Published in final edited form as:

J Neuroimmunol. 2008 October 15; 203(1): 50–57. doi:10.1016/j.jneuroim.2008.06.024.

Peripheral blood mononuclear cell infiltration and neuroinflammation in the HexB^{-/-} mouse model of neurodegeneration

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Abstract

Myeloid-derived immune cells, including microglia, macrophages and monocytes, have been previously implicated in neurodegeneration. We investigated the role of infiltrating peripheral blood mononuclear cells (PBMC) in neuroinflammation and neurodegeneration in the HexB^{-/-} mouse model of Sandhoff disease. Ablation of the chemokine receptor CCR2 in the HexB^{-/-} mouse resulted in significant inhibition of PBMC infiltration into the brain, decrease in TNF α and MHC-II mRNA abundance and retardation in clinical disease development. There was no change in the level of GM₂ storage and pro-apoptotic activity or astrocyte activation in HexB^{-/-}; Ccr2^{-/-} double knockout mice, which eventually succumbed secondary to GM₂ gangliosidosis.

INTRODUCTION

The role of myeloid immune cells, including microglia, macrophages and monocytes, in neural disease remains controversial. Activated microglia appear to contribute to the development of several neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease as well as several lysosomal storage disorders (Wada et al. 2000; Myerowitz et al. 2002; Ohmi et al. 2002; Jeyakumar et al. 2003; Wu et al. 2004; Block et al. 2007). In addition, peripheral blood mononuclear cells (PBMC) were implicated in experimental autoimmune encephalitis (EAE) in mouse models of multiple sclerosis, as blockage of their infiltration in the central nervous system ameliorated disease development (Izikson et al. 2000; Mahad et al. 2005). Conversely, inhibition of PBMC infiltration in an Alzheimer's mouse model resulted in exacerbation of the disease (Khoury et al. 2007). Recruitment of PBMCs in the brain of an Alzheimer's disease mouse model appeared to mediate reduction of plaque formation in the brain (Shaftel et al.

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2007). Moreover, healthy bone marrow transplantation into Sandhoff disease mice ameliorated neural disease and prolonged life span (Norflus et al. 1998; Wada et al. 2000).

Monocytes, macrophages, immature dendritic cells, as well as microglia, express several chemokine receptors, including the CC-chemokine receptor CCR2 (Charo and Ransohoff 2006). CCR2 is involved in PBMC infiltration in the brain of a multiple sclerosis mouse model (Izikson et al. 2000), as well as in experimental autoimmune encephalitis (Fife et al., 2000; Rottman et al. 2000; Gaupp et al., 2003). CCR2 is also required for macrophage infiltration at sites of axonal injury in the hippocampus (Babcock et al. 2003). Monocyte chemotactic protein-1 (MCP-1, also known as CC-chemokine ligand 2, CCL2), the main CCR2 chemokine ligand, is upregulated in Alzheimer disease brains, suggesting that that CCR2 may play an important role in microglial precursor trafficking into the Alzheimer disease brain (Khouri et al. 2007).

Similarly to humans, two murine genes encoding for β -hexosaminidase have been identified, *HexA* and *HexB* (Sango et al., 1995). Targeted deletion of the *HexA* locus resulted in the development of a mouse phenotype that showed only a mild degree of pathology and lack of any neurological findings (Sango et al., 1996). In contrast, disruption of the murine *HexB* locus resulted in a mouse phenotype that closely resembled that of the human disease. The mice displayed storage of GM₂ ganglioside in the CNS, and neurons with membranous cytoplasmic bodies similar to those in Tay-Sachs and Sandhoff patients (Sango et al. 1995). A phenotypic variation between humans and mice appears to result from differences in the ganglioside degradation pathway between species, with the *HexB*^{-/-} knockout mouse widely accepted as the appropriate animal model of GM₂ gangliosidosis. Progressive neuronal GM₂ storage is a cardinal characteristic of GM₂ gangliosidosis, followed by neurodegeneration associated with neuronal apoptosis occurring in the central nervous system (Huang et al. 1997). Recent studies on *HexB*^{-/-} mice also showed the presence of activated microglia and macrophages in the brain of affected animals. In addition, upregulation of several inflammation-related genes has been described in the brain of *HexB*^{-/-} concomitantly with glial activation. It has been suggested that brain inflammation contributes to neurodegeneration: The presence of activated microglia and/or macrophages in the brain preceded neuronal cell death, and were observed proximal to neurons undergoing apoptosis (Wada et al. 2000; Myerowitz et al. 2002). Furthermore, transplantation of healthy bone marrow to *HexB*^{-/-} pups attenuated the microglia/macrophage activation, reduced the extent of neuronal apoptosis and ameliorated the clinical phenotype (Norflus et al. 1998).

