Chapter 14

Oligodendrocytes: Cells of Origin for White Matter Injury in the Developing Brain

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Abstract

A prominent pattern of brain injury in preterm born infants involves damage to white matter with impaired oligodendrocyte maturation. This results in diffuse deficits in myelination that are associated with later development of cerebral palsy. While numerous experimental animal models of perinatal white matter injury have been developed, they show a spectrum of effects. This review proposes that adopting a more standard approach to defining white matter injury is important for validating experimental findings against the *bona fide* human condition. This chapter will describe the pathology of perinatal white matter injury and a general methodological approach for assessing white matter injury experimentally.

Key words Oligodendrocyte, Perinatal white matter injury, Myelination, Neuropathology, Astrogliosis, Microgliosis, Preterm, Immunohistochemistry, Histopathology, Methodology

1 Background

The white matter tracts of the brain comprise bundles of axons that establish connections between different regions. The wrapping of axons with a myelin sheath (myelination) is a cornerstone of human neurodevelopment. Myelin acts as an insulator for axons, and enables the rapid transfer of electrical information required for normal brain activity including coordinated sensory, cognitive, and motor functions. In humans, myelination begins in the second half of gestation, and progresses from deep to superficial regions of the brain in a caudal-to-rostral gradient [1]. After birth, the process of myelination progresses rapidly and peaks during the first postnatal year, but continues into adolescence and adulthood [2].

In the central nervous system (CNS), myelin is produced by oligodendrocytes. At least four stages of oligodendrocyte maturation have been described in the human brain: oligodendrocyte progenitor cells (OPCs), pre-oligodendrocytes (preOLs), immature oligodendrocytes and mature oligodendrocytes (Fig. 1) [3–5]. OPCs are highly proliferative, and develop from multipotent

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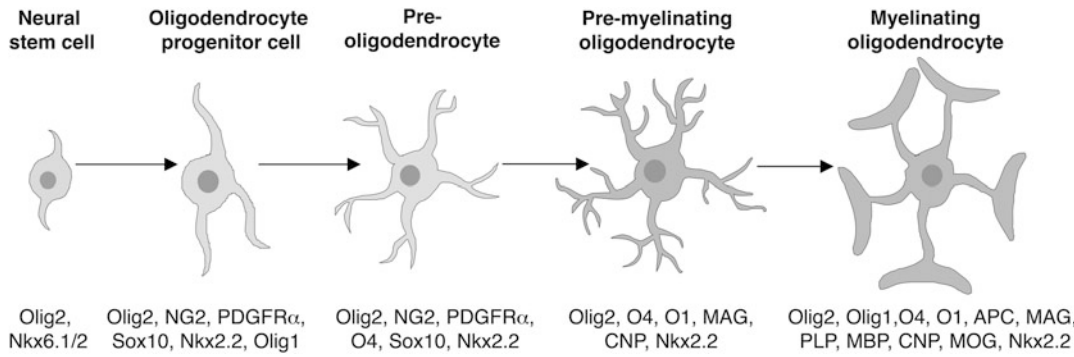

Fig. 1 Lineage and stage-specific antigens of oligodendrocytes. During development, oligodendrocytes are generated by multipotent stem cells in the proliferative zones of the brain and spinal cord. These give rise to oligodendrocyte progenitor cells that divide and migrate through the parenchyma to reach their final destination. These progenitor cells terminally differentiate into pre-oligodendrocytes, pre-myelinating oligodendrocytes and then mature myelinating oligodendrocytes that form the myelin sheath of axons (Figure provided by Dr Anzari Atik). *APC*, adenomatous polyposis coli; *CNP*, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase; *MAG*, myelin-associated glycoprotein; *MBP*, myelin basic protein; *MOG*, myelin oligodendrocyte glycoprotein; *NG2*, NG2 chondroitin sulfate proteoglycan; *Nkx2.2*, NK2 homeobox 2; *Nkx6.1/2*, NK6 homeobox 1 and 2; *O1*, O1 antigen; *O4*, O4 antigen; *Olig1*, oligodendrocyte transcription factor 1; *Olig2*, oligodendrocyte transcription factor 2; *PDGFRα*, platelet-derived growth factor receptor-α; *PLP*, proteolipid protein; *Sox10*, sex-determining region Y-box 10

stem cells in sequential waves during fetal and early postnatal life from restricted periventricular zones. OPCs first appear in the ganglionic eminence around 9 weeks of gestation in humans, and continue to be produced throughout intrauterine life, after birth and into adulthood [6–10]. Thus, even where oligodendrocyte development has been disturbed, an endogenous pool of OPCs may remain available for therapeutic targeting of repair and remyelination. Once OPCs migrate to reach their final destination, they can terminally differentiate into post-mitotic pre-myelinating oligodendrocytes, before finally forming mature oligodendrocytes capable of producing myelin and initiating axonal myelination.

