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TSPO in a Murine Model of Sandhoff Disease: Presymptomatic marker of neurodegeneration and disease pathophysiology

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Abstract

Translocator protein (18 kDa), formerly known as the peripheral benzodiazepine receptor (PBR), has been extensively used as a biomarker of active brain disease and neuroinflammation. TSPO expression increases dramatically in glial cells, particularly in microglia and astrocytes, as a result of brain injury, and this phenomenon is a component of the hallmark response of the brain to injury. In this study, we used a mouse model of Sandhoff disease (SD) to assess the longitudinal expression of TSPO as a function of disease progression and its relationship to behavioral and neuropathological endpoints. Focusing on the presymptomatic period of the disease, we used *ex vivo* [³H]DPA-713 quantitative autoradiography and *in vivo* [¹²⁵I]IodoDPA-713 small animal SPECT imaging to show that brain TSPO levels markedly increase prior to physical and behavioral manifestation of disease. We further show that TSPO upregulation coincides with early neuronal GM2 ganglioside aggregation and is associated with ongoing neurodegeneration and activation of both microglia and astrocytes. In brain regions with increased TSPO levels, there is a differential pattern of glial cell activation with astrocytes being activated earlier than microglia during the progression of disease. Immunofluorescent confocal imaging confirmed that TSPO colocalizes with both microglia and astrocyte markers, but the glial source of the TSPO response differs by brain region and age in SD mice. Notably, TSPO colocalization with the astrocyte marker GFAP was greater than with the microglia marker, Mac-1. Taken together, our findings have significant implications for understanding TSPO glial cell biology and for detecting neurodegeneration prior to clinical expression of disease.

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Keywords

Translocator protein 18 kDa; TSPO; Sandhoff disease; biomarker; microglia; astrocytes; neurodegeneration

INTRODUCTION

Sandhoff disease (SD) is an autosomal recessive disorder characterized by a deficiency in the lysosomal enzyme β -hexosaminidase, which leads to the accumulation of gangliosides and glycolipids, specifically GM2 and GA2 (Jeyakumar et al., 2002; Mahuran et al., 1999). Because gangliosides are expressed at high levels in the central nervous system (CNS), the brain is one of the most affected organs (Jeyakumar et al., 2002). Ganglioside accumulation in the brain ultimately leads to progressive and widespread neurodegeneration and motor impairments (Wada et al., 2000). In humans, the onset of the infantile form of SD occurs around 6 months of age with symptoms such as motor weakness, early blindness, macroencephaly, and seizures. Symptoms progress rapidly, and death occurs between 3 to 5 years of age (Maegawa et al., 2006).

β -hexosaminidase is formed by the dimerization of two subunits, α - and β -subunits to form β -hexosaminidase A ($\alpha\beta$) or two β subunits to form β -hexosaminidase B ($\beta\beta$). In the mouse model of SD, the gene encoding for the β subunit of β -hexosaminidase (*Hexb*) is disrupted, resulting in the deficiency of both β -hexosaminidase A and B (Yamanaka et al., 1994). This deficiency leads to impaired degradation and subsequent accumulation of GM2 and GA2 gangliosides in neurons (Sango et al., 1995; Jeyakumar et al., 2002; Mahuran et al., 1999), resulting in severe neurodegeneration in the brain (Wada et al., 2000). Sandhoff (*Hexb* KO) mice exhibit spastic and reduced hind limb movements with progressive motor deficits starting at 3 months of age (Sango et al. 1995). The life span of SD mice is approximately 5 months of age as the mice lose the ability to move and are unable to retrieve food or water (Sango et al., 1995; Tiffit and Proia, 1997; Jeyakumar et al., 2002). Histopathological studies have shown that excess storage of glycolipids in lysosomes leads to neuronal apoptosis in the cerebellum, brainstem, spinal cord, trigeminal ganglion, retina, and thalamus in SD mice (Sango et al., 1995; Wada et al., 2000). Tissues from humans diagnosed with SD have also shown neurodegeneration in spinal cord, cerebral cortex, and thalamus (Huang et al., 1997; Wada et al., 2000). Thus, the mouse model of SD shares many of the clinical symptoms and neuropathology as the human form of the disease.

Previous work has shown that microglia become activated in both the mouse model and in human cases of SD (Wada et al., 2000; Visigalli et al., 2009) and that microglial activation appears to precede neuronal degeneration. Furthermore, cDNA microarray analysis has shown increased TSPO gene expression in the SD mice (Wada et al., 2000), and using PET imaging, uptake of [^{11}C]-PK11195, a TSPO-specific ligand, was higher in SD mice at 4 months of age compared to wildtype (Visigalli et al., 2009). However, a longitudinal assessment of TSPO expression as a function of disease progression using behavioral and neuropathological endpoints has not been investigated. Furthermore, it has been previously reported that [^{11}C]-PK11195 PET imaging shows significant non-specific binding and poor

brain uptake (Boutin et al., 2007; Endres et al., 2009), and thus, better ligands are needed for *in vivo* TSPO PET/SPECT studies.

DPA-713, also known as *N,N*-diethyl-2-[2-(4[methoxy-phenyl]-5,7-dimethyl-pyrazolo[1,5-*a*]pyrimidin-3-yl)-acetamide], is a pyrazolo-pyrimidine that is 10-fold less lipophilic than PK11195 but with twice the affinity for TSPO (Wang et al., 2009). [¹¹C]DPA-713 PET imaging has been performed in a rodent model of brain injury and in a human study with higher signal-to-noise ratios than [¹¹C]-PK11195 (Doorduyn et al., 2009; Endres et al., 2009). [¹²⁵I]IodoDPA-713 is a novel TSPO-specific radioligand that has been previously used to detect TSPO *in vivo* in a mouse model of lung inflammation (Wang et al., 2009), but it is unknown whether [¹²⁵I]IodoDPA-713 can be used to detect *in vivo* TSPO binding in the brain. In this study, we used [³H]DPA713 and [¹²⁵I]IodoDPA-713 to assess the progression of TSPO expression in SD mouse model in a longitudinal fashion. The goal of this study was to examine how early in disease progression TSPO levels increased in relation to: 1) neuronal GM2 ganglioside accumulation, 2) activation of microglia and astrocytes, 3) neurodegenerative changes based on silver staining, and 4) behavioral expression of disease focusing on the presymptomatic phase of the disease.