The purpose of this study was to further investigate the role of infiltrating PBMC in neuroinflammation, neurodegeneration and neural disease development employing the *HexB*^{-/-} mouse model of GM₂ gangliosidosis. The cardinal feature of the *HexB*^{-/-} mouse, similarly to Sandhoff patients, is neuronal GM₂ storage due to β -hexosaminidase deficiency, followed by microglia activation, neuroinflammation and neuronal apoptosis in the central nervous system (Huang et al. 1997; Myerowitz et al. 2002).

MATERIAL AND METHODS

Animals and behavioral analyses

The use of mice and all manipulations and methods pertinent to this study were reviewed and approved by the University of Rochester Animal Resources committee (IACUC) as well as the Institutional Biosafety Committee and assigned protocol number 2003-142. *HexB*^{+/-} breeders were donated to us by Dr. Proia (NIH/NIDDK; Bethesda MD) and genotyped as previously described (Kyrkanides et al. 2007). *Ccr2*^{-/-} breeders (Boring et al., 1997) were purchased from the Jackson Laboratory (Bar Harbor, Maine) and were crossed into *HexB*^{-/-} to generate *HexB*^{+/-};*Ccr2*^{+/-} heterozygous mice, which were bred to generate the mice

employed in our study. Genotyping for the *Ccr2* locus was facilitated by the following primers: 5'CTCAGTTCATCCACGGCATA^{3'}, 5'GATGGCCAAGTTGAG CAGAT^{3'} and 5'TTC CAT TGC TCA GCG GTG CT^{3'} at Ta=61°C. HexB^{-/-} genotyping was performed as previously described (Kyrkanides et al. 2005, 2007). A total of 28 mice were employed in the analyses: HexB^{-/-};Ccr2^{-/-} (N=8), HexB^{-/-};Ccr2^{WT} (N=11) and WT (N=9).

Starting at 9 weeks of age, the mice were trained for two weeks on the rotarod appliance (Columbus Instruments, Columbus OH). At the age of 11 weeks, we began evaluating the behavioral performance of the mice by rotarod. Differences between the various animal groups were assessed by analysis of variance and post-hoc analysis using the Tukey method.

RNA isolation and cDNA synthesis (Reverse Transcription; RT)—Brain tissue was dissected out, frozen in isopentane chilled with dry ice, and stored in sterile tubes at -80°C until ready for RNA isolation. RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), precipitated and the concentration determined by spectrophotometry. Two µg of RNA was DNase-treated (Invitrogen) according to the manufacturer's instructions. First-strand DNA was synthesized by using 2 µg of DNase-treated RNA, random hexamers, and Superscript II (Invitrogen) according to the manufacturer's instructions.

Quantification of mRNA levels was accomplished using an iCycler (Bio-Rad) and real time qRT-PCR with Taqman probes constructed with FAM as the fluorescent marker and Blackhole I quencher (Biosearch Technologies, Novato CA). Prior to PCR of the cDNA samples, PCR conditions were optimized for each mRNA to be analyzed. Standard curve reactions were performed by varying annealing temperatures, primer concentrations, and Taqman probe concentration. Serial dilution of the starting cDNA template demonstrated linear amplification over at least 5 orders of magnitude.

