Molecular Neurobiology (2019) 56:595–610
https://doi.org/10.1007/s12035-018-1065-1

Developmental Changes in Oligodendrocyte Genesis, Myelination, and Associated Behavioral Dysfunction in a Rat Model of Intra-generational Protein Malnutrition

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Received: 20 February 2018 / Accepted: 5 April 2018 / Published online: 12 May 2018

Abstract
Impairments in oligodendrocyte development and resultant myelination deficits appear as a common denominator to all neurological diseases. An optimal in utero environment is obligatory for normal fetal brain development and later life brain functioning. Late embryonic and early postnatal brains from F1 rat born to protein malnourished mothers were studied through a combination of immunocytochemical and quantitative PCR assay for analyzing the relative expression of platelet-derived growth factor receptor-α (PDGFRα), myelin-associated glycoprotein (MAG), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) to determine oligodendrocyte genesis, differentiation, maturation, and myelination. Myelin integrity and corpus callosum caliber was assessed by Luxol fast blue (LFB) staining, whereas grip strength test and open field activity monitoring for behavioral evaluation in F1 rats. We demonstrate that intra-generational protein deprivation results in drastically low PDGFRα+ oligodendrocyte precursor (OPC) population and significantly reduced expression of myelin protein genes resulting in poor pre-myelinating and mature myelinating oligodendrocyte number, hypo-myelination, and misaligned myelinated fibers. LFB staining and MOG immunolabeling precisely revealed long-term changes in corpus callosum (CC) caliber and demyelination lesions in LP brain supporting the behavioral and cognitive changes at early adolescence and adulthood following maternal protein malnutrition (PMN). Thus, intra-generational PMN negatively affects the oligodendrocyte development and maturation resulting in myelination impairments and associated with behavioral deficits typically mimicking clinical hallmarks of neuropsychiatric disorders. Our results further strengthen and augment the hypothesis "Impaired gliogenesis is a big hit for neuropsychiatric phenotype."

Keywords Maternal protein malnutrition · Oligodendrogenesis · Demyelination · Corpus callosum

Introduction

Myelination, essential for the propagation and speedy neurotransmission within the CNS, involves interfasicular oligodendrocytes. Oligodendrocytes, first characterized by del Rio-Hortega in 1928, not only serve functions of ensheathing the axons for conduction of fast saltatory action potential but also provide integrity and metabolic support to axons and contribute to neural plasticity [1–3]. Earlier studies have identified a number of markers for oligodendrocyte lineage including PDGFRα, NKX2.2, Olig-1, Olig-2, NG2, and even the domains in brain and spinal cord from where these cells arise [4–6]. Oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system (PNS) synthesize myelin, a multi-layered, lipid-rich spiral sheath composed of sail-like extensions of plasma membrane around an axon, exclusively in vertebrates [5, 7, 8]. Myelin, a vital component of white matter, contributing ~40–50% in dry weight expands radially and longitudinally almost simultaneously in sheaths. The continuous addition of lamellae from the ab-axonal towards the ad-axonal side is followed by compaction of multiple membrane layers, necessary for fast axonal conduction [2, 9]. Any deficit in the compaction process may have negative consequences on both the transmission of the signals and axonal health.

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The formation of myelin from oligodendrocytes is a prolonged process, continuing from childhood to adolescence in humans, where myelin build-up does continue into adulthood, though at a reduced rate [10, 11]. Whereas, in rodent brain, events of myelination are programed predominantly postnatally, beginning at birth in the spinal cord and achieved throughout the brain by PND 45–60 [12, 13]. Myelination tends to proceed along with development of different brain areas. However, the start of myelination is not based on defined centers rather occurring in response to functional demand, with brain areas related to early nursing, such as suckling reflex developing first (birth to PND 10), followed by others, like motor and sensory regions (PND 10–24), and finally learning areas (PND 17–37) in rats [12, 13]. Delayed reflex maturation and other physical landmarks in F1 pups born to protein-malnourished mothers, as we have previously reported [14], incited us to investigate whether intra-generational PMN affects the spatio-temporal oligodendrocyte genesis, turnover, and myelination status in the developing rat brain.

The role of myelin in sensory and motor functions has been well established; however, the grave neurological outcome following severe myelination impairments in leukodystrophies further illustrates the pivotal role of myelin in normal brain functioning [2, 15–18]. A direct role of oligodendrocyte dysfunction in neuropsychiatric conditions is agreeable as the timing of vigorous oligodendrocyte maturation and myelin formation in developing brain coincides with the peak age for onset of clinical symptoms of anxiety disorders [12, 19]. Besides their role in myelination, oligodendrocytes also provide trophic factors and metabolites critical for neuronal function modulation and other higher order brain functions [1, 20]. A myriad of earlier studies support poor myelination and white matter dysfunction as a well-established component of large number of neurodegenerative and neuropsychiatric disorders including multiple sclerosis (MS), vascular dementia, schizophrenia, and Alzheimer's [17, 21, 22]. Insults during early brain development are considered as crucial components/causatives for aberrant and abnormal neurodevelopment resulting in later life neurological dysfunction. Early-life protein malnutrition, a major stressor involves intra-uterine growth restriction (IUGR) leading to infants born small for gestational age (SGA) resulting in perinatal mortality and postnatal morbidity [14, 23]. Not only "in utero period" is critical owing to the large-scale genesis and migration of neurons and glia, even the neonatal and early postnatal period represents an important milestone when foundation for the development of cognitive, motor, and socio-emotional skills are laid. Previous studies have reported that the normal program of oligodendrogenesis followed by myelination can be disrupted by environmental stressors leading to drastic changes in the myelination [24, 25]. Disruption of myelin and aberrant myelination as a result of malnutrition, bacterial infection, inflammation, and toxicant exposure including ethanol and heavy metals, has also been well documented [26–29]. Recent study by Tilborg et al. [30] evidenced delayed cortical myelination, oligodendrocyte maturation, and autism-like behavior in rats following combined fetal inflammation with postnatal hypoxia. Although, earlier studies have reported neuropsychiatric symptoms in patients with multiple sclerosis, contusion-type spinal cord injury (SCI), stroke, and metachromatic leukodystrophy [31, 32]; however, what role oligodendrocytes and myelin play in higher brain functions and neuropsychiatric conditions like schizophrenia and autism remains largely unclear [17]. Previous reports in schizophrenia and bipolar patients have reported reduced density of oligodendrocytes in pre-frontal cortices, loss of white matter integrity, and increased microglial density in myelinated fiber proximity along with abnormal connectivity in corpus callosum [33–36]. Autopsy studies of some mentally ill subjects have revealed subtle abnormalities in myelination as hallmarks of disease and the reason could be that such subtle myelination changes may disturb the millisecond precision critical for the signal propagation in higher cortical networks [18, 37, 38]. Recent clinical report from Chopra et al. [39] evidenced that more highly myelinated tracts are associated with increased cognitive processing speed supporting role of myelination in cognition. These findings and other reports [17, 40, 41] link myelination deficits with the psychiatric disorders. However, whether the subtle changes in the white matter and mild myelination abnormalities result in gross behavioral changes or disease phenotype is yet poorly understood. Thus, it becomes worth to understand the impact of intra-generational PMN on the genesis, density of OPCs, and their differentiation and maturation along with myelination across brain development.

As it is increasingly clear that myelination is central to neuronal signaling finally contributing to a good cognitive outcome, dysfunction of cognitive abilities like learning, memory, and attention thus coincide with the intermediate phenotype of neuropsychiatric conditions. Further support to this association is from the fact that the myelination events in the brain occur postnatally and are completed at adulthood around the same time when the incidences of the psychotic disorders like schizophrenia and autism are at peak. Therefore, whether, early-life protein deprivation induces significant deficits in oligodendrocyte formation, myelination, and resultant behavioral profile needs immediate attention. The present study assessed this oligodendrocyte and myelination impairment and gross behavioral dysfunction following intra-generational PMN, more importantly to validate early-life PMN as a hit for neuropsychiatric phenotype.

Materials and Methods

Animal Model

Seven- to eight-week-old naive Sprague Dawley female rats (160–180 g) were used in the study. Animals were housed as three females/cage in a temperature and light-controlled environment (23 ± 2 °C, 12 h light/dark cycle) with ad libitum access to water and either of the two diets: (i) low protein (LP, 8% protein, $n = 12$) or (ii) high protein (HP, 20% protein, $n = 12$) obtained from the National Institute of Nutrition, Hyderabad, India. The females were maintained on respective diets for 45 days before setting for mating with breeder males and continued on same diets during the entire period of gestation and lactation. Timed pregnancies were set and fetal brains from embryonic days (E) 16 and 18 were harvested through atraumatic measures from HP and LP dams and rest were allowed to deliver pups. Half of the embryonic brains harvested were transferred to RNA Later solution (Sigma, USA) for RNA isolation, while other half were fixed in 2% buffered paraformaldehyde (0.01 M, pH 7.4), cryoprotected with sucrose gradients (10, 20, and 30%) and cryosectioned at14 μm to obtain cross sections through the forebrain for immunocytochemical analysis.