2 Pathology of Perinatal White Matter Injury

Injury to the cerebral white matter resulting in deficits in myelination is the major pattern of brain injury observed in survivors of preterm birth, although white matter injury can also occur in term born infants, typically in combination with overt grey matter damage [11–14]. The classical pattern of perinatal white matter injury involves focal regions of macroscopic and microscopic cystic necrosis, associated with reactive gliosis [15–20]. The myelination deficits observed in these cystic lesions are related to degeneration of all cellular elements, including oligodendrocytes and axons [5, 15, 21–24]. However, in more modern cohorts of preterm infants,

imaging studies suggest that the incidence and overall burden of cystic white matter lesions is very low [25, 26], while a pattern of less severe but widespread diffuse white matter injury is now prevalent [17, 27–29].

Pathologically, diffuse white matter injury is characterized by a pattern of diffuse myelination failure associated with reactive astrocytosis and microgliosis, without major primary axonal injury [17, 22, 30, 31]. While selective death of preOLs was previously considered the predominant mechanism leading to these diffuse deficits in white matter myelination [32–34], the major consensus finding in humans is that oligodendrocytes can become arrested in their maturation, likely at the O4/O1 transition stage, and thus fail to differentiate into myelin producing cells [16, 17, 35]. Maturation arrest of oligodendrocytes has also been reported in several perinatal animal models of hypoxia-ischemia [30, 36, 37], chronic hyperoxia [38] or hypoxia [39–41], intrauterine growth restriction [42, 43], and infection/inflammation [44–46]. Thus, a role of loss of oligodendrocytes in this chronic stage of injury is unlikely [17]. Indeed, experimentally, the acute loss of oligodendrocytes in response to hypoxia-ischemia in neonatal rats [37] and preterm fetal sheep [30] is recovered by a rapid proliferative response of the OPC population. Further, the human neonatal brain exhibits vast numbers of migratory OPCs that can easily repopulate the area of injury to replace dying oligodendrocytes [47, 48]. Experimental models of neonatal white matter injury have also shown OPC proliferation and impaired maturation in the absence of acute oligodendrocyte cell death [40, 41, 45, 46]. Overall, these findings suggest that understanding the mechanisms of arrested oligodendrocyte development should be a particular focus for studies examining the pathogenic basis of neonatal white matter injury and therapeutic strategies.

3 Assessment of Experimental Perinatal White Matter Injury

As described, perinatal white matter injury is comprised of a spectrum of disorders that includes focal cystic micro/macroscopic necrotic lesions, diffuse non-cystic lesions, axonal injury, astrogliosis, microgliosis, oligodendrocyte cell death, oligodendrocyte maturation arrest, and deficits in myelination. Such injury can be assessed using a variety of techniques including magnetic resonance imaging (MRI), in particular diffusion tensor imaging (DTI), neuropathological assessment using histochemistry and immunohistochemistry, and electron microscopy, as well as molecular techniques (qPCR and in situ hybridization) to examine mechanistic injury pathways. The advantages and disadvantages of these common techniques are listed in Table 1. Below we provide an outline of neuropathological procedures that should be considered for

Table 1
Techniques used to assess experimental perinatal white matter injury: advantages and disadvantages

Technique	Application	Advantages	Disadvantages
Magnetic Resonance Imaging	Used to assess white matter volumes and macrostructure.	Noninvasive; provides details of injury (extent and localization) without having to terminate the experiment; can investigate time-course or progression of injury.	Does not provide details of cellular changes associated with the injury; animal requires sedation.
Diffusion Tensor Imaging	Used to assess white matter microstructure. e.g., integrity of axonal fibers, coherence of axon bundles, trajectory of fiber tracts.	Noninvasive	Does not provide details of cellular changes associated with the injury; animal requires sedation. Does not definitively identify myelin.
Magnetic Resonance Spectroscopy	Used to study metabolic changes in the brain.	Noninvasive	Does not provide details of cellular changes associated with the injury; animal requires sedation.
Neuropathology: Histology	Identification of gross morphological changes and injury within the brain at the microscopic level.	Provides information on degree, severity, and location of injury; quantifiable; quick.	Information on injury at one point in time. i.e., unable to assess progression of injury in the same animal; provides no information on alterations at the protein level; unable to distinguish between different specific cell types (e.g., microglia and astrocytes).
Neuropathology: Immunohistochemistry	Microscopic identification of alterations to specific cell types or protein levels.	Able to assess various neural populations (neuron, microglia, astrocyte, oligodendrocyte), subpopulations (e.g., preOL, mature oligodendrocyte), or activation states (e.g., reactive microglia: M1 or M2 phenotype).	Only provides information on changes at one point in time; densitometry assessment of immunoreactivity may not completely reflect actual protein content in tissue.