PCR reactions are performed in a volume of 25 µl and contained iQ Supermix (Bio-Rad, Hercules CA; 0.625 U Taq, 0.8 mM dNTP, 3 mM Mg²⁺, 0.2–0.6 µM concentrations of each primer, 10–100 nM probe) and 1 µl of cDNA sample. To ensure consistency, a master mix was first prepared containing all reagents except the cDNA sample. Primers were designed using the Primer Express (Applied Biosystems) and Oligo 6.83 programs (Molecular Biology Insights, Inc., Cascade, CO). In general, PCR reaction conditions were as follows: Denaturation at 95°C for 3 min, followed by 40 cycles of amplification by denaturing at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s. For each real time PCR, a standard curve was performed to insure direct linear correlation between product yield (expressed as the number of cycles to reach threshold) and the amount of starting template. The correlation was always greater than r = 0.925. PCR reaction efficiency (e) was determined for each reaction. To correct for variations in starting RNA values, the level of ribosomal 18S RNA or GAPDH RNA was determined for all samples and used to normalize all subsequent RNA determinations. Normalized threshold cycle (Ct) values were then transformed, using the function- expression = (1+ e) Ct, in order to determine the relative differences in transcript expression. Data were compared by ANOVA and Tukey's post hoc tests, and by linear regression to determine correlations using the JMP statistics program (SAS Institute). A probability of P < 0.05 was considered statistically significant. COX-2 (Ta=60°): UP-5'tgacccccaaggctcaaata^{3'}, LP-5'cccaggtcctcgcttatgatc^{3'}, PR-5'ctttgcccagcacttcacccatcagtt^{3'}; G3PDH (Ta=60°): UP-5'cccaatgtgtccgtcgtg^{3'}, LP-5'cctgcttcaccaccttcttg^{3'}, PR-5'tgtcatatacttgccaggtttctccagg^{3'}; IL1β (Ta=60°): UP-5'tcgctcagggtcacaagaaa^{3'}, LP-5'atcagaggcaaggaggaaacac^{3'}; PR-5'catggcacattctgttcaagagagcctg^{3'}; IL-6 (Ta=60°): UP-5'ccagaaaccgctatgaagttcct^{3'}; LP-5'caccagcatcagtcaccaaga^{3'}; PR-5'tctgcaagagacttccatccagttgcc^{3'}; TNFα (Ta=55°): UP-5'gacaaggctgccccgacta^{3'}; LP-5'tttctcctggtatgatagataa^{3'}; PR-5'ctcctcaccacacccgctcagcc^{3'}.

Histology

The antibody used to detect CD45⁺ in our experiment was a rat anti-CD45⁺ monoclonal antibody commercially available from Serotec (Raleigh, NC; Cat. No. MCA43G) at 1:400 dilution, coupled with a rabbit anti-rat IgG secondary antibody (Jackson Immunoresearch; West Grove, PA). Activated astrocytes were identified by a rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:1,000 dilution; Dako USA, Carpinteria, CA). Activated dendritic cells/microglia/macrophages were stained with a rat anti-major histocompatibility complex class-II (MHC-II; Bachem, Torrance, CA; clone ER-TR3). TNF α immunohistochemistry was performed using a polyclonal antibody purchased from SantaCruz (Santa Cruz, CA). GM₂ ganglioside was immunolocalized employing a mouse anti-N-acetyl GM2 monoclonal IgM antibody (Seikagaku, East Falmouth, MA; clone MK1-16). These antibodies were coupled with appropriate secondary antibodies: rabbit anti-goat IgG biotin-conjugated, goat anti-mouse IgG Fab biotin conjugated, goat anti-rat IgG biotin-conjugated antibodies and goat antimouse IgM biotin conjugated, respectively (Jackson Immunoresearch). Visualization was performed utilizing DAB (3,3-diaminobenzidine)-nickel as chromagen. The glass slides were then dehydrated through multiple ethanol solutions, cleared through xylene and cover-slipped using DPX permanent mounting medium (Fluka, NeuUlm, Switzerland). The tissue sections were then studied under a BX51 Olympus light microscope and color microphotographic images were captured as described above. The total numbers of GFAP⁺, MHC-II⁺, TNF α and CD45⁺ cells were counted in 10 random microscopic fields (40x) as follows. In each field, the number of positive cells was counted, and averages and standard errors of mean were calculated for each area of the brain.

