTATTC-3'</td>
</tr>
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</table>

F = forward primer; ICAM-1 = intercellular adhesion molecule 1; MCF-1 = macrophage colony-stimulating factor; R = reverse primer; TBP = TATA box binding protein; TGF-β1 = transforming growth factor-β1; TNFR-1 = TNF-α receptor; VCAM-1 = vascular cell adhesion molecule 1.

statistically significant. Primers for IL-12a and IL-1β were from Qiagen. All other primers are listed in Table 1.

Enzyme-linked immunosorbent assay

TNF-α levels were quantified in grey matter by enzyme-linked immunosorbent assay (Biosensis) according to the manufacturer’s instructions. Brain tissues were homogenized on ice in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 1 mM EDTA) supplemented with a protease inhibitor cocktail (1:200, Sigma). Following homogenization, samples were centrifuged at 14 000 × g for 10 min at 4°C and the supernatants were collected. Protein was quantified using the BCA protein assay reagent (Pierce Chemical Co.)

Protein extraction and western blotting

Tissues were lysed in ~6 volumes of Triton X-100 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 0.5 mM EDTA) supplemented with a protease inhibitor cocktail (1:200, Sigma). Following homogenization, samples were centrifuged at 14 000g for 10 min at 4°C and the supernatants were collected. Protein was quantified using the BCA protein assay reagent (Pierce Chemical Co.). Protein (50 μg) in sample buffer was electrophoresed on a 12% sodium dodecyl sulphate polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were incubated with a mouse anti-3-nitrotyrosine antibody (Abcam, 1:1000) or an anti-GAPDH antibody (Chemicon, 1:10000), and then incubated with a horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch). Bound antibodies were detected using the SuperSignal™ West Pico chemiluminescent substrate (Thermo Scientific).

Immunohistochemistry

Pups were sacrificed using CO2 and brains were rapidly removed. Brains were immersion-fixed in 2.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 days. IgG staining was performed on floating sections. Brains for floating sections of 16-day-old mice were cryoprotected in 30% sucrose in 50 mM Tris-buffered saline, pH 7.6, for 2–4 days. Brains were dissected along the midline and 40 μm frozen coronal sections were collected in 96-well plates containing a cryoprotectant (30% ethylene glycol, 15% sucrose, 0.05% sodium azide in Tris-buffered saline) and kept at −20°C until use. For IgG staining, endogenous peroxidase activity was quenched with 1% hydrogen peroxide in Tris-buffered saline for 30 min. After blocking with 20% horse serum in Tris-buffered saline containing 0.3% Triton X-100, floating sections were incubated overnight with a biotinylated donkey anti-mouse IgG antibody (1:200, Jackson Immunoresearch) diluted in 0.2% horse serum and 0.3% Triton X-100 in Tris-buffered saline. Sections were rinsed in Tris-buffered saline followed by incubation with avidin–biotin–peroxidase (Vectastain® Elite ABC kit, Vector, 1:1000) in Tris-buffered saline for 2 h and rinsed in Tris-buffered saline. To visualize immunoreactivity, sections were incubated with 0.05% 3,3’-diaminobenzidine tetrahydrochloride containing 0.001% hydrogen peroxide for 10 min.

For analysis of microglia morphology, paraffin coronal sections (7-μm thick) were prepared on Superfrost Plus® slides and stored at 4°C until use. The paraffin sections were deparaffinized and antigen retrieval was performed using 10 mM citric acid (pH 6.0) for 10 min. Sections were subsequently blocked with 20% normal horse serum in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 for 1 h followed by overnight incubation (at 4°C) with a rabbit anti-mouse Iba-1 antibody (1:500; Wako). After rinsing in PBS, sections were incubated for 1 h with a biotinylated anti-rabbit antibody (1:200, Jackson Immunoresearch) followed by incubation with Cy3-conjugated streptavidin (1:200, Jackson) for 60 min. Counterstaining was performed with DAPI (Molecular Probes) (1:1000 dilution, 1 min).