Electron microscopy	Ultrastructural assessment of myelin sheath thickness, axon diameter, axon number, ratio of myelinated to unmyelinated axons.	"Gold standard" quantitative assessment of myelination; high power visualization of the myelin sheath and axons; requires very small tissue pieces.	Large areas of the white matter cannot be assessed, and therefore, nonuniform changes may be missed.
Molecular techniques: qPCR	Analysis of regulators of oligodendrocyte maturation at the mRNA level.	Provides information on the putative molecular mechanisms underlying impaired oligodendrocyte development.	mRNA changes do not always translate to changes in protein; does not provide details on cellular localization.
In situ hybridization	Analysis of regulators of oligodendrocyte maturation at the mRNA level in tissue sections.	Provides information on the putative molecular mechanisms underlying impaired oligodendrocyte development; allows for phenotype analysis due to cellular localization of mRNA; works well in PFA fixed tissue.	mRNA changes do not always translate to changes in protein.

complete assessment of experimental white matter injury, focusing specifically on the use of histology, immunohistochemistry and electron microscopy.

3.1 General Considerations

3.1.1 Tissue Preparation

The most common brain fixation methods involve perfusion or immersion fixation with 10 % formalin or 4 % paraformaldehyde (PFA), followed by either (1) tissue processing (dehydration) and paraffin embedding, (2) equilibration in sucrose solution (cryoprotectant) and sectioning using the cryostat or freezing microtome, or (3) sectioning of fixed brains using a vibratome. The majority of antibodies used to assess white matter injury (*see* Table 2) will work successfully in tissues processed with any of these methods (e.g., *see* [16, 30, 37, 42, 49]). However, staining for the stage-specific cell surface antigens O4 and O1 can be markedly reduced by use of formalin solutions (likely due to methanol impurities), alcohols, solvents including xylenes, and antigen retrieval techniques, making assessment of oligodendrocyte morphology and cell counts difficult or impossible. Increasing time intervals in post-mortem fixation can also markedly reduce immunostaining for oligodendrocyte O4 and NG2 markers due to postmortem autolysis [50, 51]. Immersion fixation of blocks of brain tissues or whole rodent brains with fresh ice-cold 4 % PFA, followed by sucrose equilibration/cryostat cutting or direct vibratome cutting, then free floating immunofluorescence provides excellent staining using O4/O1 antibodies, as well as the majority of other markers required to assess white matter injury (*see* Table 1 and Sect. 3.5 for more details) [30, 37–41, 49, 52–55].

3.1.2 White Matter Assessment

White matter injury in the developing brain primarily occurs in the deep dorsal and lateral aspects to the lateral ventricles at the frontal, parietal and occipital levels, and may extend into adjacent external capsule, corona radiata, corpus callosum, and centrum ovale [5]. In rodents, the white matter constitutes only a small proportion of total brain volume, and the connecting white matter tracts encompassing the corpus callosum, overlying supracallosal radiation, and the external capsule at the level of the midseptal nuclei (frontal white matter) and the anterior hippocampal formation/anterior nuclei of the thalamus (parietal white matter) are typically used for assessment of white matter injury [37, 42, 52, 56–58]. In larger species such as sheep, white matter injury can be regionally divided into deep periventricular and more superficial regions (e.g., corona radiata, centrum ovale) at the frontal (e.g., mid-striatum) and parietal (e.g., mid-thalamic) levels [36, 59–62], while a medial versus lateral approach has also been reported [63]. Critically, irrespective of species, given that oligodendrocytes develop in a caudal to rostral direction, and can exhibit marked regional and level differences in density, maturation, and myelination, it is key

Table 2
Antibodies used for assessing perinatal white matter injury using immunohistochemistry

Antigen/marker	Labels
Oligodendrocytes	
Olig1	OPCs (nuclear) and mature oligodendrocytes (cytoplasmic)
Olig2	Nuclear pan-oligodendrocyte marker of OPCs, preOLs, pre-myelinating and myelinating oligodendrocytes. Also expressed in multipotent neural stem cells and some transit amplifying astrocyte progenitors.
O4	preOLs, pre-myelinating oligodendrocytes
O1	Pre-myelinating and myelinating oligodendrocytes
PDGFRα	OPCs and preOLs
Nkx2.2	OPCs, preOLs, pre-myelinating and myelinating oligodendrocytes
Sox10	Nuclear marker for OPCs, preOLs
NG2	OPCs and preOLs
CC1/APC	Myelinating oligodendrocyte marker; but can show overlap in cycling and NG2-positive cells.
CNP	Pre-myelinating and myelinating oligodendrocytes
NogoA	Myelinating oligodendrocytes
Myelin proteins	
MBP	Mature myelin, myelinating oligodendrocytes
MAG	Mature myelin, Pre-myelinating and myelinating oligodendrocytes
PLP	Mature myelin, myelinating oligodendrocytes
Astrocytes	
GFAP	Astrocytes (cell body and processes), radial glia, Bergman glia
S100β	Astrocytes
Glutamine synthetase	Astrocyte cell body
GLT-1	Astrocytes (and neurons)
GLAST (aka ACSA-1)	Astrocyte specific glutamate transporter; astrocytes, radial glia, Bergmann glia
AldoC	Astrocytes (and Purkinje cells)
CD44	High levels in astrocyte precursors, astrocytes; expression in other cell types including oligodendrocytes
Microglia	
Lectin	Microglia/macrophages (and blood vessels)
Iba1	Microglia/macrophages
ED1/CD68	Resting (low expression) and reactive (high expression) microglia/ macrophages