The F1 pups from LP and HP mothers were housed three per cage post weaning and maintained on the same diet until the termination of the experiment. The pups born to LP and HP females were sacrificed at postnatal ages (P) 2, 10, 15, 21, and 30 days and 3, 6, 12, 18, and 24 months ($n = 3$). On the aforementioned age points, the animals were deeply anesthetized using ether vapors and perfused transcardially with ice-cold saline (0.01 M PBS, pH 7.4), followed by 2% paraformaldehyde (PFA) and subsequently post fixed in same fixative for overnight. The tissues were subsequently cryoprotected with sucrose gradients (10, 20, and 30%) prepared in 0.01 M PBS and cryosectioned (14 μm thickness) using Leica Cryotome (CM1900, Germany) through the forebrain. The sections were collected on chromalum gelatin-coated slides, stored at − 20 °C, and subsequently used for immunocytochemical analysis. All the experiments were performed with prior approval and in accordance with the Institutional Animal Ethics Committee of Jiwaji University, Gwalior (M.P), India.

Quantitative Real-Time PCR (qRT-PCR) Assay

To assess the impact of intra-generational protein malnutrition on the dynamic changes in mRNA expression levels of myelin protein genes, the brain samples stored in RNA Later solution from E16, E18, P2, P15, and P30 from both LP and HP groups were washed in autoclaved phosphate-buffered saline. The hippocampus was micro dissected and pooled from littermates, so as to isolate total RNA from 100 mg tissue using Trizol reagent (Invitrogen, 15596-018) following the manufacturer's instructions. The RNA was quantified with a NanoDrop-1000 spectrophotometer (Biorad) and stored at − 80 °C till further use. The first-strand cDNA synthesis was carried out using Applied Biosystems cDNA Kit (4387406). All real-time RT-PCR assays were performed on ViiA7 Real-Time PCR System (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Applied Biosystems, USA). Conditions used were as follows: 95 °C for 10 min (1 cycle), 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 30s (40 cycles). Results were analyzed using comparative Ct method (2−[Δ][Δ]Ct). All reactions were performed as $n = 3$/sample and in triplicates. The gene-specific oligonucleotide primers were designed for myelin-related marker genes using Primer Quest SciTool from Integrated DNA Technologies (IDT) and are enlisted in Table 1.

Immunohistochemical Labeling

The temporal and relative expression of oligodendrocyte precursor marker (PDGFRα) and myelin proteins (PLP, MAG, MOG) were studied in embryonic and postnatal brains from both LP and HP group animals. Coronal sections of the fetal and postnatal brains were prepared for immunohistochemical localization of platelet-derived growth factor receptor-alpha (PDGFRα) for oligodendrocyte precursors (OPCs) and proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG) for myelin proteins.

Immunostaining for PDGFRα

Identical cryocut brain sections from LP and HP group animals at E18, P2, P15, and P30 were carefully selected and air-dried. Sections were then washed in PBS (3 × 5 min each) followed by immersion in 0.1% Triton X-100 in PBS for 10 min. The sections were washed thrice in PBST (0.1%

Table 1 List of primers

Gene	Primer sequence	
MAG	Forward primer	GAAACTGCACCCTGCTTCTC
	Reverse primer	CACCATGCAGCTGACCTCTA
PLP	Forward primer	GGCGACTACAAGACCACCAT
	Reverse primer	CAACTTGTCGGGATGTTCCT
PDGFRα	Forward primer	TGTTCGTGCTATTGCTCCTG
	Reverse primer	TCAGCACACTGGAGAAGGTG
18S RNA	Forward primer	AAACGGCTACCACATCCAAG
	Reverse primer	CCTCCAATGGATCCTCGTTA

18S RNA as internal control

MAG myelin-associated glycoprotein, *PLP* proteolipid protein, *PDGFRα* platelet-derived growth factor receptor-alpha

Tween-20 in PBS) for 5 min each and incubated with 10% normal goat serum (NGS) in PBS for 120 min at room temperature for non-specific protein blocking. The sections were subsequently incubated with anti-PDGFRα (rabbit polyclonal, Abcam, ab124392, 1:200 in 5% BSA in PBS) overnight at 4 °C. On the next day, the sections were washed in PBS (3 × 5 min each) followed by secondary antibody incubation, anti-rabbit FITC (Sigma Aldrich, 1:300 in 5% BSA in PBS) for 90 min at room temperature in dark. The sections were subsequently washed in PBS (5 × 10 min each) to remove unbound secondary antibody and mounted in Vectashield Hardset with DAPI and stored at 4 °C.

Anti-myelin PLP and Anti-MAG Immunolabeling

Brain sections from P2, P10, P15, P21, and P30 from LP and HP group animals were air-dried at room temperature for 1 h. Thereafter the sections were washed in PBS thrice (5 min each) and incubated with 0.2% triton X-100 (Sigma) in PBS for 20 min for antigen retrieval. This was followed by PBST washings (3 × 5 min each) and further incubation with 10% normal goat serum in PBS (non-specific protein blocking) for 120 min at room temperature in a humid chamber. After blocking, the sections were incubated with primary antibodies, i.e., anti-myelin PLP (mouse monoclonal, Abcam ab9311, 1:300) and anti-MAG (mouse monoclonal, Abcam ab89780, 1:300), overnight at 4 °C. On the next day, after bringing sections to room temperature and washing with PBST (3 × 5 min), the sections were incubated with secondary antibody, i.e., anti-mouse FITC (Sigma Aldrich, 1:200) for both anti-PLP and anti-MAG for 120 min at room temperature in dark conditions. Both primary and secondary antibodies were diluted in 5% BSA in PBST. The sections were finally washed in five changes of PBS for 10 min each to remove unbound secondary antibody if any and mounted in Vectashield Hardest with DAPI from Vector Labs.

Anti-MOG Labeling

Cryocut brain sections from P15 and P30 and 3, 6, 12, 18, and 24 months old LP and HP animals were randomly selected, air-dried, and then washed in PBS (3 × 5 min each). This was followed by incubation in 0.5% Triton X-100 in PBS for 30 min and then washing in PBST (3 × 5 min each). Non-specific protein blocking was done using 10% normal goat serum for 120 min at room temperature. Thereafter, the sections were incubated with primary antibody, rabbit polyclonal anti-MOG (Abcam ab32760, 1:100 in 5% BSA in PBST) overnight at 4 °C. Next morning, after acclimatizing the sections to room temperature, the sections were washed in PBST (3 × 5 min each) followed by incubation with secondary antibody, anti-rabbit TRITC (1:200 in 5% BSA in PBST) under dark conditions. The sections were extensively washed in PBS (5 × 10 min each) to completely remove the unbound secondary and subsequently mounted in Vectashield Hardset with DAPI.

Negative controls were performed for all experiments by omitting the primary antibody and no immunolabeling was recorded in such sections. The visualization of the slides and imaging was performed with the help of Leica DM 6000 Fluorescence microscope using appropriate filters and LASAF (Leica Application Suite Advanced Fluorescence) imaging software. Identical settings for image grabbing and processing were applied to maintain homogeneity. The quantitation of relative immunofluorescence intensity of PDGFRα, PLP, MAG and MOG was performed on the original images using intensity measurement tool of the LASAF software from Leica Microsystems, depending on the available tissue area to be imaged, images ($n = 6$)were grabbed from two different sections each from 3 individual HP and LP brains and final data is presented as mean ± SEM. The total number of images used for fluorescence quantification of a particular marker at respective time point served as "n" for statistical analysis.

Luxol Fast Blue (LFB) Staining

Cryocut sections from both HP and LP groups at 2, 3, 6, 12, 18 and 24 months were randomly selected, air-dried at 37 °C for 1 h and washed with distilled water for 5 min. This was followed by dehydration using ascending series of alcohol, i.e., 30%, 50%, 70% and 90% for 5 min each. Dehydrated sections were immersed in Luxol Fast Blue staining solution (0.1 g Luxol fast blue (Sigma Aldrich, USA) in 100 ml of 90% ethanol with 10% Glacial acetic acid) overnight at 57 °C. Next day, the sections were brought to room temperature and rinsed in 95% ethanol followed by a wash in distilled water. The differentiation of the gray and white matter tissue was obtained by immersing the slides in 0.05% lithium carbonate solution (0.25 g Lithium carbonate in 250 ml of double-distilled water) for 5–10 s, followed by 2 changes of 70% ethanol for 1 min each and then rinsed in distilled water. The sections were examined under microscope to confirm proper differentiation. Differentiation steps were repeated until a sharp contrast was achieved between blue stained white matter and colorless gray matter. Counterstaining with cresyl violet (Sigma Aldrich, USA C-1791) was performed by immersing the slides in cresyl violet solution (0.1 g of cresyl violet acetate and 5 mg of oxalic acid in 100 ml of d. H2O) for 5 min and then rinsed in distilled water. The slides were air-dried for 2 h followed by dehydration in n-butyl alcohol for 5 min, cleared in xylene for 10 min and finally mounted in DPX.

