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CORTICAL WHITE MATTER: BEYOND THE PALE

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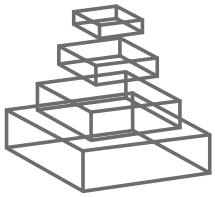

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CORTICAL WHITE MATTER: BEYOND THE PALE

Hosted By
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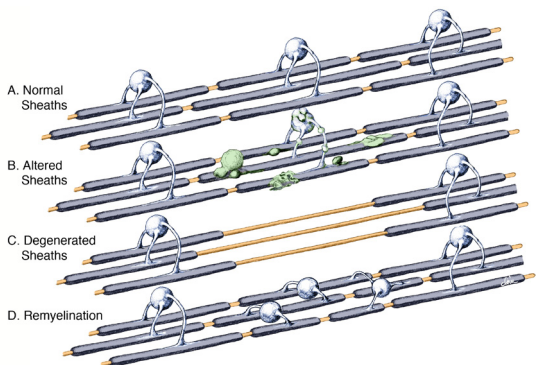

Cortical white matter, after years of arguably Cinderella status, is in the spotlight. With the advent of diffusion tensor imaging (DTI), white matter tracts offer exciting potential for functional neuroanatomy, including in humans. From another perspective, the finding that myelination continues well into adulthood has encouraged research on the contribution of myelin to learning, cognition, and psychiatric disorders (e.g., RD Fields, TINS 2008). Thus, white matter is poised, following on vasculature and glia, to take on new importance in a wide range of nervous system functions.

Image credit: Alan Peters

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EDITORIAL
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Cortical white matter: beyond the pale

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The tracts within the subcortical white matter and corpus callosum provide an anatomical connectivity that is essential for normal cognitive functioning. These structures are predominantly made up of axons that are myelinated or unmyelinated, and entering or exiting the overlying gray matter. As is increasingly recognized, however, the white matter territory is neither inert nor static. It has its own microenvironment, consisting of scattered neurons, abundant glia, and blood vessels; but at the same time it is an integrated component with the much more neuron dense gray matter.

This volume brings together 10 articles that are intended to provide a summary of some of the current thinking regarding white matter organization and the myelination process, including implications for normal and abnormal brain processes. A final article (DeFelipe et al.) is organized as a series of extended extracts and commentaries of the individual articles, each of which is followed by general comments and discussion of some of the issues raised. The order of articles in the present volume parallels the order in this final article. Topics are briefly described below.

The first two articles (Suarez-Sola et al. and Friedlander and Torres-Reveron) focus on the population of neurons in the white matter (see also Clancy et al., 2010). These are identified as "interstitial neurons," although also called by other identifiers. They are heterogeneous, and are proposed in the second of these articles to subserve varying functions, undergoing temporal re-specification of function over the lifespan. In Jakovcevski et al. the authors review the complex interplay of oligodendrocyte development, the expression of myelin proteins, and the formation and restoration of myelin. Important species differences between human and rodents are discussed. A fourth article, by Jovanov-Milosevic et al., focuses on the developmental progression of axons within the human corpus callosum, at both the molecular and morphological levels.

Another cluster of articles addresses structural–functional relationships between white matter tract microstructure and cognitive abilities. Niogi et al. provide evidence for three separable attentional networks, correlated with anatomically distinguishable networks. Paus and Toro discuss anatomical changes in the white matter, particularly as these are associated with the adolescent period, and propose a model where the *g* ratio (related to the sum of the axon diameter and thickness of the myelin sheath) may be selectively impacted in large fibers, under the influence of testosterone, with pathological consequences. Kimura and Itami describe variability in axon diameter and degree of myelination, relating this to area-specific differences in conduction velocity and implications for network integration.

Finally, white matter has long been well-known to figure prominently in multiple disease states. The article by Peters brings together data on the ultrastructural changes during normal aging of myelinated axons in the macaque, treating both underlying mechanisms and functional consequences in the context of cognitive impairment. In Hoistad et al. the authors discuss several lines of research in support of the premise that dysfunction of oligodendrocytes, triggering a cascade of myelin-related malfunctions, is a critical factor in the development of schizophrenia. The article by Lee and Fields emphasizes the active intercommunication of axons and glia, the complex interplay of gene expression and neural activity, and the implications for psychiatric disorders.

In summary, these articles present a range of data and issues that are under active investigation in the field and closely related to basic issues of white matter organization in normal and pathological conditions. We hope the reader will find this collection a useful guide to both navigating the extensive background in this area of research and interpreting the exciting new results that can be anticipated.

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REVIEW ARTICLE
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Neurons in the white matter of the adult human neocortex

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The white matter (WM) of the adult human neocortex contains the so-called "interstitial neurons". They are most numerous in the superficial WM underlying the cortical gyri, and decrease in density toward the deep WM. They are morphologically heterogeneous. A subgroup of interstitial neurons display pyramidal-cell like morphologies, characterized by a polarized dendritic tree with a dominant apical dendrite, and covered with a variable number of dendritic spines. In addition, a large contingent of interstitial neurons can be classified as interneurons based on their neurochemical profile as well as on morphological criteria. WM- interneurons have multipolar or bipolar shapes and express GABA and a variety of other neuronal markers, such as calbindin and calretinin, the extracellular matrix protein reelin, or neuropeptide Y, somatostatin, and nitric oxide synthase. The heterogeneity of interstitial neurons may be relevant for the pathogenesis of Alzheimer disease and schizophrenia. Interstitial neurons are most prominent in human brain, and only rudimentary in the brain of non-primate mammals. These evolutionary differences have precluded adequate experimental work on this cell population, which is usually considered as a relict of the subplate, a transient compartment proper of development and without a known function in the adult brain. The primate-specific prominence of the subplate in late fetal stages points to an important role in the establishment of interstitial neurons. Neurons in the adult WM may be actively involved in coordinating inter-areal connectivity and regulation of blood flow. Further studies in primates will be needed to elucidate the developmental history, adult components and activities of this large neuronal system.

Keywords: subplate, calretinin, Tbr1, nitric oxide synthase, neuropeptide Y, schizophrenia

INTRODUCTION

In the human brain, the white matter (WM) underlying the cerebral neocortex is highly developed and occupies a much larger volume than in other mammals. Although the dominant components of the WM are the complex fiber tracts, their ensheathing myelin and supporting glia, there are also large numbers of neurons dispersed among the fibers, termed the "interstitial neurons" (IN). They are prominent in the primate WM, and poorly developed in the rodent. The species differences may reflect a direct correlation between the size of the cortical gray matter, the amount of WM connecting the neocortex, and the number of IN.

In human, the border between gray and white matter is sharply defined at the bottom of the sulci and along the flanks of the gyri, but more difficult to delimit at the crowns or apices of the gyri, where radial fiber fascicles intermingle with radial rows of layer VIb neurons and IN seem to be continuous with neurons of layer VIb (see Von Economo and Koskinas, 1925). The highest density of IN is in the WM immediately subjacent to the gray matter, in the zone that contains the association or "U" fibers of the cortical convolutions, and then gradually decreases with increasing distance from the gray matter. Very few neurons lie among the long fiber tracts in the deep WM, such as internal capsule, superior and inferior longitudinal fasciculi, or corpus callosum. However, there is no sharp boundary between the superficial WM, rich in IN, and the deep WM, where IN are sparse. There may also be regional differences in the density of IN, with lowest numbers in the visual cortex, and higher numbers in the frontal and prefrontal cortex (Meyer et al., 1992; Smiley et al., 1998).

The IN display a variety of morphologies ranging from pyramidal-like to bipolar and multipolar. They can be classified into the two main neuronal categories also present in the gray matter, namely excitatory glutamatergic cells and inhibitory GABAergic neurons. A previous Golgi study in the WM of adult human cortex (Meyer et al., 1992) revealed the presence of neurons with the morphology of pyramidal cells displaying apical and basal dendrites covered with dendritic spines, which may represent the excitatory component of the WM. The second category of IN corresponds to non-pyramidal neurons similar to those described in the gray matter. Unfortunately, there are only few studies on IN of the adult human cortex, and most experimental data stem from nonprimate brains. In the following sections, we summarize the available literature on IN and point out the limitations of generalizing nonprimate data on the primate, and specifically, the human brain.

The figures of this review were taken from our human brain material in the Department of Anatomy, University of La Laguna, which was obtained from autopsies under the supervision of the ethical committees of our institutions.

DEVELOPMENTAL ASPECTS OF INTERSTITIAL NEURONS

IN of the cortical WM are often referred to as "subplate" cells. During development, the subplate is a transient cell compartment just below the future layers VI-II, or "cortical plate". Birthdating studies in rodents and carnivores revealed that subplate neurons are generated at the same time as Cajal-Retzius cells in the marginal zone (or future layer I), and prior to the birth of cortical plate neurons (Chun and Shatz, 1989a; Luskin and Shatz, 1985). Subplate cells perform multiple developmental functions: they extend pioneer fibers into the internal capsule and direct thalamo-cortical pathfinding, serve as transient synaptic targets for thalamocortical fibers, and provide a substantial glutamatergic input into the maturing cortical plate, helping in the establishment of ocular dominance columns in the primary visual cortex (reviewed by Allendoerfer and Shatz, 1994; Finney et al., 1998; Friauf et al., 1990; Kanold and Shatz, 2006; Kanold et al., 2003; McConnell et al., 1989). As the cortical plate matures, many subplate neurons degenerate and undergo programmed cell death (Allendoerfer and Shatz, 1994; Kostovic and Rakic, 1990; Wahle and Meyer, 1987). The survivors continue into adult life as IN of the WM (Chun and Shatz, 1989b; Kostovic and Rakic, 1980, 1990).

Subplate neurons are morphologically and neurochemically heterogeneous. The GABAergic subpopulations may express a variety of peptides such as neuropepide Y, somatostatin and cholecystokinin, or contain nitric oxide synthase (Chun and Shatz, 1989a; Finney et al., 1998; Judas et al., 1999; Meyer et al., 1992; Torres-Reveron and Friedlander, 2007; Uylings and Delalle, 1997; Wahle and Meyer, 1987; Wahle et al., 1987). It is not known if developmental cell death affects specific cell classes within the subplate, or whether all subpopulations are equally reduced.

To what extent is the subplate of rodents and carnivores comparable to the human subplate? In human fetuses, an initial cell condensation, the "pioneer plate", appears at 7/8 gestational weeks (GW) and is almost immediately split into superficial and deep pioneer neurons by the arrival of the first cortical plate cohorts at 8/9 GW (Meyer et al., 2000). The deep pioneer cells form the "presubplate" (Kostovic and Rakic, 1990; Meyer et al., 2000). The subplate zone proper becomes visible around 14/15 GW as a cell-poor/fiber-rich layer situated between the intermediate zone and the cortical plate. It reaches maximal width and highest cellularity from 22–36 GW, when it is four times thicker than the cortical plate. Thereafter, the subplate gradually decreases in size and becomes unrecognizable around the sixth postnatal month (Kostovic and Rakic, 1990). NPY-immunoreactive neurons attributed to the subplate appear around 14 GW in the subplate and decrease in number by the end of gestation (Bayatti et al., 2008; Uylings and Delalle, 1997). A similar time course of subplate development has been described in the monkey (Smart et al., 2002), showing that the subplate develops differently in nonprimate and primate species.

Although human subplate neurons are heterogeneous, a useful marker of the glutamatergic component is the putative transcription factor T-brain-1 (Tbr1) (Bayatti et al., 2008; Hevner et al., 2001; Kolk et al., 2005). The chronology of Tbr1 expression in human fetuses can be traced to the early cortical plate at 10 GW, which is strongly Tbr1+ (**Figure 1A**). From 14 to 25 GW, large numbers of Tbr1+ neurons are continuously added to the subplate compartment, which increases in width concurrent with the growth of the cortical plate, although the highest density is always at the border between cortical plate and subplate (**Figures 1B,C**) (Meyer, 2007). In perinatal brains, Tbr1-immunoreactivity changes from a nuclear to a cytoplasmic staining that is widely distributed in neurons in the cortical gray and white matter, and thus no longer useful as a marker molecule of the subplate. In the absence of molecules specific for the human subplate it is difficult to ascertain how many subplate cells survive as IN.

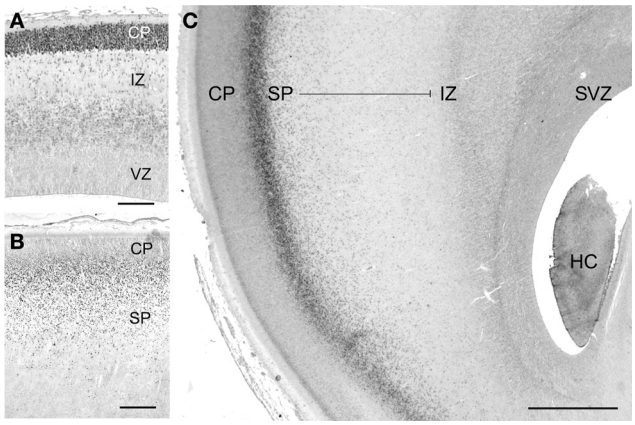

FIGURE 1 | Tbr1 marks glutamatergic neurons in the human subplate (SP). (A) At 10 gestational weeks (GW), highest Tbr1 staining is in the early cortical plate (CP). **(B)** At 16 GW, Tbr1+ cells are concentrated along the CP/SP border and distributed all over the SP. **(C)** At 25 GW, the SP, here in the temporal lobe, has increased in width and is filled with Tbr1+ neurons extending from the intermediate zone (IZ) to the deep CP border. The growth of the SP despite the expansion of the cortical wall indicates the continuous addition of new Tbr1+ neurons. HC, hippocampus; SVZ, subventricular zone; VZ, ventricular zone. Scale bar in **(A)** 100 μm, in **(B)** 200 μm, in **(C)** 1 mm.

Altogether, these data show that the IN of the human WM are not identical to the early-born subplate neurons described in rodents and cat. Rather, the cell populations in the maturing WM seem to be complemented by newly arriving neurons generated at much later stages of corticogenesis. A possible explanation for the discrepancy across species may be the extraordinary increase in cortical connectivity during evolution, which leads to an increase in size and complexity of the WM in the primate brain. In parallel to the increase of the WM compartment, a continuous supply of IN may be required during the whole period of corticogenesis. This implies that primate IN are not just incidental remnants of early-born neurons, but rather seem to belong to a distinct neuronal system that is intimately connected to the WM and may carry out activities pertinent to this location. Further studies are necessary to define the developmental origins and possible functions of IN in the adult WM of the primate cortex.

CALCIUM-BINDING PROTEINS IN INTERSTITIAL NEURONS

The GABAergic interneurons of the cortical gray matter are highly diverse, and many attempts have been undertaken to classify them according to a variety of morphological and neurochemical properties (Ascoli et al., 2008). A recent inventory of mouse cortical interneurons has led to the identification of 13 cell classes based on the combined expression of the calcium-binding proteins calbindin (CB), calretinin (CR) and parvalbumin (PV), and neuropetides,

such as vasointestinal polypeptide, NPY, cholecystokinin, somatostatin and cholinacetyltransferase (Gonchar et al., 2008). Of the three calcium-binding proteins present in the cortical gray matter, only CB and CR are expressed in IN. PV+ cells are the largest group of cortical interneurons which includes basket cells and chandelier cells (De Felipe, 2002). PV is not found in the adult human WM, and the few deep PV+ neurons occasionally found below the gray matter are more likely to represent displaced layer VI neurons. CB+ IN have been reported in the WM (Yan et al., 1996); they are concentrated in the superficial WM, often aligned along the gray/WM border and most numerous in the apex regions. They are small to medium size, have bipolar or multipolar dendritic trees (**Figures 2C,D**), and are rare in the deep WM.

CR is abundant in gray-matter interneurons mostly of supragranular layers (Gonchar et al., 2008), but its presence in the WM has not attracted much attention. This is surprising insofar as CR+ IN are the most prominent cell population in the superficial and deep adult human WM (**Figures 2A and 3**). They have diverse sizes, ranging from small to large somata, and bipolar or multipolar dendritic trees, regardless of their position in the superficial or deep WM. Small CR+ IN occur also in the internal capsule, and even in the periventricular WM of the temporal horn of the lateral ventricle. Here they form conspicuous cell clusters that give rise to local plexuses of varicose fibers, which often surround blood vessels or follow the vascular wall (**Figures 3A,B**). The small, dense fiber plexus, mostly restricted to the cluster and with few fibers spreading into the adjacent WM, suggests synaptic contacts between neighboring cells (**Figure 3C**). This staining was not observed with other markers and seems to be unique to CR.

The distribution and relative prominence of interneurons expressing calcium-binding proteins are species and area-dependent (Hof and Sherwood, 2005). CR+ cells seem to be more prominent in primate than in rodent cortex, not only in the gray matter but also in the WM. In the mouse, CR+ interneurons derive from the caudal ganglionic eminence and migrate tangentially all over the cortex (Xu et al., 2004). By contrast, primate interneurons have a double origin, with early-born cells migrating from ganglionic eminences, and later-born cells deriving from the subventricular zone (SVZ) of the cortical wall (Letinic et al., 2002; Petanjek et al., 2009). In particular, CR+ cells are very prominent in the SVZ and deep WM during late human fetal development. Characteristic clusters of doublecortin/CR+ neurons in the SVZ were interpreted as locally born interneurons destined for the superficial cortical layers (Meyer et al., 2002). The CR+ cell clusters in the adult WM suggest that not all of these cells migrate into the gray matter but may give rise to resident cells of the WM, or stay close to their place of origin. In any case, the CR+ IN in the deep WM are not derivatives of an early generated subplate, but rather late additions at a time when the fiber fascicles of the WM mature and may need positional cues. Further studies of this cell population may be interesting, particularly with regard to neuropathological alterations.

NITRIC OXIDE SYNTHASE AND NEUROPEPTIDES IN INTERSTITIAL NEURONS

Nitric oxide (NO) is a gaseous messenger molecule synthetized by several isoforms of the enzyme nitric oxide synthase (NOS). In the brain, two NOS forms are constitutively expressed, nNOS in neurons, and eNOS in endothelial cells. Activation of nNOS and

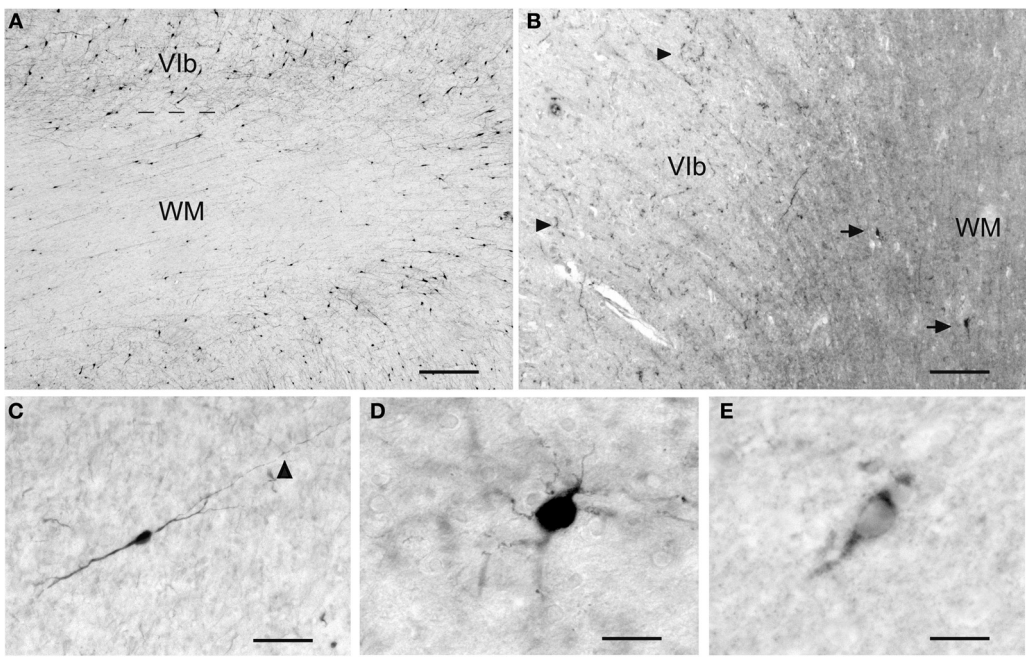

FIGURE 2 | The variety of IN in the adult human WM. (A) CR+ IN are the most abundant cell class in the superficial WM. **(B)** NPY+ IN (arrows) in the superficial WM. They may be the origin of the fiber terminals in layer VI, indicated by arrowheads. **(C)** A bipolar CB+ IN in the deep WM. The arrowhead points to an axonal ramification site. **(D)** A multipolar CB+ IN in the superficial WM. **(E)** A Reelin+ IN in the superficial WM. Scale bar in **(A)** 200 μm, in **(B)** 150 μm, in **(C)** 30 μm, in **(D)** 20 μm, in **(E)** 15 μm.

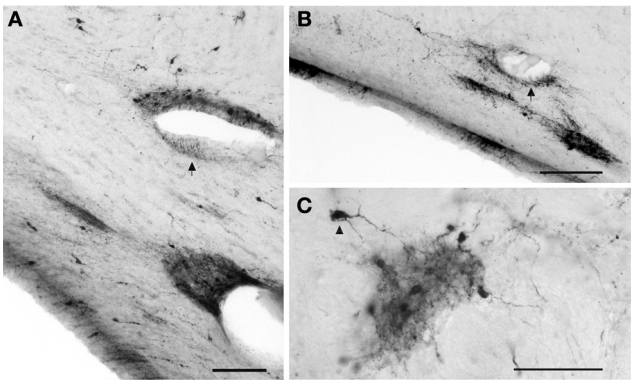

FIGURE 3 | CR+ neurons in the deep WM of the temporal lobe. (A,B) Clusters of small neurons are concentrated near blood vessels. The arrows point to fibers running along the vascular wall. **(C)** Cluster in the deep WM. The dense local axonal plexus is mostly confined to the cluster, with few fibers extending into the neighboring WM. The arrowhead points to an axon contacting a soma. Scale bar in **(A)** 50 µm, in **(B)** 100 µm, in **(C)** 50 µm.

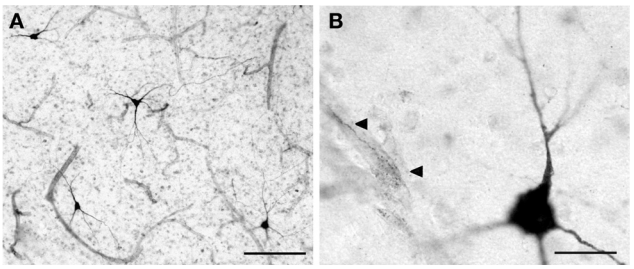

FIGURE 4 | (A) Type 1 NADPH-d+ neurons in the superficial WM. **(B)** Magnified view of a type 1 neuron near a microvessel that is contacted by NADPH-d+ axonal fibers (arrowheads). Scale bar in **(A)** 150 µm; in **(B)** 20 µm.

eNOS requires the influx of calcium ions, usually upon the activation of glutamate NMDA-receptors, and the presence of nicotinamide adenine dinucleotide phosphate (NADPH) as a co-substrate. Nitrergic, i.e. NO-producing neurons, can be visualized by NADPH-diaphorase histochemistry, as well as by immunohistochemistry using anti-nNOS antibodies (Bredt et al., 1991; Estrada and De Felipe, 1998; Hope et al., 1991; Vincent and Kimura, 1992). Due to its high diffusibility and short half-life, NO is associated with many diverse functions, such as cerebrovascular coupling, neurotransmission, neuronal survival and death, memory, and synaptic plasticity (reviewed by Calabrese et al., 2007; Garthwaite, 2008).

NADPH-d neurons in the cerebral cortex have been studied extensively in a variety of species from rodent to human (Barone and Kennedy, 2000; Estrada and De Felipe, 1998; Garbossa et al., 2005; Judas et al., 1999; Smiley et al., 1998; Yan et al., 1996). There are two main cell classes: Type 1 NADPH-d neurons are intensely stained in a Golgi-like fashion, displaying medium size to large somata and long varicose processes (**Figure 4A**). Most type 1 neurons are in the superficial WM, whereas type 2 NADPH-d neurons are restricted to the cortical gray matter. Type 2 neurons are only lightly stained and have small somata and short processes (Barone and Kennedy, 2000; Sandell, 1986). Both types express GABA, and a 4% of type 1 neurons co-express CB (Yan et al., 1996). Type 1 neurons can also express neuropeptide Y (**Figure 2B**) and somatostatin (Vincent et al., 1983). Although most GABAergic neurons are interneurons with local axons, some NADPH-d/nNOS+ neurons in the WM of rat, cat and monkey project over long distances to distant, functionally unrelated cortical areas (Higo et al., 2007; Meyer et al., 1991; Tomioka and Rockland, 2007).

One of the most interesting features of the type 1 neurons is their close association with blood vessels. Their axonal plexuses form a dense network around microvessels (**Figure 4B**), and their long processes may contact distant arterioles and capillaries (Estrada and De Felipe, 1998; Estrada et al., 1993; Iadecola et al., 1993; Yan et al., 1996). Since NO is a potent vasodilator, NOS-containing neurons are thought to be involved in the coupling of metabolic changes related to neuronal function with local increases in blood flow. Due to their strategic location just below the cortical gray matter, NOS+ IN may be contacted by corticopetal fibers and, in response, act on neighboring microvessels. On the other hand, NPY is a powerful vasoconstrictor able to antagonize the vasodilating effect of NO (Abounader and Hamel, 1997; Cauli et al., 2004) that co-localizes with NOS in a subset of IN. Somatostatin and NPY (**Figure 3B**) are expressed in IN of the superficial WM. They act directly on smooth muscle cells of cortical arterioles, and may thus constrict cortical microvessels in an activity-dependent manner (Cauli et al., 2004). A possible mechanism of the combined activity of NO and NPY in the same neuron has been proposed by Estrada and De Felipe (1998): A stimulated type 1 NADPH-d+ neuron might release NO in a diffuse way in the vicinity of the soma and main processes and increase local flow, whereas axonal branches of the same neurons ramify around more distant microvessels and release NPY, producing a spatially restricted vasoconstriction. The NOS/NPY+ IN thus form part of the neural system involved in the coupling of cortical microvessels to neuronal activity. The neurovascular interactions leading to the hemodynamic changes during enhanced or decreased cortical activity are the basis of functional neuroimaging using positron-emission tomography (PET) and functional magnetic resonance imaging (fMRI). The blood oxygen level-dependent (BOLD) signal reflects the hemodynamic response coupled to neural signalling processes (for review, see Attwell and Iadecola, 2002; Logothetis and Wandell, 2004; Ogawa et al., 1990). The NOS+ IN in the cortical WM are an important component of the vasoactive pathways which also include subcortical cholinergic and serotoninergic systems. In fact, most NOS-expressing IN are cholinoceptive, meaning that they receive cholinergic fibers from the nucleus basalis (Kocharyan et al., 2008; Smiley et al., 1998). Since the axons of NOS+ IN may spread over considerable distances into the cortical gray matter, a single IN may coordinate local blood flow in neighboring and distant cortical areas in response to corticopetal and corticofugal activation.

INTERSTITIAL NEURONS IN BRAIN PATHOLOGY

The subcortical WM and its resident IN have been associated with a variety of neurological and psychiatric disorders. Alterations of somatostatin, NPY and/or NADPH-d+ IN were observed in Alzheimer disease (e.g. Kowall and Beal, 1988; Tao et al., 1999; Van de Nes et al., 2002). However, schizophrenia is the disease

which seems to show the most dramatic abnormalities of the WM. Diffusion tensor imaging revealed disturbances of myelin function and distribution, alterations of connectivity and integrity of fiber tracts such as the cingulate bundle and uncinate fasciculus, with a higher incidence in the frontal lobes, middle temporal structures including hippocampus and amygdala, and superior temporal gyrus, as well as in subcortical centers (reviewed by Kubicki et al., 2007; Kyriakopoulos et al., 2008).

Schizophrenia also affects the IN in diverse ways. In the frontal lobe of schizophrenic patients, the IN density was decreased in the superficial WM, but increased in the deeper WM, with NADPH-d+ IN showing the same maldistribution as microtubule associated protein 2 (MAP2) positive cells in general (Akbarian et al., 1993, 1996). While some studies reported an increase in IN density in inferior parietal and dorsolateral prefrontal areas in deficit syndrome patients (Kirkpatrick et al., 1999, 2003), others observed no change neither in superficial nor in deep white matter (Beasley et al., 2002). The conflicting reports on the changes of IN density in schizophrenia were summarized by Eastwood and Harrison (2005), who observed a density increase in the superficial WM and no change in deeper compartments.

A special and rather minor subclass of IN expresses the extracellular matrix molecule Reelin, which is important for brain development and adult neuronal plasticity (reviewed by Herz and Chen, 2006; Tissir and Goffinet, 2003). In the adult cortical gray matter, Reelin is expressed by a subgroup of GABAergic interneurons (Pesold et al., 1998); in the WM, very few scattered Reelin+ cells can be visualized using immunohistochemistry (**Figure 2E**). Conversely, Reelin mRNA has been reported to be abundant in IN in the superior temporal cortex, and to be significantly reduced in schizophrenic patients (Eastwood and Harrison, 2003), in keeping with the finding that alterations of Reelin expression are a putative vulnerability factor in schizophrenia and mood disorders (reviewed by Fatemi et al., 2008).

Most discussions of IN changes in psychoses are based on the view that IN are remnants of the early-born subplate population. The early generation of IN in rodents and carnivores, and their maldistribution in schizophrenic patients, have led to the hypothesis that a migration defect of the subplate during embryonic or early fetal development underlies the pathogenesis of schizophrenia. As stated above, the developmental history of the subplate is very different in nonprimate mammals and in primates including human (Kostovic and Rakic, 1980; Meyer, 2007; Meyer et al., 2000; Smart et al., 2002). An important task for future research would be a molecular taxonomy of all neuronal populations in the WM, similar to the work done on interneurons in the gray matter. It would be particularly important to differentiate between early-born components, probably related to transient roles of the subplate, and later-appearing resident cells, which may not be important for development but rather involved in activities proper to the adult WM. The recent discovery of subplate-specific molecules in mice (Hoerder-Suabedissen et al., 2008) is a useful step in this direction, and it is hoped that similar work will shed light on the origins, categories and functional roles of human IN.

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The changing roles of neurons in the cortical subplate

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Neurons may serve different functions over the course of an organism's life. Recent evidence suggests that cortical subplate (SP) neurons including those that reside in the white matter may perform longitudinal multi-tasking at different stages of development. These cells play a key role in early cortical development in coordinating thalamocortical reciprocal innervation. At later stages of development, they become integrated within the cortical microcircuitry. This type of longitudinal multi-tasking can enhance the capacity for information processing by populations of cells serving different functions over the lifespan. Subplate cells are initially derived when cells from the ventricular zone underlying the cortex migrate to the cortical preplate that is subsequently split by the differentiating neurons of the cortical plate with some neurons locating in the marginal zone and others settling below in the SP. While the cortical plate neurons form most of the cortical layers (layers 2–6), the marginal zone neurons form layer 1 and the SP neurons become interstitial cells of the white matter as well as forming a compact sublayer along the bottom of layer 6. After serving as transient innervation targets for thalamocortical axons, most of these cells die and layer 4 neurons become innervated by thalamic axons. However, 10–20% survives, remaining into adulthood along the bottom of layer 6 and as a scattered population of interstitial neurons in the white matter. Surviving SP cells' axons project throughout the overlying laminae, reaching layer 1 and issuing axon collaterals within white matter and in lower layer 6. This suggests that they participate in local synaptic networks, as well. Moreover, they receive excitatory and inhibitory synaptic inputs, potentially monitoring outputs from axon collaterals of cortical efferents, from cortical afferents and/or from each other. We explore our understanding of the functional connectivity of these cells at different stages of development.

Keywords: subplate, white matter, cortex, subgriseal, longitudinal multi-tasking

PROPOSAL OF RE-SPECIFICATION OF NEURONAL PROPERTIES DURING DEVELOPMENT

We propose that certain types of neurons can undergo a temporal re-specification of function over the lifespan. Specifically, we suggest that the population of cortical subplate (SP) neurons does so although it is not known whether individual neurons in that cohort of cells change their function or if the surviving SP cells represent a sub-population that has different functions at different stages of the life cycle. Although we propose a long term type of multi-tasking over the lifespan, there may be other types of neuronal multi-tasking operating over shorter time scales. For example, within minutes, as information is being processed, neuromodulators and recent bouts of activity could unmask emergent functional properties such as regulation of gene expression leading to differential functions of individual neurons within the neuronal circuit within which the cell is embedded. Recording of electrical activity from large populations of interacting neurons will be required while following individual neurons' activity profiles for extended periods with tetrode arrays (Schmitzer-Torbert et al., 2005) to directly test these ideas. Current technology can apply *in vivo* optical monitoring of the dynamics of the structure of dendrites, spines and axons although this approach is generally limited to superficial cortical layers (Kerr et al., 2007). New advances in imaging technology will be required for similar tracking of deep cells such as SP neurons during development. It will also be of interest to evaluate whether changes in neuronal function occur over the course of aging. For example, the neocortex shrinks during normal aging due primarily to atrophy of cells and the neuropile (vs. neuronal loss – Freeman et al., 2008). Future studies of individual cellular and neuronal network function during aging may reveal other examples of serial neuronal multi-tasking.

Differential expression of genes plays a major role in neuronal development and functional differentiation not only from early embryonic stages (Hevner, 2006; Mallamaci and Stoykova, 2006; Mehler and Mattick, 2007; Taniura et al., 2007; Webster et al., 2006; Zimmermann, 2006) but also into senescence (Burger et al., 2007; Chu et al., 2002; Liu et al., 2009). These changes can be programmed to occur at defined stages or can be triggered by local signals, by environmental inputs or in a neuronal activity-regulated manner. Such temporally modulated regulation of gene expression can play a role in target recognition and path-finding, synaptogenesis, refinement of synaptic connections (Waites et al., 2005) as well as in cell death (Jansen et al., 2007; Lindsten et al., 2005). In addition, other influences such as sensory or motor activity, cognition, stress, infectious agents and traumatic events can alter gene expression patterns in the brain throughout life (Alfonso et al., 2005; Licino et al., 2007; McClung and Nestler, 2008).

However, after differentiation to a particular phenotype, little change is thought to occur in each neuron's fundamental properties such as their anatomical projections, location, position, chemical neurotransmitter, and the functions of the cell within the framework of the particular network where it resides. For example, a glutamatergic cortico-thalamic neuron that projects from layer 6 of the primary visual cortex to the dorsal lateral geniculate nucleus may alter composition and properties of its ion channels and neurotransmitter receptor subunits over the course of development but remain essentially the same cell "type" – an excitatory feedback visual relay pyramidal neuron processing visual information with particular receptive field properties that innervates the LGN and cortical layer 4.