(continued)

Table 2
(continued)

Antigen/marker	Labels
F4/80	Mature microglia/macrophages
CD40	Microglia/macrophages
CD45	Microglia/macrophages
CX3CR1	High levels in microglia/macrophages; lower expression in other cells types including neurons
Axons	
NF200	Phosphorylated and/or dephosphorylated 200 kDa neurofilament (high molecular weight)
SMI 312	Phosphorylated neurofilament (medium and high molecular weight)
SMI 31	Phosphorylated neurofilament (high molecular weight)
SMI 32	Non-phosphorylated neurofilament (high molecular weight)
β-APP	Damaged neurons/axons
Fractin	Apoptotic neurons/axons
TubulinΔCsp6	Damaged neurites/axons

that white matter injury be assessed at equivalent levels and region matched areas between groups.

3.2 Basic Histopathology

If the experimental insult is severe, white matter injury may be detected macroscopically at the time of tissue processing. However, it is more routine and informative to assess the tissue sections microscopically using a range of histochemical or immunohistochemical methods. Tissue sections can be stained histochemically using a neural specific stain such as thionine or cresyl violet (in acetate buffer, pH 4); these stains will detect Nissl substance, or RNA within the neurons and dendrites, but not in axons. However, conventional hematoxylin and eosin (H&E) can be used and is often beneficial for detecting the presence of other cell types within the parenchyma or meninges such as blood and inflammatory cells. Nissl stains and H&E also allow for the assessment of global injury in the brain, rather than that specific to the white matter. Alternatively, histochemical stains such as luxol fast blue (LFB) [64], eriochrome cyanine R (also called chromoxane cyanine R, solochrome cyanine R, or Mordant blue 3) [65], or Gallyas silver stain [66] can be used to specifically stain myelin. However, caution must be taken when interpreting a reduction in staining intensity as a reduction in myelin content. Silver-staining methods such as Bielschowsky or Bodian [67] identify axons within the white

matter, and provide a useful technique for the identification of axonal pathologies (*see* Sect. 3.4 for more detail).

Tissue sections stained histochemically are used to assess overt white matter injury in the form of necrotic lesions, focal or diffuse non-destructive lesions, acute axonal injury and/or areas of pallor, or for the determination of white matter volume. White matter lesions can be scored based on severity (e.g., necrotic vs. non-destructive), incidence (e.g., the proportion of experimental animals with lesions) and distribution (e.g., periventricular white matter, subcortical white matter, corpus callosum, external capsule, cingulum). The neuropathological assessment of white matter injury in human neonates is considerably more detailed [17], and often requires review by a pathologist.

The volume of the white matter also provides a surrogate measure of white matter injury and can be assessed quantitatively using imaging processing software packages such as ImageJ or Fiji (www.fiji.sc/Fiji). It must be acknowledged, however, that a reduction in the volume of the white matter (or regional volume reductions) can indicate a reduction in myelination or myelin thickness, and/or a reduction in the number of myelinated/unmyelinated axons. Therefore, to distinguish between these phenomena, further assessment using electron microscopy (*see* Sect. 3.6.3 below) is required.

3.3 Assessment of Astrogliosis and Microgliosis

Reactive astrocytosis and microglial activation are common features observed in human and experimental postmortem studies of perinatal white matter injury. Identification of areas of white matter gliosis also provides a basis for regional analysis of oligodendrocyte survival, maturation, and myelination in the lesion environment (*see* Sect. 3.5) [17, 30]. The classical antibodies used to assess astrocyte morphology include glial fibrillary acid protein (GFAP; stains major processes) and S100β (cytosolic protein), as well as glutamine synthetase and the glutamate transporters GLT-1 (aka EAAT2) and GLAST (aka EAAT1). Note that these markers do not all selectively label astrocytes. For example, GFAP is also expressed by radial glia within the cerebral cortex and Bergmann glial within the cerebellum. Further, these markers are not selective for reactive astrocytes, and thus morphological assessment is required to assess reactivity. Nevertheless, a wide range of alternative markers of normal and reactive astrocytes, including aldolase C (AldoC) and CD44 [17, 68] have been reported [69–72].