MATERIALS AND METHODS

1.1. Animal care and use statement

All animal studies were reviewed and approved by both Columbia University Medical Center and the Johns Hopkins University Animal Care and Use Committee. Studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals as stated by the United States National Institutes of Health.

1.2. Animal model and tissue preparation

Mice heterozygous for *Hexb* were generously donated by Dr. Richard Proia (National Institute of Kidney & Digestive Diseases, Bethesda, MD). Wildtype (*Hexb* +/+) or SD (*Hexb* -/-) mice were euthanized at 1, 1.5, 2, or 3 months of age by either decapitation to obtain fresh-frozen brain tissue for receptor autoradiography or by transcardiac perfusion for immunohistochemistry. Fresh frozen brains were stored at -80°C. For transcardiac perfusion, animals were deeply anesthetized with pentobarbital (100 mg/kg body weight) and perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4 at 4°C). Perfused brains were post-fixed overnight in the same fixative, cryoprotected with 25% sucrose for 48 h, flash-frozen in dry-ice-cooled isopentane, and stored at -80°C.

1.3. Behavioral assessment

1.3.1. Motor skill—Motor skill was measured using the rotarod apparatus (Columbus Instrument, Columbus, OH). The latency time the mouse remained on the rod at accelerating speeds was recorded. Each mouse was trained for 5 min at a speed of 4 RPM. Training was followed by a 30-min rest period in the home cage. Mice were then placed back on the rotarod for three trials. Each trial began at 4 RPM and accelerated every 30s by 4 RPM to a maximum of 40 RPM. Trials were separated by a 10-min rest period. Mice were tested for 3 consecutive days.

1.3.2. Locomotor activity—Mice were placed in open field activity chambers with infrared beams (San Diego Instruments Inc., San Diego, CA) for 1 h. During this time, horizontal activity and rearing were automatically recorded.

1.4. Quantitative receptor autoradiography

Fresh frozen brains were sectioned at 20 μm on a freezing cryostat (Leica, Nussloch, Germany) in the horizontal plane. Brain sections were thaw-mounted onto poly-L-lysine-coated slides (Sigma-Aldrich, St. Louis, MO) and stored at -20°C . [^3H]DPA-713 autoradiography was used to assess TSPO levels in multiple brain regions. Adjacent brain sections were used as follows: slides were thawed and warmed on a slide warmer at 37°C for 30 min and prewashed for 5 min in 50 mM Tris-HCl buffer (pH 7.4) at room temperature. Slides were incubated in a buffer containing 1.0 nM [^3H]DPA-713 for 30 min at room temperature. For non-specific binding, adjacent sections were incubated in the presence of 10 μM racemic PK11195. Slides were washed twice for 3 min each in 4°C buffer and dipped twice in 4°C deionized water. Sections were apposed to Kodak Bio-Max MR films with tritium microscopes for 4 weeks (GE Healthcare, Piscataway, NJ). Images were acquired and quantified using the MCID software (InterFocus Imaging Ltd, Cambridge, England).

1.5. Immunohistochemistry

Brain sections from PFA-perfused animals were sectioned at 40 μm using a freezing microtome (Leica SM2000R; Leica Microsystems, Wetzlar, Germany). Sections were stored in cryoprotectant consisting of 50% glycerol in 0.05M phosphate buffer at -20°C . Sections were washed with Tris-buffered saline for 30 min. For GFAP and Mac-1 (CD11b) immunohistochemistry, sections were pre-treated with 0.6% H_2O_2 in TBS for 10 min and blocked with a 5% normal goat serum and 0.2% Triton X-100 solution for 1 h. Sections were incubated with rabbit anti-GFAP antibody (1:1000, Dako Z-0334, Carpinteria, CA) or rat anti-CD11b antibody (1:250, BD PharMingen 553308, San Diego, CA) at 4°C overnight. After washing with TBS, sections were incubated with the appropriate biotinylated secondary antibodies (1:200, Vector, Burlingame, CA) for 1 h. This was followed by an incubation in ABC elite, an avidin-biotin-horseradish peroxidase (HRP) complex (Vector) for 30 min. Immunoreactivity was visualized with a 0.25 mg/mL 3,3'-diaminobenzidine (DAB) (Sigma) and 0.03% H_2O_2 solution. Sections were mounted on slides, dehydrated in increasing concentrations of ethanol, and coverslipped using Permount media (Sigma). For double and triple labeling immunofluorescence, sections were washed with TBS for 60 min and then blocked in 5% normal donkey serum with 0.2% Triton X-100 solution for 1 h. Sections were incubated in primary antibodies diluted in blocking solution overnight at 4°C using the following dilutions: rat anti-Mac-1 (1:250, BD Pharmingen 553308), rabbit anti-TSPO (1:500, Abcam ab109497), mouse anti-GFAP (1:2000, Millipore MAB3402), rabbit anti-GM2 (1:500, Millipore 345759), or mouse anti-NeuN (1:1000, Chemicon MAB377). After washing in TBS, sections were incubated with appropriate secondary antibodies (1:500, AlexaFluor488, AlexaFluor594, AlexaFluor647; Molecular Probes, Carlsbad, CA) diluted in a 2% normal donkey serum with 0.1% Triton X-100 at room temperature for 1 h. Following another series of washes in TBS, sections were mounted onto slides in ProLong Gold DAPI mounting media (Molecular Probes).

1.6. Silver staining for neuronal degeneration

Free-floating sections from PFA-perfused animals were further fixed in 4% PFA in 0.1M phosphate buffer for 48 h before staining. Silver staining was performed using the FD Neurotech NeuroSilver Kit (Ellicott City, MD) according to manufacturer's instructions.