Magnetic resonance imaging

During MRI, mice were anaesthetized with isofluorane (5% for induction, 1–2% for maintenance) mixed with O2 (1 l/min), delivered through a nasal mask. Once anaesthetized, animals were placed in a head-holder to ensure reproducible positioning inside the magnet. Respiration rates were monitored and maintained at ~60–80 breaths/min throughout the experiment. MRI experiments were performed on a 9.4 T BioSpec Magnet 94/20 USR (Bruker) equipped with a gradient coil system capable of producing pulse gradients of up to 40 gauss/cm in each of the three directions. All MRIs were acquired with a receive quadrature mouse head surface coil and a transmitter linear coil. The MRI protocol included a series of gradient echo images (T2*-weighted images) before and after the injection of gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA; Magnetol). A series of T2*-weighted images were acquired using the gradient-echo imaging sequence with the following parameters: a repetition delay of 200ms, echo time increments of 3.6ms, matrix dimension of 128 × 128 and one average. Sixteen continuous slices with slice thickness of 0.8 mm were acquired with a field of view of 2 × 2 cm². The total acquisition time per image was 13 s. At least 28 gradient-echo images were acquired in each study. After the fourth image, 0.5 mmol/kg of Gd-DTPA was injected through the tail vein via an indwelling venous catheter. The first four images were used to obtain an average baseline image. The changes in signal intensities of the
Drug treatment

Ibuprofen (Sigma, 50 or 100 mg/kg/day) was administrated daily via intraperitoneal injection to 10- or 16-day-old Gba−/− mice. Minocycline (50 mg/kg) was administered by intraperitoneal injection daily to 10-day-old Gba−/− mice. Control mice were injected with intraperitoneal PBS. Drug doses were determined as previously described (Jeyakumar et al., 2004; Henry et al., 2008).

Results

Inflammatory mediator profile

Microglia in Gba−/− mice display activation markers such as Mac-2 (Vitner et al., 2010a; Farfel-Becker et al., 2011b) and CD68 (data not shown). We have now thoroughly characterized their activation state and examined messenger RNA levels of nine inflammatory mediators by quantitative reverse transcription polymerase chain reaction. The Gba−/− mice exhibit rapid motor dysfunction associated with severe neurodegeneration and neuronal loss and develop paralysis by 21 days of age (Enquist et al., 2007). Levels of inflammatory mediators were examined in the grey matter from mice at three different stages of disease development: the presymptomatic stage (9 days of age), the time immediately prior to development of symptoms (16 days) and the end stage (21 days) (Farfel-Becker et al., 2011b). No significant differences were observed in messenger RNA levels for any of the genes at Day 9 (Fig. 1A and B), but by Day 16, levels of the proinflammatory cytokines IL-1β and TNF-α were elevated by ~4.5- and ~3.5-fold, respectively, as were levels of the TNF-α receptor (TNFR-1; ~2.5-fold) (Fig. 1A) and the proliferation factor macrophage colony-stimulating factor (~1.6-fold increase) (Fig. 1A);

\[ r_{CBV} = K \int \frac{S(t)}{S_0} \, dt \]
\[ r_{MTT} = \frac{\int t \cdot \frac{S(t)}{S_0} \, dt}{\int \frac{S(t)}{S_0} \, dt} \]
\[ r_{CBF} = \frac{r_{CBV}}{r_{MTT}} \]

where \( K \) was assumed to be 1 and \( S(t) \) and \( S_0 \) are the signal intensities in the post- and pre-contrast images, respectively. The ratios of the relative cerebral blood volume, relative mean transit time and relative cerebral blood flow values between the Gba+/− and Gba−/− mice were subsequently calculated.

Figure 1 Age-related increase in levels of inflammatory mediators in neuronopathic Gaucher’s disease mouse brain. Messenger RNA levels were measured in the grey matter of Gba−/− mice and compared with Gba+/− (A and B) and from the grey matter of K14-lnl/lnl mice compared with K14-lnl/wt mice (D and E). The symbols in D and E are the same as in A and B, respectively. The inset in B shows results from Day 9 with a larger scale for the y-axis. Results are expressed as a ratio and are mean ± SEM (n = 3 for 9- and 21-day-old mice and n = 3–7 for 16-day-old mice). Cycle threshold values were normalized to levels of HPRT or TATA box binding protein. (C) TNF-α protein levels were measured by enzyme-linked immunosorbent assay in grey matter homogenates of 16-day-old Gba−/− (−/−) mice and Gba+/− (+/−). Results are mean ± SEM (n = 4). *P < 0.05; **P < 0.01; ***P < 0.001. wt = wild-type.
protein levels of TNF-α were elevated by ~2-fold (Fig. 1C) in agreement with changes in messenger RNA levels. By Day 21, larger changes were observed in expression of all genes (Fig. 1A and B) with the exception of the proinflammatory cytokine IL-12 (Fig. 1A). Levels of the immunosuppressive cytokine, transforming growth factor-β (TGF-β1), were elevated ~2-fold at Day 16 and ~4-fold at Day 21 (Fig. 1A).