The most common markers or antibodies used to assess macrophages/microglia include lectins (e.g., biotinylated tomato lectin; lectins also stain blood vessels), ionized calcium-binding adapter molecule 1 (Iba1), ED1/CD68, as well as F4/80, CD40, CD45, and CX3CR1. It is important to note that these markers do not differentiate between macrophages (such as those entering from the periphery) versus resident CNS microglia, although new panels of

unique microglia markers are being developed [73]. Further, only ED1/CD68 is considered a marker of reactive microglia/macrophages, and morphological assessment is generally required to assess reactivity. Interestingly, there is increasing evidence that microglia can acquire multiple activation phenotypes that may have differing functions in perinatal brain injury [74, 75], as observed in adults [76–81]. The key phenotypes include the M1 classic phenotype (cytotoxic), M2a alternate phenotype (repair and regeneration), and M2b Type II-deactivating phenotype (immunomodulatory) [82, 83]. Using panels of markers for these phenotypes validated in vitro [84–86], it is possible to examine the temporal patterns and roles of activated microglia phenotypes with respect to evolution and mechanisms of perinatal white matter injury [87].

By single or double staining markers of astrocytes or microglia (e.g., GFAP/Iba1), necrotic foci can be observed microscopically as focal areas of dense astrocyte or microglial staining, with cells typically exhibiting a reactive morphology [17, 30]. Similarly, diffuse white matter injury can be characterized neuropathologically by areas of diffuse accumulation of reactive microglia and astrocytes. In the early developing white matter, the expression of GFAP-positive astrocytes is very low. For example, in our experience, widespread staining of GFAP-positive astrocytes is not present in the normal brain until around postnatal day 7 in neonatal rodents. Thus, brain lesions containing reactive astrocytes can easily be identified at these younger ages. With increasing expression of GFAP-positive astrocytes in the white matter with development, it can become difficult to assess areas of reactive astrogliosis over the normal dense astrocytic background. In such cases, low-power image montages or digital scanning (e.g., Leica Aperio ePathology) of entire brain sections can be useful in determining areas of gliosis. Counting numbers of astrocytes in brain lesions is also difficult, as astrocytes are intrinsically connected with other cell types, making accurate selection of the astrocyte cell body problematic. Further, astrocytes do not typically proliferate in response to injury, but rather exhibit thickening of their main processes with elevated GFAP expression [88]. Unbiased quantification of astrogliosis in neonatal white matter lesions has been recently reported using point-counting methods such as the Cavalieri approach [17]. In this technique, the area fraction of GFAP-positive cell soma and/or processes can be determined by overlay of a grid of known size onto a microscope image, and then counting of the grid points that fall on the item to be quantified [89].

In contrast to astrocytes, microglia can be easily visualized in the white matter during early brain development, where they exhibit an amoeboid morphology, before developing into a ramified state. Microglia typically accumulate in areas of white matter injury by migration and proliferation, and demonstrate a reactive morphology. For quantification of reactive microgliosis, counts of

numbers of microglia in white matter lesions can be determined, although with excessive microgliosis this can be challenging. An alternative approach is to use the Cavalieri approach described above for astrocytes. More detailed assessment of the temporal changes in reactive microglial phenotypes may also provide important details on potential roles of microglia in the injury process. Importantly, small clusters of amoeboid microglia can be found in the white matter during normal development in patterns that may resemble areas of injury. Thus, care needs to be taken to have appropriate matched level controls sections for assessment of injury.

3.4 Assessment of Axonal Injury

During human cortical development, growing axons (growth-associated protein 43-positive) are detected as early as 20 weeks of gestation, with extensive expression by 37 weeks [90]; thus, axons are highly vulnerable to developmental injury in preterm infants. Axonal injury is present within the focal cystic necrotic lesions in human periventricular leukomalacia (PVL) and has been established as a feature in experimental models of perinatal hypoxic-ischemic brain injury. Using fractin as a marker of apoptotic axons, degenerating axons have also been detected in diffuse non-cystic lesions within human PVL [22]; however, this finding was not replicated in a preterm fetal sheep model of global ischemia [30]. It is still unclear whether the axonal injury associated with diffuse non-cystic lesions occurs as a consequence of the underlying injury (e.g., neuronal injury) or is the primary source of the injury. Nonetheless, if axons degenerate it is reasonable to assume that myelination would also be affected leading to cerebral hypomyelination and regional volume reductions.

3.4.1 Staining Methods

One of the most well-known and utilized techniques for the pathological assessment of axonal injury is Bielschowsky's silver stain, which identifies argyrophilic axons, and is thought to stain all neurofilaments regardless of their molecular weight. This method, applied to paraffin-embedded tissue, has undergone numerous modifications, but one of the most recognizable protocols is that of Yamamoto and Hirano [91]. In models of perinatal brain injury, Bielschowsky's silver stain is used to identify regions of axonal degeneration and the presence of axonal spheroids [92]. Antibodies directed against the neurofilament proteins are also used to examine axonal pathology in human preterm brains [90] and models of perinatal white matter injury [31, 59, 93]. Neurofilaments are components of the axonal cytoskeleton and consist of a family of three polypeptide subunits: NFL (low molecular weight neurofilament; ~ 68 kDa), NFM (medium molecular weight neurofilament; 90–168 kDa), and NFH (high molecular weight neurofilament; 100–200 kDa). During axonal development, neurofilament subunits, in particular NFM and NFH, are phosphorylated at the same time that neurofilament is transported down the axon. Thus,