Statistical analysis—Differences between groups were evaluated by one way analysis of variance (ANOVA) with $\alpha=0.05$ and power equal to 0.8. Post-hoc analysis was performed by the Tukey method.

RESULTS

Deletion of the Ccr2 receptor in Sandhoff mice attenuated brain inflammation

The presence of activated microglia cells and infiltrating PBMC was scarce in the brain of wild type mice as assessed by MHC-II and CD45⁺ immunohistochemistry (Fig. 1A, D). As anticipated, we observed increased numbers of MHC-II and CD45⁺ immunoreactive cells in the adult HexB^{-/-} mouse brain (Fig. 1B, E). Conversely, we observed a significant reduction of MHC-II and CD45⁺ positive cells in the brain of HexB^{-/-};Ccr2^{-/-} double knockout mice (Fig. 1C, F; Fig 2A), demonstrating that the Ccr2 receptor is implicated in the recruitment of PBMC. Similarly, we observed lack of GFAP immunoreactivity in the cortex of wild type mice (Fig. 1G), whereas relatively large numbers of activated, GFAP-positive astrocytes were noted in the adult Sandhoff brain (Fig. 1H). Interestingly, the number of activated astrocytes remained unaltered following the deletion of the Ccr2 receptor in the brain of HexB^{-/-} mice (Fig. 1I; Fig. 2A). A significant down-regulation of TNF α and MHC-II transcript levels in the brain of HexB^{-/-};Ccr2^{-/-} double knockout mice compared to HexB^{-/-} mice was also observed (Fig. 2B). However, the transcript level for other inflammatory genes, including IL-1 β , GFAP and COX-2, remained significantly elevated in HexB^{-/-} and HexB^{-/-};Ccr2^{-/-} mice compared to WT. Further evaluation of TNF α expression in the brain of HexB^{-/-} (Fig. 3A) and HexB^{-/-};Ccr2^{-/-} (Fig. 3B) mice revealed that the number of TNF α -positive cells in HexB^{-/-} mice decreased following targeted deletion of the Ccr2 receptor, but remained significantly increased compared to wild type mice. When the animals' locomotive performance was correlated with the number of TNF α -positive cells present in their brains, a statistically significant correlation coefficient was calculated ($r=0.963$; $F=12.8$; $p=0.0001$).

Inhibition of PBMC infiltration retarded clinical deterioration

HexB^{-/-};Ccr2^{-/-} double knockout mice displayed retardation of the clinical deterioration observed otherwise in HexB^{-/-} mice, linking PBMC infiltration, in addition to neuroinflammation (Fig. 2A-B), also with clinical disease development (Fig. 2C). Specifically, targeted deletion of the Ccr2 locus in the HexB^{-/-} mouse model of GM₂ gangliosidosis significantly improved locomotive behavior, as assessed by rotarod analysis (Fig. 2C), by an average of 65% (T_{BC}-T_B/T_B). Furthermore, we observed a modest extension (28.5% on average) in life expectancy (Fig. 2C). There were no changes in gain weight among the 3 groups of mice (Fig. 2D).

Attenuation of PBMC infiltration and neuroinflammation did not alter the level of pro-apoptotic activity

Caspase-3 expression, as assessed by immunohistochemistry, was employed as a measure of pro-apoptotic activity in the brain. The number of neurons staining positively for caspase-3 remained significantly large in the HexB^{-/-};Ccr2^{-/-} double knockout and HexB^{-/-} mice compared to wild type littermates (Fig 4). The slight decrease in the number of caspase-3 immunoreactive cells following CCR2 deletion in the HexB^{-/-} mouse was not significant, the levels of which did not correlate with the decrease in numbers of PBMC infiltrating into the CNS.