1.7. [¹²⁵I]IodoDPA-713 small animal SPECT/CT for dynamic in vivo imaging of TSPO

An X-SPECT small-animal SPECT/CT system (Gamma Medica-Ideas, Northridge, CA) was used for image acquisition. Each mouse was anesthetized with isoflurane prior to imaging. About 1–2 mCi of [¹²⁵I]IodoDPA-713 was injected into each mouse via intravenous injection, and images were acquired immediately after injection. The SPECT projection data were acquired using 2 low-energy, high-resolution parallel-hole collimators with a radius of rotation of 4.65 cm (spatial resolution, 1.6 mm). The tomographic data were acquired in 64 projections over 360° at 20 s per projection. After tomography, CT was acquired in 512 projections to allow anatomic coregistration. Data were reconstructed using the ordered subsets-expectation maximization algorithm and analyzed using AMIDE software (free software provided by SourceForge). A time activity curve of the [¹²⁵I]IodoDPA713 uptake was generated for each mouse. Three mice per genotype were scanned for this study. For the blocking study, non-radioactive IodoDPA-713 (20 μM) was intravenously co-injected with [¹²⁵I]IodoDPA-713, and images were acquired immediately after [¹²⁵I] injection. Ligands were synthesized according to the methods described in Wang et al., 2009.

1.8. Imaging and image analysis

Immunohistochemistry sections stained using DAB were examined using an Olympus BX51 microscope (Proscan II, Prior, Rockland, MA) linked to an Olympus DP70 camera. High-resolution imaging and contrast microscopy using oil immersion and 40x (numerical apertures 1.00) and 100x (numerical aperture 1.35) were used. Immunofluorescently-labeled tissue was imaged at 60x or 96x magnification using a laser scanning confocal microscope (Fluoview FV10i, Olympus, Center Valley, PA), utilizing the FV10 image software. All tissues stained under the same conditions were imaged using the same scanning parameters on the same day. Five to six confocal stacks of each brain region were obtained for each age and genotype for each experimental condition. Confocal stacks were projected into single images using the maximum fluorescence. All images were analyzed using Metamorph Offline (Molecular Devices, Downingtown, PA). The threshold level was kept at the same level for analysis in images obtained from the same experiment. For colocalization analyses, gray scale images at each wavelength were used to examine the area of colocalized pixels of both wavelengths to calculate the percent colocalization of their signals. Percent colocalization was calculated as previously described by Stansfield, et al., 2012. For GM2 and NeuN association analyses, GM2 aggregates and NeuN positive cells were counted using FV10i software.

1.9. Statistical analysis

Repeated measure analysis of variance (ANOVA) was used for comparison of weekly body and brain weights across time between wildtype and Sandhoff mice. Student's t-test was used for comparison between wildtype and Sandhoff mice for SPECT imaging, rotarod test,

horizontal activity, and rearing activity within each age group due to the fact that animals were euthanized at each age and that the rotarod and activity cages were different across institutions. Therefore, for each age, we normalized the SD results to age-matched wildtype mice. The Bonferroni method was used to correct for multiple comparisons ($p = 0.0125$). A 1-way analysis of variance (ANOVA) was used for comparison between wildtype and SD mice across the 4 ages for the quantitative receptor autoradiography, and a 2-way analysis of variance was used for colocalization analyses. ANOVA with p values < 0.05 was considered significant, and these data were further subjected to *post hoc* LSD analysis.

2 RESULTS

2.1 Brain and body weights of wildtype (WT) and Sandhoff disease (SD) mice

The body weights of mice were taken weekly starting at 6 weeks and ending at 13 weeks (3 months, the oldest age used in this study) when brains were extracted. Following extraction, the brain weights of WT and SD mice (i.e., *Hexb* knockout) were measured at 3 months of age. Brain weights were not significantly different between WT (425.0 ± 15.1 mg) and SD mice (448.4 ± 20.3 mg) ($n = 13$ per group. Data are presented as mean $\pm$ s.e.m.). Figure 1A shows that the body weight gain differences between WT and SD mice were not significantly different [$F_{(1,25)}=0.12$, $p=0.7295$; $n = 13$ per group] up to 3 months of age.

2.2 Progression of motor function deficits

Previous studies have documented the progressive decline in motor function in SD mice beginning at 3 months of age (Sango et al., 1995). Here, we sought to characterize the motor coordination and grip strength of SD mice as a function of age focusing on the presymptomatic phase of the disease. This was done in order to compare the TSPO response in relation to the behavioral manifestation of disease. Figure 1B shows that at 1, 1.5, or 2 months, WT and SD mice showed no significant differences in motor skills as assessed by rotarod performance (Fig. 1B; $t_{(16)}=0.349$, $p=0.731$; $t_{(14)}=1.223$, $p=0.240$; $t_{(17)}=0.761$, $p=0.457$ for 1, 1.5, and 2 months, respectively; $n=10$ –13 mice). However, at 3 months, SD mice expressed a dramatic decrease in latency time, demonstrating decreased motor coordination and reduced grip strength (Fig. 1B; $t_{(27)}=5.131$, $p<0.001$).

We also assessed locomotor activity using activity chambers, specifically examining horizontal activity and rearing (ability to stand on hind legs). At 3 months of age, SD mice had a lower level of rearing activity compared to age-matched WT mice, indicating motor impairments (Fig. 1D; $t_{(26)}=3.405$, $p=0.0022$; $n=9$ –14 mice). Statistically significant differences in horizontal activity were not seen between WT and SD mice within each age group (Fig. 1C). However, at all ages, horizontal activity and rearing appeared to be lower in SD mice compared to age-matched WT mice (Figs 1C and D). The latter findings suggests that the generation of *Hexb*($-/-$) animals affects horizontal activity and rearing at an age when GM2 ganglioside aggregation or neurodegeneration are absent, i.e., 1 month of age (see section 2.3.1 and 2.3.3). Therefore, we inferred that the difference in horizontal and rearing behavior in SD mice is not associated with disease-induced neurodegeneration but is instead associated with a genotype effect of SD mice.

2.3 Longitudinal assessment of TSPO in relation to neuropathology

We assessed the age-dependent progression of neuropathology in SD mice by examining several pathological endpoints including 1) neuronal ganglioside aggregation (GM₂ immunohistochemistry), 2) brain injury and inflammation using TSPO levels (quantitative receptor autoradiography and *in vivo* imaging), 3) ongoing neurodegeneration using silver staining, and 4) reactive gliosis with microglia and astrocyte markers using immunohistochemistry. These studies were performed in the thalamus, brainstem, and cerebellum because they are the brain regions most severely affected in SD mice (Huang et al., 1997; Sango et al., 1995; Wada et al., 2000). We should note that we also assessed TSPO levels in other brain regions such as the hippocampus and cerebral cortex (including orbital, motor, and somatosensory cortex) and found no effect, showing the regional specificity of the TSPO response, and not a global inflammatory response (Supplementary Figs. 1A and B). However, see section 2.3.3.