phosphorylated NFH in particular, is a marker for axonal maturity [94]. Listed in Table 2 are four antibodies used to label neurofilaments: SMI 31 recognizes phosphorylated NFH; SMI 32 recognizes non-phosphorylated NFH; SMI 312 recognizes phosphorylated NFM and NFH and NF200, a pan-axonal marker that labels all 200 kDa neurofilaments (phosphorylated and dephosphorylated). Other antibodies for axonal injury/degeneration include β-amyloid precursor protein (β-APP), caspase-cleaved forms of the cytoskeletal elements actin (fractin; N-terminal fragment of actin) and tubulin (TubulinΔCsp6) (Table 2), or β-APP in combination with Caspase 6 [95, 96]. β-APP is a membrane glycoprotein present in low levels in normal neurons, but is rapidly upregulated within axons following neuronal damage [97, 98]. The fractin antibody recognizes caspase-cleaved but not intact actin, and labels apoptotic but not necrotic neurons/axons [99], and TubulinΔCsp6 labels degenerating neurites in human hypoxic-ischemic injury [100]. In human diffuse non-necrotic lesions, fractin-immunoreactivity is present, while β-APP immunostaining is rarely seen [31, 90], highlighting the need to use more than one marker of axonal injury.

3.4.2 Neuropathological Analysis

Neuropathological assessment of axonal injury in perinatal white matter injury involves analysis for the presence of reduced staining/immunoreactivity, axonal swellings and axonal spheroids. Such analysis is semi-quantitative and can involve the use of a grading system to score the amount immunoreactivity, or the proportion of cases/animals with axonal injury [22, 31, 90]. However, neither method is quantitative and thus suitable for statistical analysis. Densitometry of neurofilament immunoreactivity may be used but must be interpreted with caution for reasons outlined in Sect. 3.6.2 below.

3.4.3 Electron Microscopy

The ultrastructural assessment of axonal structure and injury is more informative and quantifiable using electron microscopy. The processing of tissue for electron microscopy is different to that for histochemistry or immunohistochemistry. Ideally, the brain is first transcardially perfused with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Alternatively, blocks (approximately 1 mm^3) can be dissected from tissue perfused with 4 % paraformaldehyde for histology, and quickly immersed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), reducing the need for additional animals. Briefly, tissue blocks are then post-fixed in 1 % osmium tetroxide in 0.2 M cacodylate buffer (pH 7.4) and stained with uranyl acetate in maleate buffer (pH 6), before being embedded in epon-araldite. Semi- and/or ultrathin sections are then cut using an ultramicrotome. These sections can be used to assess the areal density of myelinated and unmyelinated axons in non-overlapping fields from a number of sections using ImageJ software. Of note,

osmium tetroxide treatment makes many epitopes unstable, and reduces the efficacy of superimposed immunogold labeling of electron microscopy samples. New and more stable epitope tags, resistant to effects of osmium tetroxide fixation, are therefore needed to overcome this problem.

3.5 Assessment of Oligodendrocyte Stages, Death, and Maturation

3.5.1 Stage Specific Oligodendrocyte Markers

Oligodendrocytes express a range of cell surface and myelin-specific antigens that can be used to precisely identify the various stages of oligodendrocyte maturation (Fig. 1; Table 2) [4]. The pan-lineage marker Olig2 is widely used for identification of oligodendrocytes at all maturational stages. As a nuclear marker, it is particularly useful for assessing changes in total numbers of oligodendrocytes in the white matter in injury models. The OPC is the earliest stage of the committed oligodendrocyte lineage, and exhibits a simple bipolar morphology. For identification of OPCs, antibodies against platelet-derived growth factor receptor-α (PDGFRα), nuclear Olig1, Sox10, or NG2 chondroitin sulfate proteoglycan are commonly used, although some of these markers can also label a population of preOLs. As described, OPCs often exhibit marked proliferative responses in many models of white matter injury, which can be assessed by combination with cell cycle markers such as Ki67 or proliferating cell nuclear antigen (PCNA).