Corpus Callosum Caliber Measurement

The corpus callosum (CC) caliber was measured from Luxol fast blue and cresyl violet counterstained brain sections with the help of the Leica Application Suite (LAS) software using an image processing tool (distance between two points in μm's). All the images used for CC caliber measurements were of 25× magnification and for comparisons, identical sites of measurement were chosen from the corpus callosum region.

Behavioral Test Battery

The F1 progeny of HP and LP groups were subjected to a battery of tests viz., neuromuscular strength test and open field activity monitoring at 2, 3, 6, 12, 18, and 24 months using same protocols as discussed in our earlier publication [14].

Neuromuscular Strength Test

A grip strength meter (Columbus Instruments, USA) was used to assess neuromuscular function by sensing the peak amount of force an animal applies in grasping specially designed pull bar assemblies.

Open Field Test

The F1 generation rats from HP and LP fed dams were individually placed in open field arena 43 × 43 × 22 cm high walls (Columbus Instruments, Ohio USA) with infrared beam detection system for 20-min test session. Time in square analysis of individual tracks was performed and time spent in the center zone calculated for each group.

Statistical Analysis

The statistical analysis of all the data was performed using Sigma Stat 3.5. The values are expressed as mean ± standard error of the mean (SEM). Student *t* test was used for comparisons between HP and LP group at different study time points. The level of significance was set at a P value of < 0.001, indicated by asterisk (***) for highly significant and ** for < 0.01 and < 0.05 for significant (*).

Results

The present study investigates the impact of PMN on the formation and maturation of oligodendrocytes and deficits in myelination and associated motor performance. Although, most of our results are focused on the largest commissure, corpus callosum, we have also taken into account some changes in the cortex and thalamic white matter regions. Immunohistochemical localization of various oligodendrocyte specific and myelin protein markers with mean intensity quantitation has been performed. The oligodendrocyte precursors (OPCs) were labeled with PDGFRα and were characterized by their typical bipolar morphology with few ramified processes, abundantly populated along the ganglionic eminences and the cortical VZ and SVZ regions in embryonic brains. Platelet-derived growth factor acts as a mitogen and survival and differentiation factor for oligodendrocytes and by expressing PDGFRα, these OPCs maintain their highly mitogenic potential. During the early neonatal period, these PDGFRα+ OPCs were noticed to invade the dorsal areas including the cortical plate and subsequently show migratory trails to populate the forebrain and other cortical and subcortical structures during late gestation. The myelinating oligodendrocytes were characterized by the expression of proteolipid protein (PLP) and myelin-associated glycoprotein (MAG) and later the mature myelinating oligodendrocytes by myelin oligodendrocyte glycoprotein (MOG). Myelin production in the brain requires coordinated synthesis of specific proteins and lipids that facilitate spiral wrapping and compaction of the lamellae leading to fine architecture of the myelin sheaths. The myelin integrity was checked by the expression of the myelin proteins PLP and MAG. MOG immunolabeling and Luxol fast blue (LFB) staining was used to check myelination stature, corpus callosum caliber, and demyelination lesions if any.

Reduced Oligodendrocyte Precursor Population in LP Brains Speculate Compromised Oligodendrogenesis Following Maternal Protein Deprivation

Although glial restricted progenitors (GRPs) represent bi-potential progenitors for astrocyte and oligodendrocyte lineage, the PDGFRα expression marks their commitment towards oligodendrocyte lineage, giving rise to specific OPCs. The immunofluorescence labeling with anti-PDGFRα antibody revealed that PDGFRα+ OPCs appeared as bipolar cells with migratory trails towards cortex and thalamic regions. PDGFRα immunolabeling also evidenced low expression both in terms of intensity and reduced PDGFRα+ OPC population throughout the LP F1 brains. Discrete PDGFRα+ OPCs were seen in HP brains at E18 (Fig. 1a) and their number increased gradually resulting in an abundant OPC population in the thalamic and cortical areas from P2–P30 (Fig. 1b–d). However, a drastically reduced population was clearly evident in their age-matched LP counterparts (Fig. 1e–h). Mean fluorescence intensity also revealed significantly reduced PDGFRα expression in LP brains, However the difference was found to be significant at P2 (HP, 498.5 ± 37.18, LP 370.01 ± 42.35, $t = 2.28$, $p < 0.05$), P15 (HP, 683.99 ± 64.3, LP 332.17 ± 31.12, $t = 4.92$, $p < 0.05$), and P30 (HP, 690.88 ± 79.15, LP 448.57 ± 18.39, $t = 2.98$, $p < 0.05$, Fig. 1i), validating compromised oligodendrocyte development. In contrast to

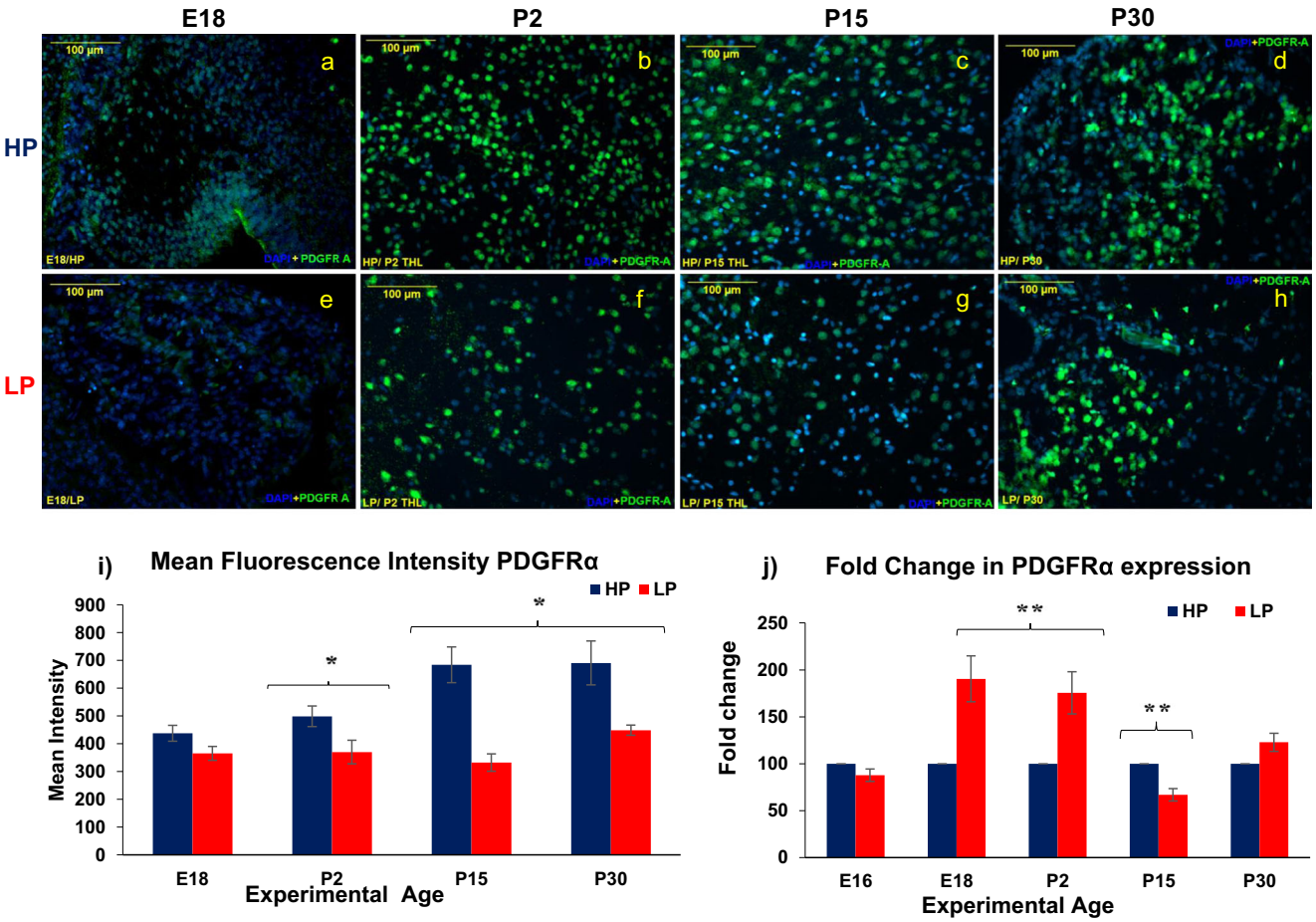

Fig. 1 Reduced oligodendrocyte precursor population in LP brains from late embryonic to postnatal period. Representative photomicrographs show PDGFRα immunolabeled oligodendrocyte precursors at embryonic day (E) 18 and postnatal days (P) 2, 15, and 30. LP brain preparations through the thalamus (THL) show significantly less PDGFRα+ OPCs at P2 (**f**), P15 (**g**), and P30 (**h**) as compared to age matched HP (**a**–**d**, respectively). **i** Quantitative mean PDGFRα fluorescence intensity measurement shows significantly decreased mean intensity in LP brains at P2, P15, and P30. **j** The G+RAPH shows fold change in PDGFRα mRNA expression detected via qRT-PCR assay evidencing a significant upregulation in LP brains at E18 and P2 to 2-fold (LP = 1.904 ± 0.65-fold, $p < 0.01$; P2, LP = 1.755 ± 2.23, $p < 0.01$ folds), with a subsequent downregulation at P15 (LP = 0.67 ± 2.2, $p < 0.01$). Data presented as mean fluorescence intensity± SEM ($n = 6$ images) and fold expression ± SEM ($n = 3$), $*p \leq 0.05$, $**p \leq 0.01$ for comparison of LP with respect to HP. THL, thalamus. Scale bar = 100 μm

this, the mRNA expression analysis through qRT-PCR revealed a significant increase in fold expression of PDGFRα in LP brains at E18 (LP = 1.904 ± 0.65-fold, $p < 0.01$) and P2 (LP = 1.755 ± 2.23, $p < 0.01$) and a subsequent downregulation at P15 (LP = 0.67 ± 2.2-fold normalized to 1 in HP controls, $p < 0.01$; Fig. 1j). The upregulation during E18–P2 can possibly be correlated to late increased PDGF transcription as a compensatory mechanism. However, a subsequent downregulation by P15 speculates low levels of adult oligodendrocyte precursors in LP brains.

