Research report

Molecular, morphometric and functional analyses demonstrate that the growth hormone deficient little mouse is not hypomyelinated

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Abstract

To study the effects of naturally occurring growth hormone deficiency type I on CNS myelination, we compared the myelination of brains from little and wild-type littermate mice using molecular, histological, morphometric, and functional analyses. The little mouse produces only 6–8% of normal levels of growth hormone GH and approximately 20% of normal circulating levels of insulin-like growth factor 1 IGF-1. Our data show that the expression of myelin basic protein MBP and proteolipid protein PLP of the little brain exhibit the same temporal pattern and amount as that of the wild-type brain. Furthermore, the density and size of myelinated axons and the myelin sheath thickness in the corpus callosum, anterior commissure and the optic nerve are comparable in the little and wild-type brains. These regions are reduced in size in the little mouse brain proportionate to the overall reduction in brain size implying a reduction in the total number of neurons. Therefore, it follows that the total myelin content is reduced, but when normalized to brain size, the myelin concentration is unchanged. Myelin staining patterns of whole brains were identical. Moreover, functional analysis of the visual pathway indicated no difference between the little and control mice. These results are inconsistent with previous reports of hypomyelination in the little mouse and suggest that this form of GH deficiency does not adversely affect the myelination process except possibly through neuronal proliferation. However, since axon size and density are maintained, the neuronal growth may conversely be inherently limited by other restricted brain growth. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Growth hormone; Myelination; Myelin basic protein; Proteolipid protein; Flash visual evoked potential

1. Introduction

Central nervous system myelination is known to be affected by growth factors including insulin-like growth factor 1 (IGF-1) [3,6,13,26,38]. There is still considerable debate as to whether growth hormone (GH) levels affect myelination. In vitro studies have shown that treatment of brain cell aggregate cultures with GH enhances myelination [1]. Comparable in vivo studies have not been done. For example, the brains of transgenic mice that are overexpressing GH [24] have not been assayed for myelin content. Moreover, the effect of GH deficiency on CNS myelination is not clear. Since IGF-1 expression in some tissues is GH-dependent, it is difficult to separate the effects of GH from those of IGF-1. It could be presumed that GH deficiency might adversely affect CNS myelination indirectly by reducing available IGF-1. Beck et al. [3] and Ye et al. [38] have reported that decreased bioavailability of IGF-1 in the brains of mice resulted in significantly decreased brain weight and regional myelin content. Further analyses of IGF-1 knockout mice suggest that its myelination is proportionate to its neuronal composition and therefore total myelin of the anterior commissure is reduced because the density of neurons is reduced [8]. Conversely, GH deficiency may affect myelination independent of IGF-1. Unfortunately, there is no ideal in vivo system in which to distinguish these possible mechanisms. GH deficient animal models have either naturally reduced
levels of IGF-1, as in little or dwarf mice, or artificially enhanced levels of IGF-1, as in IGF-1 transgenic mice [6]. Nonetheless, human patients with GH deficiency have either reduced or normal levels of circulating IGF-1. Therefore, as a first assessment of the presence of any CNS myelin abnormalities in naturally occurring GH-deficiency, we chose to study the brains of the mutant little mouse because it most closely resembles the human condition and has been widely assumed to be hypomyelinated based on previous literature [27–30]. Such studies could provide insight into brain development of children affected with GH deficiency.

The GH deficiency of the little mutant mouse is a consequence of a point mutation in the growth hormone releasing hormone receptor (Ghrhr) gene [19]. The body of the adult little mouse is about 30–40% smaller than its wild-type littermates, and its skull is approximately 20% smaller making this mutant a useful model for human isolated growth hormone deficiency type I. Furthermore, two cases of mutations in the human homolog (GHRHR) have recently been reported [36].

We have further examined the CNS myelin of little mice by assessing myelin morphology and function and the expression of major myelin proteins. From our studies, it appears that these mutants have normal CNS myelin with similar myelinated axon density, size and myelin sheath thickness as compared to their wild-type littermates. Our results do not support earlier reports of hypomyelination and suggest that this form of GH deficiency results in a smaller brain with proportionately reduced white matter regions that are otherwise normal in myelin structure, quantity and function. In contrast with the IGF-1 knockout mouse, the axon size and density are not reduced in GH-deficient little mice.