The next stage of oligodendrocyte maturation, the preOL, is also mitotically active, and exhibits a simple multipolar morphology. preOLs are most commonly identified by expression of the O4, but not O1, antibodies, although they may also express a range of markers similar to OPCs. Double staining using the mouse-monoclonal O4 and O1 IgM antibodies has been achieved by sequential primary/secondary antibody staining with a fixation step between primaries, or utilizing a biotinylated O4 antibody [3, 37, 52]. The immature oligodendrocyte is postmitotic and exhibits a complex multipolar morphology. Immature oligodendrocytes express both O4 and O1 antigens, but lack the complex myelinating morphology of mature oligodendrocytes, and do not express myelin proteins such as myelin basic protein (MBP). The progression to immature oligodendrocyte is also characterized by expression of myelin-associated glycoprotein (MAG) and 2′, 3′-cyclic-nucleotide 3′-phosphodiesterase (CNP). Mature oligodendrocytes can be identified by expression of cytoplasmic Olig1 [101], adenomatous polyposis coli (APC, aka CC-1), and myelin-associated markers, including MBP and proteolipid protein (PLP). In human tissue, NogoA is also a useful mature oligodendrocyte marker [49].

An important consideration when examining the oligodendrocyte lineage is that with advancing white matter myelination, it becomes progressively difficult to identify oligodendrocyte cell bodies using markers such as O4 and O1, due to the expression

of these antigens in oligodendrocyte processes. In such cases, double labeling using the mature oligodendrocyte marker APC with Olig2 can provide an estimate of the number of mature cells [41].

3.5.2 Oligodendrocyte Cell Death

Degenerating oligodendrocytes can be identified by cellular pyknosis (nuclear chromatin condensation) and karyorrhexis (nuclear fragmentation). When stained with the nuclear marker Hoechst 33342 or 4′,6-diamidino-2-phenylindole (DAPI), pyknotic cells in the white matter are easily identified by intensely stained, condensed nuclei. Cellular degeneration and DNA fragmentation can also be assessed with the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Confirmation of oligodendrocyte lineage or specific developmental stage can be achieved by double labeling with Olig2 or O4/O1 for example [17, 30, 32, 37]. In addition, markers of dying oligodendrocytes labeled with O4 for example, will show morphological condensation of the cell body, fragmentation of the processes, and O4-labeling of cell membrane and cytoplasm due to loss of plasma membrane integrity [57]. Note that these methods do not distinguish between apoptotic or non-apoptotic modes of cell death. Co-localization of oligodendrocyte antibodies with apoptotic cascade markers such as activated-caspase-3, along with assessment of cellular pyknosis or TUNEL, can also be used to give a broad indication of the relative contributions of different cell death pathways.

3.6 Assessment of Myelination

The white matter within the CNS is composed of ~40–50 % myelin (dry weight) [4]. Of this myelin dry weight, 70 % is composed of glycolipids and the remaining 30 % is proteins; these myelin constituents are formed within the oligodendrocyte. Oligodendrocytes and myelin are rich in glycosphingolipids, in particular galactosylceramides (GalC/O1) and their sulfate derivatives (sulfatides/O4). Each of these lipids can be localized immunohistochemically and are used to identify stages of maturation of the oligodendrocyte lineage (*see* Sect. 3.5.1).

3.6.1 Myelin Proteins

There are numerous myelin proteins, which are located at different positions within the lipid bilayer and play different roles in myelination. Thus, the choice of myelin marker is an important consideration when assessing changes in myelination. MBP and PLP are the two major CNS myelin proteins, and constitute 30 % and 50 % of the total myelin proteins, respectively [4]. MBP plays a role in myelin compaction and is localized to the major dense line, while PLP spans the entire thickness of the lipid bilayer and is localized to both the intraperiodic line and major dense line. Minor myelin constituents include: 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP, 4 %) localized to oligodendrocyte membranes during early stages of axonal ensheathment and not to compact myelin [102]; MAG (1 %) localized to periaxonal membranes of the myelin

internodes, and is thus in direct contact with the axon; myelin/oligodendrocyte glycoprotein (MOG), located on the oligodendrocyte membrane, in particular the oligodendrocyte cell processes and on outer most lamellae of the myelin sheath; and myelin associated oligodendrocyte basic protein (MOBP), located in major dense lines and involved in myelin compaction. Interestingly, MBP and MOBP mRNA are initially found within the soma of the oligodendrocyte, but once myelination proceeds they move distally into the oligodendrocyte cell processes [103, 104]. In human PVL cases [16] and in an experimental model of intrauterine growth restriction [42], MBP expression is retained with the oligodendrocyte cell soma, reflecting a deficit in the trafficking of MBP to the processes for myelin sheath wrapping. Thus, aberrant cellular localization of these two proteins should also be a consideration when assessing perinatal white matter injury in experimental models.

The large majority of experimental studies investigating the impact of perinatal insults on white matter development use MBP as a marker of myelin, and thus, any reduction in expression or aberrant cellular localization is interpreted as hypomyelination. However, MBP is only one of many myelin proteins, and myelination should be investigated in combination with other markers such as PLP or MAG. Indeed, in a model of intrauterine growth restriction in the guinea pig, we have noted a striking lack of MBP expression (i.e., trapping of MBP within the cell soma), which could be interpreted as a lack in myelination, but the presence of both PLP and MAG immunoreactivity, albeit at reduced levels [42]. Thus, conclusions on the state of myelination should not be drawn based on MBP staining alone.