Drastically Low Pre-myelinating Oligodendrocyte Population and Resultant Hypo-myelination in Developing LP F1 Brains

Proteolipid protein expression (PLP) in the cytosol of OPCs marks their differentiation to pre-myelinating oligodendrocyte during early postnatal development and the protein is later shifted from cell body to the processes to initiate myelination along with myelin-associated protein (MAG), subsequently allowing compaction of the concentric lamellae. As pre-myelinating oligodendrocytes start populating the brain during early neonatal period, PLP expression increases linearly during the first few weeks of postnatal life in a normal healthy rat brain, which was clearly evident from the preparations of HP F1 neonates presenting intense PLP immunoreactivity at P2 (Fig. 2a). Such changes were clearly demarcated in the thalamic region (Fig. 2b; from the specified area higher magnification image; yellow square image) and cortical regions (Fig. 2e). Peak PLP expressing cell population was observed at P2 only, with a subsequent reduction at P15 and P30 (Fig. 2f, g). In contrast, low PLP immunolabeling was recorded both in the thalamic (Fig. 2c, d) and cortical (Fig. 2h) regions in P2

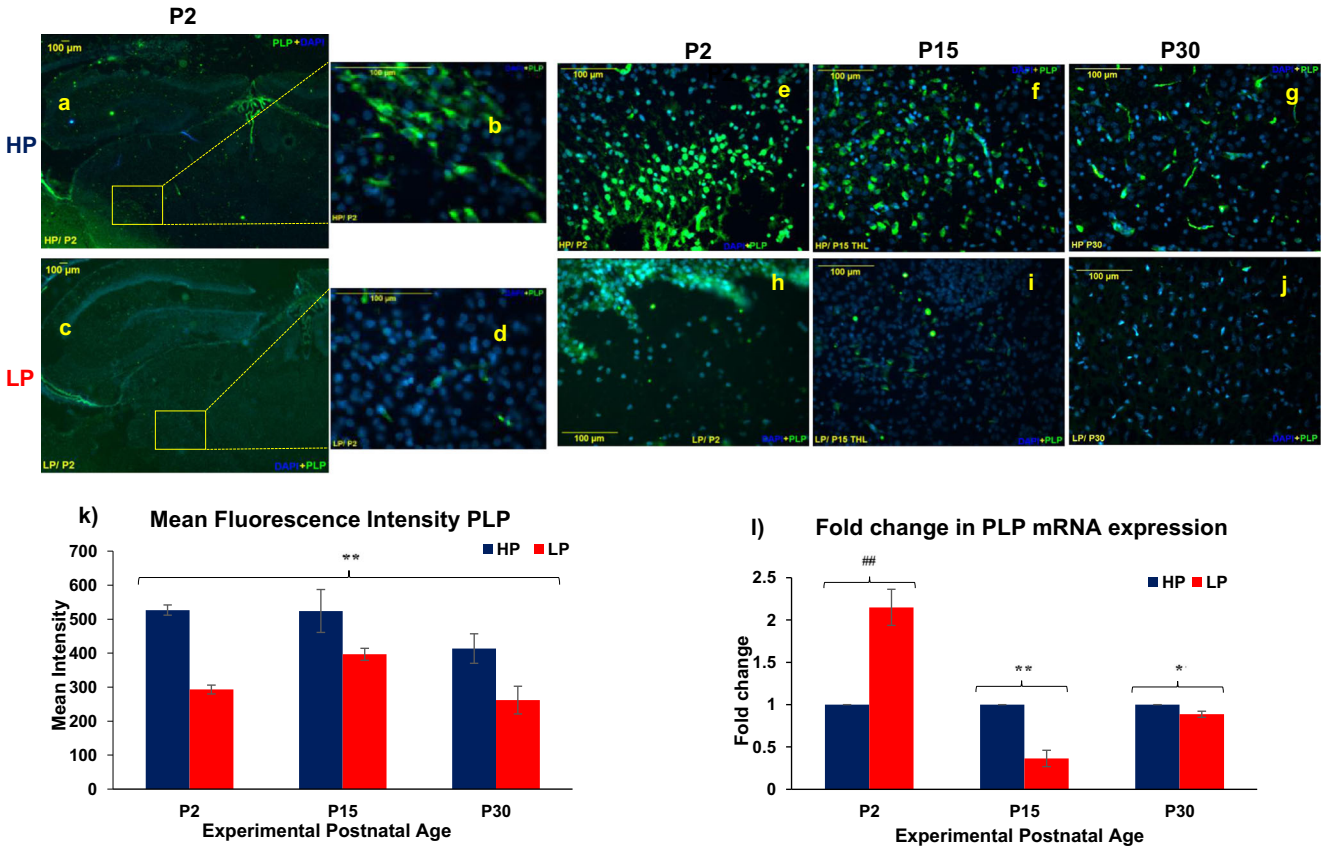

Fig. 2 Drastically low proteolipid protein expression in LP F1 brains. Low magnification merged images (**a**, **c**) from HP and LP brains show anti-PLP (green) and DAPI (blue) labeling at P2. Robust PLP expression in HP brains with less or no expression in LP counterparts at P2 is evident in higher magnification images through the thalamus (yellow rectangle) (**b**, **d**). Similar decreased PLP expression in LP brains was seen in cortical and thalamic regions at P2 (**h**), P15 (**i**), and P30 (**j**) as compared to age-matched HP controls (**e**, **f**, **g**). **k** Mean fluorescence intensity quantitation using the LASAF software revealed significantly reduced PLP intensity in LP preparations at P2, P15, and P30 with respect to controls. **l** The graph shows fold change in PLP expression detected via qRT-PCR supporting significantly increased PLP mRNA levels in LP brains at P2 (LP = 2.14 ± 0.21-fold, $p < 0.01$) followed by downregulation at P15 (LP = 0.612 ± 0.05-fold, $p < 0.01$) and P30 (LP = 0.767 ± 0.06-fold, $p < 0.05$) evidencing poor myelin-related gene expression that ultimately results in myelination deficits as evidenced in immunohistochemical staining. Data presented as mean PLP intensity ± SEM ($n = 6$ images) and fold expression ± SEM ($n = 3$), $p \leq 0.05$, $^{**}p \leq 0.01$,* for LP vs HP comparison. Scale bar = 100 μm

LP brains that continued till P30 (Fig. 2i, j) with respect to age-matched HP controls (Fig. 2f, g). In consistence with our in vivo immunohistochemical results, the quantitative mean fluorescence intensity measurements also revealed significantly low PLP expression in LP brains at P2 (HP 526.25 ± 15.16, LP 293.16 ± 12.94, $t = 5.11$, $p < 0.01$), P15 (HP 523.95 ± 62.9, LP 396.63 ± 17.77, $t = 3.80$, $p < 0.01$), and P30 (HP 413.6 ± 43.49, LP 262.14 ± 40.77, $t = 3.17$, $p < 0.01$; Fig. 2k) stating a persistent low PLP expression following maternal PMN. However, qRT-PCR results revealed an initial increase in PLP mRNA fold expression at P2 in LP brains (LP = 2.15 ± 0.21-fold, $p < 0.01$) and a subsequent downregulation at P15 (LP = 0.36 ± 0.09-fold, $p < 0.01$) and P30 (LP = 0.88 ± 0.03-fold, $p < 0.05$; Fig. 2i) further strengthening our in vivo results showing decreased PLP+ immature oligodendrocyte population in a developing postnatal brain following maternal PMN.

Hypo-myelination and Reduced Compaction Following Maternal PMN

Myelin-associated glycoprotein (MAG), a component of myelin critical for the compaction of myelin sheaths was also found to be significantly reduced following maternal PMN with a noticeable difference from P10–P30 (Fig. 3e–h). Such changes were more pronounced at P15, where a significantly low MAG immunoreactivity was observed in the cortex along the white matter tracts with only a few MAG+ diffuse myelinating fibers in LP brain sections (Fig. 3f) as compared to robust MAG expression and abundant compact myelinating fiber density in age-matched HP brains (Fig. 3b). A similar trend of hypo-myelination and loose aggregation was also noticed in the corpus callosum white matter fiber bundle at P21 in LP brains (Fig. 3g) with respect to HP controls (Fig. 3c) supporting myelination deficit following maternal PMN.