Ccr2 receptor ablation had no effect on GM₂ storage in the brain of HexB^{-/-} mice

GM₂ storage was evaluated by immunohistochemistry on histology sections harvested from HexB^{-/-}, HexB^{-/-};Ccr2^{-/-} and wild type mice employing a monoclonal antibody (Fig. 5). As anticipated, there was no change in GM₂ storage following inhibition of PBMC infiltration in the HexB^{-/-} brain. The level of caspase-3 expression correlated, however, with the storage of GM₂ ganglioside in the mouse brain.

Taken together, the aforementioned results demonstrate a partial, yet significant amelioration of brain inflammation due to the reduction of PBMC following deletion of the CCR2 receptor in the HexB^{-/-} mouse model of GM₂ gangliosidosis, leading to amelioration of the clinical phenotype. Notwithstanding this clinical improvement, HexB^{-/-};Ccr2^{-/-} double knockout mice eventually succumbed by 18 weeks of age due to neurodegeneration from increased apoptotic activity secondary to GM₂ neuronal storage.

DISCUSSION

We investigated the contribution of infiltrating PBMC to neuroinflammation, neurodegeneration and development of neural disease by deleting the CCR2 receptor (Boring et al. 1997), which is predominantly expressed by myeloid immune cells, in the HexB^{-/-} mouse model. Indeed, HexB^{-/-};Ccr2^{-/-} double knockout mice displayed significant reduction in the number of MHC-II and CD45⁺ expressing cells in the brain compared to HexB^{-/-} littermates. As monocyte chemo-attractant proteins MCP-1, MCP-2, MCP-3 and MCP-4 are the principal ligand for CCR2, our results suggest that this receptor is primarily responsible for PBMC infiltration in the brain. Since CCR2 deletion practically ablated MHC-II and CD45⁺ cells from the HexB^{-/-} brain, it suggests that a portion of reactive microglia found in the HexB^{-/-} brain originates from the peripheral blood. In the present study, CD45⁺ expressing cells were identified by immunohistochemistry using a commercially available antibody. Previous studies in our laboratories (Shaftel et al., 2007b) as well as by others (Ford et al., 1995; Sedgwick et al., 1998) employing this method as well as flow cytometry for CD45⁺ detection in mouse brain cells suggest that the sensitivity of the immunohistochemistry is such that it primarily detects cells with high levels of CD45⁺ expression, such as infiltrating macrophages (Shaftel et al., 2007b). Therefore, our results indicate that Ccr2 deletion in the HexB^{-/-} mouse model

significantly inhibited the infiltration of peripheral myeloid-derived immune cells, such as macrophages and dendritic cells, in the inflamed brain.