EARLY ROLE OF SUBPLATE NEURONS IN CORTICAL DEVELOPMENT

Pleiotropy (the ability of a single gene to influence multiple phenotypic traits) is well established (Fraser and Marcotte, 2004). Neurons can express pleiotropic genes or respond to pleiotropic gene products at different times throughout an organism's life, potentially increasing information processing ability longitudinally and responding to stimuli and stressors (de Magalhães and Sandberg, 2005; Louvi et al., 2004; Nelson et al., 2006; Russo et al., 2005). Like genes, whole neurons could increase their information processing contribution combinatorially by serving different functions over the course of the lifespan – a pleiotropy of cellular function in the temporal domain. The neurons of the cortical SP are one candidate population of cells that may behave in this manner. The SP cells emerge from the ventricular zone under the cerebral cortex, migrating below the marginal zone to the cortical preplate (Stewart and Pearlman, 1987) that is then split by the differentiating neurons of the cortical plate – some neurons taking up residence in the marginal zone and others settling below the cortical plate in the SP (König et al., 1981; Luskin and Shatz, 1985a,b; Marin-Padilla, 1971, 1978). The cortical plate neurons form most of the cortical layers (layers 2–6) while the marginal zone neurons become layer 1 and the SP neurons become interstitial cell of the cortical white matter as well as clustering at the bottom of the cortical plate just below layer 6 (layer 6b – DeDiego et al., 1994; Marin-Padilla and Marin-Padilla, 1982; Valverde et al., 1989; Woo et al., 1991). These SP cells are among the first cortical neurons to differentiate into a neuronal phenotype; they express microtubule associated protein-2 and neuropeptides before the cortical plate neurons (Arias et al., 2002; Clancy et al., 2001; Finney et al., 1998; Luskin and Shatz, 1985), they receive synaptic inputs and generate action potentials through embryonic development (Hanganu et al., 2001; Kanold, 2004; Kanold et al., 2003). These cells also serve as pioneers issuing axons into the internal capsule where they serve an important role by innervating the thalamus and providing a scaffold for the innervation of the cortex by the thalamocortical axons (Allendoerfer and Shatz, 1994; Friauf et al., 1990; Ghosh et al., 1990; Herrmann et al., 1994; Kanold et al., 2003; McConnell et al., 1989). The SP neurons are also transiently innervated by the in growing thalamocortical axons before the eventual thalamocortical target neurons within cortical layer 4 settle in their ultimate positions in the cortical plate to receive their innervation. Layer 4 neurons receive innervation by both SP neurons and thalamic axons during this period (Kanold, 2004; Kanold et al., 2003) followed by removal of inputs from the SP cells through a competitive process (Friauf et al., 1990; Kanold et al., 2003). If the SP cells are lesioned, the thalamic axons fail to innervate their correct target areas (Chun and Shatz, 1989; McConnell et al., 1989, 1994) and cortical columnar organization does not develop normally (Kanold et al., 2003). Thus, these neurons contribute to establishing functional cortical architecture during development. After performing those functions, most of these cells die (Al-Ghoul and Miller, 1989; Chun and Shatz, 1989; Wood et al., 1992).

SURVIVING SUBPLATE NEURONS

The SP neurons appear to play an important but fleeting role in orchestrating early cortical development. However, although most of these cells die soon after the innervation of the cortical plate by thalamic axons and the retraction of the SP neurons' axons that innervate layer 4, many of them (10–20%) survive (Chun and Shatz, 1989; Torres-Reveron and Friedlander, 2007). These cells remain throughout development into adulthood as a compressed band along the bottom of layer 6 (cortical layer 6b or cortical layer 7 or subgriseal cells – Valverde et al., 1989; Vandevelde et al., 1996; Reep and Goodwin, 1988; Clancy and Caullier, 1999) and as dispersed interstitial neurons scattered in the white matter. It is a matter of considerable interest to know the fate of this group of surviving cells – are they quiescent, do they serve a role in guidance in the postnatal brain as they did prenatally or do they take on an entirely new function? If they change their function and/or connectivity, this suggests a form of temporal pleiotropy for these cells. As these SP cells are greatly reduced in number during development, it is possible that they serve no major functional role after this period. However, this seems unlikely and there are other examples of numerically small neuronal types that contribute in important ways through processes such as numerical expansion of target innervation by axonal and synaptic divergence (retinogeniculocortical Y-cells – Friedlander and Martin, 1989; Friedlander et al., 1985); strategically positioned or particularly strong synaptic outputs (climbing fibers – Shinoda et al., 2000); or potent neuromodulatory outputs (Landgraf and Neumann, 2004; Rygh et al., 2006). Thus, the fact that many of these cells are lost during development should not exclude the possibility that the remaining population of these cells, although relatively small in sheer number may play some additional important role in cortical information processing. In order to evaluate such a hypothesis, it is necessary to directly evaluate the anatomical and electrophysiological properties of this reduced cohort after their initial role in cortical development and after the elimination of the majority of cells have occurred.

Figure 1 illustrates an example of a surviving white matter neuron located below the primary visual cortex of a postnatal day 20 rat that has been patched in a brain slice preparation with a biocytin filled micropipette and subsequently processed for biocytin and stained with cresyl violet.

Note that the cell's dendrites are oriented along the white matter below layer 6. An example of a surviving SP neuron located along the bottom of layer 6 that was also patched in a brain slice preparation and filled with biocytin is shown in **Figure 2**. The inset illustrates that the cell responds to a sustained direct depolarizing input with a non-decrementing train of action potentials similar to cortical interneurons.

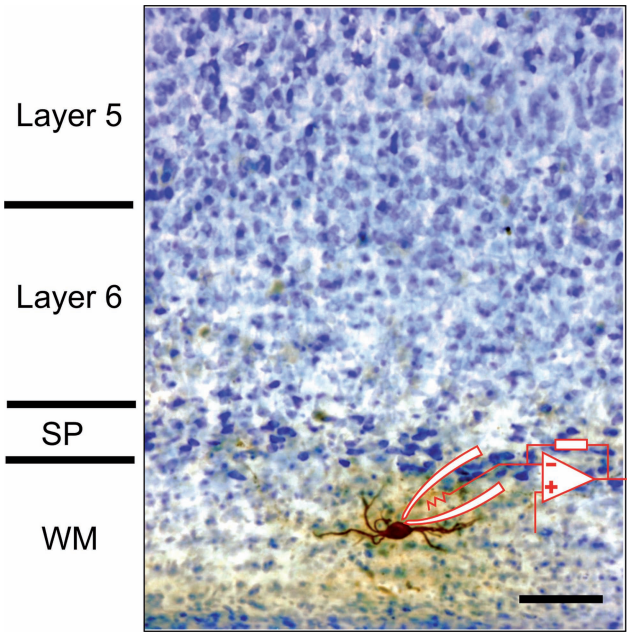

FIGURE 1 | Photomicrograph of biocytin filled white matter neuron in slice preparation from rat visual cortex. Patch pipette and recording arrangement are schematized. Tissue was counter-stained with cresyl violet after biocytin processing. Scale bar = 50 μm.

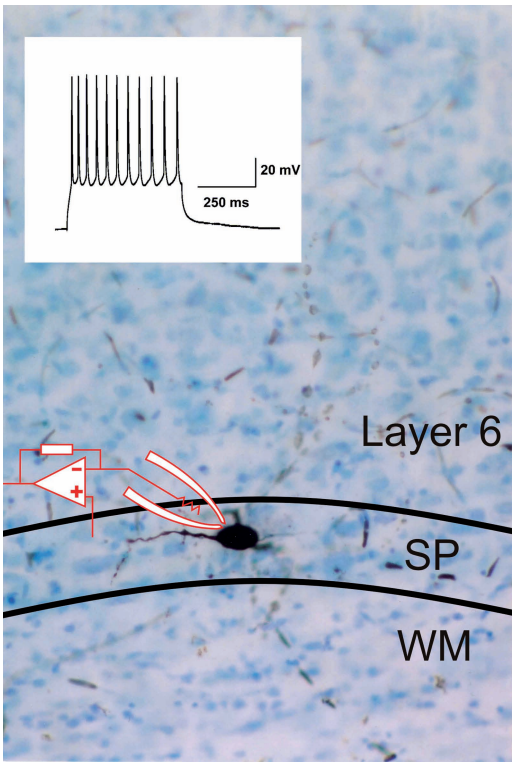

FIGURE 2 | Photomicrograph of biocytin filled subplate neuron in the subplate (SP) area at the bottom of layer 6, also from rat visual cortex acute brain slice preparation. Some of the cell's dendrites can be seen extending into layer 6. The inset shows an electrophysiological response of the cell recorded under current clamp with whole cell patch recording in response to a sustained depolarization, producing a non-decrementing train of action potentials.

MORPHOLOGY AND NITRIC OXIDE SYNTHASE

SP cells have been shown to be particularly susceptible to or play a role in the pathogenesis of disorders including early neonatal hypoxic-ischemic injury (McQuillen et al., 2003), trisomies (Cheng et al., 2004), microcephaly (Takano et al., 2006) and seizures (Kadam and Dudek, 2007). Although their potential role in such diseases has been studied, there are few studies of the functional properties of the surviving SP neuronal population in the normal brain likely due to their sparseness and location, making such studies difficult. These surviving cells express markers typical of neurons including MAP-2 and NeuN (Clancy et al., 2001; Torres-Reveron and Friedlander, 2007) and many express the synthetic enzyme for the production of nitric oxide (NO), nitric oxide synthase (NOS) that can be visualized as NADPH diaphorase (NADPHd) activity. This is illustrated in **Figure 3** where white matter neurons from human (**Figures 3A,B**) and rat brain (**Figures 3C,D**) that are positive for NADPHd are shown in tissue from young and mature brains. Human cortical tissue was obtained from resections from patients for treatment of epilepsy at the University of Alabama at Birmingham Hospital under an IRB protocol for utilization of waste tissue for histological processing and electrophysiology.

Note that not only the somata and dendrites are positive for NADPHd but that there is also considerable staining of fine processes and varicosities, suggesting the possibility that these cells may provide a diffusible signal (NO) in the white matter that could play a role in plasticity and/or pathogenesis (Garthwaite, 2008).

Retrogradely transported tracers applied to the surface of the cortex (layer 1) backfill surviving WM and SP neurons' somata, indicating that their axons reach the cortical surface (Clancy and Caullier, 1999). This projection as well as the presence of boutons on their axons in other cortical layers have been demonstrated for individual surviving white matter and SP neurons where their axonal arborizations are visualized by intracellular single cell filling (Clancy et al., 2001). An example of a SP neuron whose axon projects upward through the visual cortical layers is illustrated in **Figure 4**. Although SP and WM cells are not numerous, their axonal arborizations can be expansive, covering span up to a millimeter of cortex in the medio-lateral axis (Clancy et al., 2001). These neurons also issue axon collaterals within the white matter and deep layer 6, providing the neuroanatomical substrate for them to play a role in a local functional neuronal network.

FUNCTIONAL PROPERTIES, CONNECTIVITY AND NEUROMODULATORY PHENOTYPES

Surviving SP neurons generate action potentials; they receive both excitatory and inhibitory synaptic inputs; and they respond to sustained membrane depolarization with minimal spike frequency adaptation (Torres-Reveron and Friedlander, 2007). Thus, these cells retain a neuronal phenotype, they receive synaptic inputs from other neurons and they innervate the various cortical layers. We have also recently found (Torres-Reveron and Friedlander, 2005) that these cells provide glutamatergic excitatory synaptic inputs to neurons in cortical layer 6. An example is illustrated (**Figure 5A**) as recordings

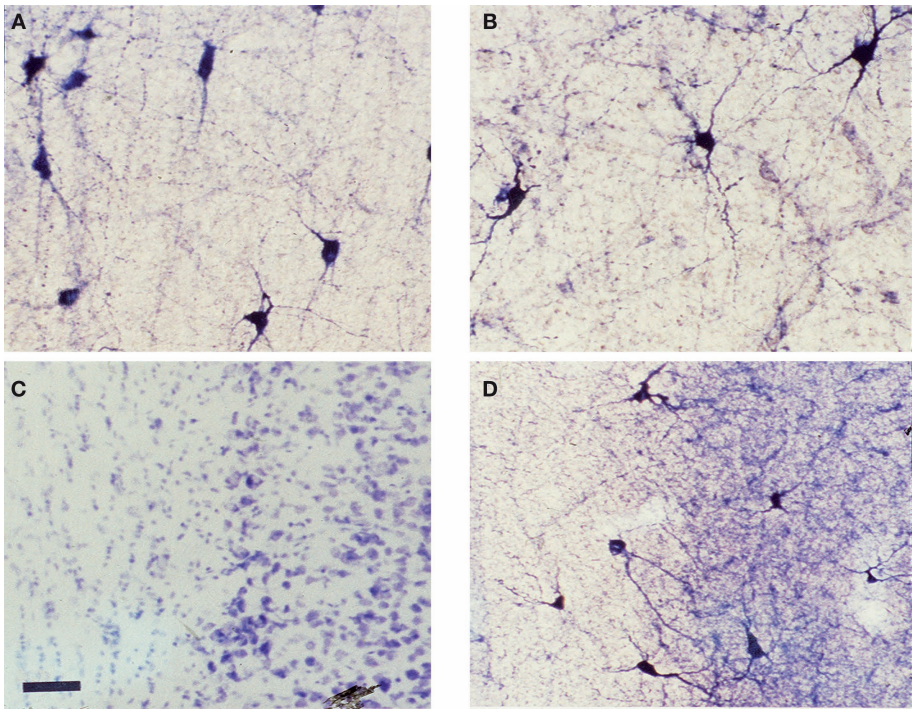

FIGURE 3 | NOS positive neurons in WM and SP. (A,B) Photomicrographs of white matter and subplate area in visual cortex from tissue obtained from human brain (after tissue resection for epilepsy surgery) from a young (3 years of age) and an older (40 years of age) subject and **(C,D)** from adult rat (42 days postnatal) visual cortex where tissue has been processed for NADPH diaphorase histochemistry. All photomicrographs are oriented with the cortical surface to the right. The human tissue shows numerous white matter neurons that are NADPHd positive in the white matter and considerable staining of processes, as well. The rat tissue sections illustrate a standard Nissl stain **(C)** where the subplate neurons can be visualized as a compressed layer at the bottom of layer 6 and the white matter is seen below. The section in **(D)** (an adjacent section) has been processed for NADPHd histochemistry – several NADPHd positive neurons can be seen in the white matter and in the subplate subgriseal area at the bottom of layer 6. Scale bar = 50 μm and applies to all four panels.

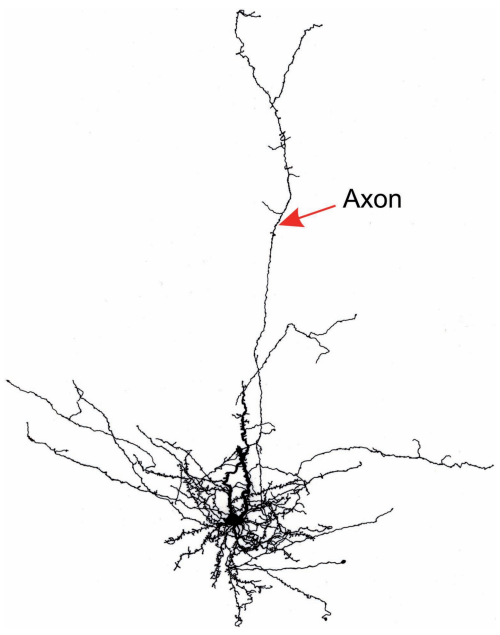

FIGURE 4 | Line drawing of a rat subplate neuron that has been filled with biocytin in a brain slice experiment. The cell's dendrites cluster around the soma at the base of layer 6 but its axon extends vertically into the cortical plate.

from a pair of synaptically connected SP – layer 6 cells recorded in dual whole cell patch clamp mode. In this case, a SP neuron at the bottom of layer 6 was patched and individual spikes were elicited every 10 s while several putative postsynaptic neurons in layer 5 and layer 6 were also patched and tested for the presence of evoked unitary postsynaptic responses. The layer 6 neuron responded with small unitary EPSPs (EPSPs evoked from a single action potential in a single presynaptic neuron) that can be seen in individual trails and as an averaged response in **Figure 5B**. Thus, surviving SP neurons not only are positioned to receive inputs from either axon collaterals of supra- or infragranular cortical projection neurons, from cortical afferents and from each other but some of them also provide excitatory synaptic input to the overlying cortical plate neurons. Interestingly, GABAergic white matter neurons with projection axons have been identified in primates (Tomioka and Rockland, 2007) and we have seen a subset of GABAergic WM and SP neurons in rat using immunohistochemistry, although we have yet to record from an identified surviving presynaptic GABAergic neuron. In addition to having fast glutamatergic excitatory synaptic output, these cells also stain positively for various neuromodulators including substance P, CCK, somatostatin and NOS. Sections that have been immuno-stained for these various substances are illustrated in **Figure 6**. The diversity of secreted chemical that these surviving cells contain together with their capacity to maintain protracted non-decrementing trains of action potentials in response to a sustained depolarizing drive may afford these surviving neurons

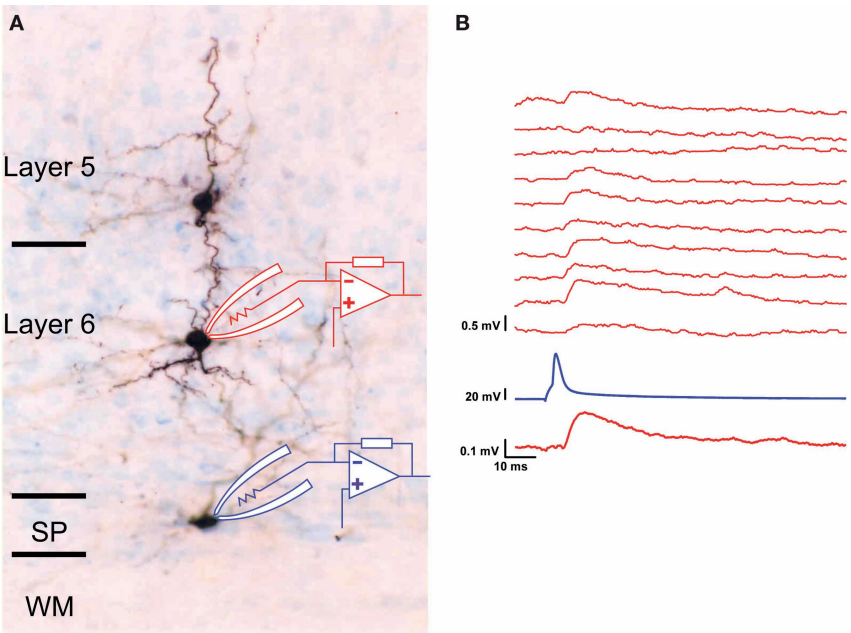

FIGURE 5 | Functional synaptic connections between SP and layer 6 neurons. (A) Photomicrograph of the brain slice preparation where three cells were patched, recorded and tested for synaptic interactions. The lower cell located in the subgriseal area (SG) of the subplate at the bottom of layer 6 functionally innervated the layer 6 neuron above it (but not the layer 5 neuron above that). **(B)** The functional synaptic connection was ascertained through dual whole cell patch clamp electrophysiological recording where a single action potential was evoked every 10 s in the subplate neuron (trace second from bottom) while the evoked unitary synaptic responses were recorded under current clamp conditions from the layer 6 cell (10 individual trials shown in top traces). Note that on some trials, there was no detectable response (an apparent transmission failure) while in most trials, a small depolarizing postsynaptic response was evoked. The resting membrane potential of the layer 6 neuron was –70 mV.

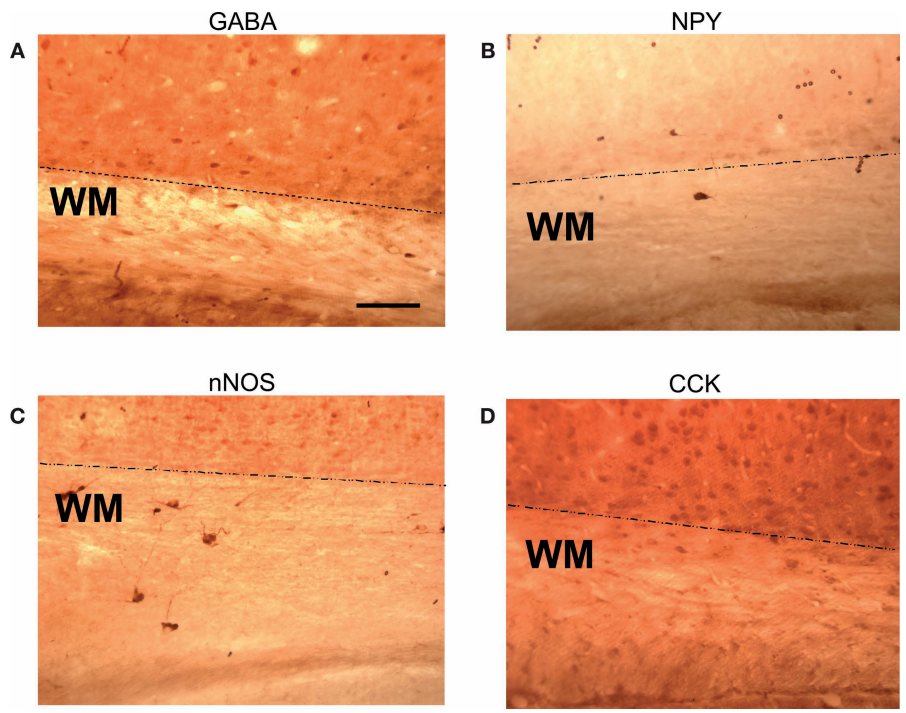

FIGURE 6 | WM and SP neuron immuno-positivity for four neuromodulatory compounds. Four sections of rat visual cortex (postnatal day 12–14) that were immuno-stained for various neuromodulators. **(A)** anti-GABA; **(B)** anti-NPY; **(C)** anti-neuronal (type 1) nitric oxide synthase (NOS); **(D)** anti-cholycystekinin (CCK). The dotted lines indicate the lower boundary of the subplate and the beginning of the white matter proper.

the capacity to provide strong neuromodulatory effects to cells in the overlying cortex.

POTENTIAL ROLE FOR SP NEURONS FOR INFORMATION PROCESSING IN THE MATURE CORTEX

That these cells remain as neurons but also have the capacity to play different roles at different stages of development is suggested by several factors. These include the persistence of intrinsic electrophysiological and synaptic properties, survival of the glutamatergic phenotype, receipt of excitatory and inhibitory synaptic inputs from other sources after the loss of their thalamocortical inputs and the re-arrangement of their axonal outputs from transiently innervating layer 4 to innervating all cortical layers. Much of the information about the properties of these surviving cells must, by necessity be obtained from *in vitro* brain slice preparations so there is little known about their properties within the circuitry of the intact brain. While their basic electrophysiological properties can be studied in the brain slice preparation, features such as how they process sensory information or identifying the sources of their synaptic inputs from distant sites are difficult to determine *in vivo* since the cells are sparse (WM interstitial cells) or compressed in a thin sheet (the SP cells at the bottom of layer 6 or subgriseal cells). Thus, although we now know somewhat more about the intrinsic and local synaptic properties of these cells in the postnatal brain, there precise function within the mature cortical network must remain somewhat speculative.

The surviving group of SP neurons may function as a sort of cortical gatekeeper, modulating information flow into and out of modules of overlying cortex to other cortical sites. Neurons of the nucleus reticularis thalami (NRT) proximal to thalamic nuclei carry out a similar function as a scattered cohort of GABAergic neurons that are embedded within the internal capsule and receive collateral excitatory innervation from thalamocortical axons as well as from cortico-thalamic axons and provide connectivity to each other within the NRT (Bokor et al., 2005; Gentet and Ulrich, 2004). They innervate thalamic neurons in inhibitory feedback projection from the thalamus and provide an inhibitory feed-forward projection from layer 6 of the cortex, as well. The NRT cells can modify the information processing state and the relay of information from the sensory periphery to the cortex by modulating membrane potential (Kim and McCormick, 1998; Ulrich and Huguenard, 1996). The cohort of surviving white matter and SP neurons may perform a related function in the cortex.

The dendritic arborizations of the WM and SP neurons within cortical layer 6 position them strategically to receive synaptic input that is otherwise destined for layer 4 from collaterals of thalamocortical axons that also arborize in layer 6 (Binzegger et al., 2005; Douglas and Martin, 2004; Molinari et al., 1995). In addition, they could also receive synaptic input from cortical layer 2 and 3 cells' axons that send axon collaterals to layer 6 (Douglas and Martin, 2004; Martin, 2002) and even into the white matter so that these neurons could also receive a copy of information that has been processed within the cortical columnar structure and is being relayed to other cortical areas. Although their somata are located within the white matter, many of the interstitial white matter neurons also have dendrites located in layer 6 where they are also positioned to potentially receive similar synaptic inputs. A difference between these neurons and the NRT cells is that most of the surviving SP and many white matter neurons are glutamatergic vs. GABAergic. However, it is interesting to note that a substantial fraction of the SP neurons are GABAergic, although we apparently have only recorded from the glutamatergic ones in our paired recordings since in all cases, the postsynaptic response was excitatory. It is not clear why our recordings should select only the glutamatergic neurons in the SP but because our results are so far limited to that sub-population, the multi-tasking behavior of these cells might be limited to certain subsets. The excitatory synaptic output of the SP and WM neurons to the cortical layers above could provide either feed-forward (for the thalamocortical inputs) and/or feedback (for the cortical efferents) information. Since the surviving SP neurons are mostly excitatory and they innervate neighboring SP cells in addition to the overlying cortical neurons, they could act as an amplification network for important signals through activating of a local network of neighboring like-type cells as well as a subset of postsynaptic targets in the overlying cortex. Recurrent excitation in such an arrangement could however, create network instability or seizures (Scharfman, 2007; Winokur et al., 2004) but depending on the types of cells that are targeted (e.g. glutamatergic excitatory vs. GABAergic inhibitory neurons), the properties of such a circuit may allow for selective amplification and contrast enhancement through feedback inhibition. The neuromodulatory chemicals in the SP and WM neurons such as NOS (Clancy et al., 2001) and various neuropeptides such as substance P (Chun and Shatz, 1989; Chun et al., 1987; Uylings and Delalle, 1997) further enhance the potential of these cells' output functions through signal gating or selective amplification that could be useful for attention, sensory learning by enhancing signal to noise ratios or changing activation thresholds and synaptic integration properties of neurons within the cortical network.

SUMMARY

There still remain considerable issues to be resolved regarding the role of these intriguing SP neurons within the mature neocortex. For example, how do the surviving cells avoid elimination during development? Which cells provide the synaptic inputs to these neurons? What are the functional properties of these neurons *in vivo*? What role do the many neuromodulators released by these cells play in information processing? Do these surviving white matter and SP neurons retain the capacity to re-enable early developmental processes in the adult cortex after injury or disease? The answers to many of these questions must await experiments where these cells are studied in the adult brain *in vivo* with selective targeting techniques. However, it is clear that SP neurons perform important functions in the cortex during early development and that a substantial number of these cells organizes into a different functional network in the postnatal brain that could contribute to cortical function in other ways. Whether such longitudinal pleiotropy of neuronal phenotype applies throughout the lifespan to the aging brain and/or to other neuronal populations remains to be evaluated. If so, this would dramatically enhance the capacity of neuronal networks throughout the lifespan.

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Individual differences in distinct components of attention are linked to anatomical variations in distinct white matter tracts

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Inter-subject variations in white matter tract properties are known to correlate with individual differences in performance in cognitive domains such as attention. The specificity of such linkages, however, is largely unexplored at the level of specific component operations of attention associated with distinct anatomical networks. This study examines individual performance variation within three functional components of attention – alerting, orienting, and conflict processing – identified by the Attention Network Task (ANT), and relates each to inter-subject variation in a distinct set of white matter tract regions. Diffusion tensor imaging data collected at 3T was used to calculate average fractional anisotropy within a set of individualized *a priori* defined regions of interest using the Reproducible Objective Quantification Scheme (ROQS) (Niogi and McCandliss, 2006; Niogi et al., 2007). Results demonstrate three functionally distinct components of attention that each correlate distinctly with three white matter tract regions. Structure–function correlations were found between alerting and the anterior limb of the internal capsule, orienting and the splenium of the corpus callosum, and conflict and the anterior corona radiata. A multiple regression/dissociation analysis demonstrated a *triple dissociation* between these three structure-function relationships that provided evidence of three anatomically and functionally separable networks. These results extend previous findings from functional imaging and lesion studies that suggest these three components of attention are subserved by dissociable networks, and suggest that variations in white matter tract microstructure may modulate the efficiency of these cognitive processes in highly specific ways.

Keywords: diffusion tensor imaging, fractional anisotropy, attention, orienting, conflict, internal capsule, anterior corona radiata, corpus callosum

INTRODUCTION

White matter tracts provide the anatomical connectivity essential for normal cognitive functioning that requires the integration of neural computation across spatially separated cortical regions such as attention and executive function abilities. This has potentially strong implications for understanding how variations in structural properties of white matter tracts from one person to another may systematically influence individual variations in efficiency across a wide range of cognitive domains, even within healthy individuals exhibiting no signs of neural or cognitive dysfunction. In support of this notion of a dimensional structure–function relationship between white matter tract microstructure and cognitive abilities, a rising number of diffusion tensor imaging (DTI) studies have shown that individual differences in white matter microstructure are systematically linked to individual differences in cognitive domains including reading and phonological processing (Klingberg et al., 2000; Beaulieu et al., 2005; Deutsch et al., 2005; Niogi and McCandliss, 2006; Dougherty et al., 2007; Qiu et al., 2008; Odegard et al., 2009), numeracy and mathematical abilities (Ewing-Cobbs et al., 2006; van Eimeren et al., 2008), executive attention (Olesen et al., 2003; Liston et al., 2006; Grieve et al., 2007; Niogi et al., 2008b), visual attention (Tuch et al., 2005; Madden et al., 2006), alerting (Nestor et al., 2007), and memory (Olesen et al., 2003; Niogi and McCandliss, 2006; Niogi et al., 2008b; Schiavone et al., 2009; Zahr et al., 2009). Additionally, it has been demonstrated that reaction time measures are sensitive to detecting variations in efficiency of cognitive domains (Tuch et al., 2005; Niogi et al., 2008b).

Moreover, a growing body of evidence suggests that DTI assessments taken from separate white matter tracts may correlate specifically with performance in different cognitive domains. As an example, DTI measures from two distinct white matter tracts in the same population of subjects have been found to correlate with performance in two distinct cognitive domains (Niogi and McCandliss, 2006; Niogi et al., 2008b). Niogi and McCandliss (2006) showed that within the same population correlations exist between reading ability and fractional anisotropy (FA) in a left superior–inferior fiber tract, but these measures were unrelated to a similar structure–function correlation between short term memory and FA in a frontal association tract. Similarly, double dissociation findings within a single population of normal healthy adults demonstrating structural and functional specificity were found for

the relationships between attention and FA in the anterior corona radiata (ACR) versus long term memory formation and the uncinate fasciculus (UF) (Niogi et al., 2008b).

Such findings serve to establish that specific, separable structure–function associations can be found across different neural networks, and variations in white matter tract properties are often closely linked to variations within these distinct cognitive domains. Such individual differences studies, however, have yet to examine whether such structure–function distinctions might also hold true for components within a specific cognitive domain. The purpose of this study is to use DTI to examine structure–function relationships of three specific cognitive processes identified as components of the general domain of attention, and to assess whether individual differences in the cognitive efficiency of each component process may be specifically linked to individual variations in microstructural properties within each of these distinct white matter tracts.

Attention is a complex cognitive domain that has been extensively investigated by employing cognitive paradigms attempting to isolate functional components as well as by employing neural studies to investigate their associated brain systems. Although many competing theories have proposed a number of potential components of attention, one highly influential approach[1] has proposed that three broad functional distinctions can be made that account for a multitude of findings across cognitive studies, neuropsychological investigations, and neuroimaging studies. Posner and Petersen (1990) proposed that investigating attention in terms of three separable component functional processes – alerting, orienting, and executive function – would help integrate a host of cognitive and neural investigations. Over the last two decades, these components of attention have been linked to separable brain networks via functional neuroimaging, electrophysiology, and lesion studies (Posner and Rothbart, 2006; Posner et al., 2006; Raz and Buhle, 2006; Fan et al., 2009).

The efficiency of the individual components of the attention system proposed by Posner and Petersen can be separately measured using the Attention Network Test (ANT) (Fan et al., 2002). The ANT is a reaction time task that measures the latency to decide whether a specific arrow symbol points leftward or rightward (the imperative stimulus). The ANT is designed to examine how different components of attention impact response times. To manipulate the alerting component of attention, the imperative stimulus which otherwise appears after a random time interval is preceded by a visual warning cue to alert the subject that a stimulus is about to appear. The orienting aspects of attention by studied placing the stimulus array above or below the fixation point, and providing or withholding a cue that is spatially predictive, thus allowing the subject to shift spatial attention to the correct location. The executive component of attention is manipulated by introducing or removing conflicting irrelevant information; the imperative stimulus is presented along with "flanking" arrows on either side that either point in the same (congruent) direction or in the conflicting (incongruent) direction. Examining how these three classes of manipulations (alerting, orienting, and conflict) impact response times provides an assay for the efficiency of each of these three components of attention. Given the simplicity and sensitivity of the ANT to three largely independent components of attention, it has been used in children and adults in normal populations, and in cohorts with neuropsychiatric disorders such as ADHD, schizophrenia, and borderline personality disorder (Posner et al., 2002; Rueda et al., 2004; Wang et al., 2005; Roberts et al., 2006; Adolfsdottir et al., 2008; Johnson et al., 2008; Fan et al., 2009).

By approaching the human attention system as one comprised of several separable networks, one can create paradigms to examine how the networks operate in a fairly independent fashion. Alternatively, different paradigms may prove useful in studying the ways in which these systems interact. Using the ANT, early studies of attention networks investigated conditions that emphasized how the networks can be studied as operating independently (Posner and Petersen, 1990; Fan et al., 2002). In fact, in the original study with the ANT, there was statistical independence between manipulations of orienting and conflict, as well as some, but not all, manipulations of alerting. Furthermore, performance scores obtained for each network were uncorrelated, suggesting that individual differences in one component cannot be easily attributed to individual differences in another component, nor attributed to an additional factor that might have influenced multiple networks together. Fan et al. extended the ANT investigation by adapting this general paradigm for use with event-related fMRI (Fan et al., 2005) and joint time–frequency analysis of electroencephalogram (EEG) data (Fan et al., 2007), thus providing further and more direct supporting evidence for the separation of these three attention components into distinct functional networks. Fan et al. (2001) also showed via twin-studies that heritable genetic variation contributes to normal individual differences in executive function presumably via dopamine rich frontal areas like the anterior cingulate, while alerting and orienting performance demonstrated no such heritability. In addition to studies using the ANT, a great deal of research on each of the three attention networks has been conducted that might help constrain our understanding of each network, as well as help generate structural hypotheses for major white matter tract structures that might be involved in these networks.

In the next section we consider such work, including neuroimaging and neuropsychological studies that may help constrain the selection of white matter tracts that may be involved in each of the three attention networks. For this, we shall consider the conflict, alerting, and orienting networks in turn with the aim of identifying white matter tract structures for further investigation for potential structure–function relationships.

Functional and neuropsychological studies have associated performance in conflict tasks (executive control) to the frontal cortex, and more specifically to a network including the anterior cingulate gyrus and lateral prefrontal cortex (Casey et al., 2000; Fan et al., 2002; Rueda et al., 2004; Callejas et al., 2005). The conflict component of attention mediates inhibitory control, resolution of conflicting stimuli impacting decision making, and, in a broader sense, can be considered necessary for decision planning and decision making. This network likely includes white matter tracts that serve to connect these regions with other structures. The frontal lobes contain numerous complex connections with different parts of the brain, many of which pass through the thalamus. As such, it is likely that tracts from the thalamus extending to the frontal lobe

[1]As of publication date, Posner and Petersen (1990) has over 2250 Web of Science citations.

and anterior cingulated gyrus, such as the ACR, may be part of the executive attention network associated with the conflict component of the ANT. Indeed, a recent study demonstrated that white matter integrity along the left ACR correlated significantly with conflict performance from the ANT in a group of normal adults and also in a cohort of adults with mild traumatic brain injury (Niogi et al., 2008b).