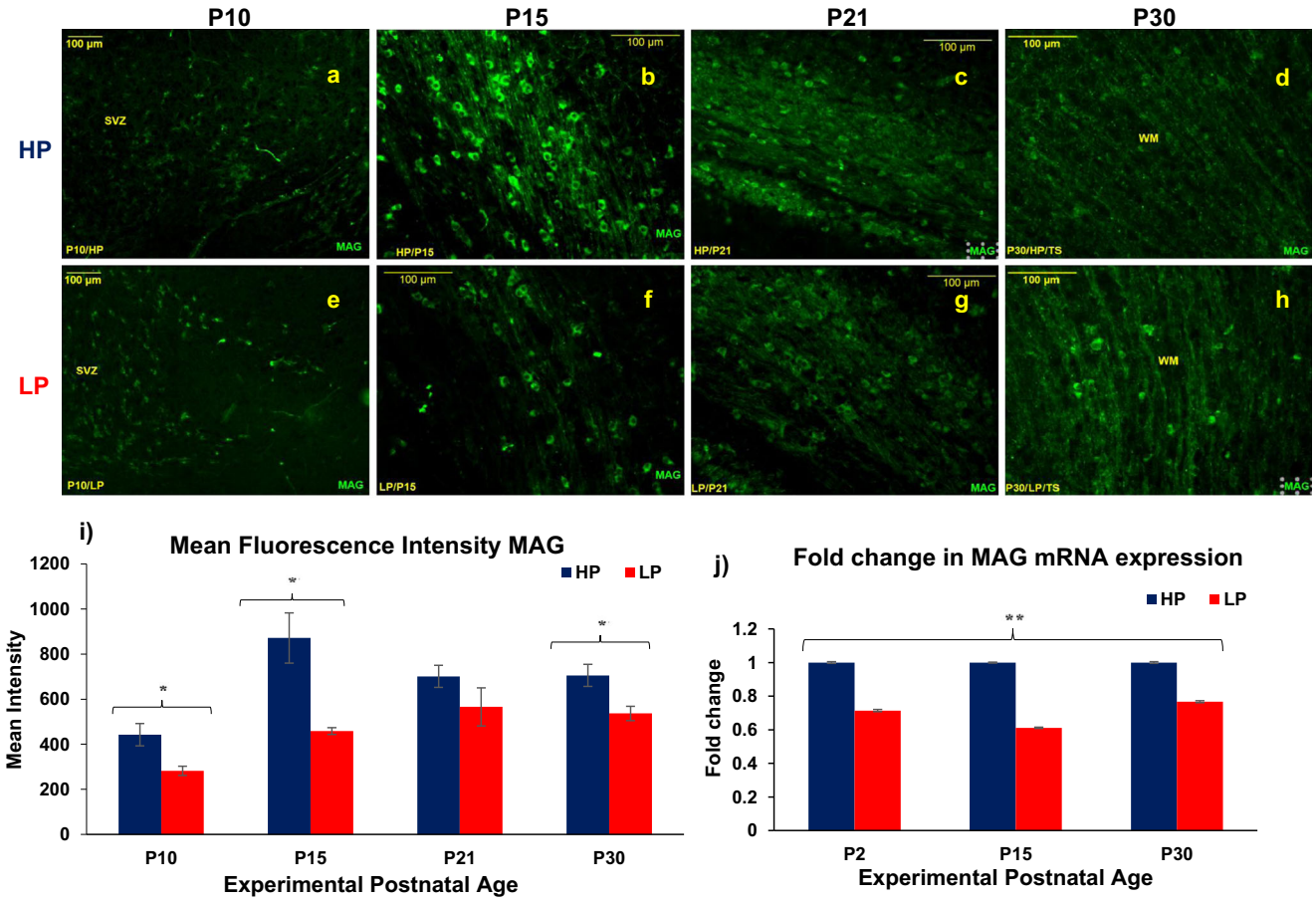

Fig. 3 Low MAG immunoreactivity in perinatal LP brains evidence compromised pre-myelinating oligo's and impaired myelination. Representative anti-MAG immunolabeled images (green, **a**–**h**) from LP and HP brains at postnatal days 10, 15, 21, and 30 evidence significantly reduced MAG expression with less number of myelinated fibers in LP brains at P15 (**f**), P21 (**f**, **g**), and P30 (**h**) as compared to age-matched HP (**b**–**d**, respectively). Crumpled and loosely arranged MAG+ myelin fibers are evident in LP brain white matter at P21 (**g**) and P30 (**h**) as compared to properly organized fibers in age-matched HP controls (**c**, **d**). **i** Quantitative measurements of MAG immunofluorescence reveal significantly decreased mean intensity in LP brains at P10, P15, and P30 as compared to HP controls. **j** The graph shows significantly decreased fold MAG mRNA expression in LP brains at P2, P15, and P30 with respect to HP controls, detected using qRT-PCR assay validating immunohistochemical findings. Data presented as mean fluorescence MAG intensity ± SEM ($n = 6$) and fold expression ± SEM ($n = 3$), $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ for LP vs HP comparison. Scale bar = 100 μm

Perfectly aligned myelinated fibers in the corpus callosum and other white matter areas were evident at P30 in HP brains (Fig. 3d), contrary to misaligned and reduced myelinated fiber density in LP counterparts (Fig. 3h) speculating hypo-myelination following PMN. Mean fluorescence intensity measurements through the LASAF software also clearly indicated the significantly decreased MAG expression in LP brain preparations at P10 (HP 442.38 ± 49.87, LP 281.95 ± 20.74, $t = 2.970$, $p = 0.014$), P15 (HP 871.71 ± 111.63, LP 458.94 ± 15.08, $t = 3.66$, $p < 0.01$), and P30 (HP 705.56 ± 49.19, LP 536.75 ± 61.37, $t = 2.14$, $p < 0.01$; Fig. 3i).These results were further supported through our qRT-PCR-mediated mRNA expression analysis revealing a significant decrement in fold expression at P2 (LP = 0.714 ± 0.07-fold, $p < 0.01$), P15 (LP = 0.612 ± 0.01-fold, $p < 0.01$), and P30 (LP = 0.767 ± 0.06-fold normalized to 1 in HP controls, $p < 0.01$) with respect to age-matched HP controls (Fig. 3j).Such deficits during the early postnatal period either in the form of hypo-myelination or reduced compaction could result in impaired myelination and white matter organization with advancing age.

Long-Term Changes in MOG Expression, Diffused Luxol Fast Blue Staining, Reduced Corpus Callosum Caliber, and Demyelination Lesions in LP F1 Brains Suggest the Persistence of Myelination Deficits Following Maternal PMN

MOG, one of the last myelin protein expressed on the surface of oligodendrocytes and outermost lamellae of compacted myelin, marks their maturation. MOG expression was significantly downregulated following maternal PMN supporting the hypo-myelination and loss of myelin compaction reported

vide supra. Such changes were constantly observed in the LP group animals from the age of 3 months to 24 months (Fig. 4a–e), in contrast to the strong MOG immunolabeling and compacted myelin in the HP counterparts (Fig. 4f–h). In addition, demyelination lesions were also observed in the corpus callosum and adjacent white matter areas in LP brains at 6 and 18 months of age (Fig. 4g, i, yellow dotted rectangle), while no such lesions were observed in their age-matched HP brains (Fig. 4b, d).These results were further confirmed by quantitation of mean fluorescence intensity for MOG through LASAF, revealing a significantly low mean intensity in LP brains at 6 months (HP 1425.04 ± 128.25, LP 932.36 ± 42.17, $t = 3.64$, $p < 0.05$) and 18 months of age (HP 1663.71 ± 77.41, LP 1328.01 ± 82.15, $t = 2.97$, $p < 0.05$) with respect to HP controls (Fig. 4k). The reduced MOG localization at the surface of myelinated fibers in LP brains speculates decreased compaction/loose arrangement of myelinated fibers and points towards functional impairments at behavioral levels.

These results were further confirmed with Luxol fast blue staining showing poor staining of the myelinating fibers of corpus callosum in LP brains at 3, 6, and 12 months of age (Fig. 5c, g, k, respectively), as compared to strongly LFB+ corpus callosum fiber bundle in their respective HP counterparts (Fig. 4a, e, i, respectively). Higher magnification images clearly reveal the diffuse LFB staining of loosely aggregated fibers in LP brains further supporting hypo-myelination and poor compactness of myelinated fibers in corpus callosum of LP brains at 3, 6, and 12 months of age (Fig. 5d–i) with respect to HP (Fig. 4b, f, j). Reduced corpus callosum and axonal damage in LP brains were prominently seen in the form of an increase in interstitial area and vacuolation between myelinated fibers at 3, 6, and 12 months of age (arrows, Fig. 5d, h, i).