3.6.2 Immunohistochemical Analysis of Myelination

Immunocytochemistry is commonly used to detected changes in the expression of myelin proteins. This can be performed using tissue sections to assess for deficits in spatial distribution and localization, as well as potentially for changes in the intensity of myelin proteins. Semi-quantification of myelin proteins localized to tissue sections may be performed using densitometry, although the sensitivity and reproducibility of this technique remain controversial. The reaction between an antibody and its antigen is not stoichiometric; therefore, the intensity of the staining product does not directly translate to the amount of that product (i.e., protein) within the tissue. This phenomenon is particularly applicable when assessing immunoreactivity using 3′,3′-diaminobenzidine (DAB) as the chromogen, because DAB does not follow the Beer–Lambert law; the brown reaction product does not absorb light but rather scatters it. Despite this, studies have shown a positive linear relationship between the intensity of immunoreactivity and the antigen concentration [105], although this may not apply to all antibodies [106]. Other chromogens such as Liquid

Permanent Red show very similar spectral profiles at both high and low levels of expression, and are considered more suitable for densitometry [107]. It should be noted that densitometry is typically used to assess relative differences in immunoreactivity between control and experimental cohorts rather than quantification of absolute protein levels.

If densitometry is the chosen form of analysis, certain technical steps need to be considered. First, it is imperative that all tissue is fixed and processed in the same way, including fixative used, duration of fixation, and section thickness. Secondly, immunohistochemistry should be performed on all tissue sections to be compared at the same time and using the same protocol. This often results in a large number of slides undergoing simultaneous immunostaining, and it is thus important that incubation times (particular of the chromogen) are consistent. Thirdly, if possible a counterstain should not be used to prevent the need for spectral unmixing [107]. Lastly, imaging should ideally be performed in a single day to maintain identical microscope parameters (e.g., intensity of light source, condenser position).

Densitometry is then performed on captured images using software packages such as Image-Pro Plus (Media Cybernetics). In preparation for densitometry measurements, the image analysis system is first calibrated using an image of a blank section of the glass slide (incident light) and an obscured section of the slide (infinite optical density). Images are then taken from matched regions of interest from immunostained tissue sections at comparable levels of the brain, converted to greyscale and the optical density (absorbance) determined. A correction is then applied to each of these images by subtracting the optical density measurement of the immunostaining from the optical density measurement from a region of background staining. Optical density units have no dimension and are logarithmic, and the value reflects the amount of photons absorbed or transmitted; an optical density of zero indicates all photons are transmitted, and an optical density of 1.0 indicates that 90 % of all photons are absorbed, while an optical density of 2.0 indicates that 99 % of all photons are absorbed.

3.6.3 Electron Microscopy

While immunohistochemical localization of myelin proteins (and oligodendrocytes) provides essential microscopic data when assessing white matter injury, electron microscopy is considered the "gold standard" technique for assessing myelination as it detects changes at the ultrastructural level. The methodology for preparing tissue for electron microscopy is presented in Sect. 3.4.3. Semi-thin and/or ultrathin sections can be used to assess the areal density of myelinated and unmyelinated axons, as well as inner axon diameter and outer axon diameter (axon + myelin sheath) in nonoverlapping fields from a number of sections using ImageJ software. The

conduction velocity of an axon is closely related to the axon diameter and myelin sheath thickness [108], thus dividing the inner axon diameter by the outer axon diameter (known as the g-ratio) provides a measure of conduction velocity. It is thought that the g-ratio of a myelinated axon is optimized to reach maximal efficiency and physiological function. A theoretical g-ratio value of 0.6 was first described by Rushton [109]; however, it is now acknowledged that different central white matter tracts have different optimal g-ratios [110].

While there is much focus on the myelinated axon, the assessment of unmyelinated tracts should not be overlooked. In a recent study in the rabbit model of cerebral palsy induced by antenatal hypoxia-ischemia, a decrease in the number and function of unmyelinated fibers was associated with hypertonia, with the loss of myelinated fibers occurring secondary to the motor deficits [111].

4 Conclusion

Here we have described a variety of methodological approaches that should be considered for the detection and assessment of perinatal white matter injury. Although not described in this chapter, a wide range of molecular techniques (e.g., qPCR and in situ hybridization) and transgenic animal models are available to complement these methods, as well as provide detailed mechanistic data of the pathogenesis of perinatal white matter injury, including regulators of oligodendrocyte proliferation and maturation [49, 54]. Behavioral assessment should also be considered in order to provide functional outcomes associated with experimental white matter injury and treatment strategies. A battery of behavioral tests, including motor, cognitive, and somatosensory paradigms, that can be used in rodents to investigate long-term outcomes are detailed in Chapter 11. Use of such combination approaches will further our understanding of the cellular and molecular mechanisms of perinatal white matter injury, with the aim to develop regenerative strategies to promote normal brain development and function in this group of infants.

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