The alerting component of attention is proposed to be responsible for activating the required cognitive systems to make the person ready to respond to a task. This form of phasic alerting is modulated by thalamic, frontal, and parietal regions (Coull et al., 1996; Sturm and Willmes, 2001; Rueda et al., 2004; Callejas et al., 2005; Fan et al., 2005, 2009). Although the reticular activating system is known to be necessary for tonic alertness, it also plays a critical role in phasic alerting (Sturm and Willmes, 2001; Oken et al., 2006; Fan et al., 2009). A likely white matter pathway that connects the proposed regions critically involves the internal capsule. The internal capsule is made up of an anterior limb (ALIC), and posterior limb (PLIC) and the bend between the two limbs referred to as the genu. The ALIC and PLIC directly relay motor and sensory information with ascending and descending fibers between the cerebral cortex and the pyramids of the medulla (Schünke et al., 2007).

The orienting network selects spatial and sensory information. Commonly, this is tested with visual cues indicating the location of an impending target (as in the ANT). The visual orienting system has been associated with brain areas such as the superior and inferior parietal lobes, frontal eye fields, and subcortical areas including the superior colliculus and reticular nuclei in the thalamus (Corbetta et al., 2000, 2002; Fan et al., 2002, 2005, 2007; Callejas et al., 2005; Wang et al., 2005; Himmelbach et al., 2006). In considering white matter tracts likely to modulate the efficiency of the orienting network, the optic radiations (OR) relay visual information via neurons from the lateral geniculate nucleus of the thalamus to the visual cortex. Such geniculate–cortical circuitry has been implicated in attention studies (Schneider and Kastner, 2009). Additionally, the orienting system must relay and compare spatial information from both visual fields which requires connectivity between hemispheres. Lesions studies of the splenium of the corpus callosum (Noudoost et al., 2006), fMRI studies of interhemispheric transfer (Weber et al., 2005), and studies examining callosal thickness in ADHD (Luders et al., 2009) suggest that commissural fibers, particularly the splenium of the corpus callosum, may play a large modulatory role in the function of the visual spatial orienting network.

Prior neuroimaging studies focused primarily on functional activations using fMRI or EEG to isolate the anatomic substrates for the attention networks. These studies have focused on the functional activations (i.e., gray matter) involved in the attention components. It remains unclear what specific white matter pathways modulate each component of attention. As has been demonstrated in several other domains reviewed above, individual differences in white matter microstructure within tracts associated with particular attention networks may closely correlate with variations in efficiency of these attentional processes.

One technique to quantify white matter integrity is DTI (Basser et al., 1994; Basser and Pierpaoli, 1996). The principle governing DTI is that water diffuses more readily along the orientation of axonal fibers than across the fibers due to hindrance from structural elements such as the axolemma and the myelin sheath. The degree of directionality is termed anisotropy. Anisotropy can be measured as the variation in the eigenvalues of the diffusion tensor (Basser et al., 1994; Ulug and van Zijl, 1999). FA, a normalized measure of anisotropy, has been shown to be sensitive to microstructural changes in white matter integrity and organization (Mukherjee and McKinstry, 2006; Niogi et al., 2008a,b). Increasing numbers of DTI studies that correlate FA with cognitive function indicate that such measurements can be used to account for a wide range skills (Moseley et al., 2002; Schmithorst et al., 2002; Medina et al., 2005; Mabbott et al., 2006; Niogi and McCandliss, 2006; Niogi et al., 2008b).

There are a variety of proposed methods to analyze DTI datasets. The current gold standard is to select regions of interest (ROIs) manually either by tracing the structure of interest with a mouse or placing a fixed geometric shaped ROI within the structure. Despite being the gold standard, these techniques are inherently tedious and prone to human error, thus decreasing inter- and intra-rater reliability, especially across laboratories that might adopt different standards and guidelines. An alternative technique of voxel-based analysis (VBA) avoids many of these difficulties, but introduces others. Automated voxel-based techniques first require data to be spatially normalized and then compared on a voxel-by-voxel basis. Thus, the data comparisons are fundamentally probabilistic, and may reflect anomalies or distortions introduced during the spatial normalization procedure (Jones et al., 2005). Studies have suggested that VBA may be inconsistent across specific regions of the brain, particularly in areas with greater anatomical variability (Quarantelli et al., 2002; Tisserand et al., 2002; Ciccarelli et al., 2003). Furthermore, given the complex directional and sensitive nature of DTI data, proper spatial normalization may prove even more difficult than originally realized (Leemans and Jones, 2009). Another difficulty with VBA is that many studies require operating on data sets that have undergone smoothing functions, which may introduce additional limitations on the spatial certainty of results. Despite being a whole-brain automated technique, VBA is prone to variability as well given the complications and non-standardized parameters used in VBA. Jones et al. (2007) conducted a study in which nine different research groups employing their own (and slightly different) voxel-based technique analyzed the same DTI data set to determine the structural difference between patient and control populations. The study showed that each of the nine research groups selected a different area of the brain that differed between the patient and control populations with minimal overlap (Jones et al., 2007).

Still, voxel-based studies are most suitable for identification of unexpected areas of white matter pathology despite the limitations previously mentioned. However, voxel-by-voxel comparisons inherently have power limitations due to the numerous multiple comparisons necessary in this approach. When *a priori* structures can be identified, a ROI approach can be employed successfully and may be better suited. In this study, we have identified in an *a priori* fashion, motivated by a literature-based review, a selection of likely white matter tracts to be involved in each component of attention. Thus, in this study we will employ a ROI technique. To counter

the inter- and intra-rater variability of manual ROI analysis, in the current study an ROI approach employing a semi-automatic segmentation tool called the Reproducible Objective Quantification Scheme (ROQS) (Niogi et al., 2007). A primary benefit of the ROQS analysis is that regions conforming to the boundaries of the tracts are selected using an objective, reproducible algorithm and in a fashion specific to each subject. Unlike VBA, this method does not require spatial normalization of tensor data, thereby operating on the original spatially untransformed data and thus avoiding confounding differences in the size and shape of an individual or template brain. Although a central drawback to this approach is that it does not lend itself to searching the entire brain for a potential correlations, it does provide a limited set of opportunities to examine well constrained *a priori* hypotheses about a small class of ROIs in white matter tract networks that can be reliably quantified and systematically related to cognitive performance (for a discussion see Niogi et al., 2007).

Here we investigate potential relationships between individual differences in the efficiency of each component of attention and individual differences in FA within specific white matter tract regions. These regions have been selected based on the existing neuroimaging literature reviewed above for the alerting, orienting, and conflict components of attention. Subsequently, we test the specificity of these findings by examining cross-correlation patterns and conducting multiple regression analyses to determine whether each of the three components of attention is related to a specific network.

MATERIALS AND METHODS

PARTICIPANTS

Subjects in this study included 26 healthy adult volunteers (19 male, 7 female) with an average age of 28.3 years (range 17–58 years; standard deviation 9.38 years)[2]. Exclusion criteria were imposed for any evidence of abnormal MRI scan, prior history of traumatic brain injury, history of neurological or psychiatric illness including drug or alcohol abuse, psychotropic medications that would affect cognitive testing, or any of a multitude of standard contraindications to MR imaging such as pregnancy or ferromagnetic implants. Written and verbal informed consent was obtained from all subjects in accordance with the Declaration of Helsinki and as approved by the authors' institutions.

ASSESSMENT OF ATTENTION COMPONENT FUNCTION

The Attention Network Task (Fan et al., 2005) was used to provide a quantitative reaction-time based assessment of each of the three major components of attention. The version of the test employed here was identical to a subset of conditions employed in the Fan et al. (2002) study, including three of the original cue conditions (no cue, central alerting cue, spatial cue) and the original two target conditions. All trials presented a target stimulus, either above or below the fixation cross. **Figure 1** illustrates the various cue and target conditions as well as their timing. All target stimuli included an imperative leftward or rightward pointing arrow centered horizontally above or below the fixation cross, with the direction of the arrow signifying whether the subject was to press the corresponding left or right index finger response key. For all target stimuli, the horizontally centered arrow was flanked on either side by two additional arrows pointing in the congruent or incongruent direction. The entire array of arrows appeared either above or below the fixation cross. Target stimuli were preceded by one of three cue conditions: (1) a no-cue condition which was not perceptively different from the preceding or following fixation stimuli (i.e., baseline, temporally and spatially non-informative), (2) a center-cue condition in which the fixation cross was temporarily replaced by an asterisk appearing directly between the two potential target locations (i.e., temporally informative but spatially uninformative), or (3) a spatial-cue condition in which an asterisk appeared in the exact position of the target arrow (i.e., temporally and spatially informative).

Reaction times were recorded and the difference in reaction times between varying conditions was used to calculate a performance score for each of the three components of attention. Specifically, a conflict score was calculated as the reaction time from all congruent conditions minus the reaction time from all incongruent conditions. Since incongruent trials typically elicit longer reaction times than congruent trials, the conflict score is expected to be negative-valued. A shorter difference in reaction times of the two trials generally indicates better executive control performance and therefore a less negative conflict score. The alerting component of attention was calculated as the reaction times for all no-cue conditions minus reaction times for all center-cue conditions. Finally, the orienting component of attention was calculated as the reaction times from all center cue conditions minus the reaction times for all spatial cue conditions. As the increasingly informative cues are predicted to enhance performance, higher alerting and orienting scores indicate increased efficiency in taking advantage of these specific forms of cue information. As in the Fan et al. (2002) study, obtaining assessments of all three attention components within the very same blocks of trials also provides the opportunity to assess the degree to which each attention component assay is correlated with, or statistically independent, of one another via a simple cross-correlation analysis.

MRI AND DTI ACQUISITION

Magnetic resonance imaging was acquired on two identical 3 T GE Signa EXCITE scanners (GE Healthcare, Waukesha, WI, USA) both equipped with the same eight-channel phased-array head coils. Whole-brain DTI was performed with an echoplanar multislice single-shot spin echo pulse sequence (TE = 63 ms, TR = 14 s) using 55 diffusion-encoding directions, isotropically distributed over the surface of a sphere with electrostatic repulsion, acquired at $b = 1000$ s/mm^2, 1 acquisition with $b = 0$ s/mm^2, 72 interleaved slices of 1.8-mm thickness each with no gap between slices, a 128×128 matrix that was zero-filled during reconstruction to 256×256, and a field of view (FOV) of 230 mm. Parallel imaging was employed using the Array Spatial Sensitivity Encoding

[2]Twenty-three of these subjects served as normal controls for a separate previously published study investigating the dissociation of attention and memory deficits following chronic mild traumatic brain injury (Niogi et al., 2008b). Please note that the prior investigation (Niogi et al., 2008b) initially reported the findings relating executive attention (conflict) to white matter integrity of the ACR using these 23 subjects. This result is replicated in this current study with the addition of three subjects. While the previous study focused on dissociating attention and memory, it did not provide an in-depth examination of all three components of attention presented here in this current study.

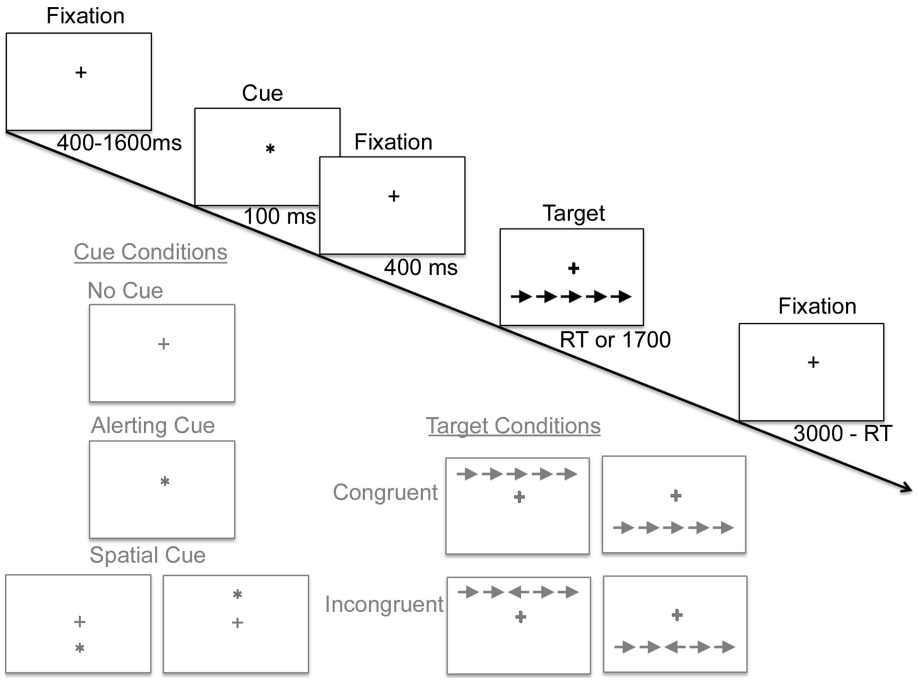

FIGURE 1 | A schematic diagram of a single trial of the Attention Network Task, in which subjects rapidly decide whether the central arrow of an array points leftward or rightward. A fixation cross at the center of the screen remains throughout the trial. After an unpredictable duration, one of three classes of cue conditions is presented (illustrated in gray). Next, following an additional 400 ms delay, a target display appears until a response is registered or a deadline expires. Sample target displays illustrate (in gray) the 2 × 2 × 2 factorial of the possible arrays of arrows containing a leftward versus rightward central arrow, congruent versus incongruent flanking arrows, and presentation above versus below fixation. The end of each trial, 3000 ms after target onset, flows seamlessly into the beginning of the next trial.

Technique (ASSET) with an acceleration factor of 2. Images were post-processed offline using DTIstudio software (Jiang et al., 2006) to obtain FA maps, apparent diffusion coefficient (ADC) maps, and directionally encoded color FA maps.

The following conventional 3 T MR imaging sequences were acquired: (1) axial 3D inversion recovery fast spoiled gradient-recalled echo (FSPGR) T1-weighted images (TE = 1.5 ms, TR = 6.3 ms, TI = 400 ms, flip angle of 15°) with 230 mm FOV, 156 1.0-mm contiguous partitions at a 256 × 256 matrix, (2) axial T2-weighted fluid-attenuated inversion recovery (FLAIR) images (TE = 126 ms, TR = 10 s, TI = 2200 ms) with 220 mm FOV, 47–48 3.0-mm contiguous slices at a 256 × 256 matrix, (3) axial magnetization prepared gradient echo (MPGR) T2*-weighted images (TE = 15 ms, TR = 500 ms, flip angle of 20°) with 220 × 170 mm FOV, 47–48 3.0 mm contiguous slices at a 256 × 192 matrix. All conventional MR images were interpreted by attending neuroradiologists certified by the American Board of Radiology to ensure participants had no clinically significant abnormal MR findings.

ROQS ANALYSIS OF INDIVIDUAL NON-TRANSFORMED FA MAPS

Anatomically defined ROIs were selected and quantified using the ROQS, which is a semi-automated process that segments white matter structures in a way that has been shown to have high inter- and intra-rater reliability (Niogi and McCandliss, 2006; Niogi et al., 2007; van Eimeren et al., 2008). The benefit of the ROQS analysis compared to manual ROI techniques is that a larger region of pixels is selected for quantification in a manner that conforms to the boundaries of a subsection of a white matter tract for each subject in highly individualized fashion that is also objective and highly reproducible. ROQS operates by using directionally encoded information from the principal eigenvector to segment structures within 2D FA maps. ROQS guidelines direct the user to select a representative seed pixel(s) within anatomically specified ROIs. This set of regions is restricted to clearly identifiable white matter tract structures that appear on two-dimensional FA maps as dominated by a single homogenous direction. Contiguous surrounding boundary pixels are selected algorithmically in the same slice of data that share the same diffusion orientation and similar diffusion properties as the seed pixel, based on individual non-normalized DTI data. The final ROI includes the boundary pixels and all the pixels within the boundary (see Niogi et al., 2007 for full methodological details).

REGION OF INTEREST SELECTION

A highly restricted set of candidate ROIs were selected for each network in an *a priori* fashion, based on the existing literature regarding the neural networks associated with each of the attention functions assayed in the ANT. **Figure 2** illustrates examples of ROQS ROIs for structures analyzed in this investigation (for a better description of the neuroanatomy refer to Mori et al., 2005; Schünke et al., 2007). Given previous results reviewed above associating executive function with FA values in ACR, we restricted selection of ROIs related to the conflict function to the left and right ACR. Prior constraints

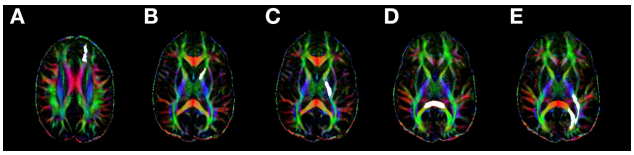

FIGURE 2 | A representative sample of the regions of interest (highlighted in white) measured for each subject using ROQS: (A) anterior corona radiata; (B) anterior limb of the internal capsule; (C) posterior limb of the internal capsule; (D) splenium of the corpus callosum; (E) optic radiations.

regarding the contribution of anterior versus posterior limbs of the internal capsule to phasic alerting functions are somewhat ambiguous, thus both ROIs were chosen bilaterally for investigation of the alerting function (left ALIC, right ALIC, left PLIC, and right PLIC). The splenium of the corpus callosum was investigated as a candidate ROI for orienting on the rationale that this readily identifiable white matter tract structure is involved in the coordination of information across left and right hemisphere posterior parietal regions. Additionally, the OR in each hemisphere were included as candidate ROIs for orienting on the rationale that these regions may mediate connections between thalamic and visual regions involved in processing information from each hemifield.

CONFIRMATORY CORRELATION ANALYSES: PREDICTED STRUCTURES AND FUNCTIONS

A Cook's *D* test was used to test for influential data points. This resulted in one subject being excluded from the study for being a statistical outlier. Subsequently, nonparametric statistical tests were then used to assess the amount of structure–function correlation between the ROIs proposed for each attention network and the corresponding ANT behavioral scores. The Spearman's rho statistic was used to test for significance of correlations for each attention function across the set of candidate ROIs proposed for that function, after applying a Bonferroni correction for multiple comparisons (alpha level set to 0.05 divided by the number of candidate ROIs proposed for each attention component).

SPECIFICITY ANALYSES

Assessing potential cross-correlations across the proposed functional networks

To assess whether positive findings for structure–function relationships were specific to each network's hypothesized attention function rather than reflecting a systemic relationship between white matter tract properties and cognitive function or specific patterns of shared variance across any two of the functional networks, we first assessed patterns of potential cross-correlations. For this, a reduced set of three candidate ROIs were selected, one for each attention function demonstrating the strongest evidence of a positive relationship. These correlations composed a 3×3 correlation matrix, with the diagonal representing the predicted set of correlations, and the off-diagonal representing the degree of specificity of those predictions. No corrections were applied to this correlation matrix to avoid any potential type II error bias that would tend to favor the predicted hypothesis of high specificity across attention functions and predicted ROI.

Assessing degree of unique variance accounted for by the proposed functional networks

The final goal of the study was to test the specificity of the attention components to their corresponding neuronal networks by attempting to establish a triple dissociation. This framework of multiple regression dissociation has proven effective in demonstrating separability of individual difference patterns of structure–function correlations across domains, such as reading versus short term memory (Niogi and McCandliss, 2006) and attention versus memory retrieval (Niogi et al., 2008b). This technique, however, has yet to be applied to dissociating component cognitive processes within a domain. Separability is directly assessed here in a regression framework that incorporates a series of three complementary multiple regression analyses, each with a specific dependent measure (FA in the respective ROIs from the specificity analysis), and three interrelated regressors: subject age in years and the two attention performance measures proposed to be unrelated to the specific functional network under investigation. Finally, the last step of each analysis included the performance scores for the proposed function for the network under investigation after controlling for the other factors.

The aim of these analyses was to assess the unique variance accounted for by each component of attention with the corresponding white matter tract. For each of the three analyses, two blocks of independent variables were applied in a step-wise fashion. The first block of each analysis contained the two attention scores proposed to be irrelevant to that ROI, as well as age in years. The second block added the proposed attention component score. Significance of the ΔR^2 change was computed using an *F* statistic. All statistical tests were performed using SPSS v.14 (SPSS Inc., Chicago, IL, USA).

RESULTS

BEHAVIORAL CROSS-CORRELATION RESULTS

Behavioral results were first examined to determine the degree of interdependence of individual differences across the three components of attention. As shown in **Table 1** the efficiency scores for each attention component were uncorrelated with one another, replicating findings from Fan et al. (2002) of functional independence across patterns of individual differences for the three components of attention function.

PREDICTED STRUCTURE–FUNCTION CORRELATIONS

Correlations between conflict scores and FA in the candidate regions of the left and right ACR provided support for this relationship (ACR Left: $r = -0.428, p = 0.012$; ACR Right: $r = -0.363, p = 0.069$). Correlations between alerting scores and FA of the PLIC and ALIC

Table 1 | Attention component correlations.

	Alerting	Orienting	Conflict
Alerting	1	–0.27 (p = 0.19)	–0.14 (p = 0.49)
Orienting	–0.27 (p = 0.19)	1	0.17 (p = 0.41)
Conflict	–0.14 (p = 0.48)	0.17 (p = 0.41)	1

Correlation coefficients between attention component scores. The correlation coefficient is followed by the significance of the correlation (two-tailed) in parenthesis. As seen here, the scores for each component were uncorrelated with one another.

provided support for a structure-function relationship restricted to the left PLIC (PLIC Left: $r = 0.474, p = 0.015$; PLIC Right: $r = 0.050$, $p = 0.81$; ALIC Left: $r = -0.153$, $p = 0.46$; ALIC Right: $r = -0.181$, $p = 0.38$). Finally, correlations between orienting scores and FA of the splenium of the corpus callosum and the OR provided support for a relationship between the splenium and orienting component (splenium: $r = 0.471$, $p = 0.015$; OR Left: $r = 0.039$, $p = 0.85$; OR Right: $r = -0.60, p = 0.77$). Before controlling for multiple comparisons across the number of predicted ROIs for each attention function, each of the three attention functions correlate significantly with at least one predicted ROI (see **Figure 3**). However, after controlling for multiple comparisons using a Bonferroni correction for the set of proposed regions, the conflict-left ACR correlation alpha values is corrected to less than 0.025 ($p < 0.05$ divided by 2 ROIs). The alerting alpha value is corrected to less than 0.0125 ($p < 0.05$ divided by four ROIs), and the orienting component alpha value is corrected to less than 0.017 ($p < 0.05$ divided by three ROIs). Using these criteria, a significant correlation remains between the left ACR

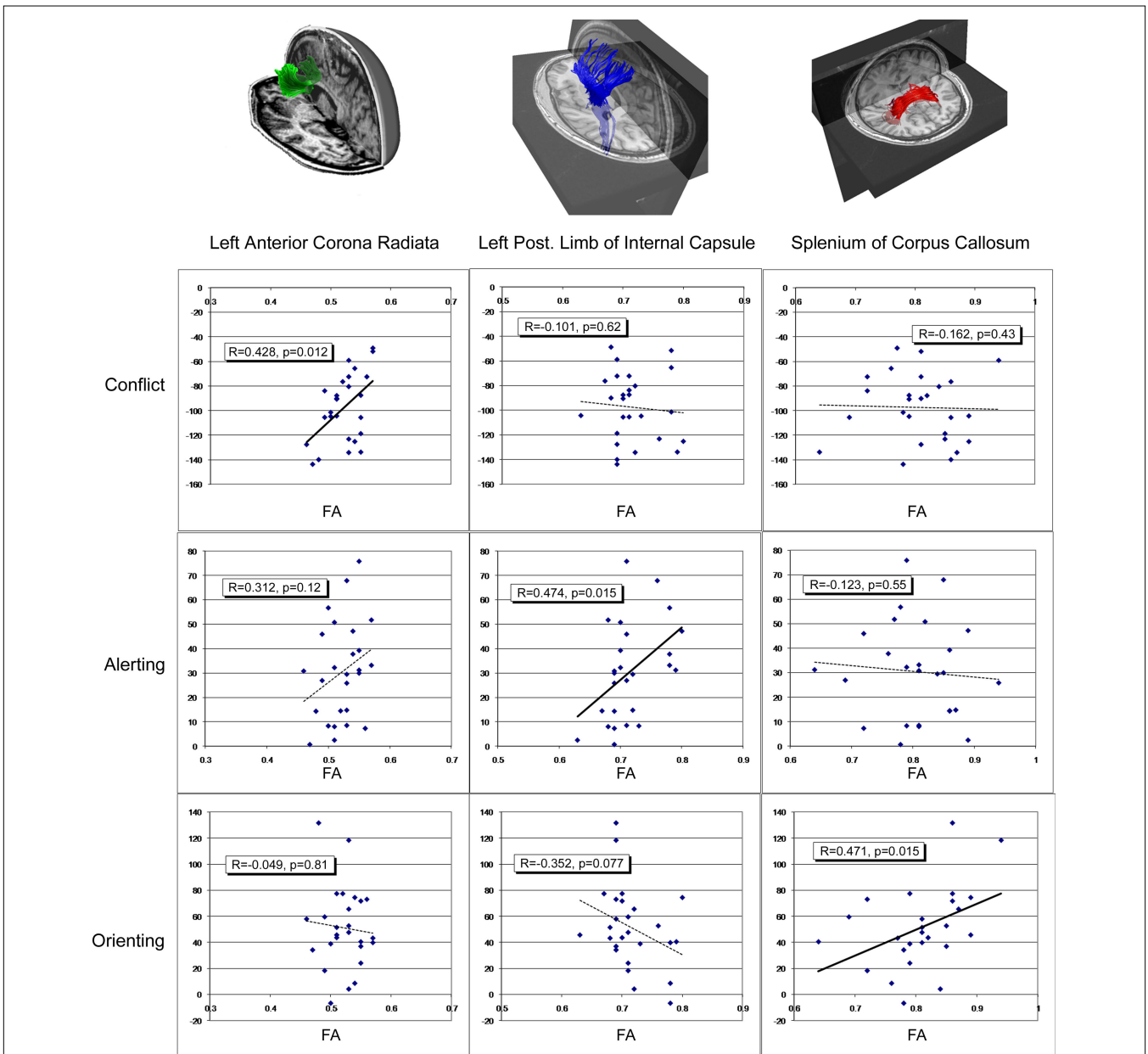

FIGURE 3 | Scatter plots demonstrating the relationship between functional components of attention identified in the Attention Network Task (ANT), and average fractional anisotropy (FA) of relevant regions of interest. Each row of graphs illustrates results for the three components of attention: the conflict component (top row), the alerting component (second row), and the orienting component (third row), each column of graphs illustrates result for a separate structure: the left anterior corona radiata (left column), the left posterior limb of the internal capsule (middle column), and the splenium of the corpus callosum (right column), each of which are illustrated via fiber tracking in the top row. The vertical axis for each graph represents the attention component score in millisecond. The horizontal axis of each graph represents FA of each ROI.

and conflict scores and between the splenium and orienting scores. The relationship between alerting and the left PLIC fails to pass this more restrictive significance threshold.

SPECIFICITY OF STRUCTURE–FUNCTION RELATIONSHIPS

Specificity of structure–function relationships I: assessment of cross-correlations

Figure 3 illustrates potential patterns of cross-correlation for the three significant structure–function relationships, using uncorrected alpha levels to ensure maximum potential sensitivity to potential cross-talk between the three purportedly functionally specific networks. As predicted, conflict scores failed to correlate with FA in left PLIC ($r = 0.101$, $p = 0.624$) or splenium ($r = 0.162$, $p = 0.43$). Alerting failed to correlate with integrity of the left ACR ($r = 0.312$, $p = 0.12$) or splenium ($r = 0.123$, $p = 0.55$). Orienting failed to correlate with integrity of the left ACR ($r = –0.049$, $p = 0.814$), yet exhibited a non-significant trend toward a correlation with left PLIC ($r = –0.352$, $p = 0.077$). This trend toward a correlation between orienting and PLIC was further evaluated via the multiple regression analysis (see below).

Specificity of structure–function relationships II: a triple dissociation

A set of three multiple regression analyses (see **Tables 2–4**) revealed that the proposed relationship between the three attention functions and three corresponding ROIs accounted for unique variance in the predicted structure–function relationships, even after accounting for the potential influences of age and the two purportedly unrelated attention performance measures. In each of the three regression analyses, R^2 change in the final block indicated that the proposed specific functional scores significantly accounted for unique variance in the analysis of all three ROIs. Specifically, after the addition of conflict in the multiple regression scheme for the left ACR (see **Table 2**) there was a significant R^2 change ($\Delta R^2 = 0.213$, $p = 0.020$) indicating that even after controlling for age and the purportedly irrelevant alerting and orienting scores, conflict still loaded heavily on the left ACR.

Likewise, in the regression analysis of the left PLIC, after the addition of alerting scores (see **Table 3**), there was a significant R^2 change ($\Delta R^2 = 0.15$, $p = 0.05$) indicating PLIC FA scores are uniquely associated with performance in the alerting component of attention even after controlling for age, conflict, and orienting performance.

Finally, after the addition of orienting scores to the analysis of the splenium of the CC (see **Table 4**), a significant R^2 change ($\Delta R^2 = 0.16$, $p = 0.044$) indicated that specificity of individual differences in FA within the splenium of the CC are specifically related to orienting, above and beyond the potential influence of age or the two other attention functions.

Interestingly, across all three analyses, the combined factors of age and the two purportedly unrelated attention components failed to account for significant variance in any of the ROIs providing additional support for functional independence across these three attention networks.

Table 2 | Multiple regression analysis for left anterior corona radiata + conflict.

Model	*R*	R^2	R^2 change	*F* change	Sig. *F* change
1	0.291[a]	0.084	0.084	0.676	0.576
2	0.545[b]	0.297	0.213	6.356	0.020

[a]*Predictors: (constant), age, orienting, alerting.*
[b]*Predictors: (constant), age, orienting, alerting, conflict.*

Table 3 | Multiple regression analysis for left posterior limb of internal capsule + alerting.

Model	*R*	R^2	R^2 change	*F* change	Sig. *F* change
1	0.359[a]	0.129	0.129	1.087	0.375
2	0.527[b]	0.278	0.149	4.330	0.050

[a]*Predictors: (constant), age, orienting, conflict.*
[b]*Predictors: (constant), age, orienting, conflict, alerting.*

Table 4 | Multiple regression analysis for splenium of corpus callosum + orienting.

Model	*R*	R^2	R^2 change	*F* change	Sig. *F* change
1	0.345[a]	0.119	0.119	0.989	0.416
2	0.526[b]	0.277	0.158	4.576	0.044

[a]*Predictors: (constant), age, alerting, conflict.*
[b]*Predictors: (constant), age, alerting, conflict, orienting.*

DISCUSSION

The attention network framework provides a key hypothesis that the general domain of attentional function, as it is instantiated in the brain, is subserved by three *functionally and anatomically separable* networks (Posner and Petersen, 1990). Clearly, the three attention networks proposed are functionally and anatomically connected via specific white matter tracts. The central hypotheses investigated in the current study are that individual variation in white matter tract microstructure is systematically linked to individual differences in cognitive efficiency, and that this structure–function relationship plays out in a highly anatomically and functionally specific manner as predicted by the separability hypothesis of the attention network framework.

To show specificity of the findings we employed a correlational dissociation (Niogi and McCandliss, 2006; Niogi et al., 2008b), which is akin to neuropsychological lesion studies. Instead of using a lesion to show specificity by loss of function, the correlational dissociation used a series of multiple regression analyses to show specificity of structure and function by correlating integrity and performance. The correlational triple dissociation has similar benefits and limitations as lesions studies. Just as a lesion study cannot make claims that other structures may be necessary or involved with the lost function, the correlational triple dissociation we employed does not claim that regions outside the ones analyzed are not involved in attention. The benefit of the correlational dissociation is that we showed that the structures examined are involved in the attention network and that, among the structures analyzed, each tract has component specificity. To establish the correlational dissociation, three criteria (similar to the criteria for a lesion study) were needed and were met. First, a control region is necessary to show specificity. This was achieved because all three

ROIs (ACR, PLIC, and splenium) correlated with one cognitive process. Secondly, the correlation must remain significant after controlling for the other domains (this was shown successfully using the multiple regression analyses). Finally, the structure–function relationship demonstrated a lack of correlation with the two other control structures (see **Figure 3**).

At the most general level, the overall pattern of results provides support for the central hypothesis that distinct white matter tract networks exist for each component of attention. In order to test these hypotheses it was first necessary to propose candidate ROIs that might uniquely capture each functional network based on existing literature and empirically establish whether any subset of these candidate ROIs related to the proposed functions. FA values from a subset of the candidate ROIs demonstrated a set of network specific brain–behavior correlations for each of the three attention components. Each of these positive correlations suggests that white matter tract microstructure accounts for substantial variance in the efficiency of attention performance. Next, examination of the cross-correlation patterns of these three positive findings of structure–function relationships revealed a dramatic pattern of specificity, suggesting that none of the three positive correlations were readily attributable to the influence of an additional common factor. Finally, the multiple regression dissociation analysis provided further evidence and critical support for the specificity hypothesis that each of the proposed structure–function relationships was quite specific in nature and accounted for unique variance even after the influence of age and the purported non-related attention components were taken into account.

Next, we consider the evidence and implications for each network, and then turn to the central question of the functional and anatomical *separability* of the three component networks of attention.

THE CONFLICT NETWORK

The correlation analysis together with the multiple regression dissociation analysis provides evidence that microstructural integrity of the ACR modulates executive attention. The finding of a frontal tract associated with executive attention is strongly supported by previous literature. Functional imaging studies have provided strong consistent support for the notion that the frontal lobes, particularly the anterior cingulate gyrus, is associated with attentional control during the Stroop Task and the conflict portion of the Attention Network Task (Casey et al., 2000; Gruber et al., 2002; Fan et al., 2005). Additionally, neuropsychological data clearly highlight the central role of the frontal lobes in executive attention (Stuss et al., 1981; Stuss and Benson, 1984; Vendrell et al., 1995; Stuss and Alexander, 2000). Previous DTI studies have also implicated frontal white matter tracts with attention performance. For example, integrity of white matter tracts associated with brain activity impacts executive attention (Olesen et al., 2003; Madden et al., 2006). Furthermore, Niogi and McCandliss (2006) demonstrated that a closely related frontal executive skill, short term memory, also demonstrated strong correlations with ACR in children. Finally, in a recent study of 43 patients suffering from mild traumatic brain injury, the left ACR integrity assessed by FA was shown to correlate with conflict scores on the ANT (Niogi et al., 2008b). It is interesting to note that in the current study, significant correlation between conflict scores and FA did not reach significance in the right ACR, but appeared only as a non-significant trend. Given this trend and no significant finding of a direct effect of laterality, it is possible that both left and right ACR regions play a role in conflict. The specific contributions of right ACR may be apparent in studies with greater power.