Quantitative measurements of relative corpus callosum caliber from LFB-stained photomicrographs through the LAS imaging software evidenced a good consistency in increasing caliber of corpus callosum with advancing age in HP controls from 3 to 24 months, while with a minimal increase in the LP brains (Fig. 5). Corpus callosum caliber of LP groups remained low throughout the life as compared to HP groups with significant differences at 12 months (HP 373.16 ± 7.25 μm, LP 281.34 ± 5.9 μm, $t = 6.297$, $p < 0.01$) and 18 months (HP 304.83 ± 4.48 μm, LP 250.93 ± 6.91 μm, $t = 5.104$, $p < 0.01$; Fig. 5m) of age.

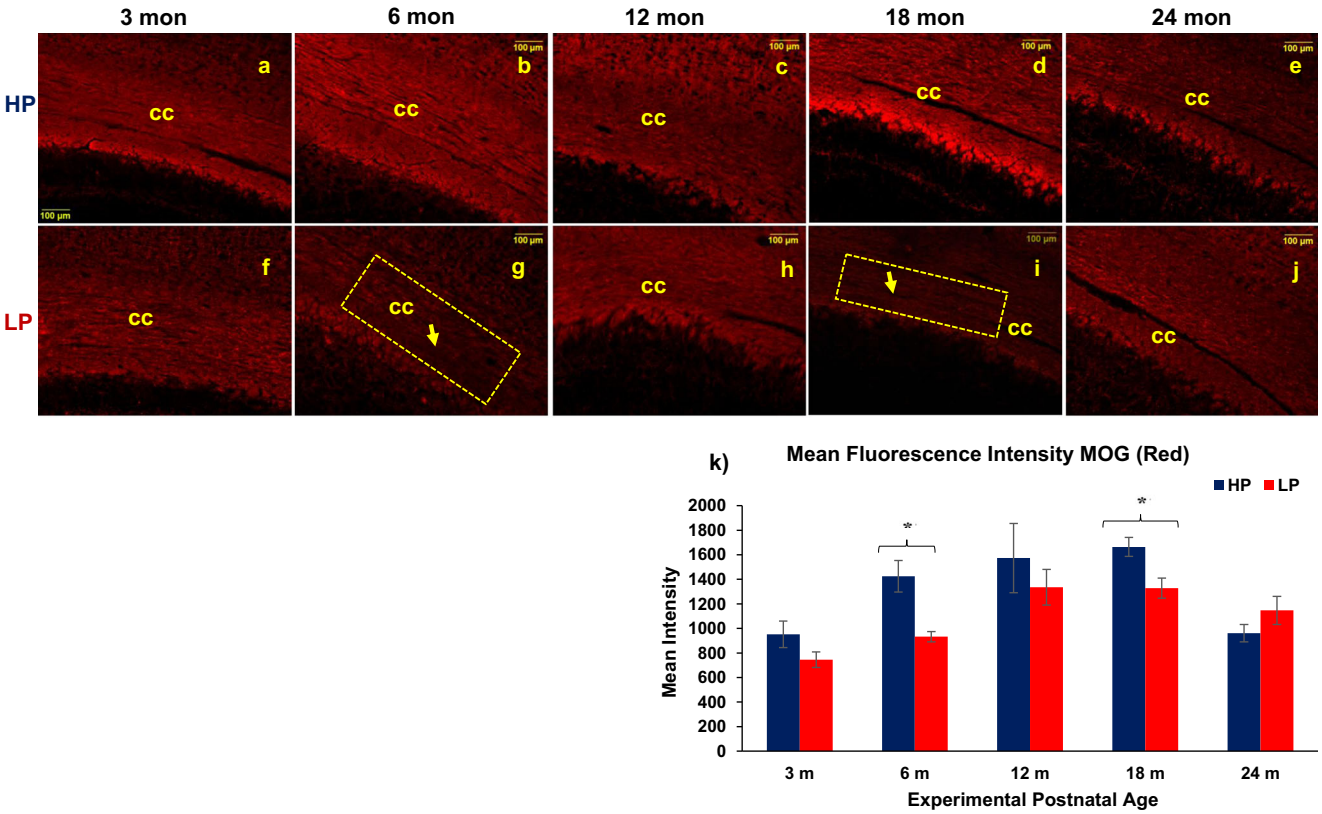

Fig. 4 **a**–**k** Poor mature oligodendrocyte glycoprotein immunolabeling with demyelination lesions in LP brains from adolescence to senility. Representative images of anti-MOG immunofluorescence labeling (red) evidence poor mature myelinating oligodendrocyte population and demyelination lesions in LP brains from 3 to 24 months (**f**–**j**), with significantly decreased MOG immunoreactivity and increased demyelination recorded at 6 months (**e**, yellow dotted rectangle) and 18 months (**g**, yellow dotted rectangle) with respect to strong MOG positivity and myelin integrity in HP controls (**a**, **c**, respectively). **k** Mean fluorescence intensity measurement using LASAF software revealed significantly decreased MOG staining in LP brains at 6 and 18 months h). *$p \leq 0.05$ for LP vs HP comparison. Scale bar = 100 μm

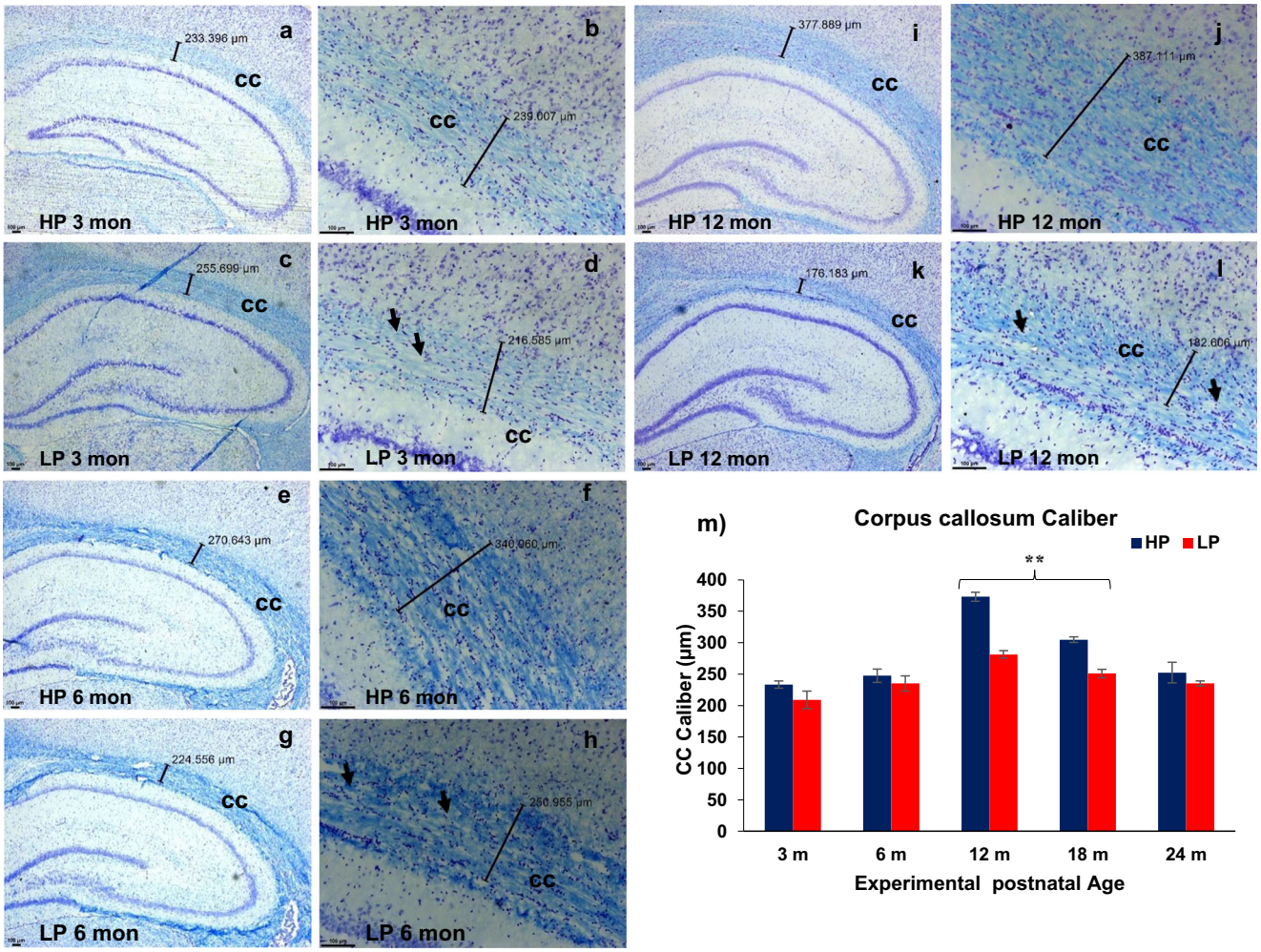

Fig. 5 Sparse myelination, reduced corpus callosum caliber, reduced fiber compaction speculate poor myelination following PMN. Photomicrographs of Luxol fast blue staining for myelin through corpus callosum (blue, **a**–**l**) in LP and HP brains at 3, 6, and 12 months of age. Diffused and sparse LFB staining is seen in LP brains at 3 months (**c**, **d**), 6 months (**g**, **h**), and 12 months (**k**, **l**) of age as compared to age-matched HP controls (**a**, **b**, **e**, **f**, and **i**, **j**, respectively). Reduced corpus callosum caliber was evidenced in LP brains with significant differences at 12 months (**i**) and 18 months as compared to age-matched HP (**m**). **m** The graph shows relative corpus callosum (CC) caliber with significantly decreased CC caliber in LP brains at 12 and 18 months of age. ***$p \leq$ 0.01 for LP vs HP comparison. Scale bar = 100 μm

This reduced CC caliber further points towards either low myelinated axon number or sparse myelination in developing brains following maternal PMN.