2. Materials and Methods

2.1. Mouse colony and identification of genotypes

Male and female heterozygous little mice were supplied by the Jackson Laboratory (Bar Harbor, ME) in the C57Bl/6J background. Mice were housed in the animal facility at the Clinical Research Squadron of Wilford Hall, Lackland Air Force Base, fed standard laboratory chow ad libitum and kept on a 12-h dark/light schedule. All protocols were approved by institutional review committees at the University of Texas Health Science Center at San Antonio. Mating of heterozygous little mice provided wild-type, heterozygous and homozygous genotypes for study. In order to ensure accurate identification of the mice, a method was developed to genotype the little locus (Ghrhr) by PCR and subsequent restriction analysis [22]. This prevented the runt of the litter from being misclassified as a little homozygote since tissues were taken as early as 18 days postnatal.

2.2. Molecular analysis of myelin proteins

Brains were harvested immediately after death induced by carbon dioxide inhalation. The left hemisphere was frozen in liquid nitrogen and stored at –20°C for protein analysis and the right hemisphere was homogenized in 4 M guanidinium thiocyanate buffer for RNA isolation. Total RNA was extracted using a protocol adapted from Chomczynski and Sacchi [9]. Five micrograms of total RNA were electrophoresed through a 1.2% denaturing agarose gel, transferred onto a GeneScreen membrane (NEN Life Science, Boston, MA) and UV crosslinked. The membrane was hybridized with a radiolabeled cDNA probe for the 14 kDa form of mouse myelin basic protein (MBP; gift of Dr. Carol Readhead, Cedars Sinai School of Medicine) according to Church and Gilbert [10]. The membrane was exposed for 24 h to a phosphorimager screen and quantitation was done using ImageQuant version 3.2 (Molecular Dynamics, Sunnyvale, CA). The membrane was subsequently stripped in 50 mM sodium phosphate in 55% formamide at 65°C for 60 min and rehybridized with a radiolabeled cDNA probe for mouse cyclophilin (Ambion, Austin TX). Following quantitation and stripping, the membrane was hybridized with a radiolabeled cDNA probe, BAS1013 [35], for mouse proteolipid protein (PLP; gift of Dr. Anthony Campagnoni, UCLA). Since it has been reported [12] that cyclophilin mRNA levels slightly decrease over the first 40 days of postnatal life, the membranes were also rehybridized with a cDNA probe for α-tubulin [12]. The message levels for MBP and PLP were normalized to the cyclophilin mRNA abundance or to the α-tubulin mRNA abundance. The relative values were the same; thus, the message levels normalized to cyclophilin abundance are reported.

MBP protein analysis was done using a radioimmunoassay kit purchased from Diagnostic Systems Laboratories (Webster, TX). The frozen left hemisphere of each brain was thawed on ice and immediately homogenized in 5 ml cold sterile H2O. The homogenate was centrifuged at 3000×g for 20 min at 4°C. The supernatant was diluted in 0.2 M Tris–acetate buffer, pH 7.2, containing 1% Triton X-100 and 0.1% aprotinin [33], and an aliquot was used to measure MBP concentration. MBP concentration was normalized to total protein concentration in the homogenate as measured by the Bio-Rad DC colorimetric assay (Richmond, CA).

2.3. Histological analysis of CNS myelin

For histological staining of myelin, 60-day old male mice were euthanized by isoflurane inhalation and brains were removed and fixed in 10% buffered formalin for 1–3 days. The brains were bisected sagittally and processed in a Shandon Lipshaw processor according to standard techniques [7] and then embedded in paraffin and sectioned. Eight-micrometer thick sagittal sections of the brains were cut, deparaffinized and hydrated to 95% ethanol and placed...
in 0.1% Luxol fast blue solution overnight at 58°C. The slides were rinsed in 95% ethanol followed by distilled water and then dipped in lithium carbonate and differentiated in 70% ethanol. They were counterstained with cresyl echt violet. Size measurements of white matter regions were made using ImagePro Plus v1.2 (Media Cybernetics, Silver Springs, MD). Anatomically matched sections were digitized and the area of each structure was calculated in pixels. The anterior commissure was measured on micrographs taken at 200× and the corpus callosum on micrographs taken at 40×. Three measurements of each brain region were taken for each of three mice per genotype.