THE ORIENTING NETWORK

Regarding the orienting network, the correlation analysis together with the multiple regression dissociation analysis demonstrates that microstructural integrity of the splenium of the corpus callosum modulates the efficiency of the orienting component of attention. This finding is consistent with previous findings that demonstrate that the visual spatial attentional orienting system is dependent on a functional network that includes left and right posterior parietal regions and is linked to a larger neural network including frontal eye fields, subcortical areas including the superior colliculus, and reticular nuclei in the thalamus (Corbetta et al., 2000, 2002; Fan et al., 2002, 2005, 2007; Wang et al., 2005; Himmelbach et al., 2006). We propose that the splenium of the corpus callosum includes white matter tracts involved in this overall network, and that this region provides a large convenient homogenous white matter region of interest that can be used to assess individual differences in this network. Our more specific findings implicating the splenium of the corpus callosum as part of the network that modulates orienting performance is consistent with Noudoost et al.'s (2006) report of a case study that demonstrates the role of interhemispheric connections in making an integral visual map across hemifields that can be used for visual spatial attention. Furthermore, splenium of the corpus callosum lesions commonly cause visual spatial neglect (Tassinari et al., 1994; Park et al., 2005; Noudoost et al., 2006), a deficit that specifically impacts orienting functions of attention. Such lesions can also cause other forms of disconnection syndromes that may be linked to spatial orienting functions as in the case or hemialexia (Sugishita et al., 1986; Suzuki et al., 1998; Le et al., 2005) and visual attention (Mayer et al., 1988).

THE ALERTING NETWORK

The correlation analysis together with the multiple regression dissociation analysis provides evidence that the PLIC modulates individual differences in the alerting component of attention. This relationship is generally consistent with the previous literature suggesting a role of the PLIC for this component of attention. For example, Sturm et al. (1999) presented PET evidence from normal volunteers that suggested alerting functions involve a vast network of regions, including inferior parietal-thalamic networks, brainstem structures, and frontal regions largely lateralized to the right hemisphere. Fimm et al. (2001) investigated alerting-related impairments of attention in 15 patients with acute circumscribed vascular lesions confined to the basal ganglia, internal capsule, and thalamus. In his study, five out of seven patients showed evidence of lesions to the posterior limb of the internal capsule. Fimm et al. (2001) suggested that thalamo-parietal projections transversing through the PLIC via the superior peduncle of the thalamus could lead to a disconnection of functionally relevant structures that impair attention. Curiously, the laterality of the PLIC-alerting effects in the present study runs counter to patterns of laterality in lesion studies. While the majority of alerting deficit findings reported above show a bias toward right

sided lesions linked to loss of attention functions associated with alerting, at least some findings suggest that left PLIC may play a role. For example, Fimm et al. (2001) reported two patients presenting with left hemisphere PLIC lesions, each demonstrating deficits in alerting-related attention skills. Although it is possible that laterality of PLIC plays out differently in the modulation of individual differences in normal control subjects and patients with rather severe damage to these structures, it is also important to note that the pattern of results reported here do not implicate significant lateralization effects *per se*, but rather demonstrate higher sensitivity to individual difference in alerting in the left PLIC.

FUNCTIONAL AND ANATOMICAL SEPARABILITY OF ATTENTION NETWORKS

Next, we turn consideration to the separability hypothesis regarding the three attentional networks. In evaluating these specificity findings, it is important to consider that the ANT was specifically designed to stress the potential conditions under which the three attention networks could be shown to be separable in their operations and individual difference patterns. More recently, several investigations have begun to explore other conditions under which these networks might interact and influence one another in important ways (for a review see Fan et al., 2009). To the degree to which the ANT exposes these networks to be anatomically and functionally separable, it is possible that white matter tract integrity is an important *independent modulator* of function such that individual differences in white matter tract microstructure in one network might be functionally associated with one component of attention relative to the other components of attention. This hypothesis was addressed by a series of multiple regressions in which the variance in function for each established white matter tract ROI was assessed for the key proposed associated function while controlling for the influence of the other component attentional functions.

Results of this multiple regression analysis demonstrated three key findings: the ACR is a unique modulator (relative to the other two ROIs) of the conflict network function, the PLIC is a unique modulator of the alerting network, and the splenium is a unique modulator of the orienting network. This correlational triple dissociation can be interpreted similarly to the "gold-standard" double-dissociation tests in neuropsychology commonly required when attempting to establish specificity of structure–function relationships. Such findings are critical in lesion studies in which the mere presence of any form of brain damage can cause widespread, non-specific difficulties in any performance assessment of cognitive function, and establishing specificity of functional and structural loss is critical. Similarly, in linking individual differences between white matter properties and cognitive performance, establishing similarly high levels of specificity between particular regions and particular functions is critical.

One non-significant trend in the cross-correlation analysis suggested a potential exception to the separability of the three networks. The trend toward correlation between the PLIC and orienting component runs counter to the specificity hypothesis that separate networks are associated with orienting and alerting functions. Critically, this trend only appeared in the zero-order correlational analysis. Further investigation of these phenomena was available from the multiple regression dissociation analysis of the PLIC. By first regressing age, alerting, and conflict on the results, this analysis demonstrated that alerting accounts for unique variance after controlling for the potential influences of other attention factors.

In addition to showing functional specificity, the correlational dissociation analysis shows that within each functional network, a gradient of individual differences in function positively correlates with a gradient of individual differences in white matter tract microstructure. Furthermore, since this study focused on normal healthy adults, rather than reflecting the impact of neural damage on related loss of function, the results may be more relevant to understanding normal population variations in white matter tract microstructure and how such differences are linked to efficiency of components of attention. As such, these findings may provide the basis of relating dimensional differences in structure–function relationships that exist within normal populations with more extreme ranges of variation of structural damage and related loss of function (for discussion see Niogi and McCandliss, 2006).

FURTHER CONSIDERATIONS

It is important to note that the ROI approach adopted here was motivated by a theoretical assumption that average FA taken in one region of a specific network may provide an index of white matter tract properties shared with other parts of this network. Claims of specificity are not meant to imply that this ROI is the only region of a network sharing these properties, but rather that this region of this network can be functionally differentiated from other regions of other networks. Furthermore, the technique used to define ROIs in this study, the ROQS method, avoids any form of spatial transformation to the DTI FA data by defining each ROI on a subject-by-subject basis.

There are a few additional limitations of this study that may be addressed in future investigations of white matter pathways involved in the components of the attention network. First, this study was a preliminary study for which it was necessary to explore a small set of ROIs that was motivated by previous literature findings and the insights of the attentional networks implicated from pervious imaging and neuropsychological studies. On one hand small set of regions explored for each function lead to fewer multiple comparison corrections and a better-powered analysis. On the other hand, only a small fraction of each network was included in each ROI, and thus the majority of white matter tracts within each network were left unexplored. The small sample size might have also limited insights into the functional significance of laterality results, such as the involvement of the right hemisphere ACR to the conflict network and the lateralization of the alerting network. Additionally, the study focused on which white matter networks are directly involved in each component. If we are to believe that there must be some sort of interaction between component networks (in addition to being individually and independently modulated by the proposed pathways), then it is possible in future studies to examine the connectivity between the white matter pathways. Future studies may benefit from combining fMRI with DTI as well as implementing tractography to study the connections between networks. Finally, the nature of the ROI approach

adopted in this study is inherently limited to *a priori* predictions of regions that might be implicated in function. Exploratory analyses examining white matter tract regions throughout the brain may play an important role in providing new hypotheses to test in future studies.

In conclusion, combining these observations with previous fMRI and neuropsychological studies suggests the components of attention are comprised of segregated functional networks and that individual differences in white matter tract microstructural integrity might modulate these functionally specific neural networks. Thus, understanding individual differences in white matter tract microstructural integrity may prove an important complement to fMRI studies of functional and anatomical organization of attention.

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The effects of normal aging on myelinated nerve fibers in monkey central nervous system

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The effects of aging on myelinated nerve fibers of the central nervous system are complex. Many myelinated nerve fibers in white matter degenerate and are lost, leading to some disconnections between various parts of the central nervous system. Other myelinated nerve fibers are affected differently, because only their sheaths degenerate, leaving the axons intact. Such axons are remyelinated by a series of internodes that are much shorter than the original ones and are composed of thinner sheaths. Thus the myelin-forming cells of the central nervous system, the oligodendrocytes, remain active during aging. Indeed, not only do these neuroglial cell remyelinate axons, with age they also continue to add lamellae to the myelin sheaths of intact nerve fibers, so that sheaths become thicker. It is presumed that the degeneration of myelin sheaths is due to the degeneration of the parent oligodendrocyte, and that the production of increased numbers of internodes as a consequence of remyelination requires additional oligodendrocytes. Whether there is a turnover of oligodendrocytes during life has not been studied in primates, but it has been established that over the life span of the monkey, there is a substantial increase in the numbers of oligodendrocytes. While the loss of some myelinated nerve fibers leads to some disconnections, the degeneration of other myelin sheaths and the subsequent remyelination of axons by shorter internodes slow down the rate conduction along nerve fibers. These changes affect the integrity and timing in neuronal circuits, and there is evidence that they contribute to cognitive decline.

Keywords: rhesus monkey, oligodendrocytes, myelin sheaths, axons, degeneration, remyelination

INTRODUCTION

There are two types of nerve fibers in the central nervous system, myelinated and unmyelinated ones. The myelinated nerve fibers are axons of neurons that are ensheathed by internodal lengths of myelin formed by oligodendrocytes. Developmentally, the internodal lengths of myelin are produced at the ends of processes of oligodendrocytes and each internode is generated by a spiral wrapping of a paired sheet of oligodendrocytic plasma membrane. Initially the successive turns of the spiral of paired membrane sheets are separated by cytoplasm, but eventually the cytoplasm is extruded from between the turns. As a result, mature, compact myelin is formed. At the ends of each internodal length of myelin are regions called paranodes, and here the turns of the spiral wraps of myelin membrane successively terminate, the innermost one terminating first (**Figure 1**). As the turns of myelin terminate the sheath gradually becomes thinner, and eventually end at the nodes of Ranvier, which separate the successive internodal lengths of myelin. At the nodes the axon is bare, but is characterized by a dense undercoating.

An oligodendrocyte forms several internodal lengths of myelin, each one on a different axon, and in general the larger the diameter of the axon, the thicker is its myelin sheath and the longer its internodes and its paranodes (see **Figure 1**). And since there seems to be some limit to the amount of myelin an individual oligodendrocyte can produce and maintain, oligodendrocytes that myelinate small diameter axons form more internodal lengths of myelin than those that myelinate larger diameter axons. Myelin contains lipoproteins, so that in unfixed brains the myelin sheaths have a white sheen. Consequently, tracts of the central nervous system that contain mostly myelinated nerve fibers and few neurons are referred to as white matter. In contrast, gray matter contains the cell bodies and dendrites of neurons and fewer myelinated nerve fibers.

Our studies have been concerned with the effects of age on myelinated nerve fibers in the central nervous system of a non-human primate, the rhesus monkey (*Macaca mulatta*). The rhesus monkey offers an excellent model in which to examine the effects of normal aging on the brain, because unlike humans, rhesus monkeys do not develop neurofibrillary tangles are not subject to the dementia that characterizes Alzheimer's disease. In humans the existence of this disease makes it difficult to study the effects of normal aging, because it is often a problem to determine if older individuals are really normal, since they may have the beginning of Alzheimer's disease, which is characterized morphologically by the presence of both senile plaques and neurofibrillary tangles that cause neurons to die. Indeed, recent studies have shown some cognitively intact persons can have substantial numbers of plaques and tangles in their brains (e.g. Bennett, 2006; Silver et al., 2002). Added difficulties in determining which of the morphological aging changes that occur in the human brain are responsible for normal cognitive decline, is that most older people have not been behaviorally tested before their brains become available for examination, so that their real cognitive status is usually not known. And even when a brain

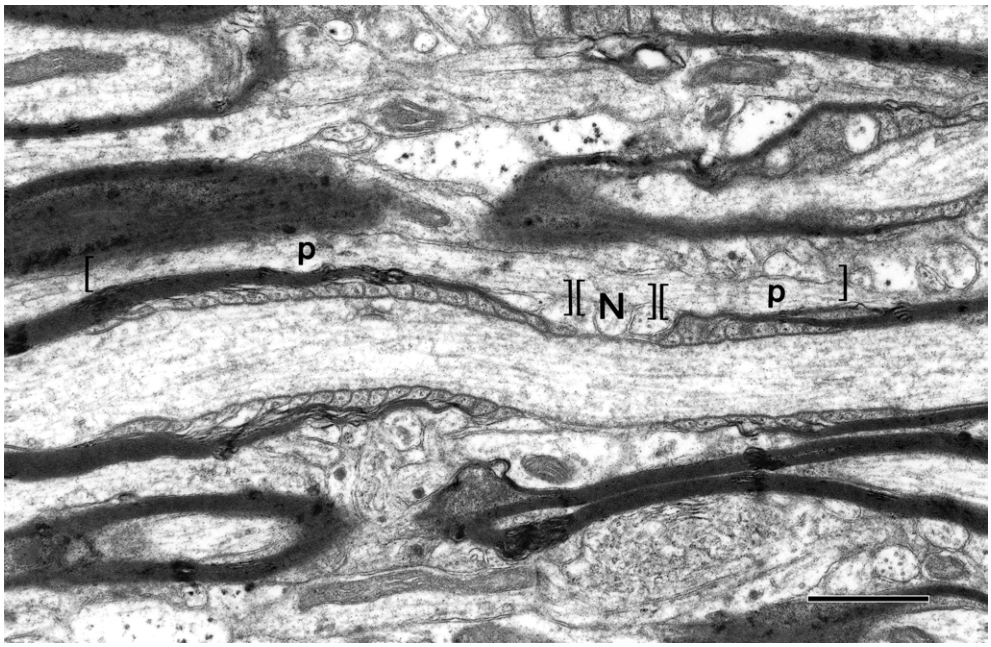

FIGURE 1 | A longitudinal section of a myelinated nerve fiber in primary visual cortex of a rhesus monkey. The section passes through a node of Ranvier (N) where the axolemma has a characteristic undercoating. On each side of the node are the paranodes (p). The sheath on the right has 8 lamellae, while the one on the left is thicker with 15 lamellae, so that it has a longer paranode. Scale bar = 1 micron. (From Peters and Sethares, 2003).

becomes available for examination, the delay in fixing the tissue usually leaves the structural preservation less than optimal.

Rhesus monkeys live for a maximum of 35 years (Tigges et al., 1988) and the advantage of using them as a model for normal aging is that they are not subject to Alzheimer's disease, and overall, do not lose significant numbers of neurons from their cerebral cortex with age (see Merrill et al., 2000; Morrison and Hof, 1997; Peters et al., 1998), However, Smith et al. (2004) have recently claimed that there may be a focal loss of neurons from cortical area 8A of the prefrontal cortex. None of the neurons in the aging monkey cortex acquire neurofibrillary tangles, and although some senile plaques may be present, particularly in the frontal and primary somatosensory cortices of older monkeys, they are few in number (Heilbroner and Kemper, 1990; Struble et al., 1985). The small numbers of plaques do increase with age in monkeys, but there is no correlation between plaque burden and cognitive decline (Sloane et al., 1997). The other advantage of using monkeys to study normal cognitive decline is that over the entire range of their life span, monkeys can be behaviorally tested to determine their cognitive status, and in our studies their cognitive status is defined by a cognitive impairment index (CII). Their brains can then be properly prepared for morphological, physiological or biochemical analyses.

The first hint that there are age-related changes in myelinated nerve fibers came from the observation that in old humans and monkeys there is a decrease in the intensity of haemotoxylin staining of white matter (e.g., Kemper, 1994; Lintl and Braak, 1983). The underlying reason for this increased myelin staining pallor is still not clear, but it is now know that there are a number of age-related alterations of myelinated nerve fibers in the primate central nervous system, such as a loss of some myelinated nerve fibers and alterations in the morphology and composition of myelin sheaths, that could account for the decrease in staining intensity. Some of these alterations will be considered in the next sections, which will concentrate primarily on age changes that have been encountered in non-human primates. The correlations that occur between age-related morphological changes in myelinated nerve fibers and cognitive decline will also be considered.

LOSS OF MYELINATED NERVE FIBERS

Magnetic resonance imaging (MRI) studies of both human (e.g. Albert, 1993; Guttmann et al., 1998) and monkey (Lai et al., 1995; Wisco et al., 2008) brains have shown there is a loss of white matter from the cerebral hemispheres with age. For example, Wisco et al. (2008) calculate that in rhesus monkeys there is a 11.5% loss of white matter from the forebrain with age, in contrast to only a 2% loss of gray matter. However, it should be noted that other studies on humans (e.g. Pfefferbaum et al., 1994; Resnick et al., 2003; Sullivan et al., 2004) and monkeys (Andersen et al., 1999) suggest that loss of volume of the hemispheres is mainly due to a thinning of the cerebral cortex. Nevertheless, there seems to be general agreement that there is some loss of white matter with age and this is supported by stereological studies on cognitively normal human brains. The first of these studies was that of Pakkenberg and Gundersen (1997) who examined brains from humans between 20 and 95 years of age, and using the Cavalieri's principle to determine volume changes, they concluded there is a 28% decrease in the volume of white matter from the cerebral hemispheres. In another study from this same laboratory, Tang et al. (1997) using light and electron microscopy concluded that this loss is due to a 27% overall loss in the lengths of myelinated nerve fibers from white matter. Later Marner et al.

(2003) extended these studies by examining samples that were taken systematically and randomly from the white matter of 36 normal brains of males and females aged between 18 and 93 years. They concluded that though the overall loss of white matter from the human cerebral hemispheres is 23%, the overall decrease in total myelinated nerve fiber length is even greater, being 45%. A similar loss of myelinated nerve fibers from the human brain has been reported by Meier-Ruge et al. (1992), who examined the brains of cognitively normal humans and concluded there is a 16% loss of myelinated nerve fibers from white matter of the precentral gyrus and an 11% loss from the corpus callosum.

Studies of the effects of age on the monkey brain support the contention that there is a loss of myelinated nerve fibers with age. In each of the white matter tracts we have examined some loss of myelinated nerve fibers has been found. Over the life span of the monkey the average number of myelinated nerve fibers lost from the optic nerve (Sandell and Peters, 2001), and from the anterior commissure (Sandell and Peters, 2003) is about 45%, while from the fornix and the splenium of the corpus callosum (unpublished data), the loss is about 25%. In all four structures the correlations between the decreasing numbers of myelinated nerve fibers and increasing age are significant.

In contrast, there is no measurable loss of myelinated nerve fibers from the visual cortex (Nielsen and Peters, 2000), but the inability to detect a loss may be due to the relatively sparse numbers of myelinated nerve fibers present in cortex, because a few myelinated nerve fibers with degenerating axons have been seen in cortex (**Figure 2**). Indeed myelinated nerve fibers with degenerating axons, as indicated in the electron microscope by the presence of dense axoplasm with a loss identifiable organelles, or the presence of empty myelin sheaths (**Figure 2**), have been encountered in all of the parts of the aging monkey brain that we have examined, suggesting that myelinated nerve fiber loss is ubiquitous. And based on earlier studies of Wallerian nerve fiber degeneration, there is little doubt that once an axon degenerates, breakdown and degeneration of its myelin sheath inexorably follows (e.g. Guillery, 1970).

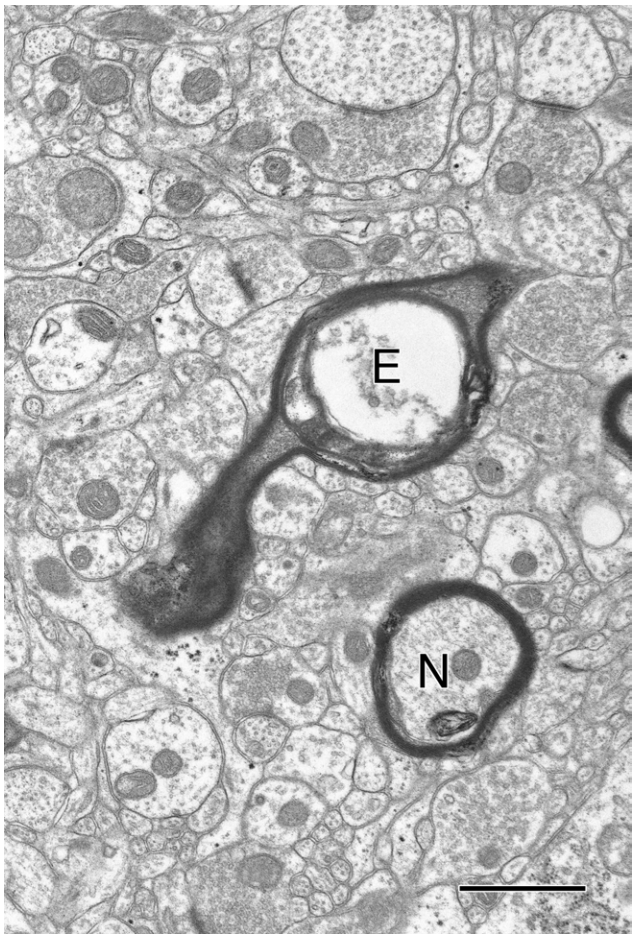

FIGURE 2 | In the field is a normal nerve fiber (N) and another nerve fiber (E) in which the axon has degenerated, leaving an empty sheath. Layer 4 from area 46 of a 27-year-old rhesus monkey. Scale bar = 1 micron.

Myelinated nerve fiber loss from white matter in pathways must result in some disconnection between various parts of the central nervous system. But interestingly, although there are no significant correlations between the extent of myelinated nerve fiber loss from the splenium of the corpus callosum and the cognitive decline shown by monkeys (Peters and Sethares, 2002), there are correlations between cognitive decline and myelinated nerve fiber loss from the anterior commissure (Sandell and Peters, 2003) and the fornix (unpublished). In this context, it is interesting that cutting the splenium of the corpus callosum, which is the principal fiber pathway connecting the occipital cortices, has little effect on cognition (Innocenti, 1986). The anterior commissure, provides the interhemispheric connection between the entire temporal lobe, as well as parts of the orbitofrontal cortex prepiriform cortex and the amygdala (Demeter et al., 1990; Jouandet and Gazzaniga, 1979; Sullivan and Hamilton, 1973a,b), and numerous studies have shown that the anterior commissure provides a pathway whereby visual information can reach the opposite hemisphere and contribute to behavioral responses, such as two-choice discrimination (Doty et al., 1994; Gross et al., 1977; Sobotka and Ringo, 1996). The fornix, on the other hand, carries the main output from the hippocampus, and studies of the effects of lesioning the fornix in both monkeys (Fletcher et al., 2006; Gaffan et al., 2001; Owen and Butler, 1981; Wilson et al., 2007) and humans (e.g. D'Esposito et al., 1995; Gaffan et al., 1991) have revealed the role of the fornix in memory and have described amnesia as a major consequence of making such lesions.

It might be assumed that since myelinated nerve fibers are lost from white matter with age, that there must be a concomitant loss of the neurons from which the nerve fibers arise. For the optic nerve, this may be the case, since retinal ganglion cells are subject to damage from ocular changes and systemic disease that occurs frequently in the elderly (Garner et al., 1994). But for the other central nervous system pathways, in which the myelinated nerve fibers arise from cortical neurons, a different reason has to be sought, because, as stated above, recent studies have shown that in normal aging few neurons are lost from the cerebral cortices of either monkeys or humans (e.g., Hof et al., 2000; Merrill et al., 2000; Morrison and Hof, 1997; Pakkenberg and Gundersen, 1997; Peters et al., 1998), and Freeman et al. (2008) have recently shown that in normally aging humans cortical neuron numbers are preserved even when there is cortical atrophy. To account for the age-related loss of myelinated

nerve fibers from white matter, we have suggested that only the portion of the axonal plexus of a pyramidal cell that enters the white matter, degenerates by a dying back process, leaving the more extensive local axonal plexus in the cortex intact (Peters and Rosene, 2003). This scenario would account both for the loss of some myelinated nerve fibers from white matter and for the failure to detect myelinated nerve fiber loss from the cerebral cortex itself.

DEGENERATIVE CHANGES IN MYELIN SHEATHS

Obviously, in normal aging some myelin sheaths degenerate as a consequence of their axons degenerating, but in other cases myelin sheaths degenerate even though the axon is intact. In the latter category there are two kinds of myelin sheath alterations. The most common age-related degenerative alteration is an accumulation of dark cytoplasm in pockets that are produced by a splitting of the major dense line (e.g. Peters et al., 1994; Peters and Sethares, 2002; Peters et al., 2000; Sandell and Peters, 2003). Examples of what will be referred to as dense sheaths are shown in **Figure 3**. The location of the dense cytoplasm in splits of the major dense line implies that the cytoplasm must be derived from the parent oligodendrocyte, because the major dense line of the myelin sheath is produced by apposition of the cytoplasmic faces of the plasma membrane of the oligodendrocyte forming the myelin sheath. The amount of dense cytoplasm can vary from a small amount contained in a local split of the sheath to an accumulation that is extensive, causing the sheath to bulge out into the surrounding intercellular space. Longitudinal sections of affected sheaths show that the accumulations of dense cytoplasm are localized, although there may be several such loci along an internodal length of myelin. Proof of the fact that the accumulation of dense cytoplasm in normal aging is a degenerative change comes from studies of Curprizone toxicity, which leads to oligodendrocyte death, resulting in the formation of dense cytoplasm in the cytoplasmic process on the inner face of the myelin sheath (Ludwin, 1978). A similar dense cytoplasm also occurs in the sheaths of mice with a myelin-associated glycoprotein deficiency (e.g. Lassmann et al., 1997).

Another, but less common myelin alteration associated with aging is the formation of myelin balloons (e.g. Feldman and Peters, 1998; Peters and Sethares, 2003). These balloons can be as large as 10 μm in diameter, so that even by light microscopy the larger balloons they are visible as holes in the neuropil of the aging cortex. Electron microscopic analyses show that these holes are really localized fluid-filled cavities that are accommodated by splits in the intraperiod line of the affected sheaths, and since the intraperiod line is produced by apposition of the outer faces of the cytoplasmic membrane of the oligodendrocyte, the fluid-filled sacs are potentially in contact with the extracellular space. In larger balloons, the axon of the nerve fiber is pushed to one side of the sheath,

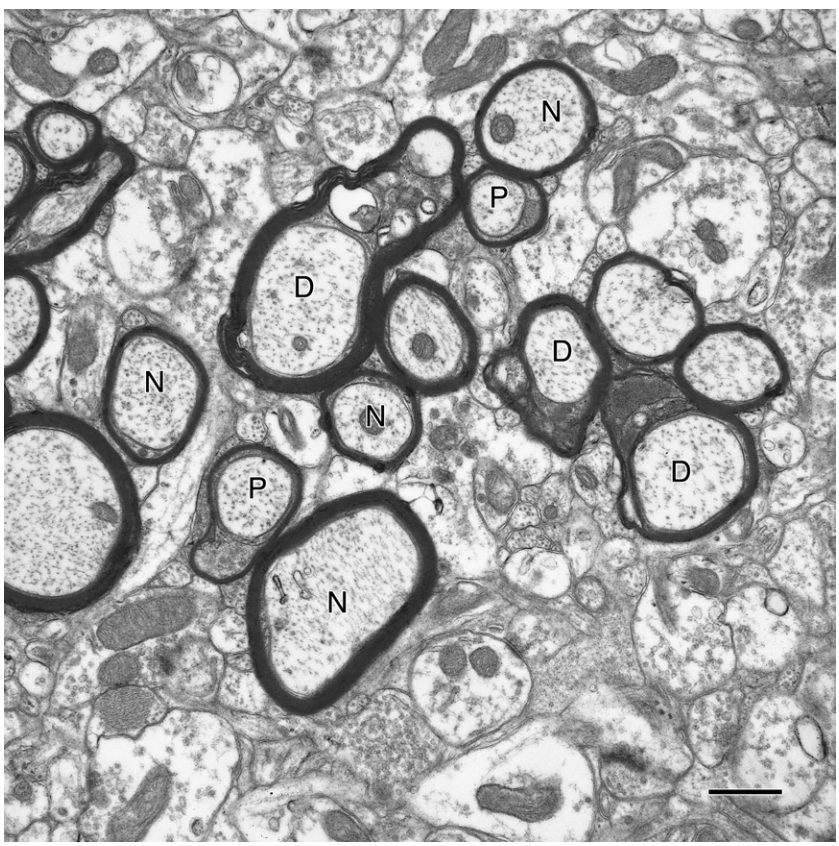

FIGURE 3 | A cross-sectioned nerve fiber bundle in primary visual cortex of a 29-year-old rhesus monkey. In this bundle some of the nerve fibers (N) have normal sheaths and are sectioned through internodes, and others (P) are sectioned through paranodes. Three nerve fibers (D) have degenerating sheaths, as shown by the accumulation of dense cytoplasm in splits between lamellae. Scale bar = 1 micron.

suggesting that the fluid in the sac must be exerting some pressure. But despite this, the sheath is of the same thickness all around the balloon, and there is no obvious change in the periodicity of the myelin lamellae. Consequently it does not appear that the myelin sheath is elastic, and so the formation of a balloon must entail the production of excess myelin by the parent oligodendrocyte. It should be emphasized that dense sheaths and balloons are not entirely separate entities, since it is not uncommon for a balloon to have some dense cytoplasm at its base, or for a sheath with a balloon to have some dense cytoplasm between its lamellae.

Again, there is evidence that the formation of balloons is a degenerative process, since myelin balloons can be produced by Cuprizone (Ludwin, 1978) and tetraethyl tin (Malamud and Hirano, 1973) toxicity, and by chronic copper poisoning (Hull and Blakemore, 1974). Balloons can also occur in early phases of Wallerian degeneration (Franson and Ronnevi, 1989), and in severe diabetes (Tamura and Parry, 1994).

When the percentage of myelinated nerve fibers showing either the presence of dense cytoplasm or of balloons is examined, it is found that the frequency of such profiles increases significantly with age (e.g. Feldman and Peters, 1998). More importantly there are significant correlations between cognitive declines and the frequency of profiles of degenerating sheaths in cortical area 46 (Peters and Sethares, 2002), splenium of the corpus callosum (Peters and Sethares, 2002), anterior commissure (Sandell and Peters, 2003), and fornix (unpublished data). An exception is primary visual cortex, in which there is no correlation between cognitive decline and myelin sheath degeneration (Peters et al., 2000). This may be because primary visual cortex has little role in cognition. It is presumed that the correlations between myelin degeneration and cognition are due to the degenerating slowing down conduction velocity, and thus affecting the timing in neuronal circuits.

Duce et al. (2007) have identified a number of genes that might produce cytotoxicity in white matter. These genes range from ones that can affect life span, to ones that can affect the reorganization of glial cytoskeleton, others that can produce oxidative and proteolytic injury, and yet others that are cell cycle inhibitors. But these authors focus particular attention on a gene called Klotho, a multifunctional gene that is known to defend against oxidative stress, and suggest that with a decrease in the activity of Klotho there is a loss of this protection, which may result in the death of oligodendrocytes.

THE CONTINUED FORMATION OF MYELIN

There are other age-related alterations in myelin sheaths, which indicate that myelin continues to form with age. The first is an increase in the overall thickness of normal myelin sheaths with age. Thus, in the primary visual cortex of the monkey the mean number of lamellae in sheaths of myelinated nerve fibers in layer 4Cβ of young monkeys is 5.6, while in old monkeys it is 7.0 (Peters et al., 2001). However, the increase in thickness of sheaths is not uniform, because the mean increase in the numbers of lamellae is largely because thick sheaths, with more than 10 lamellae, become more common in old monkeys. This increase in the numbers of lamellae on nerve fibers with thicker myelin sheaths often affects their paranodes, so that whereas longitudinal sections of young nerve fibers show the paranodal pockets of cytoplasm to terminate is a regular sequence and to be all in contact with the underlying axolemma (see **Figure 1**), in nerve fibers of old monkeys with thicker sheaths the paranodes can be disarrayed. They can be piled up on one another, so that only some of the paranodal loops are in contact with the axolemma (Hinman et al., 2006). A similar situation has been reported in the myelinated nerve fibers of old rats (Sugiyama et al., 2002), and it seems likely that such disruption of the paranodal region could affect conduction velocity. As yet, not determination seems to have been made about whether myelin sheaths in white matter also become thicker with age.

Another change that is considered to indicate the continued formation of myelin is the formation of sheaths that contain redundant myelin, so that the sheaths are too large for their enclosed axons. When such sheaths are cross-sectioned and examined by electron microscopy the axon is seen to be located at one end of an excessively large sheath that loops off into the surrounding neuropil. Such sheaths were first described by Rosenbluth (1966) in the cerebellum of the toad. Sturrock (1976) described such sheaths in anterior commissures of old mice, and later Cullen and Webster (1979) found them to be common in the optic nerves of metamorphosing toads. During metamorphosis the optic nerves become shorter, and the myelin sheaths undergo extensive remodeling, producing redundant sheaths that disappear later in development. These events led Cullen and Webster to suggest that the overproduction of myelin is to allow the sheaths to enlarge so that they can accommodate subsequent increases in the diameters of the enclosed axons. However, the role of redundant sheaths in the aging process is not yet evident, although axons in these sheaths generally have small diameters.

REMYELINATION

When the frequencies of various kinds of profiles of myelinated nerve fibers are quantified in the vertical bundles of nerve fibers in the cerebral cortex it becomes evident that the frequency of profiles of paranodes increases with age (Peters and Sethares, 2003). As pointed out earlier, for the present purposes the paranodes are defined as those regions at the ends of internodes where the spiraled myelin lamellae gradually terminate as the sheath approaches a node of Ranvier. As the myelin lamellae terminate, the major dense line opens up to accommodate a spiral of cytoplasm, which in longitudinal sections through paranodes appears as a series of pockets of cytoplasm on each side of the axon (see **Figure 1**). Where the plasma membrane on the inner faces of these pockets meet the axolemma, the two membranes, become very close to each other and form a junctional complex. This membrane apposition makes it quite easy to identify profiles of myelinated nerve fibers sectioned through paranodes (**Figure 3**), and it turns out there is a 57% increase in the frequency of paranodal profiles in the aging visual cortex, a 90% increase in area 46 of prefrontal cortex (Peters and Sethares, 2003), and a 60% increase in the anterior commissure (Sandell and Peters, 2003).

There could be two reasons for these age-related increases in paranodal profiles. One is an increase in the lengths of the paranodes with age. The second is an overall increase in the numbers of paranodes. Paranodes do become slightly longer as the numbers of lamellae in myelin sheaths increase with age, but not enough to account for the 60% or more increase in the frequency of paranodal profiles. Consequently, the increase in frequency has to be due to an increase in the overall numbers of internodal lengths of myelin. This

would occur if remyelination were taking place, such that some of the original internodal lengths of myelin degenerate and are replaced by shorter internodes. The accepted hallmarks of remyelination are the presence of short internodes and of sheaths that are inappropriately thin for the size of the enclosed axons (e.g. Brück et al., 2003; Hirano, 1989; Kreutzeberg et al., 1997; Ludwin, 1995; Prineas and McDonald, 1997). Such internodes have been found in the aging central nervous system. Internodes of myelin, as short as 3–8 μm, are present in old monkeys, as well as sheaths that are inappropriately thin for the size of the enclosed axon (see Peters and Sethares, 2003).