TNF α has been previously associated with activated microglia and is implicated in neurodegeneration from GM₂ gangliosidosis (Wada et al. 2000). In our study, the levels of TNF α transcript correlated with PBMC infiltration into the brain, as assessed by presence of MHC-II and CD45⁺ cells as well as MHC-II mRNA abundance in the HexB^{-/-} brain: Blockage of PBMC infiltration resulted in significant reduction of TNF α transcript abundance as well as of the number of TNF α -expressing cells in the brain. Therefore, our data indicate that infiltrating PBMC and activated microglia are a major source of TNF α in the HexB^{-/-} brain. To this end, we observed only a partial decrease (about 50%) in TNF α transcript levels after Ccr2 deletion in the HexB^{-/-} mouse model. This finding suggests that the residual TNF α expression may be derived from the resident, MHC-II⁺ microglia cells found in the HexB^{-/-};Ccr2^{-/-} mouse brain, as suggested by our results. Moreover, we observed modest but significant attenuation of the clinical phenotype in HexB^{-/-}/Ccr2^{-/-} double knockout mice. To this end, animal motor behavior correlated with the number of TNF α -positive cortical cells at a statistical significant degree. This finding implies that the TNF α expression seen in HexB^{-/-};Ccr2^{-/-} mouse brains etiologically contributes to the residual behavioral impairment that the mice display. Motor deterioration also corroborated with the level of caspase-3 expression in the brain, a measure of pro-apoptotic activity. In a previous study, a modest clinical attenuation was also observed when MIP-1 α was deleted in the HexB^{-/-} mouse model; in the latter experiment, however, a significant reduction of neuronal apoptosis was reported (Wu & Proia, 2004). TNF α is known to contribute to neurodegeneration through the silencing of cell survival signals as well as the induction caspase-dependent cascades (Li et al., 2004). Recently, TNF α was shown to stimulate extensive microglial glutamate release in an autocrine manner by up-regulating glutaminase, ultimately leading to neurotoxicity. However, TNF α also activates neuroprotective factors, including MAPK and expression of nuclear factor-KB, which in turn moderate neurotoxicity (Takeuchi et al., 2006). Notwithstanding TNF α 's unequivocal role in neurotoxicity, the exact mechanism mediating its effects, therefore, remains unclear.

The presence of reactive astrocytes in the HexB^{-/-} mouse brain has been previously described (Myerowitz et al., 2002; Kyrkanides et al., 2005). In our studies, we observed insignificant changes in the number of GFAP-positive cells in the HexB^{-/-}/Ccr2^{-/-} brain compared to HexB^{-/-} littermates. Moreover, GFAP and IL-1 β transcript levels did not change in the HexB^{-/-}/Ccr2^{-/-} brain, and it is possible that astrocytes are inherently activated by GM₂ gangliosidosis in the HexB^{-/-} mouse model. IL-1 β is a potent pro-inflammatory cytokine released from astrocytes, microglia and neurons and has been implicated in CNS injury and neurotoxicity for conditions ranging from stroke to Alzheimer's disease (Griffin et al., 1989, 1995; Boutin et al. 2001; Mrak et al. 1995; Toulmond & Rothwell 2000). However, previous reports demonstrated that pro-inflammatory cytokines IL-1 α and IL-1 β are neuroprotective in an NMDA model of excitotoxicity, an effect inhibited by the IL-1 receptor antagonist (Carlson et al 1999). Moreover, IL-1 signaling was critical for repair in a mouse model of cuperzone-induce demyelination (Mason et al. 2001). Recent work in our laboratory offered compelling evidence that IL-1 β , through the IL1RI receptor, plays a neuroprotective role in a mouse model of Alzheimer's disease (Shaftelet et al. 2007a, b). Evaluation of possible underlying mechanisms mediating the aforementioned neuroprotection suggested the clearance of A β plaques in this model by PBMC after their recruitment in the inflamed brain. To this end, healthy bone marrow transplantation into HexB^{-/-} mice resulted in significant clinical attenuation, amelioration of neurodegeneration and extension of life expectancy (Norflus et al. 1998). These data suggest that PBMC recruitment in the inflamed brain is an adaptive immune response. In the HexB^{-/-} mouse model, however, recruitment of β -hexosaminidase deficient PBMC can not facilitate GM₂ clearance; conversely, bone marrow transplantation harvested from wild type

mice provides a source of mononuclear cells that contribute to GM₂ clearance in the HexB^{-/-} brain (Norflus et al. 1998). Taken together, the aforementioned data suggest that IL-1 β upregulation in the brain may be part of an inherent neuroprotective response. Additional studies are needed, however, to further elucidate the role of IL-1 β signaling in neurodegeneration.

In conclusion, inhibition of PBMC infiltration in the HexB^{-/-} brain retards disease progression and neurodegeneration in this mouse model of GM₂ gangliosidosis. Conversely, transplantation of β -hexosaminidase – competent myeloid precursors in patients may provide the basis for developing new therapies for lysosomal storage and other neurodegenerative disorders.