2.4. Electron microscopy of optic nerves and brain

Sixty-day little male mice and controls (n = 5 for each) were deeply anesthetized with sodium pentobarbital (100 mg/kg), and transcardially perfused with 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 25°C. The brain and the optic nerve connected to the eye were removed and fixed overnight in the same fixative at 4°C. The brain was sagittally sliced in ~0.5 mm of thickness in the region containing the anterior commissure and the corpus callosum. Each region was then dissected from the thick section. The tissues were washed in 0.1 M phosphate buffer, postfixed in 1% OsO4 in the same buffer, rinsed with H2O, dehydrated through a graded series of ethanol and propylene oxide, and directionally embedded in epoxy resin (Polybed 812, Polysciences, Warrington, PA). Semithin sections of ~1 μm of the optic nerves were cut perpendicular to the longitudinal axis of the nerves at a point 1 mm behind the retina. Sections were stained with 7% aqueous uranyl acetate and counterstained with Reynolds’ lead citrate [20] using a microwave staining procedure [15]. Semithin sections of the brain tissues containing the anterior commissure or the corpus callosum were cut sagittally and processed in the same manner. The sections were examined with a Jeol 100 CX transmission electron microscope (J.E.O.L., Peabody, MA).

Six to eight photographs were randomly taken from each anterior and posterior part of the anterior commissure and from each corpus callosum at 10,000×. Each negative was printed to give a final 30,000× magnification. The axon size and thickness of myelin sheaths were measured with the aid of the Image-Pro analysis system. Only those axons which were perpendicular to their long axis and in which both the inner and outer surface of the myelin sheath were sharp and distinct were used in this analysis. The diameters of axons were calculated from the circumference of the inner surface of the myelin sheaths. The thickness of myelin sheaths was measured by determining the circumference of the outer surface of the sheath and using the approximation as calculated by ImagePro which subtracts the axon circumference and corrects for the shape of the image. ImagePro determines the size in pixels and subsequently converts to nanometers, for example, using a micrograph of a calibration grid. The optic nerve measurements were not converted from pixels to nanometers, but these data can be compared between genotypes to determine relative size. Axon density was determined from photos taken at 2000×. Two to three photos per mouse (n = 3 for each genotype) were counted for each brain region. Only those myelinated axons completely contained within the photo border were counted.

2.5. Visual evoked potentials

Flash visual evoked potentials (FVEPs) were recorded from 6-week old little and wild-type control (n = 11 for
Each mouse. The mice were anesthetized with ketamine and xylazine (75 and 10 mg/kg, respectively) under normal room illumination. Normal body temperature was maintained by use of a surrounding hot water bag, and corneas were kept moist with saline. Stainless steel needle electrodes were placed subdermally in the occipital midline (active) and the ear (reference). A subdermal needle electrode in the midline near the tail served as the ground electrode. The signals were amplified by 10,000 and filtered from 1 to 1000 Hz, and were digitized and recorded by a Nicolet 4094 digital oscilloscope (Nicolet Instruments Technology, Madison, WI) set for averaging (0.396 s trace, digitization rate = 10,000/s).

During recording of FVEPs, ambient illuminance was dim (2.9 lx) as measured at the eye. The surrounding luminance was 0.3 to 0.6 fL. The mice were placed prone facing a Grass PS22 flash lamp diffusing face plate (Grass Instruments, Quincy MA) at a distance of 20 cm. The mice were stimulated binocularly at 1 flash/s. Flash illuminance at the eyes was 88 lx s, and flash duration was 10 μs. One-hundred twenty traces synchronized with flash onset were averaged to produce one FVEP. Four FVEPs were recorded from each mouse. Noise controls were produced in the same way but with the flash occluded by a black opaque cloth. The digitized waveforms were saved as ASCII files with Vu-Point II (Maxwell Laboratories, La Jolla, CA). The average of the four FVEPs was computed and used as the measured datum for each mouse. Since the positive peaks at approximately 50 ms are contaminated by the electroretinogram because of the small size of the mouse heads, the following negative peak and the subsequent positive peak were measured as the cortical response. The implicit time to this negative and the following positive were compared in the little and control mice. The amplitudes of the positive peaks, measured from the trough of the preceding negative, were also compared.