The most significant elevations (~120–400-fold) were detected for the chemokines CCL2/MCP-1, CCL3/MIP1α and CCL5/RANTES (Fig. 1B), which regulate immune cell infiltration and blood–brain barrier breakdown. Since microglia in Gba−/− mice have normal glucocerebrosidase activity (Enquist et al., 2007), we also analysed levels of these cytokines in K14-lnl mice, a neuronopathic Gaucher’s disease mouse model that has a large reduction in glucocerebrosidase levels in all tissues (except the skin), including microglia (Enquist et al., 2007). Similar changes in cytokine and chemokine levels were detected in K14-lnl mice as in Gba−/− mice (Fig. 1D and E), suggesting that

**Figure 2** Morphology of microglia activation. Immunohistochemical staining of microglia using an anti-Iba-1 antibody (red) in the cortex of 21-day-old Gba+/− and Gba−/− mice. The top panels show low magnification images of layer V of the somatosensory barrel field cortex, and the panels below show progressively higher magnification of specific cells in the same region. Normal, ramified microglia are seen in Gba+/− cortex, whereas activated microglia with hypertrophic cell bodies and thickened processes are seen in neuronopathic Gaucher’s disease cortex. Scale bars: top panel = 40 μm; middle and lower panels = 10 μm.
glucosylceramide accumulation in microglia is not involved in the mechanism by which they become activated.

In addition to changes in cytokine levels, activation of microglia is accompanied by alterations in their morphology (Streit et al., 1999). Microglia from 21-day old Gba<sup>−/−</sup> mice displayed hypertrophic cell bodies and thickened processes (Fig. 2) compared with the ramified morphology and numerous processes observed in microglia from Gba<sup>+/−</sup>/C<sup>0</sup> brains (Fig. 2), confirming the altered activation state of the microglia.

**Blood–brain barrier integrity**

The chemokine profile suggested impairment of the blood–brain barrier and activation of endothelial cells in Gba<sup>−/−</sup> mice. Blood–brain barrier integrity was assessed by IgG immunostaining. No IgG staining was detected in 12-day old Gba<sup>−/−</sup> mouse brain (data not shown) but was readily apparent from Day 16 (Fig. 3). Interestingly, IgG staining was detected in brain regions known to be affected in the disease (Farfel-Becker et al., 2011b), such as layer V of the somatosensory barrel field cortex (S1BF), hippocampus and the thalamus. Scale bars: top panel = 1 mm; lower panels = 20 μm. Arrows indicate IgG staining.

Blood–brain barrier integrity was further examined by contrast-enhanced MRI after intravenous injection (via the tail vein) of Gd-DTPA (Fig. 4). Signal intensities were reduced to 68 ± 10% of the initial signal in Gba<sup>+/−</sup> brains and to 48 ± 12% in Gba<sup>−/−</sup> brains within the same time frame (P < 0.05) (Fig. 4A). The ratios of relative cerebral blood volume, relative mean transit time and relative cerebral blood flow for Gba<sup>+/−</sup> and Gba<sup>−/−</sup> mice were 2.19 ± 1.2 (P < 0.05), 1.04 ± 0.18 (P > 0.05) and 2.15 ± 1.4 (P > 0.05), respectively. Moreover, for up to 100 s after Gd-DTPA injection, signal recovery in Gba<sup>−/−</sup> brains was considerably slower than in Gba<sup>+/−</sup> mice (Fig. 4A), supporting the notion of attenuated leakage from the microvasculature.

Endothelial cell activation is accompanied by upregulation of vascular cell adhesion molecule-1 and intercellular adhesion molecule 1 (Min et al., 2005). No significant differences were detected in messenger RNA levels of these genes at Day 9 (Fig. 5), but by Day 16, messenger RNA levels of vascular cell adhesion molecule-1 and intercellular adhesion molecule 1 were elevated by ~1.5- and ~2.5-fold, respectively, and larger changes were observed at 21 days of age (Fig. 5), suggesting the involvement of endothelial cell activation in blood–brain barrier permeability.
Levels of 3-nitrotyrosine

A major mechanism by which microglia induce neurotoxicity is via release of reactive oxygen species (Block et al., 2007). High levels of both superoxide and nitric oxide result in formation of peroxynitrite, a potent oxidant that modifies tyrosine residues in proteins to form 3-nitrotyrosine (Szabo et al., 2007). Western blot analysis demonstrated higher 3-nitrotyrosine levels (~4-fold as...
determined by densitometry) in the grey matter of 21-day-old Gba−/− mice (Fig. 6) than in that of control mice.