2.3.1 Longitudinal assessment of neuronal aggregation of GM2 gangliosides

—To determine the age at which neuronal ganglioside aggregation begins in this mouse model, we stained for GM2 gangliosides at each of the four different ages (1, 1.5, 2, and 3 months) in both WT and SD mice. We stained for GM2 instead of GA2 as GM2 precedes GA2 in the ganglioside catabolic process in mice (Phaneuf et al., 1996; Jeyakumar et al., 2002). We co-stained for GM2-positive gangliosides and NeuN-positive neurons and counted the number of neurons that contained GM2 aggregates in different brain regions. In the thalamus, we observed neuronal GM2 ganglioside aggregation beginning at 1.5 months, peaking at 2 months, and subsiding at 3 months of age, possibly due to neuronal loss (Fig. 2A; $F_{(4,14)}=18.825$; $p < 0.001$). Significant accumulation of neuronal GM2 ganglioside aggregation was not observed in the brainstem or cerebellum until 2 months of age (Fig. 3A; $F_{(4,14)}=8.711$; $p = 0.003$ for brainstem and Fig. 4A; $F_{(4,14)}=22.150$; $p < 0.001$ for cerebellum). The level of GM2 ganglioside aggregation at 3 months of age remained similar to levels observed at 2 months in these two brain regions. This suggests that there is a regional and temporal specific accumulation of gangliosides and that one of the first brain regions affected by this disease is the thalamus.

2.3.2 Age-dependent analysis of brain TSPO levels using [3H]DPA-713

quantitative autoradiography—We examined TSPO levels in different brain regions in SD mice as a function of age to determine the association between the initiation of GM2 ganglioside aggregation (the primary initiating event that leads to neuronal degeneration) and the TSPO response to this neuronal injury. We performed [³H]DPA-713 TSPO autoradiography and analyzed 5 brain regions: the cerebral cortex (including the orbital, motor, and somatosensory cortex), hippocampus, thalamus (ventral posteromedial thalamic nucleus), brainstem (parvicellular reticular nucleus), and cerebellum (deep cerebellar nuclei) in age-matched WT and SD mice. In WT mice, we found no significant differences in [³H]DPA-713 binding to TSPO as a function of age for any of the brain regions examined; thus, we combined the [³H]DPA-713 binding data for WT mice from each age and brain region (thalamus: $F_{(3,34)}=0.066$; $p=0.978$; brainstem: $F_{(3,29)}=0.110$; $p=0.954$; cerebellum: $F_{(3,32)}=0.027$; $p = 0.994$; hippocampus: $F_{(3,34)}=0.153$; $p=0.927$; cortex: $F_{(3,33)}=0.128$; $p=0.943$; $n=9-14$ /age group). In the thalamus of SD mice, TSPO levels measured using

[³H]DPA-713 autoradiography were markedly elevated relative to WT beginning at 1.5 months of age (Fig. 2B). TSPO levels progressively increased as a function of age in SD mice, reaching nearly 5-fold higher levels relative to WT at 3 months of age (Fig. 2B; $F_{(4, 62)}=139.144$; $p < 0.001$). It should be noted that the earliest significant increase in TSPO levels in the thalamus occurred at 1.5 months of age, a time when there was minimal, non-significant GM2 ganglioside aggregation in neurons (Fig. 2A and B). Therefore, the TSPO response in the thalamus was measured at the earliest age when GM2 ganglioside aggregation was observed.

In the brainstem, we observed a significant increase in TSPO binding beginning at 2 months of age with further increases at 3 months of age (Fig. 3B; $F_{(4, 53)}=13.002$; $p < 0.001$). The significant increase in TSPO levels in the brainstem coincided with a significant increase in neuronal GM2 accumulation in this brain region. In the cerebellum, TSPO binding was significantly elevated only at 3 months in SD mice as compared to WT and all other ages (Fig. 4B; $F_{(4, 59)}=6.692$; $p < 0.001$). This was in contrast to the significant ganglioside accumulation seen in the cerebellum at 2 months. It is possible that the TSPO glial response in the deep cerebellar nuclei may require a greater amount of time than in other brain regions. Additionally, we found no statistically significant differences in TSPO binding in the hippocampus or the orbital, motor, and somatosensory cortex at any age (Supplementary Figs. 1A and B; $F_{(4, 62)}=0.559$; $p=0.693$ for hippocampus and $F_{(4, 60)}=1.191$; $p=0.324$ for cortex).

2.3.3 Longitudinal assessment of neurodegeneration—To assess the relationship between TSPO expression and neurodegeneration in the SD mouse brain, we used silver staining as a means to evaluate ongoing neurodegeneration. At 1 month of age, SD mice showed no significant silver accumulation in the thalamus (Fig. 2C), cerebellum (Fig. 4C) or brainstem (Fig. 3C) compared to WT. At 1.5 months, there was significant silver accumulation in the thalamus of SD mice compared to WT. At 2 and 3 months, SD mice showed robust silver accumulation in the thalamus (Fig. 2C) and in both the brainstem (Fig. 3C) and cerebellum (Fig. 4C). The cerebral cortex (including the orbital, motor, and somatosensory cortex) exhibited minimal silver accumulation by 3 months (Supplementary Fig 2A). Collectively, these results indicate that TSPO expression increases in brain areas undergoing neurodegeneration. It is notable that we detected significant and selective neurodegeneration in the CA2 region of the hippocampus beginning at 1.5 months and increasing with disease progression (Fig. 5A). This is in contrast to the fact that we did not detect increased TSPO levels or glial cell activation via quantitative autoradiography or immunohistochemistry in the CA2 region of the hippocampus at any age. These findings suggest that the CA2 region of the hippocampus is not able to mount a glial/TSPO response to ongoing neurodegeneration. Further studies are needed in order to understand this unusual and unexpected finding.