2.6. Statistical analysis

Data were analyzed statistically using GraphPad Prism (San Diego, CA). Myelin protein quantities were compared with one-way analysis of variance (ANOVA). Electron microscopy counts and measures as well as FVEP data were compared with parametric t-tests.

![Fig. 2. Luxol blue myelin staining in wild-type (A,C,E) and little (B,D,F) brains. A and B show the corpus callosum, and C and D show the anterior commissure. The anterior limb is more intensely stained than the posterior limb in each mouse. E and F show the cerebellum. Scale bar: A–B, 373 μm; C–D, 82 μm; E–F, 658 μm.](image-url)
3. Results

3.1. Myelin proteins

The period of maximal MBP mRNA expression in mice is between 10 and 30 days after birth with the peak at about 15 days [5]. Thereafter, mRNA levels decline and plateau at a much lower level. PLP expression is similar [35]. Therefore, MBP and PLP expression was determined at 18, 25 and 60 days postnatal in little mice. As seen in Fig. 1, MBP mRNA expression in little mice exhibited the same developmental pattern as that of the wild-type controls. The expression is lower than wild-type mice, but is only statistically significant at 60 days ($p = 0.022$, ANOVA). However, there is no difference in MBP protein levels between little and wild-type mice at any age tested. Although MBP protein concentration appears lower in little mice at postnatal day 18, the difference is not signifi- cant ($p = 0.104$ using ANOVA; $p = 0.08$ using unpaired t-test). Since MBP protein levels were found to be normal in little mice, expression levels of PLP were also determined. Fig. 1 also shows that PLP mRNA levels in little mice were no different from wild-type; therefore, protein levels were not measured.

3.2. Myelin morphometry

Our molecular data of myelin proteins in the little mouse are inconsistent with reduced myelination. Therefore, histologic and morphometric analyses of the major CNS myelinated regions were done in order to determine if there were structural abnormalities despite normal expression of these key myelin genes. The Luxol blue histochemical staining shown in Fig. 2 indicates no change in myelin concentration in the forebrain regions examined and in the cerebellum. The anterior limb of the anterior

Fig. 3. Electron micrographs of wild-type (A,C,E) and little (B,D,F) brains. A and B show the corpus callosum, C and D show the anterior commissure (posterior limb), and E and F show the optic nerve. Brains from 60-day male mice were perfused with 2.5% glutaraldehyde and 1% paraformaldehyde and sagittally sectioned. Optic nerves were cross-sectioned. Scale bars: A–B, 0.24 μm; C–D, 0.48 μm; E–F, 0.73 μm.
commissure demonstrates equal Luxol staining in the little and wild-type brain. The posterior limb is also stained equally between the genotypes but less than the anterior limb.

Fig. 3 shows representative micrographs of the corpus callosum, anterior commissure and optic nerve from the little and wild-type mice. The axon and myelin structure in these regions of the little mouse appeared normal with a distinct major dense line. The thickness of myelin sheaths, axon sizes, and density of myelinated axons were measured in each of these regions in little and wild-type mice. The corpus callosum and anterior commissure were selected for study because these regions have previously been shown to have reduced axon density or to be abnormally myelinated in IGF-1 knockout and insulin-like growth factor binding protein 1 (IGFBP-1) transgenic mice [3,8,38]. Since myelin thickness increases with increasing axon diameter, the myelin sheaths of comparable axon sizes were compared. As shown in Figs. 4 and 5, our results indicate no difference in either the thickness of myelin sheaths or the density of myelinated axons in the corpus callosum of mature mice. Additionally, no difference was seen in average axon diameter in the corpus callosum (724.1 ± 15.5 nm, S.E.M. for the little, and 692.0 ± 11.9 nm, S.E.M. for the wild-type; p = 0.101). The results for the anterior commissure are also shown in Figs. 4 and 5. The density of the myelinated axons is the same between little mice and wild-type controls in both the anterior and the posterior limb. Moreover, the thickness of the myelin sheaths in both regions is not reduced in the little mice. Instead, our data indicate a statistically significant increase in average myelin thickness of axons less than 700 nm in diameter within the posterior limb of the anterior commissure of the little mouse as compared to the wild-type controls.