**Effect of non-steroidal anti-inflammatory drugs on symptom onset and lifespan**

Despite the fact that inflammation appears to be a downstream event in the pathogenic cascade, it may nevertheless be a target for adjunctive therapy in multiple lysosomal storage disorders (Jeyakumar et al., 2004; Wu and Proia, 2004). We recently demonstrated that treating Gba−/− mice prior to symptom onset (at 10 days of age) with the non-steroidal anti-inflammatory drug ibuprofen significantly decreased the extent of elevation of cathepsin D and Z messenger RNA levels (Vitner et al., 2010a). We therefore examined if anti-inflammatory agents such as minocycline and ibuprofen could improve symptoms in Gba−/− mice. Mice were injected intraperitoneally with ibuprofen (100 mg/kg), minocycline (50 mg/kg) or PBS (control) starting prior to symptom onset (at 10 days of age). Ibuprofen (100 mg/kg) was also injected from Day 16 onwards. No changes in weight gain or lifespan were observed (Fig. 7A). However, TNF-α and macrophage inflammatory protein-1α messenger RNA levels were decreased in the grey matter of 16-day-old mice after ibuprofen treatment from Day 10 (Fig. 7B), and TNF-α and RANTES messenger RNA levels were decreased in the grey matter of 21-day-old mice after ibuprofen treatment from Day 16 (Fig. 7C). No changes were observed in levels of the other inflammatory mediators (data not shown).

**Figure 5** Endothelial cell activation in neuronopathic Gaucher’s disease mouse brain. Messenger RNA levels were measured in the grey matter of Gba−/− (Gbaflox/flox;nestin-Cre) mice and compared with Gba+/− (Gbaflox/+;nestin-Cre) mice. Results are expressed as a ratio and are mean ± SEM (n = 3–4). Cycle threshold values were normalized to levels of HPRT or TATA box binding protein. *P < 0.05; **P < 0.01. ICAM-1 = intercellular adhesion molecule 1; VCAM-1 = vascular cell adhesion molecule 1.

**Figure 6** 3-Nitrotyrosine (NITT) levels. Western blots of homogenates (50 μg of protein) from the grey matter of 21-day-old Gba−/− and Gba+/− mice. GAPDH was used as loading control. Triplicate samples from different mice are shown.

**Discussion**

In this study, we have demonstrated a chronic inflammatory response in brains of a mouse model of neuronopathic Gaucher’s disease, which becomes more pronounced with disease progression. Unlike other studies on neuroinflammation in lysosomal storage disorders, the mice used in our study only have defective enzyme (glucocerebrosidase) levels in nestin-expressing cells, i.e. neurons, astrocytes and oligodendrocytes, with normal levels in microglia. Thus, any inflammatory response must occur as a result of the defect in neurons and astrocytes and not due to a primary defect in microglia.

Microglia are activated in various neuronal insults and can be neuroprotective, or can promote neurodegeneration by production of proinflammatory or neurotoxic effectors, including IL-1β, nitric oxide (NO•) and reactive oxygen species (Block and Hong, 2005). In the current study, we have shown that cytokine levels increase as symptoms become more severe, suggesting they are produced in response to, or contribute to, local brain inflammation. Elevation of the proinflammatory cytokines IL-1β and TNF-α may be largely responsible for neurotoxicity. In contrast, the immunosuppressive cytokine, TGF-β1, which acts by inhibiting TNF-α, IL-1, IL-6 and IL-12 production by microglia/macrophages (Vitkovic et al., 2001), was elevated in the initial stages of the inflammatory reaction and became more prominent at later stages, which may be a response to the increase in TNF-α and IL-1β. Elevation of the immunosuppressive cytokines may be too low and occur too late to have an impact on disease progression.

The upregulation of TNF-α and TNF-α receptor-1, a death receptor, suggests a role for TNF-α signalling in neuronopathic Gaucher’s disease. TNF-α secreted by activated glia induces neuronal death through receptor-mediated caspase activation and through caspase-dependent and caspase-independent components of the mitochondrial cell death pathway, which includes
increased reactive oxygen species generation (Tezel, 2008). Further studies will focus on elucidating the various signalling cascades downstream to TNF-α/C11 in neuronopathic Gaucher’s disease.