2.3.4 Longitudinal assessment of reactive gliosis—Another aspect of this study was to determine the cellular sources of the TSPO response in SD mouse brain. It is known that increased TSPO expression in the injured brain is associated with both microglia and astrocyte activation (Chen and Guilarte, 2008; Chen et al. 2004; Consenza-Nashat et al.,

2009; Raghavendra et al., 2000; Veenman and Gavish 2000; Venneti et al., 2004). Thus, we performed immunohistochemistry using Mac-1 as a microglia marker and GFAP as an astrocyte marker. Wildtype mice exhibit morphologically normal microglia and astrocytes at all ages examined. On the other hand, in the thalamus of SD mice, microglia activation was first observed at 1.5 months of age, peaked at 2 months of age, and decreased at 3 months of age (Fig. 2D). In both the cerebellum and brainstem, SD mice expressed a progressive age-dependent increase in Mac-1 labeling with maximal activation at 3 months relative to WT (Figs. 3D and 4D for cerebellum and brainstem, respectively). Neither the cortex nor the hippocampus showed activated microglia as demonstrated by Mac-1 labeling (Supplementary Fig. 2B and Fig. 5B).

For astrocytes, we found that in the thalamus, brainstem, cerebellum, and cerebral cortex of SD mice, there was an increased number of astrocytes and increased GFAP levels at all ages relative to WT (Fig. 2E, 3E, 4E, and Supplementary Fig. 2C for thalamus, brainstem, cerebellum, and cortex respectively). Therefore, it appears that in SD mice, astrocytosis occurs very early in time and prior to microglia activation. In all brain regions examined in SD mice, activated astrocytes were observed as early as 1 month of age (Fig. 2E, 3E, 4E, and Supplementary Fig. 2C). Astrocyte activation became more pronounced with disease progression in the regions of the brain that exhibited increasing levels of TSPO as the disease progresses, i.e., the thalamus, brainstem, and cerebellum. The astrocytes in the cerebral cortex, which did not show increased TSPO levels with disease progression, demonstrated an increase in number based on GFAP-immunohistochemistry; however, these astrocytes did not appear to be hypertrophic. In contrast, GFAP staining was seen throughout the entire hippocampus in both WT and SD mice (Fig. 5C) as is normal for this brain region.

2.4 In vivo TSPO imaging using [¹²⁵I]IodoDPA-713 SPECT

We used [¹²⁵I]IodoDPA-713 small animal SPECT imaging to measure TSPO expression *in vivo* in SD mice. Small animal SPECT images were co-registered with small animal CT to select the appropriate brain regions for analysis. There was a higher brain uptake of [¹²⁵I]IodoDPA-713 in the SD mouse compared to WT (Fig. 6A). Time-activity curves in the thalamus showed that SD mice had higher uptake of [¹²⁵I]IodoDPA-713 than WT mice across all time points (data not shown). Quantitative analysis of the SPECT imaging showed a trend of increased uptake of [¹²⁵I]IodoDPA-713 in the thalamus, cerebellum, and brainstem with a statistically significant increase of [¹²⁵I]IodoDPA713 uptake in thalamus at 2 months ($t_{(4)}=2.81$, $p=0.0484$) and in the brainstem at 3 months ($t_{(6)}=3.263$, $p=0.0172$) compared to WT (Fig. 6B). To determine that the [¹²⁵I]IodoDPA713 brain uptake was selective for TSPO, we used a pharmacological block in SD mice using nonradioactive IodoDPA713 (20 μ M), resulting in complete blockade of [¹²⁵I]IodoDPA713 uptake in the brain of SD mouse (Fig. 6A). These findings indicate that the increased [¹²⁵I]IodoDPA713 in the mouse brain is directly related to increased TSPO brain levels.

2.5 Temporal TSPO expression in glial cell types

An important aspect in understanding the TSPO response to brain injury is to determine the cellular sources of the TSPO response since both microglia and astrocytes express and

increase TSPO levels following injury (Chen and Guilarte, 2008; Chen and Guilarte, 2006; Kuhlmann and Guilarte, 2000; Maeda et al., 2007; Vowinckel et al., 1997). In Figures 2, 3, and 4, we show the temporal pattern of TSPO expression and the activation of microglia and astrocytes in SD mice. However, this data does not show the temporal expression of TSPO within each glial cell type. In order to understand the contribution of microglia and astrocytes to the TSPO signal during the course of neurodegeneration obtained by [³H]DPA-713 receptor autoradiography and [¹²⁵I]DPA-713 small animal SPECT, we performed triple-labeled immunohistochemistry and confocal imaging of TSPO with Mac-1 and GFAP.

Figure 7 depicts triple-labeled immunofluorescent confocal images of TSPO, Mac-1, and GFAP in both WT and SD mice. Triple-labeled immunofluorescent confocal imaging confirmed that TSPO colocalized with both microglia and astrocytes in the thalamus (Fig. 7A–C), brainstem, and cerebellum. We also measured the percent colocalization of TSPO with Mac-1 and TSPO with GFAP in the thalamus (Figs. 7B and 7C), brainstem (Figs. 8A and B), and cerebellum (Figs. 9A and B) at the various ages. In the thalamus, the percent colocalization between TSPO and Mac-1 followed a similar pattern in both WT and SD mice with a more pronounced effect in the SD mice (Fig. 7B). The percent colocalization between TSPO and Mac-1 increased with age in both WT and SD mice and peaked at 2 months (Fig. 7B; 2-way ANOVA: Age: $F_{(3,27)}=4.386$; $p=0.016$. Genotype: $F_{(1,27)}=10.740$; $p=0.004$. Age x Genotype: $F_{(3,27)}=2.106$; $p < 0.132$. $n=3-4$ mice per age group and genotype). In the brainstem and cerebellum, percent colocalization patterns of TSPO and Mac-1 mirrored those seen in the thalamus with levels of percent colocalization being highest in the thalamus (Figs. 8A and 9A).