These morphometric data suggest that the little mice have CNS myelination of comparable thickness and density to the wild-type mouse. To estimate the relative total number of myelinated axons in these regions, the cross-sectional sizes of the regions were compared by histological methods. The area of each region was calculated from

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<td>Avg. myelin thickness</td>
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<td>Avg. axon diameter</td>
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Fig. 4. Myelinated axon density in the brain regions examined in the little and wild-type mice. Myelinated axons were counted in 2–3 photos per mouse (n = 3 for each genotype) taken at 2000× magnification for each brain region. Values are means ± S.E.M. A.C., anterior commissure; al, anterior limb; pl, posterior limb.

Fig. 5. Thickness of myelin sheaths of axons in the forebrain regions of little and wild-type mice. Sections shown in Fig. 3 were photographed at 10000× and the axon diameter and myelin sheath thickness were measured using ImagePro. (A) Myelin measurements in the corpus callosum. (B) Myelin measurements in the anterior commissure. *p < 0.01. (C) Myelin measurements in the optic nerve (measurements not converted to nm). Values are means ± S.E.M. The percentage of axons analyzed is shown in brackets.
comparable serial sections in the little and control mice that were stained with Luxol fast blue as shown in Fig. 2. The area of the anterior commissure of the little mouse is calculated to be approximately 75.9% that of the wild-type mouse, and the area of the corpus callosum is about 82.1% that of the wild-type mouse. Thus, the corpus callosum and anterior commissure are proportionately smaller relative to brain size. So it follows that the total neuronal number is likewise reduced proportionately.

Analysis of the optic nerve myelination also indicated no difference between the little mice and wild-type controls. The distribution and average axon size were similar (Fig. 5), and the density of myelinated axons was identical (Fig. 4). In addition, no difference was observed in the thickness of myelin sheaths of comparable axon sizes.

3.3. Neurologic function

We selected a noninvasive FVEP test procedure in order to test the functionality of the myelin. We initially established the feasibility of distinguishing hypomyelinated mice using this procedure by testing a group of shiverer mutants and their wild-type littermates. The homozygous shiverer mutant is MBP deficient and consequently lacks CNS myelin [2,25,32]. The cortical response was significantly delayed in the shiverer mice [23]. The averaged plots of the FVEPs from the little mice and wild-type controls are shown in Fig. 6. Because of the possibility of detection of the electrical response of the eye (electroretinogram) due to the small head size, the early signals culminating at around 60 ms were not measured. The implicit times to the first negative peak (98.7 ± 3.4 ms, S.E. for little, and 91.3 ± 3.4 ms, S.E. for wild-type) and to the first positive peak (153.7 ± 16.2 ms, S.E. for little, and 153.3 ± 11.5 ms, S.E. for wild-type) of the FVEPs were very reproducible and showed no difference between the little and control mice. The average amplitudes of the positive peaks in the little mice were reduced, but the difference was not statistically significant (7.65 ± 1.7 μV, S.E. for little, and 13.2 ± 2.5 μV, S.E. for wild-type; p = 0.083).

4. Discussion

This study shows that brain myelin in GH-deficient little mice is normal in structure and quantity and is proportionate to regional brain mass. The thickness of myelin sheaths surrounding comparable axons is identical between the little mouse and wild-type controls in all regions examined. Because myelinated axon size and density are preserved in the little mouse, the reduction in total myelin content cannot be attributed to a reduction in the size or density of projection neurons, but rather to a reduced total size of the white matter tracts. There is a concomitant reduction in the total number of neurons, but there is no change in the composition of the white matter region. Thus, it appears that having only 6–8% normal levels of GH in mice results in a smaller brain with proportionately reduced white matter regions whose myelinated axons are not proportionately reduced in size or density.