We have not been able to identify demyelinated nerve fibers in the monkey brain, but this should not be a surprise, since such demyelinated nerve fibers would be expected to resemble unmyelinated nerve fibers. However, in support of the fact that demyelination is taking place, we have seen fragments of degenerating myelin within the cytoplasm of both microglia, and more commonly within astrocytes in the brains of aging monkeys. Also some of the amorphous phagocytosed material within the cytoplasm of astrocytes in the cerebral cortex of old monkeys labels for antibodies to myelin basic protein (Peters and Sethares, 2003).

A recent article on the remyelination of rubrospinal nerve fibers that remyelinate after a contusion lesion of the spinal cords of mice serves to shed some light on what is happening during aging. Lasiene et al. (2008) have shown that remyelinated nerve fibers in the rubrospinal tract of mice have much shorter internodal lengths than in control mice, and that these remyelinated axons conduct at a lower rate than the controls. There is also evidence that there are reductions in conduction velocity in the nerve fibers of aging cats (Morales et al., 1987; Xi et al., 1999). A reduction in conduction velocity also occurs in demyelinating diseases (e.g. Felts et al., 1997; Waxman et al., 1995).

Consequently, it can be assumed that remyelinated nerve fibers with shorter internodes in the aging monkey also have a slower conduction rate than the nerve fibers that remain unaffected by age, and that this would affect the timing in neuronal circuits (e.g. see Wang et al., 2005). However, when correlations between the frequency of occurrence of profiles of paranodes and the overall cognitive status of individual monkeys, as measured by the Cognitive Impairment Index, CII, are examined, there is no significant correlation between the two measures, for visual cortex and anterior commissure, but there is a correlation for area 46 of prefrontal cortex ($p < 0.01$; Peters and Sethares, 2002). The reason for this correlation between CII and paranodal frequency may have to do with the unique role of prefrontal cortex in cognition.

EFFECTS OF AGE ON OLIGODENDROCYTES

In monkey cerebral cortex stained with Perl's reaction for ferric iron the processes of some oligodendrocytes in old monkeys show swellings along their lengths (**Figure 4**; insert), and when these swellings

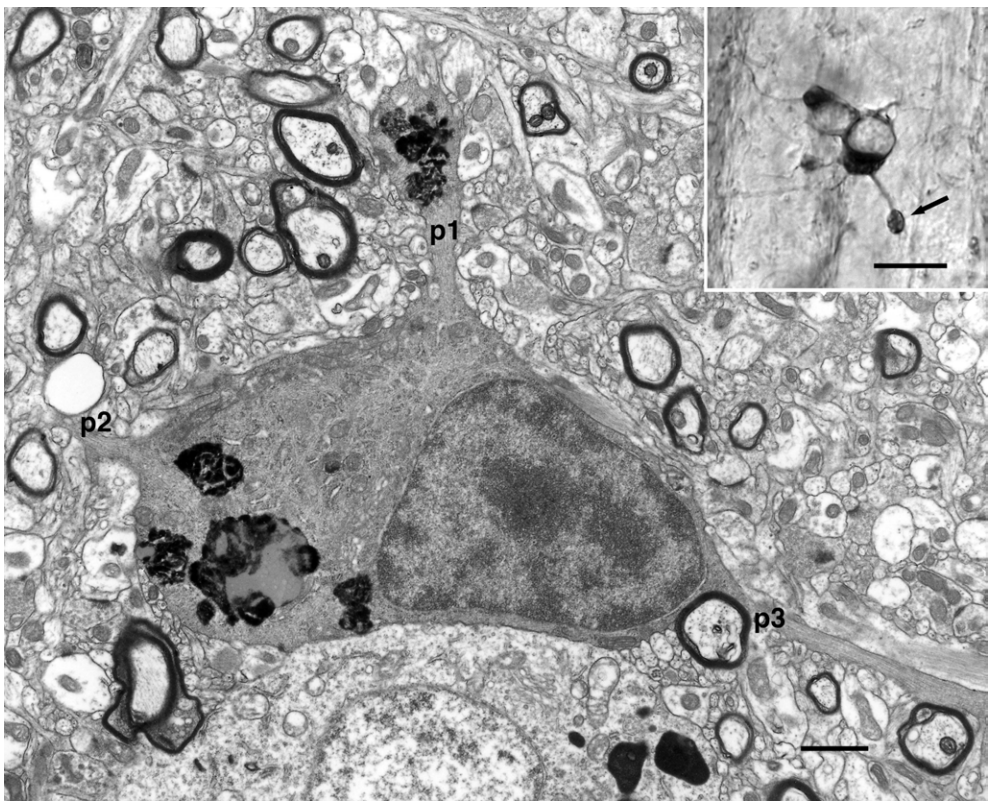

FIGURE 4 | Electron micrograph of an oligodendrocyte in layer 6 of area 17 of a 35-year-old rhesus monkey. Three processes, p1–p3, extend from the cell body. One of the processes, p1, has a swelling that contains dense inclusions, which are similar to the dense inclusions in the cell body. Scale bar = 1 micron. The **insert** shows a light microscopic image of an oligodendrocyte stained with Perl's reaction for iron compounds. Note the large swelling (arrow) on one of the processes. It is similar to the one seen on process p1 in the accompanying electron micrograph. Area 46 of a 28-year-old monkey. Scale bar = 10 microns.

are examined in the electron microscope it is seen that they contain dense inclusions (**Figure 4**). Similar dense inclusions also occur in the perikarya of old oligodendrocytes (Peters, 1996; Peters and Sethares, 2004; Peters et al., 1991), and since the dense material is not membrane bound, it is unlikely to be produced by phagocytosis. Similar swellings along oligodendrocyte processes have been reported in twitcher mice, which are a model for globoid leukodystrophy, and Levine and Torres (1992) suggest that the material in the swellings comes from components of myelin sheaths that are being renewed. Most probably the material is produced by degeneration of some components of the myelin sheaths that belong to the oligodendrocytes, and it is tempting to suggest that the material is related to the dense cytoplasm that accumulates between the lamellae of some sheaths in old monkeys.

It is also common in old monkeys to find oligodendrocytes in pairs, rows and groups, suggesting that oligodendrocytes may be proliferating with age (Peters and Sethares, 2004; Peters et al., 1991), and when comparisons are made between the numbers of oligodendrocytes in young and old primary visual cortices it is evident that there is an increase in the numbers of oligodendrocytes with age (Peters et al., 1991). Thus in layer 4Cβ for example, in which oligodendrocytes account for about 55% of the total population of neuroglial cells, there is a 50% increase in the numbers of oligodendrocytes with age (Peters and Sethares, 2004). In a more recent study an assessment was made of the effects of age on the populations of neuroglial cells throughout the depth of monkey primary visual cortex (Peters et al., 2008). It was seen that the numbers of oligodendroglial cells in the various layers essentially reflects the frequency of myelinated nerve fibers within them, the greatest numbers of oligodendrocytes being in the deeper layers. Again, with age the numbers of oligodendrocytes in all layers was found to increase by about 50%. In contrast, there are no changes in the frequency of either astrocytes or microglial cells with age. There is also an increase in the frequency of oligodendrocytes in monkey optic nerve with age (Sandell and Peters, 2002), as well as in the fornix (unpublished data), but not in the anterior commissure (Sandell and Peters, 2003). The reason for this difference is not yet apparent.

What is the origin of the increased numbers of oligodendrocytes that are generated, and why are they necessary? The formation of groups and rows of oligodendrocytes during aging could be taken to suggest that oligodendrocytes are dividing, but the prevailing view is that mature oligodendrocytes do not divide (see Keirstead and Blakemore, 1997; Ludwin, 1995; Norton, 1994), and in a study of the generation of new cells in the adult dentate gyrus of the hippocampus in old monkeys using BrdU labeling no labeled oligodendrocytes were found (Ngwenya et al., 2008). It is more likely that new oligodendrocytes originate from the oligodendroglial precursor cells which express NG2 chondroitin sulfate, These cells are scattered throughout the central nervous system, and in adult rodents they account for about 5% of all neuroglial cells (e.g. Levine et al., 2001).

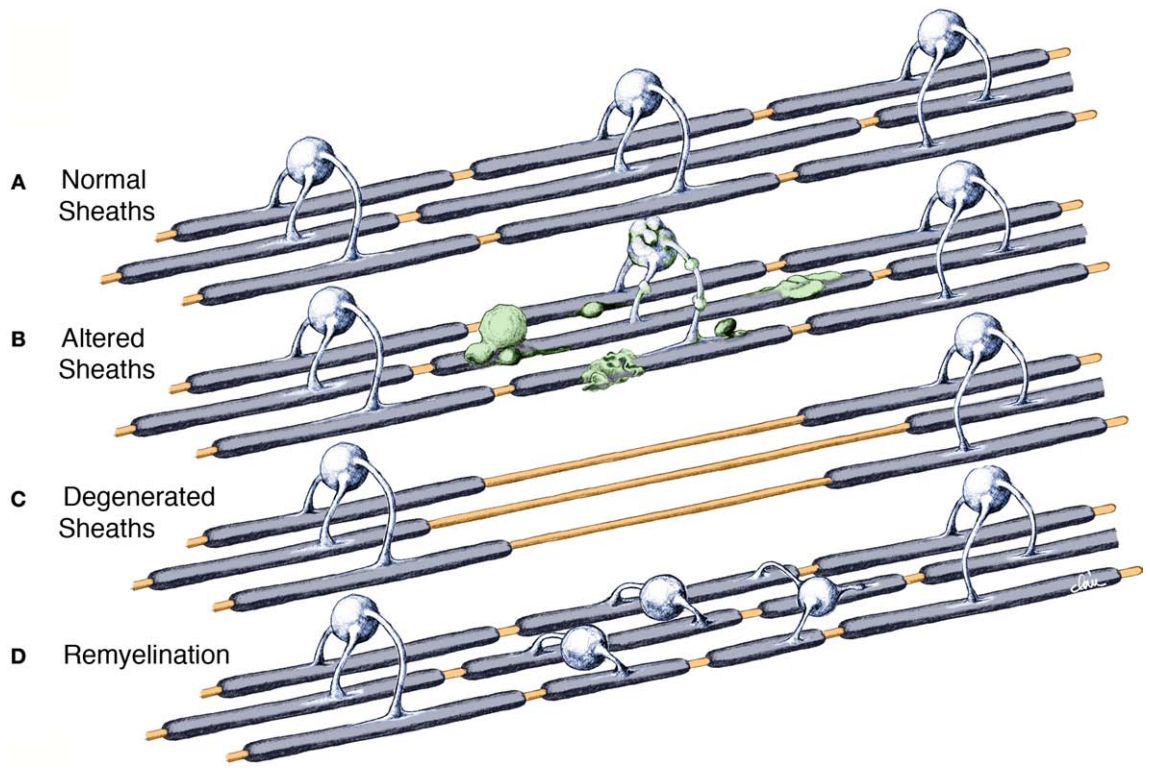

FIGURE 5 | Diagrammatic representation of the degeneration of sheaths with age, and the subsequent remyelination of axons. (A) Normal state. **(B)** Some sheaths become altered by the presence of dense cytoplasm and the formation of balloons. This is believed to occur when the oligodendrocyte accumulates dense inclusions within its cell body and within swellings along its processes. **(C)** The degeneration of myelin sheaths leaves axons bare. **(D)** The bare axons are remyelinated by newly generated oligodendrocytes that form short internodal lengths with thin sheaths.

Studies such as that by Cerghet et al. (2006) have shown that there is a turnover of oligodendroglial cells in the adult mouse, such that some oligodendrocytes undergo apoptosis, and die, while new oligodendrocytes are being generated. And interestingly, Cerghet et al. (2006) found the turnover of oligodendrocytes in the corpus callosum to be greater in female mice than in males, indicating that the lifespan of oligodendrocytes is shorter in females than in males. Moreover, Rivers et al. (2008) have recently shown that in adult mice many of the newly generated oligodendrocytes that arise from the oligodendrocytic precursor cells during adult hood are involved in myelination. They calculate that about 20% of all oligodendrocytes in the adult corpus callosum are generated during adulthood and that many of these cells form myelin. In contrast, the same group calculates that only about 5% of the adult-born oligodendrocytes in the cerebral cortex appear to be involved in the elaboration of myelin sheaths, but Rivers et al. (2008) were not able to determine the function of the other adult generated oligodendrocytes present in cortex.

Unfortunately there is no information about the rate of turnover of oligodendrocytes in the adult monkey, but there is no reason to doubt that it is basically different from in rodents.

A SYNTHESIS

It is proposed that the following scenario can explain the available data on the effects of age on myelinated nerve fibers in the central nervous system of the monkey. During aging some neurons lose their long projecting myelinated axons that enter white matter, while retaining their local plexuses so that the parent neuron does not die. The consequence of this is that, as has been demonstrated, some myelinated nerve fibers are lost from white matter, even though there is no significant loss of neurons from the cerebral cortex. For other neurons the effects of aging are less severe (see **Figure 5**), since their axons remains intact, even though some of the internodal lengths of myelin that ensheath them degenerate (**Figure 5B**). The process of demyelination probably begins as an oligodendrocyte shows stress and starts to accumulate dense inclusions in swellings of its processes and in its perikaryon, as well as in spaces between the lamellae of the myelin sheaths for which the oligodendrocyte is responsible. Ultimately the oligodendrocyte dies, which results in the degeneration and loss of the internodal lengths of myelin belonging to that oligodendrocyte (**Figure 5C**). Oligodendrocyte precursor cells are then activated and generate new oligodendrocytes that repair the damage by remyelinating the bare lengths of axons. In the process of remyelination, several new oligodendrocytes are involved in the replacement of the original internode of myelin. These oligodendrocytes produce shorter internodal lengths than the original one, and the new sheaths are thinner (**Figure 5D**). Thus, when profiles of sectioned myelin sheaths in older monkeys are examined, it is found there is an increase in the number of profiles of paranodes, and this is accompanied by an increase in the total number of oligodendrocytes. This breakdown of myelin sheaths, together with the formation of shorter internodal lengths of myelin and the consequent increase in the number of nodes of Ranvier, would result in a slowing down of the rate of conduction along affected myelinated nerve fibers. Consequently the timing in neuronal circuits would be affected and contribute to cognitive impairment that occurs with increasing age.

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Oligodendrocyte development and the onset of myelination in the human fetal brain

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Oligodendrocytes are cells that myelinate axons, providing saltatory conduction of action potentials and proper function of the central nervous system. Myelination begins prenatally in the human, and the sequence of oligodendrocyte development and the onset of myelination are not thoroughly investigated. This knowledge is important to better understand human diseases, such as periventricular leukomalacia, one of the leading causes of motor deficit in premature babies, and demyelinating disorders such as multiple sclerosis (MS). In this review we discuss the spatial and temporal progression of oligodendrocyte lineage characterized by the expression of specific markers and transcription factors in the human fetal brain from the early embryonic period (5 gestational weeks, gw) until midgestation (24 gw). Our *in vitro* evidence indicated that a subpopulation of human oligodendrocytes may have dorsal origin, from cortical radial glia cells, in addition to their ventral telencephalic origin. Furthermore, we demonstrated that the regulation of myelination in the human fetal brain includes positive and negative regulators. Chemokines, such as CXCL1, abundant in proliferative zones during brain development and in regions of remyelination in adult, are discussed in the view of their potential roles in stimulating oligodendrocyte development. Other signals are inhibitory and may include, but are not limited to, polysialic acid modification of the neural cell adhesion molecule on axons. Overall, important differences in temporal and spatial distribution and regulatory signals for oligodendrocyte differentiation exist between human and rodent brains. Those differences may underlie the unique susceptibility of humans to demyelinating diseases, such as MS.

Keywords: human brain development, immunohistochemistry, myelination, oligodendrocyte progenitor cells, organotypic slice cultures, chemokines, PSA-NCAM, transcription factors

INTRODUCTION

Oligodendrocytes are glia cells that produce myelin, the lipid-enriched axon-ensheathing membrane, which is essential for saltatory conduction of action potentials in the central nervous system (CNS). They were first discovered by Robertson (1899), but named and classified by Rio Hortega (1921). The origin and differentiation of oligodendrocytes have been extensively studied in animal models, and are especially well documented in rodents, thanks to advances in various molecular biology techniques that have provided the means of genetic mapping of cell lineages in the developing mouse brain (He et al., 2001; Kessaris et al., 2006; Marshall and Goldman, 2002; Rivers et al., 2008; Tekki-Kessaris et al., 2001). Along the neural tube oligodendrocytes are produced ventrally under the influence of the morphogen Sonic Hedgehog (Shh), and migrate at the progenitor stage to dorsal regions (as reviewed in Miller, 1996; Richardson et al., 2000; Spassky et al., 2000). It has been shown, however, that after the initial wave of ventrally derived oligodendrocytes, they are completely replaced by a dorsally derived population (Kessaris et al., 2006; Vallstedt et al., 2005). Progression of cells of oligodendrocyte lineage through various stages of differentiation has been described in depth in rodents using primary oligodendrocyte cell cultures (e.g. Miller, 1996; Pfeiffer et al., 1993; Raff, 1989; Reynolds and Hardy, 1997). Furthermore, extensive studies have shown transcription factors essential for oligodendrocyte specification as well as growth factors that influence oligodendrocyte proliferation and differentiation (Lu et al., 2000, 2002; Orentas and Miller, 1996; Takebayashi et al., 2000). In the ventral diencephalon and telencephalon, Shh influences the expression of oligodendrocyte lineage genes, Olig1 and Olig2, which are basic helix-loop-helix (bHLH) transcription factors (Lu et al., 2000; Pringle et al., 1996; Takebayashi et al., 2000; Tekki-Kessaris et al., 2001; Zhou et al., 2000). The question, however, remains to which extent could observations obtained on these animal models relate to human brain development.

In the recent years our laboratory has published several studies based on immunohistochemical analysis of the human fetal brain describing early oligodendrocyte specification (Filipovic et al., 2003; Jakovcevski and Zecevic, 2005a,b; Rakic and Zecevic, 2003a) and the onset of myelination (Jakovcevski et al., 2007). We have also pioneered the use of organotypic slice cultures, as well as primary cell cultures from human fetal brains, in addressing questions of the capacity of radial glia cells to generate oligodendrocytes (Mo and Zecevic, 2009), the interplay of other cell types and oligodendrocyte progenitor cells during brain development (Filipovic and Zecevic, 2005, 2008), and axon-oligodendrocyte interactions preceding myelination (Jakovcevski et al., 2007). Here we will summarize

results of those studies, compare them with other work published on developing human brain and put it all in the perspective of knowledge obtained from studies on animal models.

METHODOLOGICAL REMARKS

Several issues, ethical and methodological, need to be addressed regarding human fetal brain tissue. All of the tissue used in our studies was obtained after legal abortions performed either at the Obstetrics and Gynecology Clinic, University of Belgrade (Serbia), or from the Brain Bank, Albert Einstein College of Medicine (Bronx, NY). The Institutional Ethics Committees of these institutions and the University of Connecticut approved the tissue collection, and informed consent was obtained from the parents. The handling of tissue was performed in accordance with all regulations set forth by the Institutional Ethics Committees and the Helsinki Convention. No evidence of disease or developmental abnormalities was discovered after ultrasonic and neuropathological examination of fetal brains. The ages of the embryos and fetuses were estimated on the basis of weeks after ovulation, crown-rump length (Olivier and Pineau, 1962), and anatomical landmarks (O'Rahilly et al., 1987).

Tissue was fixed by submersion into 4% formaldehyde in 0.1 M phosphate buffer, typically within 15 min, but never longer than 2 h *post-mortem*, which is among the shortest reported in the literature. Fixation times varied depending on the size of the tissue block, but were never shorter than overnight. Fixation was always done at 4°C, followed by cryoprotection in 30% sucrose solution in the same buffer, and freezing of tissue in isopentane cooled to −70°C. Despite very careful attempts made to standardize the conditions for each case, considerable differences in immunoreactivity to certain antibodies could be detected. Each immunostaining has to be evaluated by an experienced observer and antibody reactivity has to be assessed on case-to-case basis. Only cells that displayed typical morphology and distribution of the antigen as predicted (i.e. transcription factors are nuclear, receptors are on the cell membrane) were used in evaluation. Another source of difficulties in working with human tissue is that optimal antibody dilutions are difficult to determine, since each individual sample may vary in reactivity to some antibodies. However, for most of the antibodies used in our studies we have been able to get fairly reproducible staining intensities. The antibodies and tissue samples that resulted in great variability in staining intensity and/or staining pattern were thus excluded from our analyses.

To be able to do experimental manipulation with human brain tissue, we have developed primary cell cultures and slice cultures from human fetal forebrains (**Figure 1**). These cultures are different from cultures taken from experimental animals in several respects. Firstly, human fetal cells are harvested from only one brain specimen at the time, unlike in animals where one whole, or even several litters can be pooled in order to obtain cells in optimal numbers. Additionally, the factors that greatly increase case-to-case variability of these cultures include genetic differences (humans are rarely "inbred"), age differences (age of the fetus is never completely precise within 1–2 weeks), and different conditions of tissue before cultures are made. In order to standardize to the maximum tissue quality as the only addressable problem among those mentioned above, we have devised a protocol to keep tissue in oxygenated buffered saline solution supplemented with glucose and glutamine on ice during transport. Transportation time is typically <2 h. In this way after preparation of cultures usually less then 10% of cells are dead as assessed by trypan-blue staining (Mo et al., 2007).

In addition to long-term cell cultures (min. and max. time, 12 h to 5 days), which we used to study radial glia cells (Mo and Zecevic, 2008, 2009; Mo et al., 2007), and oligodendrocytes (Filipovic and Zecevic, 2005, 2008), we also established acute cell cultures (Howard et al., 2006; Jakovcevski and Zecevic, 2005b). Acute cultures are dissociated cells from well-defined brain regions, maintained for 4–6 h

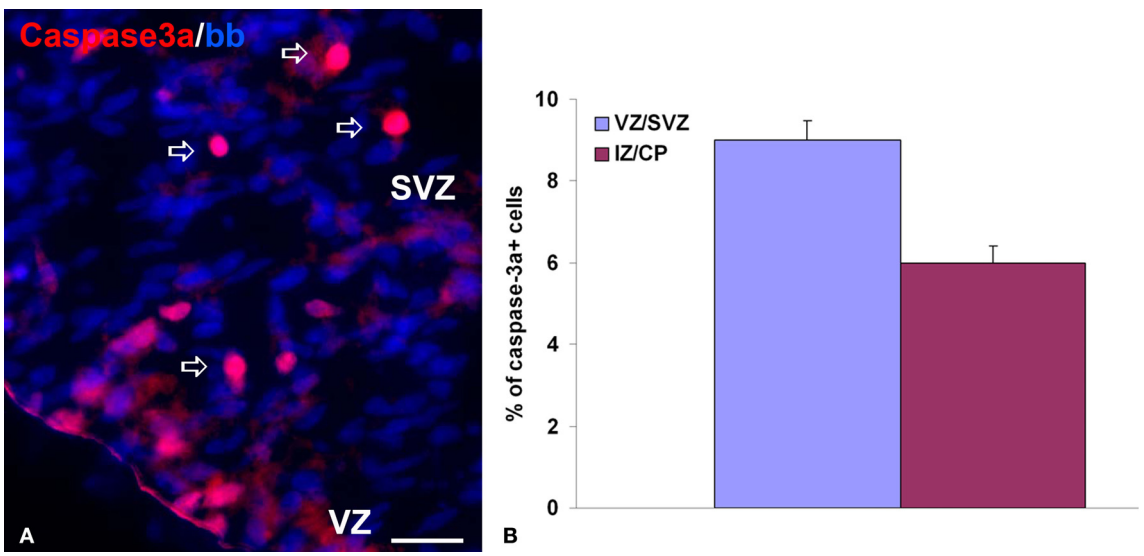

FIGURE 1 | Cell death in chronic slice cultures. (A) The representative image of activated caspase-3 staining (red, arrows) on the ventricular/subventricular zone (VZ/SVZ) of the slice culture from a 19-week-old human fetus after 5 days *in vitro*. Nuclei are counterstained with bisbenzamide (blue). Scale bar: 20 μm. **(B)** Proportion of caspase-3a$^+$ cells in all bisbenzamide-labeled nuclei in the VZ/SVZ and intermediate zone/cortical plate (IZ/CP) of 19-week-old human fetus. Note the higher percentage of apoptotic cells in the VZ/SVZ, similar to results obtained by TUNEL staining on frozen sections (Rakic and Zecevic, 2000).

before fixation and immunostaining. They were used to estimate the proportions of marker-defined cell populations among all cells in the region (**Figures 6C,D**).

In order to manipulate experimental conditions, but still preserve cell-to-cell interactions and extracellular matrix composition present *in vivo*, we have established organotypic slice culture from the human fetal forebrains. Brain slices were kept for 3–5 days *in vitro*. Under culture conditions cells in slices retained viability after 5 days with very little cell death as shown by activated caspase-3 staining (**Figure 1**). In addition, both cell proliferation and normal cellular composition, judged by the staining patterns with the cell-type specific antibodies, closely resembles *in vivo* sections from the same age. However, there are a few exceptions to this, most notably the oligodendrocyte maturation seems to be accelerated in slices, as discussed below (Jakovcevski and Zecevic, 2005a; Jakovcevski et al., 2007).

SEQUENTIAL EXPRESSION OF IMMUNOHISTOCHEMICAL MARKERS DURING PROGRESSION OF OLIGODENDROCYTE LINEAGE IN HUMANS

Oligodendrocyte lineage has been described in detail in rodents by expression of specific proteins used as immunomarkers of various stages in oligodendrocyte development (e.g., Pfeiffer et al., 1993). Oligodendrocytes are also the cell population in the central nervous system with the most significant turnover, and as such, all stages of precursor cells exist in the CNS throughout adult life (Dawson et al., 2003). Oligodendrocyte development in humans begins during the second trimester of gestation and progresses towards birth, and further into adulthood (Back et al., 2001; Jakovcevski and Zecevic, 2005a; Rakic and Zecevic, 2003a; Rivkin et al., 1995; Yakovlev and Lecourse, 1967). We focused recently on elucidating progression of oligodendrocyte lineage in humans in the first half of gestation (**Figure 2**).

Early oligodendrocyte progenitor cells are characterized by their expression of platelet derived growth factor receptor alpha (PDGFRα) and NG2 proteoglycans (Pringle and Richardson, 1993; Pringle et al., 1992; Stallcup and Beasley, 1987) and typical morphology with few ramified processes (**Figure 3A**; also **Figures 4 and 5**; for more elaborate description see Jakovcevski and Zecevic, 2005a; **Figure 2**). These cells are still mitotic, so we consider them "progenitors". Later along the oligodendrocyte lineage cells are not considered to be proliferative any more, thus in this review we use the term "precursors" for these cell types. We detected the first PDGFRα expressing (PDGFRα$^+$) cells in the forebrain of 10 gw old fetus, but they appear in higher numbers only around 15 gw, when they are most numerous in the ganglionic eminences and in the cortical VZ/SVZ (**Figure 4**). By midgestation (19–22 gw) oligodendrocyte precursor cells invade more dorsal areas of the telencephalic wall, including the cortical plate. During the whole period of our observation (from the onset of neurogenesis, at 5 gw, until the beginning of the third trimester, 24 gw) early oligodendrocyte progenitors were most dense in the cortical subventricular

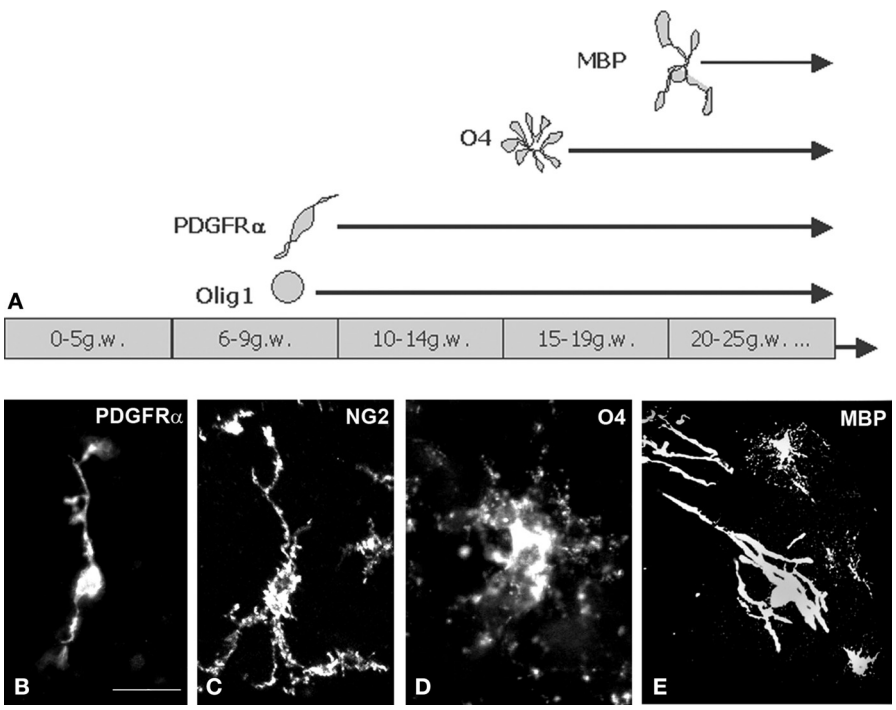

FIGURE 2 |Time-course of various oligodendrocyte lineage markers expression in the human forebrain. (A) Early oligodendrocyte precursor cells appear around 9 gw in the ganglionic eminence, and during the next few weeks spread to cortex. Late oligodendrocyte precursors appear in modest numbers around 15 gw. MBP$^+$ cells with typical oligodendrocyte morphology are very sparse at midgestation, but their population steadily enlarges. The first myelin sheaths can be demonstrated by MBP staining at 18 gw in thalamus, and around 21 gw in the internal capsule. During the whole period of our observation (up to 24 gw) cortical white matter was not myelinated, i.e. not "white". **(B–E)** Examples of 3D reconstructions from serial confocal sections stained with anti-PDGFRα **(B)**, anti-NG2 **(C)**, anti-O4 **(D)** and anti-MBP **(E)** monoclonal antibodies. Image on panel B was taken from ganglionic eminence subventricular zone at 15 gw, panels **(C)** and **(D)** from cortical subplate zone at 22 gw and panel **(E)** from internal capsule, 24 gw. Scale bar: 20 μm.

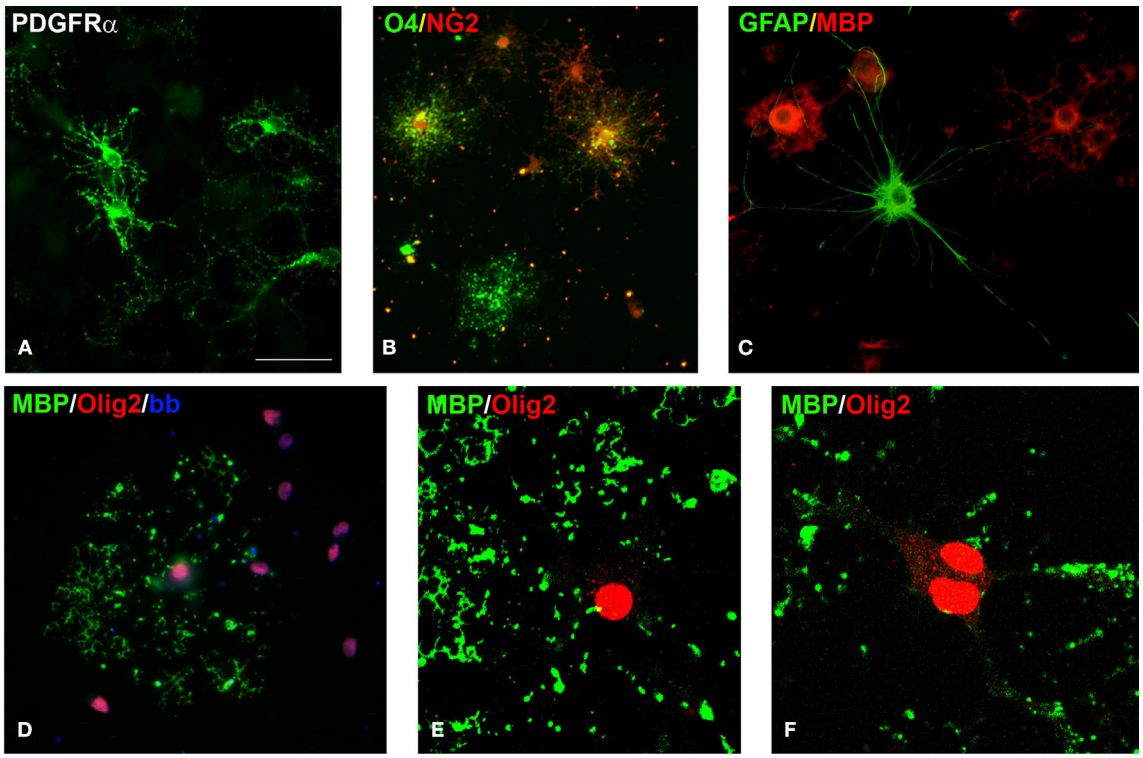

FIGURE 3 | Cells of oligodendrocyte lineage in culture. (A) Early oligodendrocyte precursors expressing PDGFRα. **(B)** O4$^+$ late oligodendrocyte precursors (green), some of them double-labeled with NG2 (yellow in overlay) and some early oligodendrocyte precursors labeled only with NG2 (red). **(C)** GFAP$^+$ astrocyte (green) among MBP$^+$ oligodendrocytes (red). **(D)** All cultured oligodendrocytes express nuclear Olig2 (red), here shown in the MBP$^+$ mature oligodendrocytes (green). **(E, F)** Confocal image of an oligodendrocyte with Olig2$^+$ nucleus that produces MBP$^+$ myelin-like membrane (green). Interestingly, Olig2 expression is not localized only to the cell nucleus, but it is also seen in the cytoplasm. Scale bar: **(A–D)** 20 μm, **(E, F)** 10 μm.

zone, consistent with their origin from this secondary proliferative zone in the human cortex (Jakovcevski and Zecevic, 2005a; **Figures 4 and 6**).

In the next stage in oligodendrocyte development, late oligodendrocyte precursor cells are characterized by O4 immunoreactivity, whereas pre-myelinating oligodendrocytes are reactive to O1 antibody (**Figures 2D and 3B**). O4 and O1 antibodies were raised against glycoproteins in the oligodendrocyte membrane and are shown to specifically label cultured late oligodendrocyte precursor cells (Bansal et al., 1989; Sommer and Schachner, 1981; Warrington and Pfeiffer, 1992). Staining of tissue sections with these antibodies is often hampered by the instability of the glycan epitopes to fixation. Earlier study on rat brain sections has shown that, indeed, it is not fixation but freezing that disperses the antigens and hampers immunostainings (Warrington and Pfeiffer, 1992). In our studies, however, we have been able to detect O4 and O1 expressing cells in human brains when we omitted detergent from the blocking solution. Similar results were reported by Back et al. (2001). In the human forebrain at midgestation (20–22 gw), we reported that O4$^+$ and O1$^+$ cells are especially dense in the subplate layer, immediately bellow the cortical plate (Jakovcevski and Zecevic, 2005a; Rakic and Zecevic, 2003a). This distribution of late oligodendrocyte precursors raises a possibility that transient subplate layer is important for maturation of oligodendrocytes. This is not inconceivable, since subplate is the target of numerous afferent fibers (Kostovic and Judas, 2002; Kostovic and Rakic, 1990) and has a rich extracellular matrix (Sheppard et al., 1991), also attracting numerous glial fibrillary acidic protein expressing astrocytes at midgestation (Zecevic, 2004). Considering the importance of transient subplate layer which receives the thalamic afferents essential for proper wiring of cortical neurons (McKellar and Shatz, 2008; Moore et al., 2008), it is tempting to speculate that oligodendrocytes come to the subplate to obtain yet unknown signals necessary for their maturation and myelination of the axons in correct sequence.