Early-Life PMN Induced Neuromuscular Deficits and Impulsive/Hyperactive Behavior in LP F1 Rats

The body weight of LP-fed rats remained significantly low throughout life as compared to the respective HP-fed rat animals (reported earlier by Aijaz et al. 2015). Neuromuscular strength of both HP and LP group animals increased linearly with advancing age. However, the neuromuscular deficits were clearly evident in LP group rats as indicated by significantly reduced forelimb grip strength at all age points in comparison to their age matched HP groups (Fig. 6). The difference was significant at 3 months ($n = 12$, $t = 9.622$, $p \leq 0.001$), 6 months ($n = 12$, $t = 14.272$, $p \leq 0.001$), 12 months ($n = 12$, $t = 2.953$, $p = 0.007$), 18 months ($n = 12$, $t = 2.478$, $p = 0.021$), and 24 months ($n = 12$, $t = 2.994$, $p = 0.007$) of age.

Open field activity monitoring data from LP group rats suggest impulsive and low anxiety-like behavior from early adolescence to late adulthood. Time in square analysis from the 20-min test session revealed that LP group animals spent significantly more time in the center zone at 2 months ($n = 8$, $t = -6.974$, $p \leq 0.001$), 3 months ($n = 8$, $t = 3.753$, $p = 0.002$), 6 months ($n = 8$, $t = -5.975$, $p \leq 0.001$), 12 months ($n = 8$, $t = -6.038$, $p \leq 0.001$), 18 months ($n = 8$, $t = -4.719$, $p \leq 0.001$), and 24 months ($n = 5$, $t = -1.010$, $p = 0.342$) of age with respect to age-matched HP controls (Fig. 7). Increased center time indicates impulsiveness towards anxiety provoking the center zone supporting low basal anxiety and loss of habituation in LP F1 progeny.

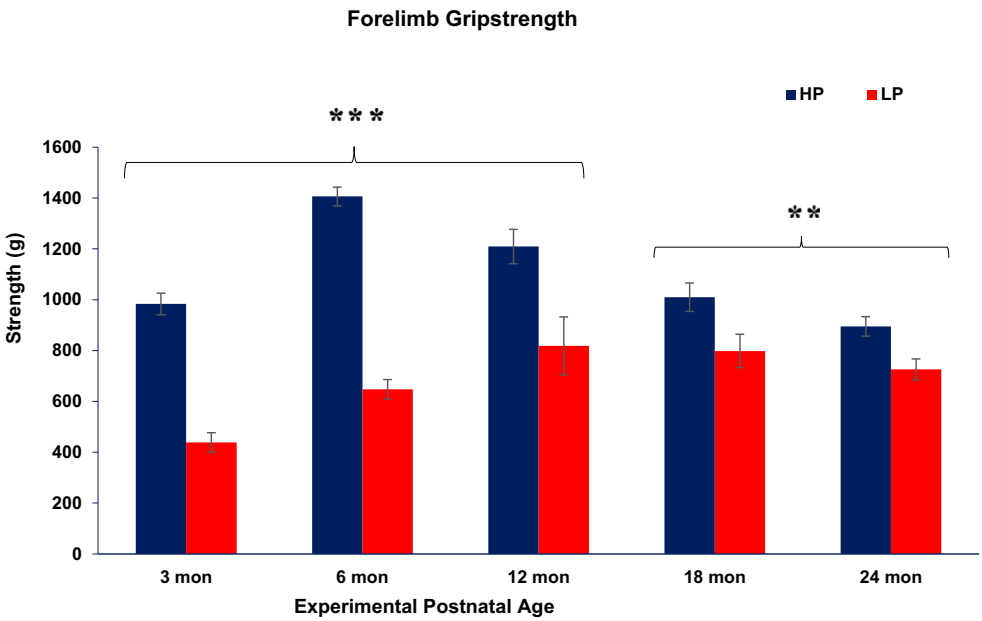

Fig. 6 Reduced neuromuscular strength following intra-generational protein malnutrition continues till senility in rats. The graph shows significantly reduced forelimb grip strength in LP F1 rats at 3, 6, 12, 18, and 24 months with respect to age-matched controls. Data presented as mean ± SEM ($n = 12$). ***$p \leq 0.001$, *$p \leq 0.05$ for comparison of LP F1 with respect to HP F1 controls

Discussion

Neuronal changes in structure and function have largely been focused in neuropsychiatric disorder studies. However, evidences postulate that astrocytes, microglia, and even oligodendrocytes contribute to the pathophysiology of neuropsychiatric phenotype including clinical disorders like major depressive disorder (MDD), schizophrenia, autism, and attention-deficit hyperactivity disorder (ADHD) [35, 42–45]. Early nutritional insults results in impaired brain function not only while the nutrient is in deficit but also after repletion [46]. Thus, early-life stress may have deleterious effects on oligodendrocyte genesis and myelination. Oligodendrocytes, the myelinating cells of the CNS, are derived from OPCs that arise from multipotent neuroepithelial progenitors in the neurogenic niches of the developing CNS. They are highly proliferative and motile cells, dividing as they migrate to populate the developing CNS [47, 48] and subsequently differentiate into highly arborized oligodendrocytes by expressing myelin-specific proteins that contact and ensheathe axons and provide trophic support to allow fast conduction velocity [49, 50].

The acquisition of platelet-derived growth factor receptor-α (PDGFRα), considered to be the most reliable marker of OPCs, marks the commitment of OPCs towards oligodendrocyte lineage. PDGFRα promotes their survival and proliferation via binding to its secreted ligand and potent mitogen, PDGF [51, 52].

In the present study, PDGFRα+ OPCs were noticed as early as E14 and peaked during first 2 weeks of postnatal life

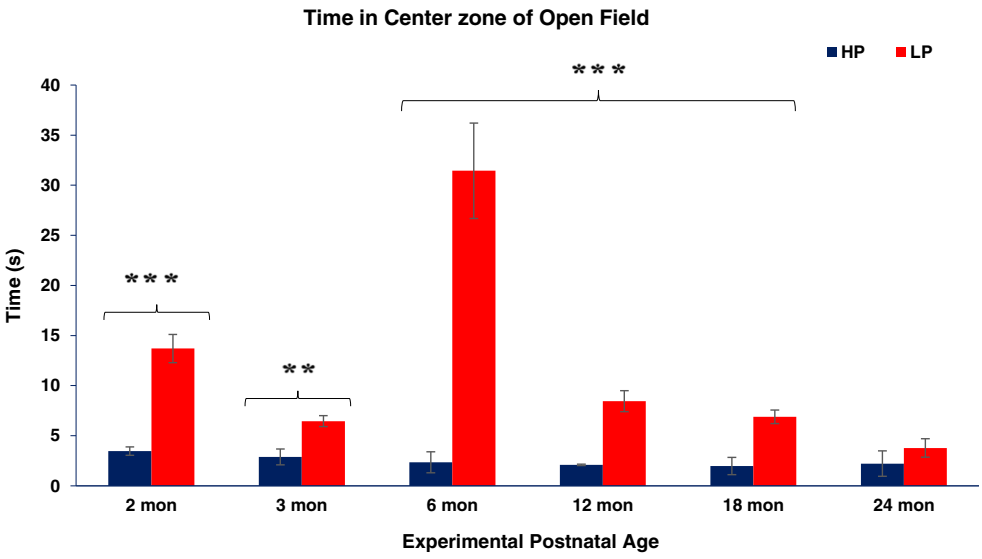

Fig. 7 Impulsive, anxious, and hyperactive-like behavior in open field test continues to late adulthood in LP F1 rats. The graph shows time spent in the center zone of the open field arena in a 20-min test session. Time in square analysis from individual tracks evidence significantly increased center time in LP F1 rats at 2, 3, 6, 12, and 18 months of age supporting the low anxiety and impulsive-like behavior in open field test. Data presented as mean ± SEM ($n = 8$). ***$p \leq 0.001$, **$p \leq 0.01$ for comparison of LP F1 with respect to HP F1 controls

in normal HP brains, initially lying near the ventricles and later homogenously distributed in the cortical and subcortical areas. Impact of intra-generational protein deprivation was clearly and consistently evident in terms of decreased PDGFRα+ cell population in both thalamic and cortical regions from E18 to P30, reflecting a depletion of the OPC pool, otherwise required to generate abundant oligodendrocytes for proper myelination. Surprisingly, our real-time qRT-PCR results revealed significantly increased PDGFRα fold expression in LP brains at E18 and P2, despite low immunoreactivity in the in vivo experiments, suggesting a possible delayed/or blocked differentiation of OPCs into pre-myelinating oligodendrocytes to cope with the increased demand despite reduced OPC pool.