Both TNF-α and IL-1β lead to upregulation of adhesion molecules on the surface of endothelial cells and increase blood–brain barrier permeability (Min et al., 2005; Brabers and Nottet, 2006). In the Gba−/− mouse, messenger RNA levels of CCL2/MCP-1, CCL3/MIP1α and CCL5/RANTES were highly elevated; these chemokines are specifically involved in leucocyte recruitment at sites of inflammation (Stamatovic et al., 2005; Ransohoff, 2009) and in activation of endothelial cells and expression of adhesion molecules (Andjelkovic and Pachter, 2000). Changes in blood–brain barrier permeability in Gba−/− mice are likely to result from the increases in TNF-α, IL-1β and CCL2/MCP-1 levels, which occur concomitantly with changes in blood–brain barrier permeability. Although we cannot categorically exclude the possibility that endothelial cells themselves accumulate glucosylceramide and become activated in response to glucosylceramide accumulation, this seems unlikely in the Gba−/− mouse since endothelial cells remain unrecombined under the nestin promoter (Graus-Porta et al., 2001). The accumulation of glucosylceramide in blood vessels and the activation state of endothelial cells in neuronopathic Gaucher’s disease needs to be further elucidated.

Unfortunately, the rapid onset and extreme severity of disease development in this mouse make it difficult to conclude whether the loss of blood–brain barrier integrity is a cause of disease development or rather a late outcome of the disease. Nonetheless, the detection of altered blood–brain barrier permeability by MRI suggests that MRI might be a useful tool to evaluate clinical progression in human patients with Gaucher’s disease, where disease progression is less rapid than in the Gba−/− mouse.

Macrophage/microglia activation also causes oxidative damage, which was apparent in Gba−/− mice. Peroxynitrite is a potent trigger of oxidative protein and DNA damage, including DNA strand breakage and base modification (Ahmad et al., 2009), and induces cell death by both apoptosis and necrosis (Szabo et al., 2007). Peroxynitrite also plays a role in the pathogenesis of neurodegenerative disorders such as Parkinson’s and Alzheimer’s diseases, amyotrophic lateral sclerosis (Szabo et al., 2007).
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Extrapolated from a somewhat similar inflammatory response in other lysosomal storage disorders. For instance, in Sandhoff disease, it was suggested that microglia recognize damaged and dying glycolipid-accumulating neurons and remove them by phagocytosis. The inability of the microglia to degrade the endocytosed glycolipids because of their own enzyme deficiency may lead to exacerbation of the inflammatory process (Wada et al., 2000). Moreover, it was suggested that cellular inflammation might be initiated by the stored glycosphingolipids at the blood–microvascular endothelial cell interface, as observed in Sandhoff mice (Jeyakumar et al., 2003). The appearance of activated microglia in Gba⁻/⁻ mice may help to understand the mechanism leading to neuroinflammation in neuronopathic Gaucher’s disease, since microglia express normal glucocerebrosidase levels (Enquist et al., 2007). Nevertheless, microglia become activated, indicating that the changes in the state of the microglia are not the result of glucosylceramide accumulation in the microglia themselves, but rather a downstream consequence of glucosylceramide accumulation in other cell types such as neurons and/or astrocytes. It should be stressed that any mechanism uncovered in Gba⁻/⁻ mice may differ somewhat from the situation in human patients (in which all cells, including microglia, carry the glucocerebrosidase mutation). However, these differences are unlikely to be that extensive, since Gba⁻/⁻ mice develop symptoms similar to K14-lnl/lnl mice (which do carry the mutation in macrophages) and display a similar profile of inflammatory mediators.

In summary, we have demonstrated microglial activation as a possible contributor to neuronal death in neuronopathic Gaucher’s disease. In addition to studies showing that neuronal glucosylceramide accumulation (at least in cell culture) does not lead directly to neuronal death (Pelled et al., 2000), our data support the notion that once a critical threshold of glucosylceramide storage is reached in neurons, the neurons become dysfunctional, which triggers a signalling cascade thereby activating surrounding microglia (Fig. 8). This subsequently initiates a neuroinflammatory cascade involving elevation of cytokines, release of neurotoxic agents and reactive oxygen/nitrogen species and blood–brain barrier permeabilization. The persistence of glucosylceramide accumulation in neurons and the continuous activation of glia results in chronic inflammation contributing to neuronal cell death. An open question remains concerning the mechanism(s) by which neurons that accumulate glucosylceramide signal microglia. Intervention in this signalling pathway might pave the way to new therapeutic approaches for neuronopathic Gaucher’s disease.

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**References**