Maturation of oligodendrocytes is marked by the expression of myelin proteins, and the two major myelin proteins, myelin basic protein (MBP) and proteolipid protein (PLP), are the first to be expressed at detectable levels (Verity and Campagnoni, 1988). MBP was detected very early in human embryonic brains (around 5 gw), but this expression was attributed to Golli/MBP splice variants (Tosic et al., 2002; Zecevic et al., 1998). In the forebrain, first MBP$^+$ cells of typical oligodendrocyte morphology (**Figures 3C–F**) were found at 18 gw, around the middle of intrauterine development (Back et al., 2001; Jakovcevski and Zecevic, 2005a). MBP$^+$ cells are scattered through the intermediate zone, the future white matter, and increase in numbers with progression of development (**Figure 2E**; Jakovcevski and Zecevic, 2005a). Our evidence supports the ventral to dorsal progression of oligodendrogenesis, also reported in rodents (Pringle and Richardson, 1993; Timsit et al., 1995). Indeed, a ventro-dorsal

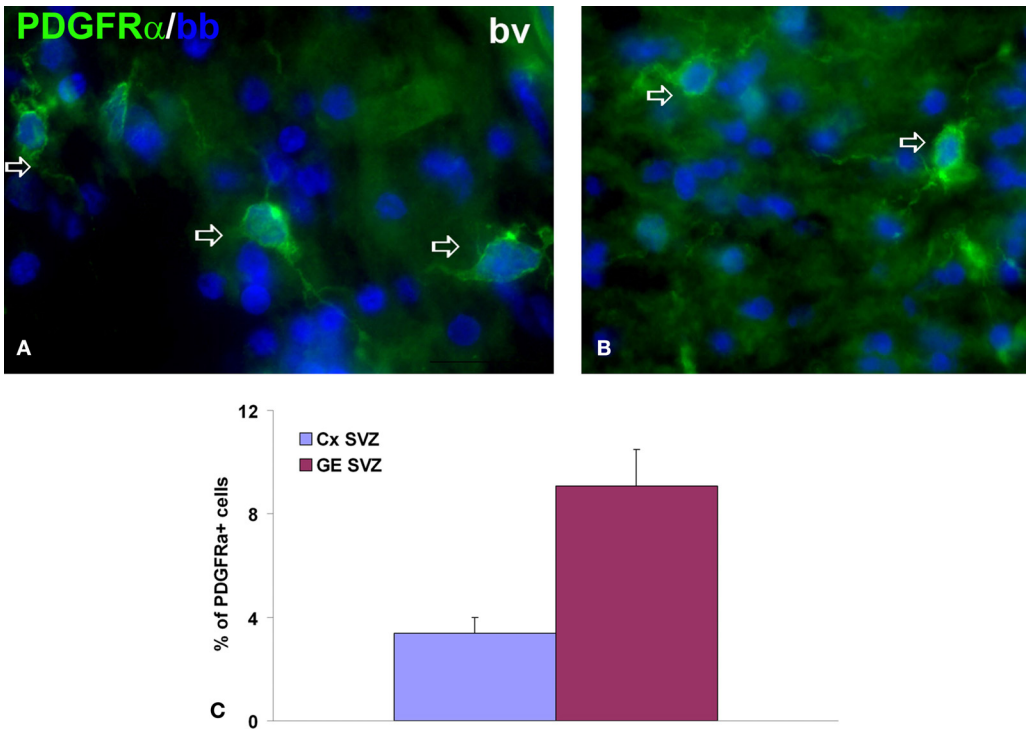

FIGURE 4 | Early oligodendrocyte progenitors in 15-week-old human forebrain. (A, B) Representative image of PDGFRα+ early oligodendrocyte progenitors (green, arrows) in ganglionic eminence subventricular zone (GE SVZ, **(A)**) and cortical SVZ (Cx SVZ, **(B)**). Nuclei are counterstained with bisbenzamide (bb, blue). **(C)** Proportion of PDGFRα+ cells from all cells in these two regions at 15 gestation weeks. bv, blood vessel.

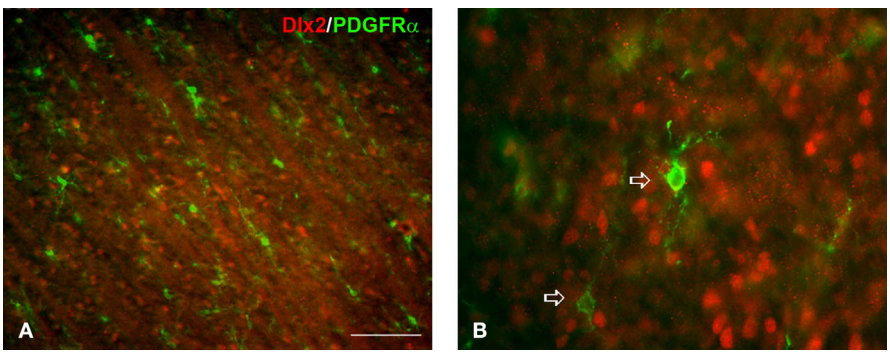

FIGURE 5 |The expression of Dlx2 and PDGFRα in 15 weeks old human fetal brain. Dlx2 (red) and PDGFRα (green, arrows) are usually not co-expressed in the emerging cortical white matter **(A)** and ganglionic eminence **(B)**. Scale bar: 100 μm **(A)**, 20 μm **(B)**.

gradient in the extent of myelination (Jakovcevski et al., 2007) and in oligodendrocyte precursor cells density (Jakovcevski and Zecevic, 2005a,b; Rakic and Zecevic, 2003a) was seen in the human fetal forebrain.

Finally, in the rodent forebrain oligodendrocytes are initially derived from ventrally positioned ganglionic eminences (GE), whereas later they originate from the dorsal cortical subventricular zone (Kessaris et al., 2006; Marshall and Goldman, 2002). In human fetal forebrains at midgestation a subpopulation of cortical oligodendrocyte progenitor cells was expressing Dlx2 and Nkx2.1 (Rakic and Zecevic, 2003a), transcription factors specific for ventrally derived cells in rodents (Anderson et al., 1997; He et al., 2001; Marin and Rubenstein, 2001). However, as these transcription factors show a strong signal in the human proliferative zones of both GE and cortex (Rakic and Zecevic, 2003b), the origin of human Dlx2- and Nkx2.1-expressing oligodendrocyte progenitor cells could not be simply determined. Another large population of oligodendrocyte progenitor cells at this stage did not express Dlx2 and Nkx2.1 transcription factors, and could represent population of dorsally derived oligodendrocytes in the human brain (**Figure 5**; Rakic and Zecevic, 2003a). Yet a third population of cells co-labeled with oligodendrocyte progenitor markers (PDGFRα, NG2, Olig1),

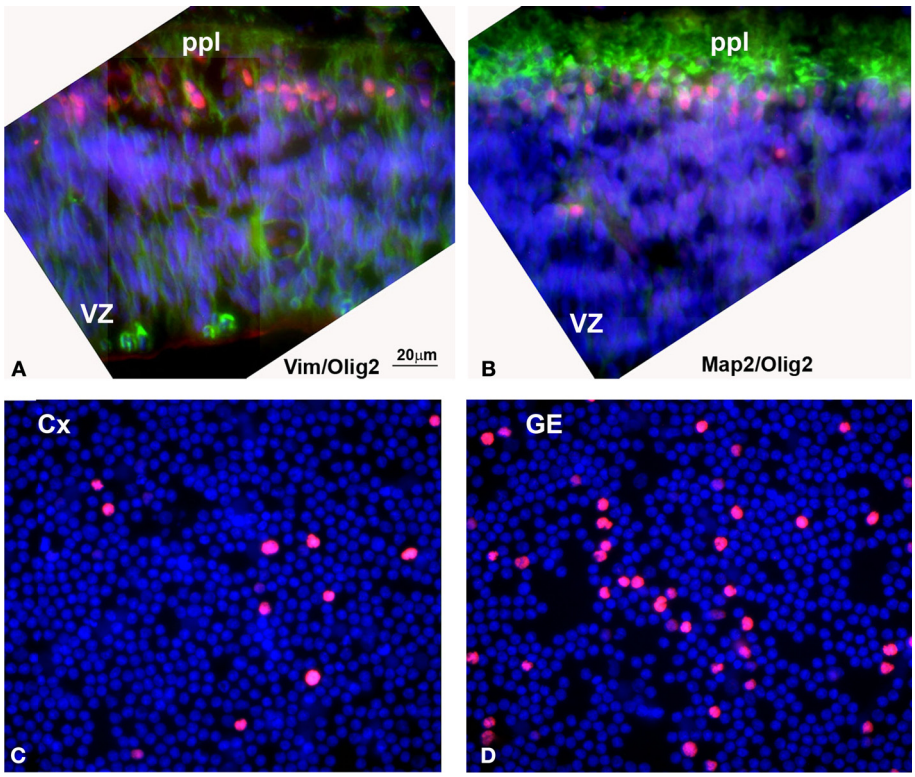

FIGURE 6 | (A, B) Olig2 expression in human embryonic (5 gw) forebrain. (A) Olig2 cells (red) in the primordial plexiform layer (ppl), but not in the ventricular zone (VZ), where vimentin$^+$ radial glia cells (green) are dividing. **(B)** Olig2 is expressed in nuclei of MAP2$^+$ young neurons (green) at this stage. **(C, D)** Densities of Olig2$^+$ cells (red) in the acute (4 h *in vitro*) dissociated cell cultures from human cortical (Cx) and ganglionic eminence (GE) ventricular/subventricular zones at midgestation (20 gw). Nuclei are counterstained by bisbenzamide (blue). Detailed quantification was published by Jakovcevski and Zecevic (2005b).

stem cell marker nestin, and markers of cells from hematopoietic lineage (CD34, CD68) was present in what appears to be a stream of cells migrating between the ganglionic eminences and cortical subventricular zone (Rakic and Zecevic, 2003a). These combined findings suggest multiple origins of human cortical oligodendrocytes (Jakovcevski and Zecevic, 2005a,b; Rakic and Zecevic, 2003a) and have broad implications for normal brain development as well as white matter pathologies.

It is possible that oligodendrocytes derived from various sources have different roles, or myelinate different axonal pathways. This becomes especially important and demands further investigation in the light of reports that implicate oligodendrocytes in other functions in addition to "traditional" myelin formation, including synaptic regulation and signaling at the nodes of Ranvier (Bergles et al., 2000; Butt et al., 1999; Lin et al., 2005; Nishiyama et al., 2009).

OLIG GENES AND THE COMMON OLIGODENDROCYTE – NEURONAL PROGENITOR CELL

Studies of origin of oligodendrocytes in rodents have shown that ventral morphogen Shh is essential for early specification of oligodendrocyte progenitors in the neural tube (Nery et al., 2001; Tekki-Kessaris et al., 2001). Shh activates a cascade of transcription factors including Pax10, Dlx2, Nkx2, Olig1 and Olig2 (Nery et al., 2001; Pringle et al., 1996; Tekki-Kessaris et al., 2001). Olig2 was described in neural progenitors that give rise to both motor neurons and oligodendrocytes in the ventral spinal cord region (Lu et al., 2000, 2002; Takebayashi et al., 2000; Zhou et al., 2000). Olig genes belong to a bHLH group of transcription factors that are necessary and sufficient for generation of oligodendrocytes and for myelination in mice (Lu et al., 2000, 2002; Takebayashi et al., 2002; Tekki-Kessaris et al., 2001; Zhou and Anderson, 2002; Zhou et al., 2000; 2001). In the human fetal brain we demonstrated the expression of Olig2 in the ganglionic eminences and the medial cerebral cortex starting as early as 5 gw, before the onset of either neurogenesis or oligodendrogenesis (**Figures 6A,B**; Jakovcevski and Zecevic, 2005b). Our study also revealed that various cell types later in development express both Olig1 and Olig2. Most notably, Olig2 is expressed in all MBP$^+$ cells in the human fetal forebrain and spinal cord at midgestation, and in around 50% of early oligodendrocyte progenitors in the SVZ. At the same time and at the same location, cortical SVZ, a subpopulation of MAP2$^+$ neuronal progenitors is also Olig2$^+$. In contrast, mature neurons in the cortical plate labelled with either NeuN or GABA antibodies, do not co-express Olig2 (Jakovcevski and Zecevic, 2005b). Taken together these observations suggest existence of a common progenitor cell for oligodendrocytes and at least some neuronal classes in the human forebrain. Similar relationship was extensively reported in rodent spinal cord where oligodendrocytes and motoneurons

develop from the same Olig2 expressing domain, as seen by various methods including Olig2-deficient mice (reviewed in Lu et al., 2002; Richardson et al., 2000; Rowitch, 2004). In the forebrain, however, the situation appears to be more complex. Common progenitor cell has been suggested for cortical interneurons and oligodendrocytes derived from ganglionic eminences (He et al., 2001), and a recent fate-mapping study shows that oligodendrocyte precursors in adult mice give rise to projection neurons in the piriform cortex (Rivers et al., 2008). On the basis of our studies of olig transcription factors' expression in the fetal human central nervous system, we propose the model linking oligodendrocyte and neuronal lineages in brain and spinal cord through common Olig2-expressing progenitors (**Figure 7**).

Genetic mapping and heterologous transplantation fate mapping studies in mice have shown that Olig2 domain within medial ganglionic eminence gives rise to cortical interneurons (Butt et al., 2005; Miyoshi et al., 2007). There is, however a controversy about the necessity of Olig2 for cortical interneuron fate determination. When Olig2 was ablated from cortical progenitors no change in interneuronal populations was observed (Miyoshi et al., 2007). One possible explanation for this finding could be that other bHLH genes, like Olig1, compensate for it. In our study we found Olig1 co-localized in vimentin-expressing cortical radial glia cells, a cell type demonstrated to be a multiple neural progenitor (Mo and Zecevic, 2009; Mo et al., 2007). We have no evidence that in human brain Olig1 is co-expressed in neuronal populations, but that does not preclude the possibility that by the time neurons acquire their fate this gene gets down-regulated (Jakovcevski and Zecevic, 2005b). This inability to draw conclusions about temporal developmental events is an intrinsic limitation of all studies on fixed human tissue, and is the most important reason why we resorted to different *in vitro* systems.

In vitro, Olig2 plays a role in self-renewal of mouse neurosphere cultures, and in differentiation of neurons and oligodendrocytes in

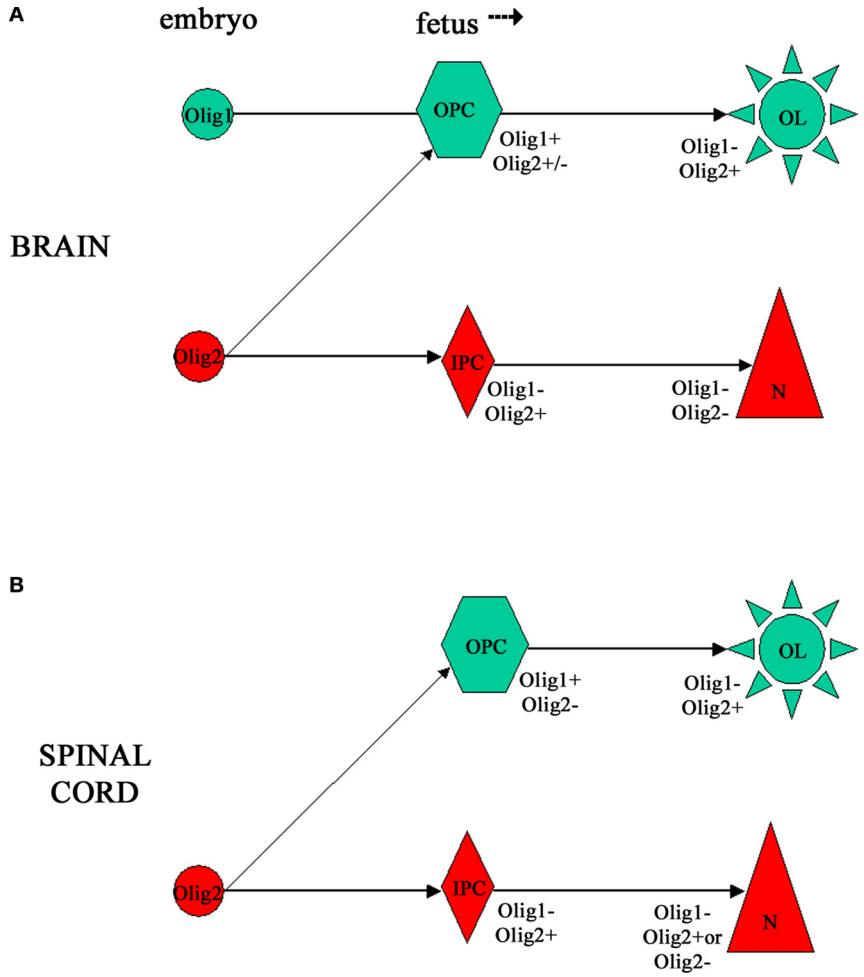

FIGURE 7 | Schematic representation of possible lineage relationships between neurons and oligodendrocytes linked via Olig transcription factors in human CNS development, based on our results (Jakovcevski and Zecevic, 2005b). (A) In the embryonic forebrain Olig1 and Olig2 appear to be expressed by separate progenitor cell pools, and our evidence suggests that Olig2$^+$ progenitors give rise to neurons. Later during fetal development early oligodendrocyte progenitors express both Olig1 and Olig2, but mature MBP$^+$ oligodendrocytes in the fetal forebrain are Olig1$^-$/Olig2$^+$. **(B)** In the embryonic human spinal cord we detected only Olig2 expression. At the later age (15 gw) Olig1 is expressed in early oligodendrocyte progenitors, whereas Olig2 is expressed in MBP$^+$ oligodendrocytes. Interestingly, Olig2 was expressed in many NeuN$^+$ postmitotic neurons in the dorsal, but not ventral horn.

appropriate condition media (Hack et al., 2004). In cultures with growth factors (FGF2 and EGF), Olig2 was expressed in almost all progenitors derived from mouse ganglionic eminences or cortex, whereas dorsal transcription factors, Pax6 and Emx1, were downregulated (Hack et al., 2004). In contrast, in cryosections of human lateral ganglionic eminence at 15–20 gw, Olig2 and Pax6 were co-expressed in the same progenitor cells. This finding was extended to cell cultures from cortical VZ/SVZ. Co-expression of these two transcription factors in dividing progenitor cells is consistent with an increased complexity of human neural progenitor cells in comparison to other mammals (Mo and Zecevic, 2008).

RADIAL GLIA DERIVED SUBPOPULATION OF FOREBRAIN OLIGODENDROCYTES

As has been discussed above, we reported that in the VZ/SVZ of the fetal forebrain, cells occasionally co-express radial glia cells markers and oligodendrocyte lineage markers, suggesting lineage relationship (Jakovcevski and Zecevic, 2005a,b). Radial glia are multipotent progenitor cells in rodents, generating projection neurons (Miyata et al., 2001, 2004; Noctor et al., 2001) and a subpopulation of forebrain oligodendrocytes (Casper and McCarthy, 2006; Malatesta, et al., 2003). Similar to these findings, radial glia cells are also neuronal and oligodendrocyte progenitor cells in human fetal brain (Mo and Zecevic, 2009; Mo et al., 2007). *In vivo* loss- and gain-of-function studies that are successfully used in animal models to show linage relationship are not possible in humans. To study cortical neural progenitors and their progeny, we established an *in vitro* system with human fetal brain tissue. This approach enabled us to use some of experimental methods not commonly applied to human brain. For example, the Cre/loxP co-transfection was used to study the relationship of two cell types: radial glia and oligodendrocytes. We first isolated human fetal radial glia in culture and then transfected them with a BLBP-Cre/Floxed-YFP to study cell fate of their progeny. Thus, specificity of BLBP (brain lipid binding protein) plasmid to radial glia cells was combined with YFP (yellow fluorescent protein) reporter signaling related to β-actin-like promoter that remains active in cells after differentiation. The Cre/loxP method made it possible to see the progeny of BLBP$^+$ radial glia even after BLBP expression was downregulated during cell differentiation to another cell type (Mo and Zecevic, 2009; Mo et al., 2007). As we expected from the initial findings of double-labeled cells in cryosections of fetal forebrain, *in vitro* results directly demonstrated that a small subset of fetal radial glia cells differentiates along oligodendrocyte linage following the same path as described *in vivo* (Jakovcevski and Zecevic, 2005a). Transfected cells expressed first NG2 and O4 (**Figure 8**), and subsequently markers and morphology of premyelinating oligodendrocytes labeled with MBP and MOG (myelin oligodendrocyte glycoprotein) (Figure 5 in Mo and Zecevic, 2009).

We further demonstrated that this *in vitro* generation of oligodendrocyte progenitors from isolated radial glia cells was under Shh influence. This is not surprising considering a well-described role of this morphogen in brain development in rodents (Nery et al., 2001; Tekki-Kessaris et al., 2001). However the role of Shh in normal human forebrain development, and specifically in dorsal oligodendrogenesis, is less well documented. From this aspect, our finding that radial glia enriched from cortical VZ/SVZ of midgestational (20 gw) fetuses expressed both Shh receptors, Ptc1 (patched) and Smo (smoothened), was consistent with the idea that Shh influences human forebrain development in a similar way that was reported in rodents. We found that adding Shh in culture of radial glia cells, increased the number of generated O4$^+$ oligodendrocyte precursor cells, whereas adding Shh inhibitor, cyclopamine reduced the number of O4$^+$ cells in these cultures. Taken together these findings support the idea that Shh usually thought of as a ventral morphogen, also may play a role in dorsal oligodendrogenesis in human forebrain.

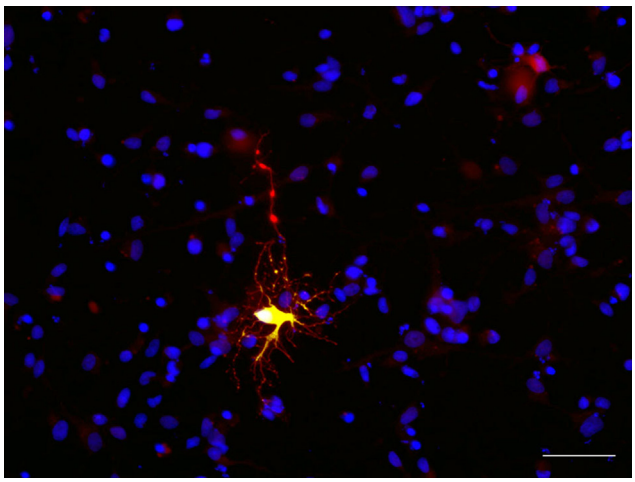

FIGURE 8 | Human fetal radial glia cell in culture differentiates as an oligodendrocyte progenitor. BLBP-Cre/Floxed-YFP transfected cell (green) co-labeled with O4 (red) after 7 days in culture. Scale bar: 20 μm.

Dorsal transcription factor Pax6 has been reported to be important for generation of neurons in rodents (Götz et al., 1998; Heins et al., 2002), and also in humans (Mo and Zecevic, 2008). Pax6 however may also have a role in oligodendrogenesis (Mo and Zecevic, 2008). Other transcription factors, such as Olig1 and Olig2 may be necessary to specify radial glia as oligodendrocyte progenitors. Indeed we have observed that Olig1 and Olig2 can be co-expressed with radial glia marker vimentin along the ventricular zone at 15 gw (Mo and Zecevic, 2009). It would be important to determine whether a majority or only a specific subtype of human oligodendrocytes is generated from radial glia cells and whether radial glia are the only early oligodendrocyte progenitors in the cortical VZ/SVZ. Alternatively radial glia cells could generate oligodendrocytes only at a specific developmental stage or at a particular brain region, such as cortical VZ/SVZ. However, without careful quantification on a larger sample size, we do not know how widespread these phenomena are. Equally unknown is how they change during the span of development? This is particularly important to establish, since developmental potential of progenitor cells becomes more and more restricted over time.

ASTROCYTE-OLIGODENDROCYTE INTERACTIONS DURING OLIGODENDROCYTE DEVELOPMENT

Formation of myelin during primary myelination and its restoration in remyelination, involves proliferation, migration and differentiation of oligodendrocyte progenitors into myelin-forming oligodendrocytes. Growth factors, cytokine members of the interleukin (IL) superfamily, chemokines, such as growth-related oncogene alpha (GRO-α) also referred to as CXCL1, and other

mediators influence oligodendrocyte progenitor cells proliferation and/or differentiation, and are also very important for remyelination (e.g. Dubois-Dalcq and Murray, 2000; Gard and Pfeiffer, 1993; Grinspan, 2002; Mason et al., 2003; Ransohoff et al., 2002; Wilson et al., 2003). Moreover, it has been demonstrated that this chemokine is upregulated during experimental autoimmune encephalomyelitis (EAE) (Glabinski et al., 2000) and around lesions in multiple sclerosis (Filipovic et al., 2002, 2003; Omari et al., 2005, 2006). We studied the distribution of chemokine CXCL1 and its receptor CXCR2 in human developing forebrain, and reported that it is highly expressed in cortical VZ/SVZ (Filipovic et al., 2003) in accord with an earlier study in rodents (Robinson and Franic, 2001). The SVZ is one of the largest proliferative regions with multiple progenitor classes in developing rodent (Lachapelle et al., 2002; Luskin, 1993) and human brain (Rakic and Zecevic, 2003a,b; Sanai et al., 2004; Zecevic et al., 2005). The SVZ in adult brain is also a source of newly generated oligodendrocytes in MS (Nait-Oumesmar et al., 2007). In rats, CXCL1 induces proliferation of oligodendrocyte progenitors and limits their migration (Robinson et al., 1998). The fact that human and rodent oligodendrocyte progenitors differently respond to various mitogens (Armstrong et al., 1992; Scolding et al., 1995; Wilson et al., 2003; Zhang et al., 2000) led us to explore the role of CXCL1 in the human fetal forebrain. Moreover, we were intrigued by the finding that during midgestation very few oligodendrocyte progenitors express CXCR2 receptor (Filipovic et al., 2003). Our data suggested that if this chemokine has a role in proliferation of oligodendrocyte progenitor cells, it may exert its action trough other cell types present in the brain. To study cell-to-cell interactions we used organotypic slice cultures from cortical VZ/SVZ zones and demonstrated that proliferation of human fetal oligodendrocyte progenitor cells is indeed dependent on CXCL1/CXCR2 signaling (Filipovic and Zecevic, 2008). Moreover, we identified that this cell signaling involves the activation of the ERK1/2 pathway and release of IL-6 from astrocytes. Notably, when astrocytes are stimulated with CXCL1, they secrete IL-6, which in turn acts on oligodendrocyte progenitors and induces their proliferation (Filipovic and Zecevic, 2008). Thus, we demonstrated that in contrast to rodents where CXCL1 directly induces oligodendrocyte proliferation (Robinson et al., 1998), in human fetal brain CXCL1 has an indirect effect, acting through astrocyte secretion of IL-6, to increase oligodendrocyte proliferation.

At this point it became important to determine whether CXCL1 promotes only proliferation or affects also differentiation of oligodendrocyte progenitors. To study this, we treated human fetal slices, isolated from midgestational VZ/SVZ, with CXCL1 (10 ng/ml) for various lengths of time (12, 24, or 48 h). After treatment the number of MBP$^+$ premyelinating oligodendrocytes was quantified in these brain slices. Although the number of MBP$^+$ cells increased gradually, it was still not significantly different than in control slices. However, when slices treated with CXCL1 (10 ng/ml, 48 h), were cultured for additional 1 or 3 days, the number of MBP$^+$ oligodendrocytes was significantly higher than in controls slices, suggesting a delayed action of CXCL1 on differentiation of oligodendrocyte progenitors (**Figure 9A**). To better assess differentiation of oligodendrocytes, we calculated a differentiation index, defined as the ratio between MBP$^+$ cells and the sum of MBP$^+$ and PDGFRα^+ cells. A higher value of the differentiation index indicates more advanced differentiation of oligodendrocyte progenitors (Sakurai et al., 1998). In the first 48 h *in vitro*, CXCL1 treated slices had lower differentiation index than the control ones (0.17 ± 0.02 vs. 0.24 ± 0.03, $n = 3$, $P < 5$), while at 5 days the differentiation index was significantly higher in treated slices than in the controls (0.6 ± 0.07 vs. 0.4 ± 0.05, $P < 0.05$). Moreover, at 5 days *in vitro* MBP$^+$ oligodendrocytes in treated slices had longer and more branched processes as compared to controls (**Figures 9D,E**). Western blots revealed increased levels of MBP after CXCL1 treatment, as compared to controls (**Figure 9I**). In contrast, in the presence of either the CXCR2 antagonist (100 nM, 24 h) or after blocking ERK1/2 activation with PD98059 (50 μM, 24 h), the number of MBP$^+$ oligodendrocytes was significantly decreased (**Figure 9B**). Taken together these results confirmed the role of CXCL1 not only in proliferation, but also in oligodendrocyte differentiation.

To test if this positive effect was due to secreted factors from different cell types, we isolated the effect of microglia and astrocytes by selectively eliminating each cell type with appropriate toxins (Filipovic and Zecevic, 2008). We collected conditioned media from these cell type specific cultures, and treated slices with either astrocyte or microglia conditioned media for 24 h. Four days later the number of MBP$^+$ oligodendrocytes was quantified. Only the conditioned medium from CXCL1 treated astrocytes (CXCL1–ACM) increased the number of MBP$^+$ oligodendrocytes, as compared to control media. In contrast, the number of MBP$^+$ oligodendocytes significantly declined when ERK1/2 or IL-6 were blocked (**Figure 9C**). In addition, blocking of either CXCR2, ERK1/2 activation or IL-6 release from astrocytes, changed the morphology of MBP$^+$ cells, which exhibited less elaborate processes (**Figures 9F–H**).

This finding supported our hypothesis that the CXCL1/ERK1/2/IL-6 pathway had an important role in oligodendrocyte progenitor proliferation and their subsequent differentiation into premyelinating oligodendrocytes. In the absence of microglia or astrocytes, MBP$^+$ oligodendrocytes had less branched processes and their number was reduced as well. Similar to findings on proliferation, the differentiation of oligodendrocyte progenitors was decreased by neutralization of IL-6, but not of CXCL1 secreted from astrocytes (**Figure 9C**). We propose that a novel interaction between astrocytes and oligodendrocytes, the one that includes CXCL1/IL-6 signaling pathway, enhances development of human fetal oligodendrocytes.

During remyelination in MS, basic processes of oligodendrocyte development including proliferation, migration and differentiation of oligodendrocytes progenitors are recapitulated. For that reason it is of extreme importance to understand processes during normal oligodendrogenesis. We describe here that constitutive expression of CXCL1 has physiological relevance, whereas exogenous CXCL1 might be relevant in pathological conditions, such as developmental disorders of white matter, or in MS. This is supported by the finding that CXCL1 is abundant around MS lesions (Filipovic et al. 2002, 2003), the site where attempts of remyelination are found (Chang et al., 2002; Franklin, 2002; Levine et al., 2001; Raine et al., 1981;Wolswijk, 1998). We discovered that CXCL1 acts in synergy with IL-6. The function of IL-6 is not well understood, but it was reported to range from protracting relapses (Diab et al., 1997) to limiting demyelination in EAE (Di Marco et al., 2001; Willenborg et al., 1995). Future studies should address the role of this pathway

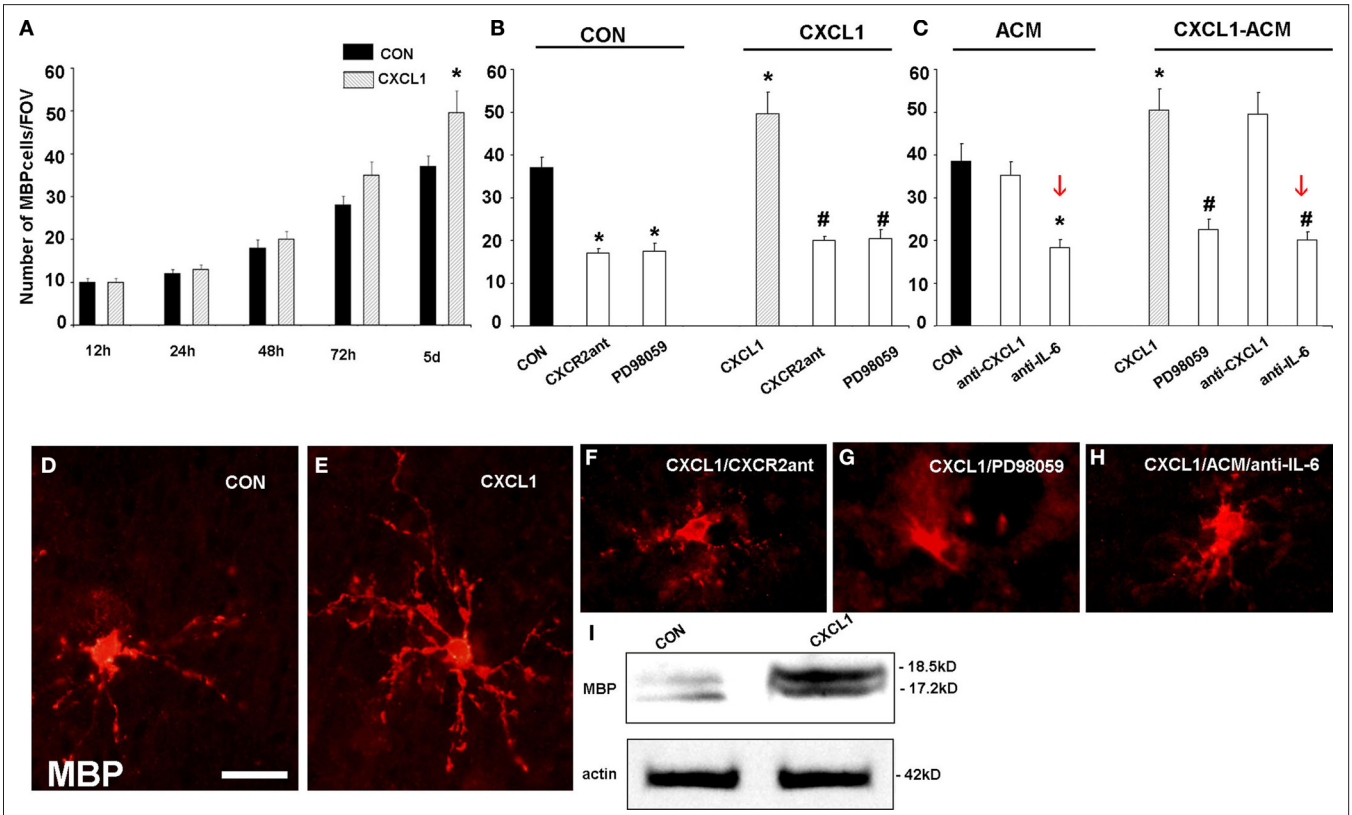

FIGURE 9 | Effect of CXCL1 on the differentiation of oligodendrocyte progenitors in slice cultures from VZ/SVZ region at midgestation. (A) Time-dependent effect of CXCL1 on the number of MBP$^+$ oligodendrocytes (OLs) per field of view (FOV). OL number is significantly different between control (CON) and CXCL1 treated slices after 5 div ($n = 7$, $^*P < 0.05$ vs. control slices) **(B, C)** The number of OLs in different experimental conditions after 120 h *in vitro*. **(B)** Decreased number of OLs is observed after inhibition of CXCR2 receptor or ERK1/2 pathway in CON and CXCL1 treated slices ($n = 7$, $^*P < 0.05$ vs. controls, $^\#P < 0.05$ vs. CXCL1 treated slices). **(C)** Treatment of slices with CXCL1 stimulated astrocyte conditioned medium (CXCL1/ACM) significantly increases the number of OLs, as compared to conditioned medium from unstimulated astrocytes (ACM). Reduced number of OLs cells is observed after ERK1/2 inhibition, or after neutralization of IL-6, in ACM or in CXCL1-ACM. ($n = 3$, $P < 0.05$ vs. ACM; $^\#P < 0.05$ vs. CXCL1–ACM). Error bars indicate SEM. **(D–H)** The morphology of MBP$^+$ cells in the different conditions at 5div. **(D)** MBP$^+$ oligodendrocytes in control slices. **(E)** OLs with multiple processes after CXCL1 (10 ng/ml, 48 h) treatment. **(F, G)** OLs have fewer and shorter processes after CXCL1 treatment in a presence of CXCR2 antagonist (100 nM, 24 h) or PD98059 (50 μM, 24 h), **(H)** Similar morphology is obtained when IL-6 is neutralized in CXCL1-ACM. **(I)** Western blot demonstrating upregulation of MBP (18.5 kD and 17 kD isoforms) in CXCL1 (10 ng/ml, 48 h) treated cultures after 5div. Equal loading of proteins is confirmed by actin. Scale bar: in **(D)** for **(D–H)**, 20 μM.

in disorders of the white matter during normal human brain development.