The percent colocalization between TSPO and GFAP differed significantly between WT and SD mice, partially due to the low signal of both proteins in the WT mice. In SD mice, we observed high levels of colocalization in the thalamus beginning at 1.5 months and remaining elevated until 3 months of age (Fig. 7C; 2-way ANOVA: Age: $F_{(3,29)}=2.122$; $p=0.126$. Genotype: $F_{(1,29)}=41.309$; $p < 0.001$. Age x Genotype: $F_{(3,29)}=4.136$; $p = 0.018$. 1-way ANOVA: $F_{(7,29)}=8.401$; $p < 0.001$. $n=3-4$ per age group and genotype). Similarly, the temporal progression of TSPO and GFAP colocalization observed in the brainstem mirrored what was observed in the thalamus (Fig. 8B). In the cerebellum, we observed high levels of TSPO and GFAP colocalization beginning at 1.5 month of age and remained elevated throughout the progression of disease (Fig. 9B). In all regions and at all ages examined, TSPO colocalization with astrocytes was greater than its colocalization with microglia.

DISCUSSION

The present work provides novel information on the relationship between the TSPO response to the initiation and progression of neurodegeneration and glial cell activation during the presymptomatic phase of disease in SD mice. The SD mouse provides a well-characterized animal model of neurodegeneration where the initiating pathological event (aggregation of GM2 ganglioside) can be assessed and the magnitude and temporal course of the TSPO response can be compared to neuronal GM2 aggregation, as well as

pathological and behavioral endpoints. Based on the *ex vivo* [³H]DPA-713 quantitative autoradiography and *in vivo* [¹²⁵I]IodoDPA-713 small animal SPECT imaging, we were able to map the regional TSPO expression in the SD mouse brain as a function of age and pathology. Our findings indicate that the thalamus is the brain region where the earliest increases in GM2 ganglioside aggregation and TSPO expression occur, and that these increases occur prior to physical and behavioral manifestation of disease. Additionally, the magnitude of the TSPO response markedly increases as a function of age as neurodegeneration progresses. Our finding that the thalamus is one of the earliest affected brain regions is consistent with other studies using this animal model (Maegawa et al., 2006) and in the clinical setting with patients that express the disease (Kumar et al., 2014, Wu et al., 2013, Saouab et al., 2011, Yun et al., 2005).

Importantly, the regional expression of TSPO in the SD brain was consistent with affected (thalamus, brainstem, and cerebellum) brain regions. The finding that increased TSPO expression in the thalamus occurs with the first observable evidence of GM2 aggregation and prior to behavioral manifestation of disease strongly suggests that TSPO can be used for the early detection of neurodegenerative disease and to assess its progression. These characteristics make TSPO an ideal biomarker to assess the effectiveness of therapeutic interventions by tracking the TSPO signal during the presymptomatic stage of the disease.

It is notable that we were not able to detect an increase in TSPO or glial cell activation in the CA2 region of the hippocampus in SD mice despite significant and progressive neurodegeneration measured by silver staining. To our knowledge, this pathology has not been described previously in either the SD mouse model or in human postmortem tissue from SD subjects. As previously noted, one possibility for this discrepancy could be related to the fact that the CA2 region of the hippocampus is a very small area and the methods used do not have sufficient spatial resolution to detect a TSPO or glial response. Studies using emulsion autoradiography with resolution at the cellular level could be useful to provide an explanation to this question. Alternatively, it is possible that the CA2 region is unique from other brain regions and is not able to mount a TSPO/glial response to injury. For example, a study by Kipp et al., demonstrated that astrocytes cultured from different brain areas not only have differences in cytokine profile expression and capacity in basal unstimulated conditions, but also have regional and temporal specific patterns of activation and cytokine upregulation in response to an inflammatory stimuli, such as LPS (Kipp et al., 2008). Taken together, these findings suggest that glial cell activation and thus the TSPO response to injury is likely to differ by brain region and one cannot assume that different types of injuries will generate the same TSPO/glial cell response.

Progressive reactive gliosis is known to occur in this mouse model of SD as demonstrated in the present study and by other studies (Myerowitz et al., 2002; Wada et al., 2000; Kyrkanides et al., 2005; Kyrkanides et al., 2007). However, no previous study has examined the age-dependent activation of both microglia and astrocytes. Here, we established the regional and temporal pattern of microglia and astrocyte activation over the course of disease progression with astrocyte activation occurring earlier in time than microglial activation and confirm that both microglia and astrocytes are the source of the observed TSPO response although they express different temporal profiles (Figs. 2, 3, and 4).

To further understand the function of TSPO and its role in the SD process, we first confirmed that both microglia and astrocytes expressed TSPO with specific regional and temporal patterns. Notably, in this neurodegenerative animal model, TSPO exhibited a higher degree of colocalization with astrocytes than with microglia, which is different with has been previously observed in other brain injury models (Chen and Guilarte, 2008; Cosenza-Nashat M et al. 2009). The greater degree of TSPO expression and colocalization with astrocytes in the SD mouse brain may be related to the early (i.e., 1 month) and sustained progression of astrocytosis present in several brain regions examined. The early astrocytic response occurred before the upregulation of TSPO and before neurodegeneration as assessed by silver staining, indicating that the generation of the SD mouse by knocking out the HexB gene may alter astrocyte physiology. This is consistent with the fact that abnormal growth patterns in astrocytes cultured from SD mice have been documented (Kawishima et al., 2009). Specifically, these studies found significantly faster rates of proliferation, increased ERK phosphorylation, and decreased Akt phosphorylation in astrocytes cultured from SD mice compared to WT. Another study found that despite conditionally expressing β -hexosaminidase in neurons from SD mice, the number of GFAP-positive astrocytes did not decrease and GFAP gene expression remained elevated, whereas the number of MHC-II and CD45 positive cells and gene expression levels decreased as a result of expressing β -hexosaminidase in neurons from SD mice (Kyrkanides et al., 2012). These results suggest that the early proliferation response of the astrocytes is independent of GM2 aggregation in neurons and that there is likely a disruption of neuron-astrocyte signaling. Taken together, these studies suggest that SD mice may have abnormally-functioning astrocytes, potentially explaining the early degree of GFAP expression and hypertrophy observed in the present study. However, it is important to note that despite the early activation of astrocytes, the TSPO signal does not increase until later in the disease process. This finding suggests that the TSPO increase in activated astrocytes starting at 1.5 months in the thalamus is the result of injury due to the disease, and not due to alterations in astrocyte physiology observed as early as 1 month of age.