Whether the reduction in total numbers of neurons in the brain is due to insufficient actions of GH on neuronal proliferation cannot be concluded. Neuronal proliferation under these conditions could be limited by inherent brain size and, therefore, may be an indirect consequence of GH deficiency. We also recognize that the cause of reduced neuronal proliferation cannot be distinguished between GH deficiency and reduced IGF-1 levels in this mouse. It is unclear whether little mice have reduced brain levels of IGF-1, although our data indicate that they have sufficient...
brain IGF-1 to escape hypoplasia of the olfactory tract as observed in IGF-1 knockout mice [8]. That is, our data indicate no reduction in myelin staining of the anterior limb of the anterior commissure as compared to the wild-type mouse.

GH has previously been implicated as a potentiator of MBP expression as shown in vitro [1]. As demonstrated here, a deficiency of GH appears to cause a slight reduction in steady state MBP mRNA, but the brain may compensate for this possibly by either upregulating translation or stabilizing the protein itself possibly by efficiently compartmentalizing in the myelin. The expression of MBP protein in the whole brain of little mice is equivalent to that of the wild-type mouse when normalized to whole brain protein. We did not quantitate the number of oligodendrocytes, so MBP expression per cell remains unknown, but previous experiments have shown that oligodendrocytes have flexibility in the amount of myelin, and possibly MBP, that they produce [34,38]. Given that total MBP protein and total myelin are equivalent between the little and wild-type mice when normalized to brain size, any potential reduction in oligodendrocyte number appears to be compensated for in the little mice with regard to myelination.

That the little mouse suffers no CNS dysmyelination is further supported by our functional assay. The FVEP test has proven useful for detecting myelin disorders in both mice and humans [4,14,23]. Our results show that the little mice have normal function of the visual pathway as measured by the amplitude and implicit time of the FVEP. The timing of the FVEP is the same between the little and wild-type mice. Also, the average amplitudes of the peak responses in the little mice were slightly lower than those of the wild-type, but not significantly. It is tempting to speculate that this minor reduction is due to fewer axons. The optic nerve diameter of the little mouse has been shown by Noguchi et al. [28] to be smaller than that of the control mouse. Therefore, the number of axons must be reduced since our data show no change in axon density or size or in the thickness of the myelin sheaths. However, animal studies show that a huge number of optic nerve axons must be lost to produce a significant loss of amplitude. One estimate is that over 95% of the optic nerve must be lost before there is a significantly depressed FVEP amplitude [16]. An alternate explanation could be that the density of synaptic terminals on the cell bodies and dendrites and the number of dendritic branches of cells in the cortex could be affecting the amplitude of the FVEP. These could possibly be reduced in the little mouse.

This study was initiated in order to explore the effect of GH deficiency on brain development in vivo. The morphology and myelination of white matter regions in humans with isolated growth hormone deficiency who have not received GH therapy has not been documented. There is a case report of a patient with a homozygous partial deletion of the IGF-1 gene who does not show signs of CNS dysmyelination as estimated by MRI [37], but this patient’s serum GH was elevated and it is possible that GH has effects on neuronal growth and myelination independent of IGF-1 [31]. In addition, children with 18q-syndrome, in which a portion of the long arm of one chromosome 18 is deleted, are missing one copy of the MBP gene as a result and are dysmyelinated [17]. The shiverer mouse mutant has demonstrated that a loss of one copy of MBP is not sufficient to cause dysmyelination in the mouse [32]. Therefore, it is noteworthy that 18q-children have a high frequency of GH insufficiency or deficiency, but their circulating IGF-1 levels are in the normal range [11,18,21]. It is possible that haploinsufficiency of MBP in these patients is further exacerbated by low GH levels which together result in the CNS dysmyelination observed. Our results from the little mouse suggest that GH deficiency alone does not result in dysmyelination, but does appear to cause slightly reduced steady-state levels of MBP mRNA. The mouse appears able to compensate when there are two functional copies of the MBP gene as evidenced by normal levels of MBP protein. The compensation mechanism may be impaired when only one functional copy is present and may result in less MBP protein produced. Possible synergistic effects of low GH levels and haploinsufficiency of MBP during development are being investigated.

Acknowledgements

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