AXONAL SIGNALING AND MYELINATION IN THE HUMAN FETAL BRAIN

A wide variety of signals that may be involved in initiation of myelination of CNS axons has been reported. Although mouse oligodendrocytes *in vitro* have the capacity to form myelin-like membranes in the absence of neurons (Temple and Raff, 1986), co-culture with neurons significantly increases MBP gene expression in these cultures (Macklin et al., 1986), suggesting that axons are necessary for efficient myelination. *In vivo* experiments in the rat optic nerve demonstrated that oligodendrocyte proliferation and subsequent myelination depend on the electrical activity in axons (Barres and Raff, 1993). Other studies have confirmed that ion channel activation or electrical activity in axons can regulate myelination (Demerens et al., 1996; Ishibashi et al., 2006; Stevens et al., 1998), suggesting that oligodendrocytes preferentially myelinate axons which fire action potentials. This is not surprising, considering that the most important role of myelin sheath is to provide saltatory conduction of action potentials.

Myelination in the human brain progresses over several decades, which is much longer than a complete lifespan of the commonly studied animals. Recently emerged data suggest that myelination may play an important role in data processing by neurons (Fields, 2008). On the one hand, it is now clear that human white matter changes with experience, i.e. that myelinating glia respond to environmental cues, as shown by magnetic resonance imaging of neglected children (Teicher et al., 2004). On the other hand, myelin thickness influences conduction velocity, which is in turn instrumental in regulation of synchronous firing of action potentials and proper function of the brain (Fields, 2008).

Finally, several myelin proteins, including Nogo-A, MAG and MOG cause the growth-cone collapse that prevents axons from reaching their targets (for review see Filbin, 2003). This feature of myelin is now appreciated as an important regulatory mechanism to suppress sprouting and formation of abnormal connections in development (Huang et al., 2005). It is thus essential to understand

cues and signals that oligodendrocytes need to initiate unsheathing of axons in order to get insight into normal organization, and especially disorders of the human brain. Several uniquely human diseases, including multiple sclerosis and psychiatric disorders are linked to defects in myelination (Aston et al., 2005; Franklin, 2002; Georgieva et al., 2006; Tkachev et al., 2003; Trapp and Nave, 2008).

Before myelination is initiated, oligodendrocyte precursors transform first to pre-myelinating oligodendrocytes and then into mature myelin-producing cells (Pfeiffer et al., 1993). During this process, myelin proteins, MBP and PLP, shift their expression from cell bodies to processes that form myelin sheaths (Hardy and Reynolds, 1991). In humans there is a clear dissociation between the time of oligodendrocyte differentiation and the beginning of myelination in the fetal forebrain (Back et al., 2001; Jakovcevski and Zecevic, 2005a). Pre-myelinating oligodendrocytes ($O1^+$ cells) were present already at the beginning of midgestation (17–20 gw). The onset of myelination, however, was described only several months later, during the last trimester, first in the basal ganglia and somewhat later in the corpus callosum (Back et al., 2001; Jakovcevski et al., 2007; Ulfig et al., 1998). Biochemical methods, such as detection of sulfatide by thin-layer chromatography and detection of MBP by SDS-PAGE, showed compact myelin in the human forebrain only after birth (Kinney et al., 1994).

We observed that the number of MBP^+ oligodendrocytes dramatically increased in slice cultures of the human fetal forebrain, in comparison with their number in the frozen sections of the same fetal brains. Moreover, MBP^+ cells in cultures appeared to be forming myelin sheaths (Jakovcevski and Zecevic, 2005a; Jakovcevski et al., 2007). This may be attributed to the lack of inhibition of the oligodendrocyte differentiation in culture conditions and/or the presence of growth factors in the culturing medium. To explore the first possibility, we demonstrated that a prominent increase in myelination coincided with the down-regulation of a polysialic acid conjugated form of the neural cell adhesion molecule (PSA-NCAM). This finding is consistent with the notion that a PSA-mediated signaling mechanism might be one of the regulators of primary myelination in the human fetal brain (Jakovcevski et al., 2007). Notably, in multiple sclerosis patients, axons that fail to regenerate myelin sheathes, also re-express PSA-NCAM (Charles et al., 2002). There are, however, reports that in experimental CNS regeneration paradigms PSA promoted myelination (Mehanna et al., 2009; Papastefanaki et al., 2007). This indicates that a negative correlation between PSA-NCAM and myelination may be development-specific, and dependent on the context in which myelination occurs.

It is important to stress that PSA-NCAM is not the only cell adhesion molecule to be implicated as an axonal inhibitor of primary myelination. Many other molecules, for example neural adhesion molecule L1, have been shown to be downregulated from axonal surface upon the onset of myelination. Furthermore, interfering with L1 prior to myelination onset inhibited myelination (Barbin et al., 2004). This suggests that adhesion molecules are an important signal during initial contact between axon and oligodendrocyte, but their downregulation is needed for myelination to proceed. Other types of molecules that are inhibitory for myelination comprise oligodendrocyte myelin proteins and their receptors. For example the Nogo-receptor interacting protein, LINGO-1, expressed by both axons and oligodendrocytes, negatively regulates myelination and its antagonist promoted spinal cord remyelination in the EAE model of MS (Mi et al., 2005, 2007).

Inhibitory signals in the brain act not only through cell-to-cell interactions, but also could travel through the cerebrospinal fluid and the circulation, and thus are absent from the slice culture. Supporting this notion, although Nkx2.1- and Shh-deficient mice lack mature oligodendrocytes, the cell cultures from these animals produce sufficient numbers of pre-myelinating oligodendrocytes (Nery et al., 2001). This indicates that inhibitors present *in vivo* are lost when cells are grown in culture. The existence of possible inhibitors of myelination during normal development has great clinical importance, since the observed lack of functional remyelination in the MS plaques may be due to specific inhibition of oligodendrocyte differentiation, despite the fact that oligodendrocyte precursors are present in and around the lesions (Chang et al., 2002; Filipovic et al., 2002; Levine et al., 2001; Scolding et al., 1998).

OLIGODENDROCYTE DEVELOPMENT: RODENT VS. HUMAN

Since most studies of mammalian brain development are done on rodents, we find it useful to understand how various time-points in rodent brain development correlate with much longer human brain development. In the literature there are just a few attempts to relate processes during rodent brain development with those in humans (Bayer et al., 1993; Clancy et al., 2007; Verley, 1977). Previous attempt to draw parallels between development of experimental animals and humans resulted in designation of Carnegie stages (O'Rahilly, 1979). This classification is based on somatic morphology of embryos and it presumes that brain development is linearly predictable from somatic development, and that all brain regions develop at equivalent rates across species. Other attempts were done to make rat/primate comparisons based on neuroanatomy, comparing rat embryonic days 11–21 with human weeks 4–16 (Bayer et al., 1993). However, most of these comparisons do not take into account disparities in relative sizes of human brain regions. After a closer examination of our schematics of oligodendrocyte lineage markers expression during human development given in **Figure 2**, it is clear that comparisons between human and rodent brains are neither easy to make, nor linear. Studies of human brain development from our laboratory suggest that processes like neurogenesis and oligodendrogliogenesis progress at different pace in the human brain, so every process has to be compared separately. Whereas neocortical neurogenesis does begin at 5 gw, corresponding to mouse embryonic day 10, the ending of neurogenesis is not that clear, since at the last time-point of our study, 24 gw neurons are still being born out of cells isolated from the cortical VZ/SVZ (Mo et al., 2007; 2008). Cortical oligodendrogenesis begins around 10 gw in humans, but it progresses well into adulthood in humans (Yakovlev and Lecourse, 1967). Based on the expression of markers of oligodendrocyte lineage, we will try to draw parallels between human and mouse development, focusing on the forebrain. PDGFRα mRNA in the mouse brain was first detected at embryonic day 15 (E15), reached a peak at postnatal day 14 (P14), and fell to lower levels in adults (Yeh et al., 1993). In contrast, mRNA for MBP was detected later, at P3–P4 in the mouse corpus callosum and the internal capsule, respectively

(Verity and Campagnoni, 1988). MBP expression reaches peak in the mouse cortical and subcortical white matter at P20, when primary myelination is completed (Campagnoni and Macklin, 1988; Verity and Campagnoni, 1988). In the human forebrain, the first early oligodendrocyte progenitors appear at 10 gw, which then can be compared to E15 in mice. The first MBP$^+$ premyelinating oligodendrocytes are detected at 18 gw in humans, which then is comparable to P3 mouse (**Figure 2**). Our observations on early oligodendrocyte development and myelination onset, however, roughly fit into the previously proposed model of human-to-rodent development comparison (Bayer et al., 1993). Subsequently human primary forebrain myelination takes decades, compared to weeks in rodents (Yakovlev and Lecourse, 1967). The most important reason for this prolonged development is that much larger human brain has entire neocortical regions which rodent brains completely lack, whereas some of the key regions of rodent brains are in human relatively underdeveloped (e.g. olfactory bulbs).

Another interesting issue is concerning the overall number of oligodendrocytes in human versus rodent brain. Although numbers of oligodendrocytes needed to myelinate large human brains are much higher than in rodents, it is not as clear if the densities and proportions of oligodendrocytes are different. In mouse cerebral and cerebellar cortex, for example, the density of oligodendrocytes was estimated to be $12.5 \times 10^3/\text{mm}^3$, which makes for approximately 5% of all cells detected by the nuclear staining (Irintchev et al., 2005; Jakovcevski et al., 2009). Comparable stereological studies of the human prefrontal cortex suggest very similar densities for oligodendroglia in humans (Ongur et al., 1998; Stark et al., 2004). These studies also demonstrated decreased density of oligodendrocytes in patients with schizophrenia and mood disorders (Ongur et al., 1998; Stark et al., 2004).

In conclusion, greater complexity of the human brain, including its longer development, larger size, and the existence of unique regions and functions, as well as uniquely human pathologies calls for more studies on human brain development. The fact that many more oligodendrocytes need to be generated to myelinate a huge amount of axons in the large white matter of the human brain, supports the idea that oligodendrocytes may be generated for a longer time period and also from various progenitor sources than in the far smaller rodent brain. Inter-species differences, thus, should be taken into consideration when extrapolating results from animal models to primate and particularly human brain. The main difficulty in conducting studies on human brain development is the limited source of human brain tissue, but hopefully this will change with more groups working in the field of human development enabling easier comparisons of results (e.g. Bayatti et al., 2008; Fertuzinhos et al., 2009; Petanjek et al., 2008; Stillman et al., 2009). Another promising new direction is the use of human embryonic stem cells-derived tissues that would allow *in vitro* studies of developmental processes on a much larger scale and in a more reproducible manner.

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Growth of the human corpus callosum: modular and laminar morphogenetic zones

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The purpose of this focused review is to present and discuss recent data on the changing organization of cerebral midline structures that support the growth and development of the largest commissure in humans, the corpus callosum. We will put an emphasis on the callosal growth during the period between 20 and 45 postconceptual weeks (PCW) and focus on the advantages of a correlated histological/magnetic resonance imaging (MRI) approach. The midline structures that mediate development of the corpus callosum in rodents, also mediate its early growth in humans. However, later phases of callosal growth in humans show additional medial transient structures: grooves made up of callosal septa and the subcallosal zone. These modular (septa) and laminar (subcallosal zone) structures enable the growth of axons along the ventral callosal tier after 18 PCW, during the rapid increase in size of the callosal midsagittal cross-section area. Glial fibrillary acidic protein positive cells, neurons, guidance molecule semaphorin3A in cells and extracellular matrix (ECM), and chondroitin sulfate proteoglycan in the ECM have been identified along the ventral callosal tier in the protruding septa and subcallosal zone. Postmortem MRI at 3 T can demonstrate transient structures based on higher water content in ECM, and give us the possibility to follow the growth of the corpus callosum in vivo, due to the characteristic MR signal. Knowledge about structural properties of midline morphogenetic structures may facilitate analysis of the development of interhemispheric connections in the normal and abnormal fetal human brain.

Keywords: callosal septa, midline structures, semaphorin3A, glia of indusium griseum, fetal brain, magnetic resonance imaging

INTRODUCTION

To accomplish complex tasks, mammals require coordinated brain activity, based on precise and efficient connections between the two hemispheres. These connections consist of axons that traverse the telencephalic midline, principally in three commissural tracts: the corpus callosum, the hippocampal commissure and the anterior commissure. Among these, the corpus callosum is the most voluminous fiber tract, and in the human species reaches its maximum complexity and size relative to brain volume (Gazzaniga, 2000). Anatomical studies in experimental rodents demonstrated that the majority of contralaterally projecting (callosal) neurons are located in cortical layers II/III and layer V (Innocenti and Price, 2005), while in the primate brain fibers of the corpus callosum predominantly originate from layer III pyramidal neurons of the neocortex (Mrzljak et al., 1988; Schwartz and Goldman-Rakic, 1991; Schwartz et al., 1991). Axons of the callosal neurons elongate to the intermediate zone, then navigate medially through a well-defined pathway along the medial wall of the ipsilateral ventricle, cross the midline, grow further into the contralateral hemisphere towards the target region and area (usually homotopic), and finally enter the appropriate cortical layer to establish functional connections. Members of the Netrin, Slit, Semaphorin, Ephrin and Wnt families of guidance molecules and their receptors, coordinate this extremely demanding navigation. As secreted and diffusible molecules, they have long-range effect on the growth cone and axons, or as molecules attached to the cellular membranes or extracellular matrix (ECM), they have short-range effects (see reviews Judas et al., 2003; Lindwall et al., 2007; Plachez and Richards, 2005). Recent findings suggest that the axis of axon elongation is determined even prior to axon outgrowth by the manner in which Netrin, Slit and Wnt receptors are localized within the neuron (Killeen and Sybingco, 2008). Expression of these molecules is regulated by both intrinsic cell-autonomous factors (transcription factors that coordinate receptor expression and signaling at the growth cone membrane) and by extrinsic factors in the extracellular environment.

Several well-known developmental mechanisms, such as guidance by pioneering axons, guidance by pre-existing axonal tracts and guidance by cellular structures have been ascribed to various morphogenetic zones involved in the complicated pathfinding during the formation of commissures in a mammalian brain. However, these different morphogenetic zones have some principal properties in common: (1) strategic location, (2) sequential appearance and dissolution, i.e. particular developmental window, (3) modular or laminar appearance, (4) versatile expression of guidance cues and (5) abundance of ECM. Disturbances in finely tuned expression of guidance and ECM molecules in the morphogenetic zones might cause structural or functional anomalies ranging from subtle cognitive impairment to severe developmental abnormalities, including dysgenesis and agenesis of the corpus callosum and other commissures (reviewed by Paul et al., 2007; Richards et al., 2004).

In this review, we discuss dynamic changes of the properties of morphogenetic zones related to corpus callosum growth, revealed by a new application of classical histological methods, molecular biology techniques and advanced high-resolution brain imaging, focusing on the human midline and its specificities.

THE MORPHOGENETIC ZONES OF EARLY CORPUS CALLOSUM: FIRST HALF OF GESTATION IN HUMANS

As soon as they arise from the soma of future cortical neurons, the predetermined cortical efferents grow toward the intermediate zone guided by gradients of different guidance molecules (for review on the spatio-temporal distribution of guidance cues in the transient embryonic and fetal zones see Judas et al., 2003). When they reach the intermediate zone, the axonal populations have to decide to grow medially around the ipsilateral ventricle as future callosal axons, or to grow laterally toward the internal capsule as long subcortically projecting axons, in both cases avoiding proliferative zones which express repelling cues, e.g., semaphorin3A (SEMA3A) (Bagnard et al., 1998). Data regarding this decision point in human callosal formation are still missing, but exuberant axonal bifurcations have been reported in mice. Bifurcations are supposed to be a developmental mechanism for some cortical neurons to choose elongation toward the midline after cutting off the lateral branch if it never reaches a viable target (Garcez et al., 2007). Moreover, it has recently been demonstrated in mice that the expression of the guidance cue neuropilin1 (Npn1, a receptor for SEMA3A) by the growing callosal axons, has a critical role in the choice of tangential extension toward the midline (Hatanaka et al., 2009).

The early growth of future callosal axons in humans and the subsequent morphogenesis of the callosum were firstly demonstrated by histological methods and described in the classical embryological studies (for review see Rakic and Yakovlev, 1968). Prior to corpus callosum formation, at 11 postconceptual weeks (PCW), a new structure designated as *massa commissuralis* is rapidly formed after the fusion of median groove banks above the septal area in the so called "*commissural plate* of Hochstetter" (Rakic and Yakovlev, 1968). The first axons, named pioneering, approach and penetrate the massa commissuralis at the mediosagittal plane after 11 PCW (Rakic and Yakovlev, 1968). Therefore, the medially positioned massa commisuralis is probably the first midline structure that expresses specific molecules and morphogenes for guidance and nurture of pioneering callosal axons in humans. Moreover, it has recently been shown that a portion of developing callosal axons at 17 PCW expresses Npn1, and that a considerable number of those originate from the cingulate cortex (Ren et al., 2006). These axons most likely represent the pioneering axons in human callosal development, considering the newly established central role of Npn1 in the development of pioneer projections from the cingulate cortex of mice (Piper et al., 2009). Correlation of in vitro magnetic resonance imaging (MRI) and histological analysis of the developing human cerebrum revealed that the commissural plate, as well as other transient fetal zones, can be visualized on T1-weighted images already at 10 PCW (**Figure 1**, Rados et al., 2006). The commissural plate is then at the onset of its development and visibility on MRI scans as a thickened dorsal part of the telencephalon impar (**Figure 1B** double arrows, Rados et al., 2006). The callosal fibers that penetrate massa commissuralis and form the *callosal plate*, can be demonstrated by histological methods at 12–13 PCW (Rakic and Yakovlev, 1968), while the earliest stage of their visualization by diffusion tensor imaging (DTI) is at 14–15 PCW (Huang et al., 2009; Ren et al., 2006). Some reports have suggested that the first axons are the ones that will form the rostrum, the genu and the body (Ren et al., 2006), while others suggest that the callosum will grow in both anterior and posterior directions, with a more prominent anterior growth (Huang et al., 2009).

With the formation of the callosal plate, several morphogenetic zones appear along the midline and subsequently develop into transient cellular structures: the midline sling, the glial wedge and the glia of indusium griseum (indusial glia). These developmental structures were first recognized and described in morphological studies of experimental animal models (Silver et al., 1982, 1993). Later they were further explored in mice by modern molecular methods, and for each structure a critical role in the morphogenesis of the corpus callosum has been established (Shen et al., 2006; Shu and Richards, 2001; Shu et al., 2003a,b,c; Smith et al., 2006; Tole et al., 2006). Recently, histological and correlated histological/MRI studies demonstrated that these transient structures are also present during the period of early development of the human forebrain midline (Lent et al., 2005; Ren et al., 2006), indicating conservation of developmental mechanisms and structures during mammalian evolution. These morphogenetic structures situated in strategic locations have overlapping developmental windows between 13 and 20 PCW (Lent et al., 2005; Ren et al., 2006).

The midline sling was originally described as a thin concave lamina consisting of migrating glia-like cells that tightly underline the ventral surface of the developing corpus callosum (Silver et al., 1982, 1993). It was later proven that, at least in mice, a substantial portion of cells that forms the sling are neurons (Shu et al., 2003b). Neuronal nuclear antigen (NeuN), calretinin and glial fibrillary acidic protein (GFAP) have been demonstrated in the human midline sling, indicating that it also consists of neuronal and glial cells (Ren et al., 2006). The first clearly NeuN positive reactivity was shown to be located paramedially on the corticoseptal border at 13 PCW, but at a later stage (15 PCW) numerous neurons were observed more medially (Ren et al., 2006), suggesting that this population of cells migrates toward the midline. The origin of the cells that compose the midline sling in humans is complex and not clearly understood. However, one can speculate that similarly to the sling-cells in mice (Shu et al., 2003b), they are coming from the adjacent subventricular zone (Ren et al., 2006). Although, the pioneer callosal axons of mice (Ozaki and Wahlsten, 1998; Rash and Richards, 2001) cross the midline before the development of the sling at embryonic day (E) 17 (Silver et al., 1982), prenatal experimental lesioning causes a failure in corpus callosum formation, thus confirming the significance of midline sling in this developmental point (Silver and Ogawa, 1983).

The glial wedge at the gross structural level is described as a bilaterally symmetrical modular structure located at the corticoseptal boundary between the cingulate cortex (dorsal telencephalon) and the septum (ventral telencephalon). The corticoseptal boundary is genetically defined (Shen et al., 2006), and its correct dorsoventral position is critical for the formation of both the glial wedge and the corpus callosum. In the human brain, the glial wedge is present at 14 PCW, flanking the ventral side of the corpus callosum. It is

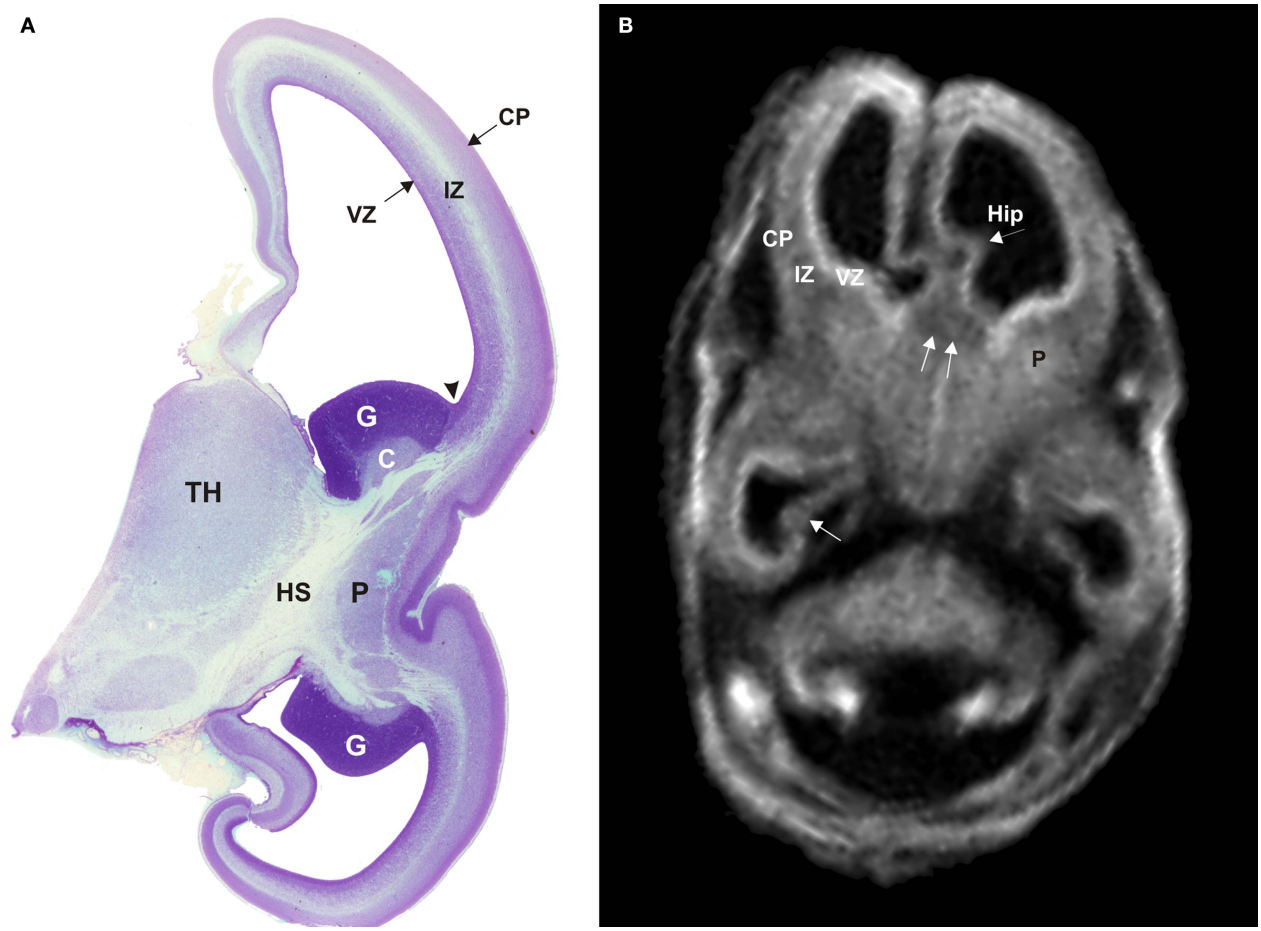

FIGURE 1 | Semi-horizontal Nissl-stained slide (A) and T1-weighted MRI slice (B) through the fetal telencephalon at 11.5 PCW (A) and 12.5 PCW (B). The cerebral wall consists of the ventricular zone (VZ), the intermediate zone (IZ) and the cortical plate (CP). Trilaminar organization of the cerebral wall, the ganglionic eminence with developing caudate and putamen, and the hippocampal anlage (arrowhead Hip) are all clearly visible on both histological and MRI sections. The two fornix bundles merge toward the septal area and contribute to the triangular shape of the telencephalon impar in MRI scans. Double arrows in **(B)** point to the commissural plate. C, caudate nucleus; CP, cortical plate; G, ganglionic eminence; Hip, hippocampus; HS, hemispheric stalk; IZ, intermediate zone; P, putamen; TH, thalamus; VZ, ventricular zone. Modified and reproduced with permission from Rados et al. (2006). Copyright Elsevier Ltd.

densely populated by cells that are positive for nuclear factor Ia (Nfia), vimentin and GFAP. These cells are in continuity with the radial glial cells of the ventricular zone of the cerebral wall (Lent et al., 2005; Ren et al., 2006). Differentiation of the glial wedge cells into astrocytes begin at 14 PCW, earlier than in the rest of the cortical wall, and lasts at least through the first half of gestation (Rezaie et al., 2003). Experimental work in mice, has pointed out that guidance by the glial wedge occurs through SLIT–ROBO and WNT–RYK signaling (Andrews et al., 2006; Keeble et al., 2006; Shu and Richards, 2001; Shu et al., 2003d).

In the human brain, the indusial glia begins to develop at about 14 PCW, when discernible Nfia (Ren et al., 2006), GFAP (Lent et al., 2005; Ren et al., 2006) and vimentin (Lent et al., 2005) expression has been shown dorsally to the developing corpus callosum. The importance of indusial glia for callosal development has been convincingly demonstrated by *Nfia*- and *Nfib*-knockout mice. These have significantly reduced or absent indusial glia and glial wedge, and fail to form the corpus callosum (Shu et al., 2003a; Steele-Perkins et al., 2005). In the conditional knockout mice for fibroblast growth factor receptor 1/GFAP (*Fgfr1*/*Gfap*), it has been shown that indusial glia originates from ventricular radial glial cells (*Fgfr1* gene is critical for dorsally directed migration of radial glial cells in the midline) (Smith et al., 2006). When *Fgfr1* is knocked out earlier in development, all midline glial structures that derive from the corticoseptal boundary and corpus callosum, fail to develop (Tole et al., 2006).

The list of guidance molecules, transcription factors and morphogens involved in corpus callosum formation in mice and humans, which are expressed either in the midline structures or by growing callosal axons, is continuously expanding (see review articles Judas et al., 2003; Lindwall et al., 2007; Plachez and Richards, 2005; Richards et al., 2004). However, evidence for expression of these molecules in the human brain midline is still very scanty. Northern blot analysis of total RNA and mRNA for *Netrin1*, its receptor *deleted in colon cancer*, *Slit1*, *Slit2* and *Slit3*, their receptors *Robo1* and *Robo2*, and the transcription factors *Nfia* and *Emx1*, confirms

that these nine genes are expressed in the human forebrain at 17 PCW, and that *Netrin1*, *Slit2* and *Slit3* have increased expression in ventrocaudal regions of the forebrain, compared to the dorsal neocortex (Ren et al., 2006). The most informative part of the study was demonstration of the presence of Npn1 in axons of the human corpus callosum at 17 PCW (Ren et al., 2006).

Studies in experimental rodents have demonstrated that ECM molecules such as laminin, fibronectin, proteoglycan NG2, heparan and chondroitin sulfate proteoglycans play an important role in the guidance of commissural axons (Braga-de-Souza and Lent, 2004; Inatani et al., 2003; Yang et al., 2006). So far, in the human brain only tenascin-C was convincingly shown to be present dorsally and ventrally to the corpus callosum until 20 PCW (Lent et al., 2005). ECM molecules rather than just constituting a structural scaffold, interact with guidance cues and can critically modulate axonal guidance function (reviewed by de Wit and Verhaagen, 2007; Kleene and Schachner 2004).

Taken together, studies on human brain development have confirmed that developmental mechanisms governing the early formation of the forebrain midline and corpus callosum are indeed very similar to, if not the same as those described in experimental rodents. However, the human fetal brain is significantly larger than the brain of prenatal rodents and the distances the growing commissural axons have to cover are notably longer in every segment of their complex journey. Therefore, it is reasonable that commissural connections in humans require a longer period of contemporaneous persistence of supporting midline structures and probably additional morphogenetic zones.

MODULES AND LAMINA FOR LATER GROWTH OF CORPUS CALLOSUM: SECOND HALF OF GESTATION IN HUMANS

There are only a few histological studies describing the later stages of corpus callosum development in humans. In fetuses at 18–20 PCW, it is a well-developed fibrillar structure, which in coronal planes, runs transversally toward the midline and after crossing curves along the roof and lateral wall of the lateral cerebral ventricle. In the midsagittal plane, the outlines of the major callosal parts (genu, body and splenium) are clearly visible and assume the same shape and position as in the adult brain, with the exception of being much smaller in their rostrocaudal extent and thickness (Rakic and Yakovlev, 1968). DTI studies of the fetal brain at 19 PCW show the callosal radiation with its typical shape, similar to the one seen in neonates, with axons from all parts of the callosum forming a mohawk-shaped structure (Huang et al., 2009). At this stage the callosal cross-sectional area is only 5% of the size seen in a 5-year-old infant. At the neonatal stage, just before apparent myelinization of the callosum starts, it is half the size (Huang et al., 2006).

Beside the early midline structures, a morphogenetic role in formation of the human corpus callosum during the second half of gestation has been described for two additional zones: callosal septa (Jovanov-Milošević et al., 2006) and subcallosal zone (SCZ) (Kostović et al., 2002). These two midline structures jointly form "grooves" in which callosal bundles are laid down during the second half of gestation.

Postmortem analysis of the corpus callosum (age range from the 18 PCW to adult), immunostained for GFAP, NeuN and chondroitin sulfate proteoglycan (CS-56), in addition to classical histological methods, revealed the existence of modular cellular structures; namely, the callosal septa, which are most prominent during the second half of gestation (**Figure 2**) (Jovanov-Milošević et al., 2006). Callosal septa of variable sizes radially invade the corpus callosum from its ventral surface, dividing it into irregular segments (**Figures 2A,B**). In horizontal sections, septa appear as thin railway slippers along the anteroposterior axis of the corpus callosum. In midsagittal sections, the callosal septa appear as radial striations wider in the ventral portion. In addition, thinner striations, one to three cell rows wide, are evenly spaced between the larger septa (**Figures 2G,H**, arrowheads). Such modular structure and segmentation of the corpus callosum is visible in illustrations of midsagittal sections of the developing monkey (Killackey and Chalupa, 1986) and human brain (Bayer and Altman, 2005a,b; Rakic and Zecevic, 2003). However, callosal septa have not been explicitly described, and their importance in the protracted development of the corpus callosum in primates has been essentially overlooked. The reasons why the importance of these structures was not perceived in recent studies of the human midline might lie in the fact that the septa are not discernible in coronal sections through the corpus callosum, and the fact that the latest fetal age examined in these studies was 20 PCW.

During the developmental window of 18–34 PCW, the number of callosal septa is individually variable. Usually 15–20 thicker and longer septa and numerous smaller septa are unevenly distributed along the anteroposterior axis (**Figures 2A–D**). In the genu and the anterior part of the callosal body (**Figure 2B**, framed area), the septa are more numerous and more regularly spaced (**Figure 2D**, arrow). In the rest of the callosal body, their number declines, while in the splenium it increases again, but the septa are still less numerous and less prominent there, in comparison with the anterior portion of the callosum. At the cellular level, the callosal septa contain: GFAP reactive meshwork, NeuN positive neurons, CS-56 immunoreactive ECM (**Figure 2**, Jovanov-Milošević et al., 2006) and expression of the guidance molecule SEMA3A in cells and ECM (Judas et al., 2005). The intensive GFAP staining of septa is associated with perivascular astrocyte elements, fine glial fibers and scattered GFAP positive astrocytes. The ventral, wider portion of the septa has a larger number of neurons (**Figures 2E,F**) as well as a higher content of chondroitin sulfate proteoglycan (**Figures 2G,2H**). At midgestation, SEMA3A is expressed in septa of the anterior third of the callosum and above the fornix (**Figure 3**, Judas et al., 2005). It is important to note that chondroitin sulfate proteoglycan in the ECM plays an important role in the cellular localization of SEMA3A, and their interaction can modulate the biological activity of this guidance molecule (de Wit et al., 2005).