In the present study, we used the second-generation TSPO ligand DPA-713 in the tritiated and iodinated forms for TSPO autoradiography and *in vivo* imaging using small animal SPECT. Overall, one advantage of using [^3H]DPA-713 for TSPO autoradiography studies is the fact that there is virtually no background; thus, a very high signal to noise ratio. Furthermore, using the [^{125}I]IodoDPA-713 allows for *in vivo* SPECT imaging studies in which a single synthesis can be used for multiple studies due to the relatively long half-life of ^{125}I . Recent work has shown that [^{125}I]IodoDPA-713 can be used for imaging TSPO in the lungs using small animal SPECT (Wang et al., 2009; Ordonez et al., 2015). Our current study is the first to demonstrate *in vivo* [^{125}I]IodoDPA-713 small animal SPECT imaging in the brain, with results similar to those measured with [^3H]DPA-713 autoradiography despite the fact that [^{125}I]IodoDPA-713 small animal SPECT does not have the same degree of spatial resolution as [^3H]DPA-713 autoradiography. Our findings strengthen the use of [^{125}I]IodoDPA-713 as an attractive radioligand for TSPO imaging using small animal SPECT.

Overall, we have shown that the regional expression of TSPO in SD mice is brain region-specific and depends on the age of the SD animal as disease progresses with the thalamus providing the earliest signal of disease based on TSPO expression and other markers of neurodegeneration. Therefore, it is possible that PET, SPECT, or magnetic resonance imaging techniques (if developed) of brain TSPO could serve as an early indicator of brain pathology in the clinical setting. However, while PET and SPECT imaging of TSPO may be useful in the clinical setting as they provide real-time information of ongoing brain inflammation and injury, the potential risks of radiation exposure in children expressing the disease must be taken into consideration. From a translational perspective, the present work sets the stage to determine if TSPO expression in peripheral cells could serve as a surrogate marker for TSPO levels in the brain. For example, using this progressive animal model of SD, one can examine the relationship between increases in TSPO expression in the CNS and those in TSPO-expressing peripheral cells such as platelets, monocytes, and macrophages. The discovery of an early peripheral biomarker of CNS disease would be a significant advance for clinical use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Characterization of Sandhoff Mice

A) Weekly body weights of wildtype and Sandhoff mice from 6 to 13 weeks of age (3 months of age). There was no significant difference of body weight between wildtype and Sandhoff mice at any age (Repeated ANOVA. $p = 0.7295$). Data is expressed as the mean of the body weights per week $\pm$ SEM. $n = 13$ for both wildtype and Sandhoff mice.

B) Wildtype and SD mice show no significant differences in motor skills as assessed by rotarod performance at 1, 1.5, or 2 months of age. However, at 3 months of age, SD mice expressed a dramatic decrease in latency time demonstrating their decreased motor coordination and reduced grip strength. Data is expressed as mean $\pm$ SEM. $n = 10$ –13 mice per group.

C) Horizontal activity and D) rearing of WT and SD mice were measured for 1 hour using the open field activity chamber at each of the 4 ages. When correcting for multiple comparisons, a significant difference between age-matched 3 month wildtype and SD mice was seen for rearing activity ($t = 3.405$; $p = 0.0022$). No significant differences were seen in horizontal activity when applying the Bonferroni method to correct for multiple comparisons. Data is expressed as mean $\pm$ SEM. $n = 9$ –14 mice per group.

FIGURE 2. TSPO Levels and Neuropathological of Sandhoff disease mice in the thalamus

A) GM₂ ganglioside aggregation (green) in neurons stained with NeuN (red).

Representative images show increasing GM₂ aggregates with age in SD mice as compared to WT. GM₂ aggregation begins at 1.5 months and reaches significant levels by 2 months ($F_{4,14} = 18.825$; $p < 0.001$).

B) TSPO levels measured by [³H]DPA-713 quantitative autoradiography progressively increased as a function of age in SD mice. TSPO levels were significantly increased beginning at 1.5 months, the earliest age at which GM₂ aggregation is observed, and increased with age ($F_{4,62} = 139.144$; $p < 0.001$).

C) Representative horizontal brain photomicrographs of active neurodegeneration as detected with silver staining. The top row represents low magnification images with the boxes indicating the area of which the high magnification images in the bottom row were generated. At 1 month, SD mice show no significant silver accumulation in the thalamus

compared to WT. At 1.5 months, there is slight silver accumulation in the thalamus in SD mice compared to WT, and at 2 and 3 months, SD mice show robust silver accumulation in the fiber tracks of the thalamus.

D) Microglia activation as indicated by Mac-1 labeling occurs as early as 1.5 months, with 2 month SD mice exhibiting the highest levels of Mac-1 labeling..

E) Activated astrocytes are observed as early as 1 month of age in SD mice, and activation becomes more pronounced with age and disease progression.

FIGURE 3. Neuropathological Assessment of Sandhoff disease in the brainstem

A) Representative images showing GM2 accumulation (green) in SD mice compared to WT. Slight increase in the levels of GM2 aggregation begins at 1.5 months, reach significant levels by 2 months, and stay consistent at 3 months ($F_{4,14} = 8.711$; $p = 0.003$)

B) TSPO levels in the brainstem, as determined by [³H]-DPA autoradiography, is significant compared to WT mice at 2 months, and increases to higher levels at 3 months of age (B; $F_{4,53} = 13.002$; $p < 0.001$).

C) At both 2 and 3 months, silver staining shows ongoing neurodegeneration. The top row represents low magnification images with the boxes indicating the area of which the high magnification images of the bottom row were generated.

D) WT mice exhibit morphologically normal microglia and astrocytes. Microglia activation, as indicated by Mac-1 labeling, is seen at low levels in the brainstem at 1.5 months. SD mice demonstrate a progressive increase over time in Mac-1 labeling compared to WT mice.

E) Activated astrocytes are observed beginning as early as 1 month of age in SD mice, and activation becomes more pronounced with age and disease progression in SD mice.

FIGURE 4. Neuropathological Assessment of Sandhoff disease in the cerebellum

A) Representative images of GM2 accumulation in SD mice compared to WT. Low levels of GM2 aggregation (green) begin at 1.5 months, reach significant levels by 2 months, and stay consistent at 3 months of age ($F_{4,14} = 22.150$; $p < 0.001$).

B) TSPO levels in the cerebellum, as determined by [³H]-DPA autoradiography, become significantly increased compared to WT mice at 3 months of age ($F_{4,59} = 6.692$; $p < 0.001$).