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Figure 1. Ccr2 deletion results in attenuation of mononuclear cell activation but not of astrocytes in the HexB^{-/-} mouse brain

(A) Adult wild type brains lacked any MHC-II immunoreactivity, whereas (B) HexB^{-/-} brains were characterized by the presence of MHC-II positive cells identified as activated microglia-macrophage-monocytes. Conversely, (C) 4 month old HexB^{-/-};Ccr2^{-/-} double knockout mice displayed only traces of MHC-II immunostaining in their brain, indicating that a large portion of the MHC-II activated cells commonly observed in HexB^{-/-} mouse brain are infiltrating peripheral blood mononuclear cells. (D) Wild type mice were also characterized by the lack of CD45⁺-positive cells, whereas (E) HexB^{-/-} mice displayed high numbers of infiltrating mononuclear cells, which (F) were absent in the brains of adult HexB^{-/-};ccr2^{-/-} double knockout mice. (G) Wild type mice also lacked GFAP-positive cells (reactive astrocytes), and (H) as expected, 4 month old HexB^{-/-} mice displayed large numbers of activated astrocytes. Interestingly, (I) CCR2 deletion did not alter the levels of GFAP expression, suggesting that astrocyte activation is inherent to β -hexosaminidase deficiency and is not affected by reactive microglia and infiltrating mononuclear cells. Bar=100 μ m.

Figure 2. Inhibition of PBMC infiltration in the HexB^{-/-} brain reduced neuroinflammation, ameliorated the neurological phenotype but did not treat the disease
 CCR2 deletion in Sandhoff mice resulted in (A) significant decrease in the number of MHC-II and CD45⁺-positive mononuclear cells in the brain, but not of GFAP-expressing astrocytes. (B) Furthermore, we observed a significant down-regulation of a number of inflammatory genes in the adult HexB^{-/-};ccr2^{-/-} double knockout brain, in particular MHC-II and TNF α , but not IL-1 β or GFAP transcript. It also led to (C) significant attenuation of their neurological phenotype as assessed by rotarod. The HexB^{-/-};Ccr2^{-/-} double knockout mice eventually succumbed from GM₂ gangliosidosis by the age of 18 weeks on the 129S4/C57 mixed strain background. (D) There were no differences in weight gain between the various animal groups. *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$.

Figure 3. TNF α expression in the mouse brain

TNF α expression was evaluated by immunohistochemistry in histology sections harvested from (A) HexB^{-/-} knockout mice as well as (B) HexB^{-/-};Ccr2^{-/-} and (C) wild type littermates. (D) The number of TNF α -expressing cells in 10 consecutive histology sections are presented. Overall deletion of the Ccr2 receptor in HexB^{-/-} mice resulted in a decrease of the number of TNF α -expressing cells in the brain. *= p <0.05; **= p <0.01; ***= p <0.001. Bar=100 μ m.

Figure 4. Inhibition of PBMC infiltration did not change pro-apoptotic activity

Caspase-3 expression was employed as a measure of pro-apoptotic activity in histology sections harvested from mouse brains. (A) HexB^{-/-} mice display widespread caspase-3 immunoreactivity, localized primarily in neurons. The example shown depicts cortical neuronal staining. (B) HexB^{-/-}; Ccr2^{-/-} double knockout mice also demonstrated extensive caspase-3 staining in cortical neurons. (C) Conversely, we did not observe caspase-3 immunostaining in wild type littermates. (D) Numerical representation of caspase-3 immunostaining. ***=p<0.001. Bar=100μm.

Figure 5. GM₂ storage was not altered by CCR2 deletion in the HexB^{-/-} mouse brain

GM₂ storage is a cardinal feature of HexB^{-/-} mouse neuropathology as was assessed by immunohistochemistry. (A) HexB^{-/-} mice demonstrate, as expected, widespread neuronal GM₂ storage, the distribution of which (B) did not change after CCR2 deletion. (C) There is lack of GM₂ storage in wild type mice.