In our recent publication [53], using the same model, we have reported a compromised astrocytogenesis in terms of delayed spatio-temporal appearance and differentiation, precocious maturation, and low turnover rate throughout life. The astrocytes upon differentiation start synthesizing PDGF, a signaling molecule that by interacting with PDGFRα expressed on the surface of OPCs, induce and promote their proliferation to meet the required number of oligodendrocyte population in CNS. Thus, the compromised astrogenesis and the low PDGFRα expression following maternal PMN could have been the primary cause leading to reduced oligodendrocyte population and hypo-myelination in the F1 PMN rats.

Although, we have recorded an elevated levels of PDGFRα mRNA expression in LP brains during late embryonic and early postnatal life, i.e., E18 and P2, but low PDGFRα immunoreactivity in LP brain sections. Such a difference suggest that although the PDGFRα gene is transcribed leading to an elevated mRNA levels, but in conditions of temporal delay of astrocyte differentiation, the mRNA is not translated into protein leading to low PDGFRα immunoreactivity. Low PDGFRα expression of OPCs may also account for the aberrant migration might bypassing the WM leading to poor myelination. Such decreased OPC population and PDGFRα expression indicate the proliferative defects imposed by maternal PMN.

Reduction in PDGFRα+ OPCs in the cortical and thalamic regions of LP brain implicate decreased OPC genesis in the SVZ and OPC migration following maternal PMN. Furthermore, as migration of OPCs involves the blood capillaries, whether poor vasculature in protein malnourished brains is also a factor for reduced migration needs to be addressed in future studies.

Furthermore, the precocious maturation of astrocytes was also reported in terms of precocious S100β co-expression in the GFAP expressing astrocytes leading to their temporally early maturation [53]. In addition, the S100β upregulation is reported to impair oligodendrogenesis in terms of their differentiation and maturation [54] and myelin formation as well as neuronal integrity in an inflammatory environment [55], putting S100β as a surrogate biomarker of brain injury in perinatal conditions with white matter (WM) injury. Such compromised maturation lead to the reduction in the proportion of myelinated axons and thinner myelin sheaths leading to axonal hypo-myelination.

The progressive stages of maturation of oligodendrocyte precursors involve downregulation of PDGFRα and acquisition of pre-myelinating oligodendrocyte markers. Differentiation and maturation of the oligodendrocytes involves the induction of genes encoding myelin-associated proteins such as PLP, MAG, and MBP which together are strongly induced following oligodendrocyte differentiation [50, 56, 57]. Young oligodendrocytes remain in intimate contact with the axons and myelinate them. The initiation of the myelination involves the transformation of the pre-myelinating OPCs into the mature myelinating oligodendrocytes involving shifting of the PLP and MBP expression from cell body to the processes that form myelin sheaths [56–58]. Such a shift from pre-oligodendrocytes to myelinating phenotype (immature oligodendrocytes) occurs between 28 and 40 weeks in humans [59, 60], while in rats from postnatal week 2 onwards [61, 62]. PLP, an integral membrane protein, constitutes more than 50% of the total myelin protein in the CNS and is specifically involved in the differentiation of the myelinating oligodendrocytes. The present study evidenced a significant reduction in the PLP and MAG +ve pre-myelinating oligodendrocytes in the LP brains from P2–P30, pointing towards the differentiation defects leading to a poor pre-myelinating oligodendrocyte pool in the LP brains that ultimately result in hypo-myelination.

Myelin thickness has been reported to affect conduction velocity which is pivotal for proper brain functioning and synchronous action potential firing [41]. Earlier studies have also reported a strong correlation between the integrity of corpus callosum white matter and the performance of neural circuitry [63, 64]. Our MAG immunoreactivity results revealed decreased MAG expression evidencing loose arrangement with low compaction of the myelin fibers in the LP brains. The disorganized and loose compaction of corpus callosum myelin fibers following maternal PMN, points to impaired behavioral functioning. The reduction in the corpus callosum caliber evidenced in LP rat brains from adulthood to senility showing retarded myelination indicate the long-term impact of intra-generational PMN. Pacagnella et al. [65] also hypothesized that the proportion of myelinated fibers do not increase as quickly in malnourished animals as in normal animals and even the density remains low. The structural integrity of white matter tracts associated with temporal and parietal cortices are pivotal to cognitive processing [66]; thus, reduced compaction, demyelination, or lesions in these tracts following maternal PMN directly correlate the impaired cognitive processing.

Myelin oligodendrocyte glycoprotein (MOG) present only in mammalian species has been regarded as a putative candidate target in demyelination for almost three decades [67–70]. Our findings reveal significantly low levels of MOG expression in the corpus callosum and other white matter areas of the thalamus in LP brains along with disorganization of the normal cyto-architecture of the myelinated fibers along with damaged fibers and deformation of their contours across age. Myelin oligodendrocyte glycoprotein has a crucial role in myelin integrity and also in aiding adhesion and cell surface interactions [50, 71]. Thus, reduced MOG levels following PMN appear to be associated with the loose compaction, hypo-myelination, and reduced corpus callosum caliber as noticed in LP brains with age advancement. The increased interstitial area and vacuolation of the corpus callosum of LP brains at 3, 6, 12, and 18 months with sparse Luxol fast blue staining evidence decreased myelination and low myelinated fiber density in the corpus callosum and other thalamic white matter tracts of LP brains. A reduction in the number of myelinated axons and the myelin was also reported in malnourished rats by Almeida et al. [27] and Miyata et al. [25]. Our results are in line with previous reports from Morell et al. [72] and Almeida et al. [24, 27] showing axonal disorganization with anomalous myelination, many axons being enveloped by myelin sheath and many axons without myelin following PMN. Such long-term changes in the myelin status of LP brain might be the resultant of proliferative and differentiation deficits leading to poor maturation and low oligodendroglial population. WM injury due to selective maturation arrest of pro-oligodendrocytes has been reported in pre-term infants [73, 74], models of hypoxia [75, 76], postnatal inflammation [77, 78], hyperbilirubinemia [79, 80], and fetal growth restriction [81, 82]. Such cellular maturational deficits due to perinatal insults cause myelin deficits [83]. The hypo-myelination of the proportion of myelinated axons and thinner myelin sheath formation due to inhibition of proliferation, differentiation, and maturation of OPCs was also reported following LPS-induced inflammatory response [84].

The rapid impulse propagation is pivotal for sensory, motor, and cognitive functions in vertebrates and is facilitated by myelin through axonal insulation thereby reducing the transverse capacitance and increasing resistance of the axonal plasma membrane [2, 85]. Thus, the large-scale myelin impairments and irreversible demyelination lesions evidenced in present study are predicative of greater cognitive impairments reported in our earlier publication [14].

The other behavioral tests, like grip strength performance, also evidenced poor neuromuscular strength throughout the life, validating the deleterious effects of myelination deficits on brain function and motor output. In addition, the LP F1 rats also presented hyperactivity and impulsivity from pre-adolescence to late adulthood, a typical symptom in ADHD and schizophrenic patients. The autopsy samples of schizophrenic and autistic patients also revealed decreased expression levels of myelin-associated human proteins [86–89] showing an association between poor myelination and neuropsychiatric phenotype. Takahashi et al. [17] have clearly indicated that oligodendrocytes and myelin dysfunction is a primary change in schizophrenia, resulting in alteration in the maintenance of axonal tracts and circuitry abnormalities, affecting normal synaptic function and frequently presenting behavioral and cognitive dysfunctions. Thus, the oligodendrocyte/myelin dysfunction can be a primary cause of schizophrenia rather than a consequence of illness.

In conclusion, intra-generational protein malnutrition leads to drastic changes in oligodendrocyte progenitor pooling, reduced expression of genes associated with oligodendrocytes and myelin leading to hypo-myelination, disorganized myelin fiber alignments, reduced corpus callosum caliber, and increased demyelination in the developing brain. These deleterious changes in oligodendrogenesis and myelination result in behavioral deficits persisting through pre-adolescence to late adulthood, thus strengthening the statement that early-life protein deprivation can be a big risk for neurodevelopmental disorders and associated neuropsychiatric phenotype.

Acknowledgements The authors are thankful to Prof. Pankaj Seth, National Brain Research Centre, Manesar, India, for the RT-PCR study.

Funding Information This study was supported by the Department of Biotechnology, Govt. of India, New Delhi, through a project grant (BT/PR4001/MED/30/669/2011) under the National Initiative on Glial Cell Research in Health and Disease.

Compliance with Ethical Standards

All experiments on SD rats were performed in accordance with the Institutional Animal Ethics Committee of Jiwaji University and in compliance with the National Institutes of Health Guide for the care and use of laboratory animals.

Conflict of Interest The authors declare that they have no conflict of interest.

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