C) At both 2 and 3 months, representative horizontal brain photomicrographs reveal robust diffuse neurodegeneration as detected through silver staining. The top row represents low magnification images with the boxes indicating the area of which the high magnification images of the bottom row were generated.

D) WT mice exhibit morphologically normal microglia and astrocytes. Microglia activation, as indicated by Mac-1 labeling, is observed at 1.5 months and progressively increasing as animals age and the disease progresses.

E) Activated astrocytes are observed beginning as early as 1 month of age in SD mice, and activation becomes more pronounced with age and disease progression in SD mice.

FIGURE 5. Neuropathological Assessment of Sandhoff disease in hippocampus

A) Representative horizontal brain photomicrographs reveal neurodegeneration in the CA2 region of the hippocampus beginning at 1.5 months and continuing as the disease progresses. The top row represents low magnification images with the boxes indicating the area of which the high magnification images of the bottom row were generated.

B) WT and SD mice exhibit morphologically normal microglia. Microglia activation, as indicated by Mac-1 labeling, does not appear to occur as animals age or the disease progresses in this brain region.

C) Activated astrocytes are observed at all ages in SD mice at comparable levels to those seen in WT mice.

FIGURE 6. *In vivo* TSPO imaging using [^{125}I]-IodoDPA713 SPECT in the Sandhoff disease mouse brain

A) Representative brain uptake of [^{125}I]IodoDPA-713 in wildtype (WT), 3-month SD mouse, and 3-month SD mouse with pharmacological block using non-radioactive IodoDPA-713. The CT, for orientation purposes, indicates that slices are at the level of the thalamus. Tracer uptake is higher in the brain of the SD mouse, and the uptake was blocked by co-injection of 20 μM non-radiolabeled IodoDPA-713.

B) Analysis of [^{125}I]-IodoDPA713 uptake in SD mouse brain. At 2-months of age SD mice exhibited a significant increase in [^{125}I]-IodoDPA713 uptake in the thalamus ($t_4=2.81$, $p=0.0484$). At 3-months of age, SD mice expressed a significant increase in [^{125}I]-IodoDPA713 uptake in the brainstem ($t_6=3.263$, $p=0.0172$). Images are expressed in units of Standardized Unit Value (SUV) and are normalized for weight and injected dose. Data is expressed as the mean of $\text{SUV} \pm \text{SEM}$. $n = 3-4$ mice per group. * $p < 0.05$ compared to WT.

FIGURE 7. Temporal colocalization of TSPO with glial markers in wildtype (WT) and Sandhoff disease (SD) mice in the thalamus

A) Representative triple labeled immunofluorescent confocal images in the thalamus of WT and SD mice at the four different ages. Triple labeled immunofluorescent confocal imaging confirmed that TSPO colocalized with the microglial marker Mac-1 as indicated by the purple and magenta colors and astrocyte marker GFAP as indicated by the yellow color.

B) In the thalamus, the percent colocalization of TSPO with Mac-1 follows a similar pattern in both the WT and SD mice, with a more pronounced effect in the SD mice. The percent colocalization between TSPO and Mac-1 increases with age in both WT and SD mice and peaks at the 2 months of age.

C) The percent colocalization between TSPO and GFAP also differs significantly between WT and SD mice, due to the low signal of both in the WT mice. In the SD mice, we observed a high degree of colocalization beginning at 1.5 months and remaining at 3 months of age. Each value represents the mean $\pm$ SEM. $n = 3 - 4$ animals and experiments.

FIGURE 8. Temporal colocalization of TSPO expression with glial markers in wildtype (WT) and Sandhoff disease (SD) mice in the brainstem

A) In the brainstem, the percent colocalization between TSPO and Mac-1 increases with age and follows a similar pattern in both the WT and SD mice, with a more pronounced effect in the SD mice (2 way ANOVA: Age: $F_{3,29} = 11.843$; $p < 0.001$. Genotype: $F_{1,29} = 18.117$. $p < 0.001$. Age x Genotype: $F_{3,29} = 4.089$; $p = 0.019$. 1 way ANOVA: $F_{7,29} = 9.470$; $p < 0.001$. Each value represents the mean $\pm$ SEM. $n = 3 - 4$).

B) The percent colocalization between TSPO and GFAP differs significantly between WT and SD mice. In the SD mice, we observed high levels of colocalization beginning at 1.5 months and this amount of colocalization remained elevated up until 3 months of age (2 way ANOVA: Age: $F_{3,28} = 6.457$; $p = 0.003$. Genotype: $F_{1,28} = 130.46$. $p < 0.001$. Age x Genotype: $F_{3,28} = 18.319$; $p < 0.0001$. 1 way ANOVA: $F_{7,28} = 26.807$; $p < 0.001$. Each value represents the mean $\pm$ SEM. $n = 3 - 4$). Within a group, bars with different letters are significantly different at $p < 0.05$.

FIGURE 9. Temporal colocalization of TSPO expression with glial markers in wildtype (WT) and Sandhoff disease (SD) mice in the cerebellum

A) In the cerebellum, the percent colocalization between TSPO and Mac-1 increases with age and follows a similar pattern in both the WT and SD mice, with a more pronounced effect in the SD mice (2 way ANOVA: Age: $F_{3,26} = 2.8953$; $p = 0.062$. Genotype: $F_{1,26} = 16.433$. $p < 0.001$. Age x Genotype: $F_{3,26} = 3.778$; $p = 0.028$. 1 way ANOVA: $F_{7,26} = 5.315$; $p = 0.002$. Each value represents the mean $\pm$ SEM. $n = 3 - 4$).

B) The percent colocalization between TSPO and GFAP differs significantly between WT and SD mice. In the SD mice, we observed high levels of colocalization beginning at 1 month and these levels of colocalization remained elevated up until 3 months of age (2 way ANOVA: Age: $F_{3,27} = 2.560$; $p = 0.084$. Genotype: $F_{1,27} = 61.830$. $p < 0.001$. Age x Genotype: $F_{3,27} = 1.315$; $p = 0.297$. Each value represents the mean $\pm$ SEM. $n = 3 - 4$). Within a group, bars with different letters are significantly different at $p < 0.05$.