The callosal septa have not been shown by conventional MRI, yet the abundance of ECM and proteoglycans in septa, as well as their radial orientation between callosal bundles, should influence the MRI signal in diffusion-weighted imaging (DWI) of the human fetal brain. In vivo DWI of infants born between the ages of 25 and 34 PCW, with some having follow-up scans at term equivalent age, showed lower fractional anisotropy (FA) values in the genu than in splenium. Decreased FA values generally indicate a less coherent parallel organization of axons, which in this case may be due to the presence of septa in the genu. The study also showed higher apparent diffusion in the genu, indicating higher water content

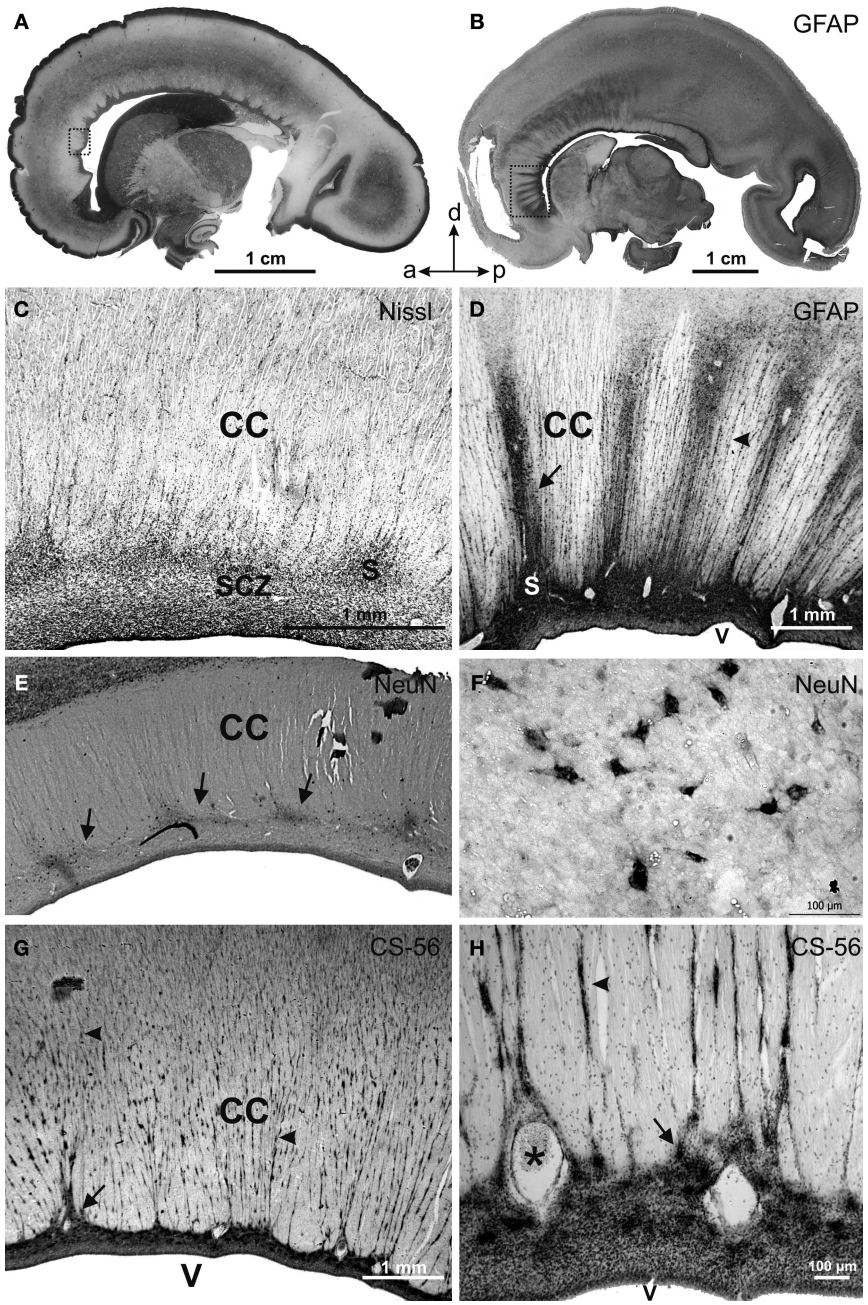

FIGURE 2 | Parasagittal (A) and midsagittal (B) sections of the human fetal brain showing the transient cellular structures callosal septa at 21 (A), 24 (E–H) and 25 (B–D) PCW stained with Nissl (A,C), glial fibrillary acidic protein (GFAP) (B,D), neuronal-specific nuclear protein (NeuN) (E,F) and chondroitin sulfate proteoglycan (CS-56) (G,H) immunocytochemistry. Framed areas in **(A)** and **(B)** are enlarged correspondingly in **(C)** and **(D)**; **(F)** and **(H)** represent high magnification of callosal septa shown in **(E)** and **(G)**. Arrows point at thicker callosal septa, arrowheads at striations. s, callosal septa; CC, corpus callosum; SCZ, subcallosal zone; V, cerebral ventricle; a, anterior; p, posterior; d, dorsal. Modified and reproduced with permission from Jovanov-Milošević et al. (2006). Copyright Collegium Antropologicum.

and smaller fiber density (Partridge et al., 2004), possibly due to chondroitin sulfate proteoglycan in the callosal septa and their remnants. In utero DWI of fetal white matter between 18 and 37 PCW also showed lower FA values in the genu. As the authors themselves suggested, this might be a consequence of a specific geometric microstructure of the callosal septa. The predominance of radially oriented septa within the genu would decrease its FA value (Kasprian et al., 2008).

The ventral part of the callosal septa is in continuation with the SCZ, which like a thin lamina occupies the median and paramedian territories situated between the developing corpus callosum (dorsally) and the fornix bundles (ventrally) (Kostović et al., 2002). The

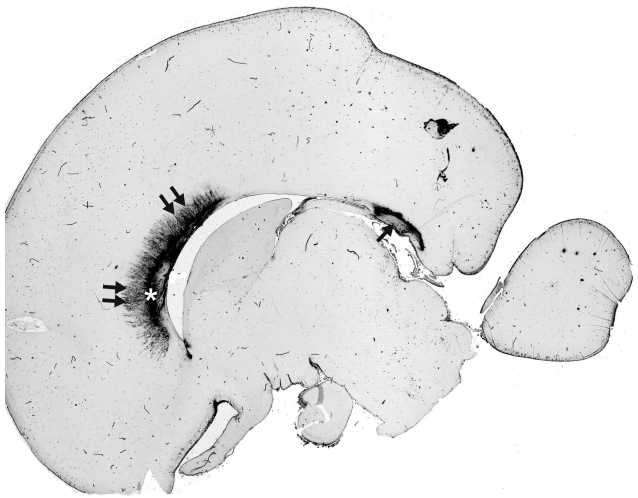

FIGURE 3 | SEMA3A immunoreactivity in human brain midline at 20 PCW is restricted to ventral area of anterior callosal portions, where it is located in both cell bodies and the extracellular matrix. The SEMA3A is prominent in callosal grooves: in septa (double arrows) and in the subcallosal zone (asterisk). In addition, strong SEMA3A immunoreactivity is visible dorsally to the fornix system (arrow). Modified and reproduced with permission from Judas et al. (2005). Copyright American Society of Neuroradiology.

cellular composition of the SCZ (revealed by acetylcholinesterase histochemistry and Golgi staining) is characterized by area-specific neuron-like cells with long and wavy processes, large glia-like cells, maturing neurons, migratory-like neurons and radial glial cells (**Figure 4**), with a difference in distribution between the medial (nucleus septohippocampalis) and lateral portion (allocortical counterpart of the subventricular zone). In fetuses at midgestation, the lateral paramedian portions of the subcallosal region gradually transforms into the subventricular zone of the dorsal neocortical telencephalic wall, both described in detail by Zecevic group (Zecevic et al., 2005). Some of the cells of SCZ also express SEMA3A (Judas et al., 2005).

During the early postnatal period, the callosal septa become thinner and shorter, lose their neuronal and chondroitin sulfate proteoglycan content. At the same time, the number of cells in the SCZ decreases.

Several lines of evidence support the morphogenetic role of the callosal grooves. The period of developmental peaks of the callosal septa and SCZ (between 18 and 34 PCW) corresponds to the period of intensive growth of callosal fibers (Huang et al., 2006). The septa are appropriately shaped to support the transverse growth of callosal fibers, similarly to the midline sling during early callosal development in mice and humans (Ren et al., 2006; Shu et al., 2003b; Silver et al., 1982). The complexity and preferential orientation of the processes of subcallosal cells, as well as those of cells in the septa, suggest that they may be involved in the guidance of callosal axons. The cells of the septohippocampal continuum may have a pivotal role in the bidirectional guidance of fornix fibers. The cells of the callosal septa produce the axonal guidance molecule SEMA3A (Judas et al., 2005), shown to be necessary for active guidance of callosal axons across the midline towards the opposite hemisphere in mice (Piper et al., 2009). If the Npn1 expression, which was demonstrated at 17 PCW (Ren et al., 2006), still persists during the second half of gestation, it is reasonable to presume that it would continue to interact with its ligand, such as SEMA3A. Finally, the ECM and chondroitin sulfate proteoglycan in septa and SCZ can interact with guidance molecules and serve to guide growing callosal axons along the ventral callosal moiety during the second half of gestation. A dual developmental role has also been shown for the chondroitin sulfate proteoglycan: in the subplate it is a favorable substrate for growth of thalamocortical axons, but at the same time, it is an inhibitory substrate for developing cortical efferents forcing them to grow in a chondroitin sulfate proteoglycan negative intermediate zone (Bicknese et al., 1994; Miller et al., 1995). In that respect, proteoglycans in the brain midline may guide the growth of callosal axons along the ventral callosal tier, similarly to the role of subplate-proteoglycans in the growth of thalamocortical connections. Or they may act as an unsuitable growing substrate, confining callosal axons to the mainstream of growth (Jovanov-Milošević et al., 2006; Judas et al., 2005; Lent et al., 2005). An additional role of the ECM is maintaining diffusible guidance molecules within callosal grooves (Jovanov-Milošević et al., 2006).

The corpus callosum of adult primates consists of "segments" which contain topographically segregated callosal fibers for a given cortical area (Moses et al., 2000; Pandya et al., 1971; Witelson, 1989). Thus, callosal septa with their topographic arrangement, shape and content most likely provide a basis for topographically ordered commissural projections from one hemisphere to another, or at least maintain the topographical relationship during development. Confirmation of this suggestion can be found in the fact that the basic topography and terminal field patterns of callosal projections in monkey brain are established already by E133, well before birth (Dehay et al., 1988; Killackey and Chalupa, 1986; Schwartz and Goldman-Rakic, 1990, 1991). It should be noted that in mice, newly added axons grow along the ventral tier of the corpus callosum (Ozaki and Wahlsten, 1992). In addition, tractography studies in humans showed an evident dorsoventral fiber distribution in the adult human brain, with earlier developed medial cortical areas sending their fibers dorsally through the corpus callosum, while later developed laterodorsal cortical regions send them ventrally (Tovar-Moll et al., 2007). In this respect, callosal septa and SCZ with their abundance of ECM and guidance molecules along the ventral aspect of the corpus callosum are strategically located for influencing the growth of callosal axons.

One of the principal mechanisms of cortical development is an overproduction of axons, axonal branches and synapses, the so-called developmental exuberance, which is followed by a subsequent selection and refinement based on adequate target region recognition and activity of the functional connections (reviewed by Innocenti and Price, 2005). The corpus callosum is a pivotal example for such developmental exuberance, since the number of callosal axons in monkeys at E165, exceeds the number of callosal axons present in the adult by at least 3.5 times (LaMantia and Rakic, 1990a,b). Thus, the later morphogenesis of the corpus callosum is even more complex due to the processes of retraction of exuberant callosal fibers. In the primate brain, this continues during the

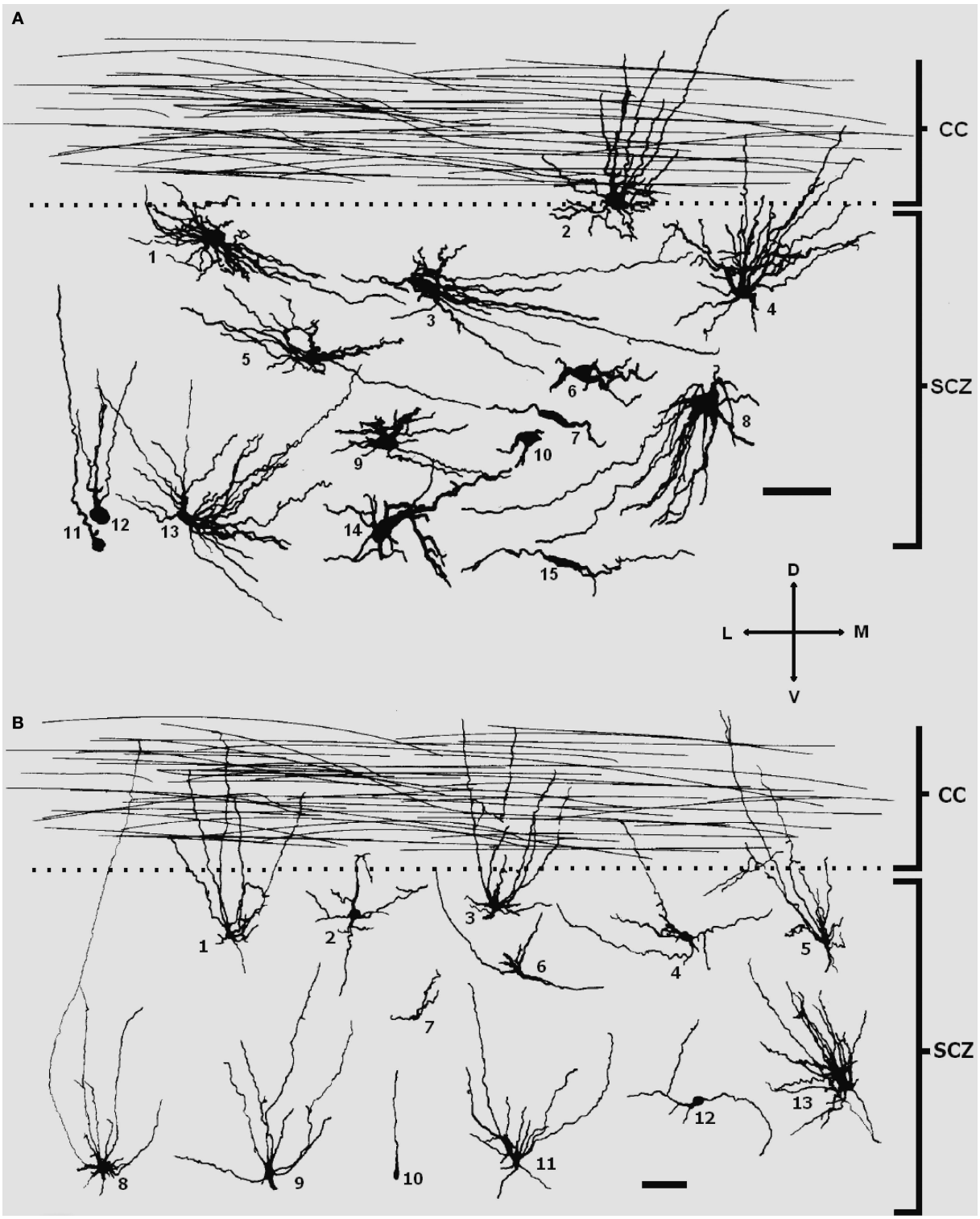

FIGURE 4 | Different types of cells in the subcallosal region of a 22-PCW (A) and a 28-PCW human fetus (B), as reconstructed by Neurolucida software. The subcallosal zone (SCZ) at both stages contain radial glial cells (B10), glia-like cells (A1, A3), migratory-like neurons (A7), immature polymorphic neurons (A6, 8, 9, 10, 12, 14,15 and B2, 4, 6, 7, 12), and region-specific large neuron-like cells with long wavy dendrites oriented predominantly towards the corpus callosum (CC), partly penetrating it (A2, 4, 5, 13 and B1, 3, 5, 8, 9, 11). Note that processes of large neuron-like cells seem to be less profusely branched in the older specimen. Scale bar: 50 μm. Modified and reproduced with permission from Kostović et al. (2002). Copyright S. Karger AG, Basel.

first three postnatal months (LaMantia and Rakic, 1990a). In the human brain, decrease in size of midsagittal cross-sectional area was observed after 32 PCW and this lasts to the second postnatal month. This suggests the beginning of a retraction of exuberant callosal axons (Clarke et al., 1989), concomitantly with the resolution of waiting compartments (Kostovic and Rakic, 1990) and the resolution of the majority of the callosal septa (Jovanov-Milošević et al., 2006). The remnants of septa that continue for some time after birth may help in the process of withdrawal of exuberant callosal axons, since these structures and processes temporally overlap.

In addition, the septa may also provide corridors for migration of later-born neurons, since thick callosal bundles on the roof of the lateral ventricle represent a structural barrier. The groups of neurons originating from the subventricular zone may use glial fibers in the callosal septa to "climb", while guidance molecules and ECM facilitate migration towards the cortex (Jovanov-Milošević et al., 2006).

CONCLUDING REMARKS

Recent progress in gene targeting methods, advances in axonal tracing, and high-resolution MRI techniques have revealed the morphogenetic zones and their role in guiding callosal axons across the midline. Although, the corpus callosum at midgestation assumes a shape and position not essentially different from that in the adult brain, it is still far from its definitive rostrocaudal extent and thickness (Huang et al., 2006; Rakic and Yakovlev, 1968). For each forebrain connectivity system, the growth of fibers (extension, accumulation and ingrowth) may last from 4 to 8 weeks with significant overlap among systems, but also with regional differences in timing, of up to 2 weeks (Kostovic and Jovanov-Milošević, 2006). In the developing brain, all histogenetic events (neurogenesis, gliogenesis, migration, cell differentiation, axonal extension and synaptogenesis) proceed within laminar or modular compartments or zones. These do not have an equivalent in the adult brain (Kostovic et al., 2002; Rakic et al., 2004). Therefore, different structures and processes have to be interpreted in a precisely defined spatial and temporal context. In that respect, it is important to note that in humans the complex event of interhemispheric integration through the corpus callosum continues throughout gestation and well after birth. The second half of gestation and the early neonatal period are important for the multiple-fold increase of callosal axonal number, selection of functional axons, withdrawal of exuberant axons and targeting of the region, area, layer and cells of the cortical plate. Insight into the organization of the morphogenetic zones involved in development of cortico-cortical pathways is a prerequisite for the studies of preterm and term-born infants in normal development and pathological conditions. It has been previously suggested that in preterm infants the periventricular areas are vulnerable, but also show vigorous structural plasticity due to the abundance of ECM and guidance molecules, in addition to a "waiting" position of fibers (Judas et al., 2005). Analogously, callosal septa with their content of ECM and guidance molecules give the callosum a potential for structural plasticity in infants when their development is disturbed prenatally. Contributing to that idea are the known adverse effects of preterm birth on cross-sectional callosal size, measured on structural MRI. Differences in size have been particularly notable in the posterior callosal portions (Nosarti et al., 2004), where the septa are not so prominent. It is not yet clear whether defects in callosal septa formation can effect corpus callosum size and contribute to callosal hypoplasia in humans. MRI parameters such as FA may provide valuable information for prognostication and therapy management in prenatal brain injury, since neurodevelopmental impairments in infants born preterm are related to microstructural abnormalities in specific regions of the corpus callosum (Counsell et al., 2008). Therefore, the knowledge of the sequence of events and the composition of developing forebrain midline enables a more reliable understanding of normative parameters necessary for studies of structural plasticity after perinatal brain injury.

Considering the clinical significance of the corpus callosum (over 50 different syndromes display dysgenesis of the corpus callosum and over 40 genes are linked to these anomalies, reviewed by Richards et al., 2004), its importance for cognitive functions (Gazzaniga, 2000; Teicher et al., 2004) and its frequent lesioning in the perinatal period (Stewart et al., 1999), we would strongly encourage long-term studies of the formation of commissural pathways in humans.

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Could sex differences in white matter be explained by *g* ratio?

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Recent studies with magnetic resonance imaging suggest that age-related changes in white matter during male adolescence may indicate an increase in *g* ratio wherein the radial growth of an axon outpaces a corresponding increase in myelin thickness. We review the original Rushton (1951) model where a *g* ratio of ~0.6 represents an optimal relationship between the axon and fibre diameters vis-à-vis conduction velocity, and point out evidence indicating slightly higher *g* ratio in large-diameter fibres. We estimate that fibres with a diameter larger than 9.6 μm will have a relatively thinner myelin sheath, and brains with increasingly larger proportions of such large-diameter fibres will have progressively lower concentration of myelin. We conclude by pointing out possible implications of "suboptimal" *g* ratio for the emergence of "disconnection" disorders, such as schizophrenia, in late adolescence.

Keywords: myelin, axon, connectivity, adolescence, schizophrenia, brain development

INTRODUCTION

In vivo studies of the human brain have revealed the presence of striking sex differences in the volume and microstructure of white matter (WM). In adults, the overall volume of WM is higher in men than women; in most studies, this is true even after accounting for sex differences in brain size (e.g. Gur et al., 2002). On the other hand, sex differences in the "density" (e.g. Good et al., 2001; the term "density" or "concentration" refers to the probability of classifying a voxel as belonging to a specific type of tissue without making any inference about possible underlying neurobiological processes) and fractional anisotropy (e.g. Hsu et al., 2008) of WM constituting various fibre tracts appear to vary across the tracts, being higher in women than men in some WM pathways and *vice versa* in others.

Many of these sex differences in WM emerge during adolescence. We (Perrin et al., 2009) and others (De Bellis et al., 2001; Giedd et al., 1999; Lenroot et al., 2007) have shown a steep increase in the WM volume during adolescence in boys but not girls. In our sample of 400 adolescents, relative lobar volumes of WM were very similar in boys and girls at the age of 12 years, after which they diverged to reach—by the age of 18 years—a sex difference varying between 9% in the frontal lobe and 25% in the occipital lobe (Perrin et al., 2009; see **Figure 1**).

The total volume of WM is determined by the number of axons, their calibre and the thickness of myelin sheath produced by the oligodendrocytes. Given the known elimination of axons in the early post-natal period (e.g. LaMantia and Rakic, 1990, 1994), age-related increases in the volume of WM during brain development in childhood and adolescence can be accounted for by increases in axonal calibre and/or thickness of the myelin sheath. Recent data from our laboratory provide indirect evidence in favour of age-related changes in axonal calibre during male adolescence. First, we have obtained values of magnetization-transfer ratio (MTR), which provides an indirect index of myelination (Kucharczyk et al., 1994; Schmierer et al., 2004). As can be seen in **Figure 2**, MTR values in lobar WM were about the same in boys and girls at the age of 12 years after which they also diverged, *increasing* slightly in girls (in the frontal lobe) while *decreasing* in boys (in the parietal and occipital lobes); the sex by age interaction was significant in all lobes indicating the opposite effect of age on MTR in boys and girls throughout the brain (Perrin et al., 2009). Second, we have examined WM density in the cortico-spinal tract (CST) at the level of the internal capsule and observed a similar divergence, namely age-related increases and decreases in WM density in girls and boys, respectively (Perrin et al., 2009; **Figure 3**).

Thus, in the same sample of typically developing adolescents, we have observed the following three phenomena: (1) WM volume increases steeply during adolescence in boys but not girls; (2) MTR decreases with age in boys while it does not change (or increases slightly) in girls; and (3) WM density in the CST decreases with age in adolescent boys but increases in girls. We have proposed that these phenomena may be best explained by a disproportionate growth of the axon compared with the growth of its myelin sheath and, as such, by changes in *g* ratio (axon diameter/fibre diameter; see below). We have speculated that a subtle shift in *g* ratio in WM of the male brain during adolescence would affect the proportion of WM tissue occupied by axons and myelin, thus explaining the emergence of sex differences in WM density and MTR described above (Herve et al., 2009; Perrin et al., 2008, 2009). Here we will formalize this proposal by considering in more detail the relationship between fibre diameter and *g* ratio.

G RATIO

G ratio refers to the ratio between axon diameter *d* and fibre diameter *D*; fibre diameter is the sum of the axon diameter (or calibre) and the thickness of the myelin sheath (**Figure 4**). **Figure 5** illustrates the wide variations in axonal morphology vis-à-vis axon diameter and the thickness of myelin sheath. In the monkey corpus callosum (CC), unmyelinated axons, accounting for about 30% of all axons in the genu and <10% elsewhere in

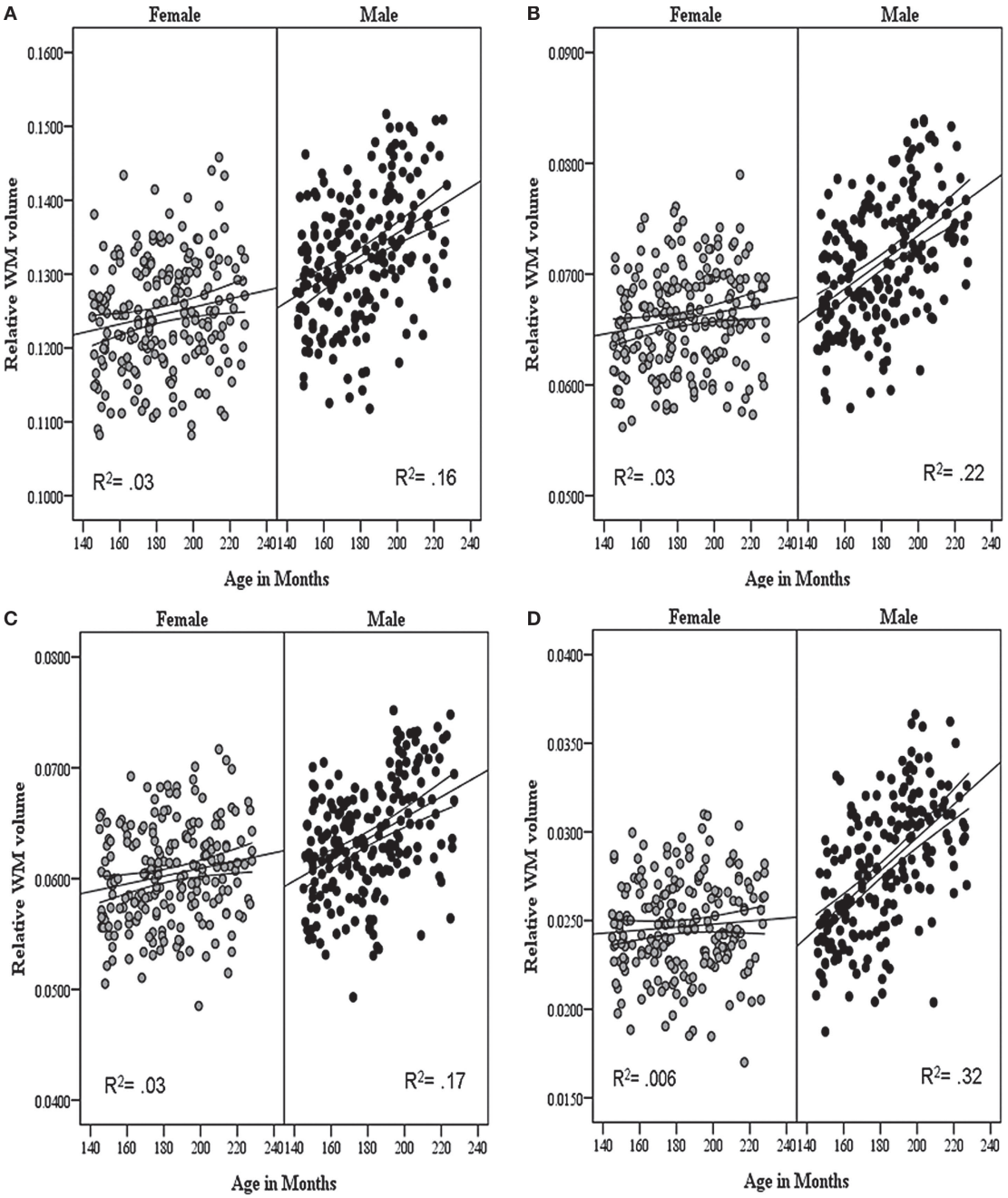

FIGURE 1 | The relationship between age and relative white matter (WM) volume in male and female adolescents in the (A) frontal, (B) parietal, (C) temporal and (D) occipital lobes. The lines represent the regression equation with 95% confidence intervals. Reprinted from Perrin et al. (2009).

the CC, have a small diameter (~0.1 μm), whereas the diameter of myelinated axons varies between 0.1 and 2.5 μm, with the largest axons (2.5 μm) found in the CC midbody (LaMantia and Rakic, 1990; see also Aboitiz et al., 1992 for similar findings in the human CC). The largest axons in the human brain are found in the internal capsule and, most likely, correspond to the cortico-spinal neurons (Lassek, 1942; Verhaart, 1950); the size of these axons increases from birth (~1 μm), through childhood (12 μm at 7 years of age) into adulthood (24 μm). These numbers correspond to the fibre diameter and, as such, reflect both the radial growth of the cortico-spinal axons and their increased myelination with age. We have suggested that changes in the WM density in the CST observed in our studies in relation to plasma levels of testosterone reflect changes in the diameter of these axons (Herve et al., 2009).

In general, axons of large diameter have a thick myelin sheath. It turns out that the *g* ratio is relatively stable at ~0.6 (Rushton, 1951). This observation, together with the small diameter of unmyelinated

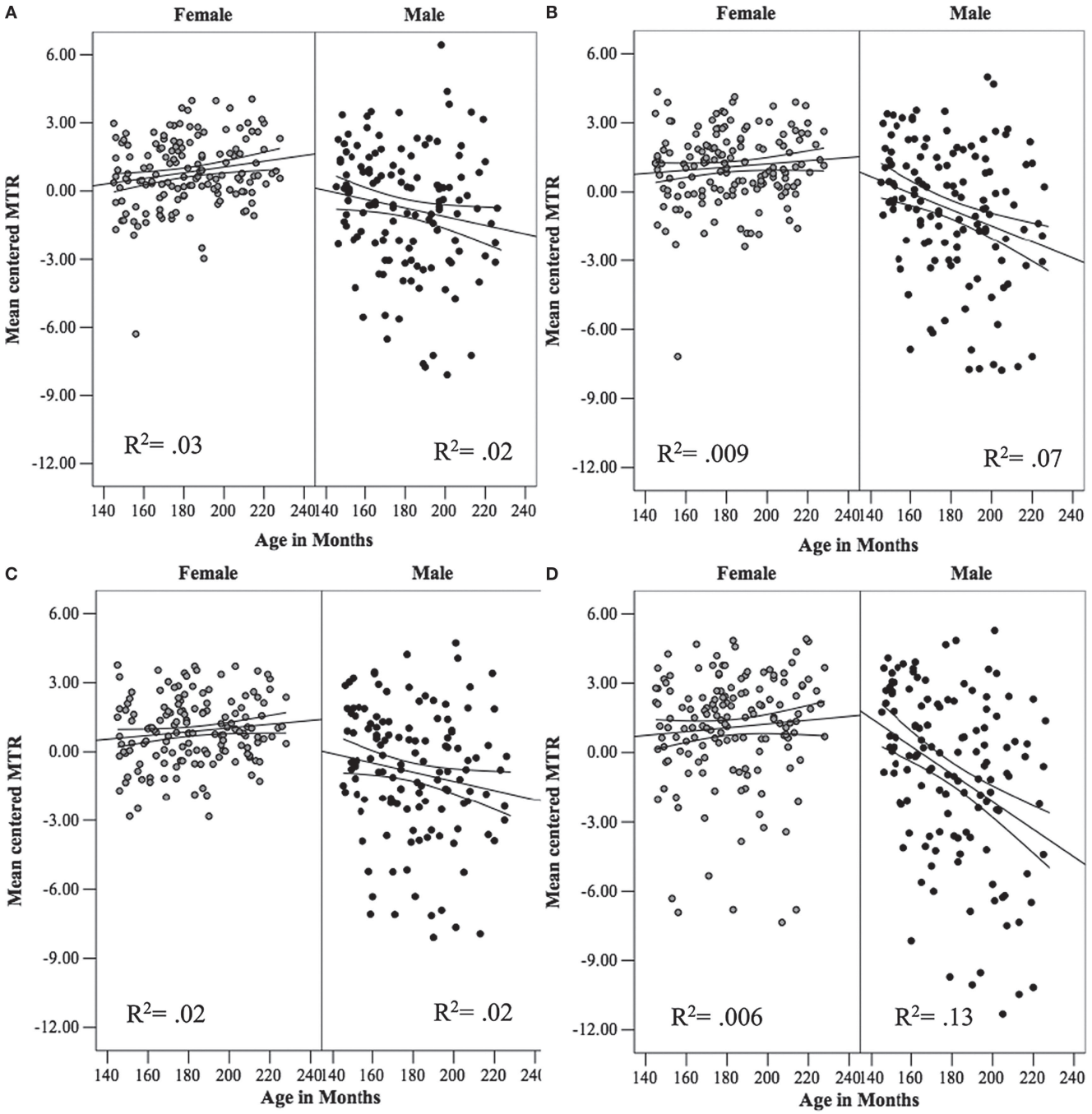

FIGURE 2 | The relationship between age and mean values of magnetization-transfer ratio (MTR) in white matter (WM) in male and female adolescents in the (A) frontal, (B) parietal, (C) temporal and (D) occipital lobes. The lines represent the regression equation with the 95% confidence intervals. Reprinted from Perrin et al. (2009).

axons, suggests possible coupling between myelination and axon diameter (see below). But the range of *g* ratio varies considerably across axons of the same fibre tract (Berthold et al., 1983), as a function of age (Berthold et al., 1983; Jeronimo et al., 2008), mouse strain (Little and Heath, 1994) or disease (Friede and Beuche, 1985). Importantly, *g* ratio appears to increase as a function of axon diameter, indicating a relatively thinner myelin sheath in large axons (Berthold et al., 1983; Chatzopoulou et al., 2008; Gillespie and Stein, 1983). Even though most of the above morphometric studies of *g* ratio were carried out in peripheral nerves, the most recent and comprehensive study of *g* ratio was carried out in the optic nerve, which is a part of the central nervous system and whose myelination is generated by oligodendrocytes (Chatzopoulou et al., 2008). As mentioned above, a subtle shift

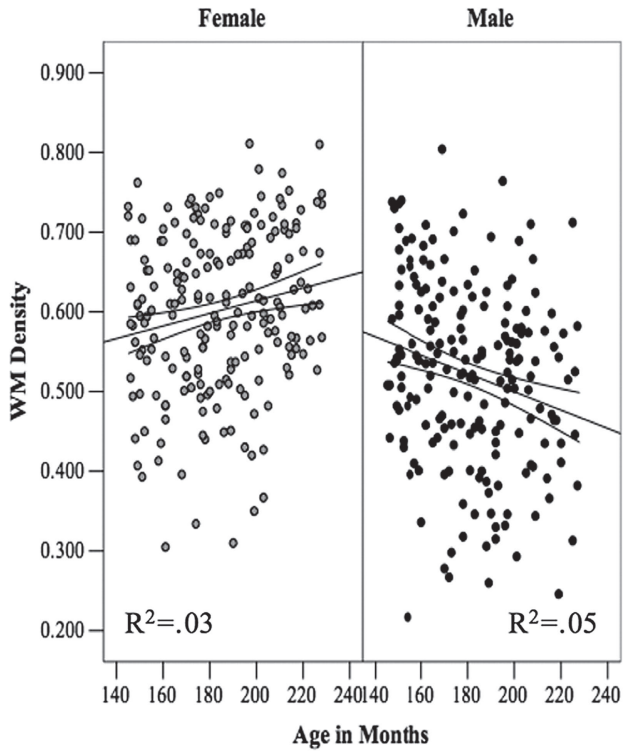

FIGURE 3 | The relationship between Age and white-matter (WM) density in the left cortico-spinal tract in female and male adolescents. The lines represent the regression equation with the 95% confidence intervals. Reprinted from Perrin et al. (2009).

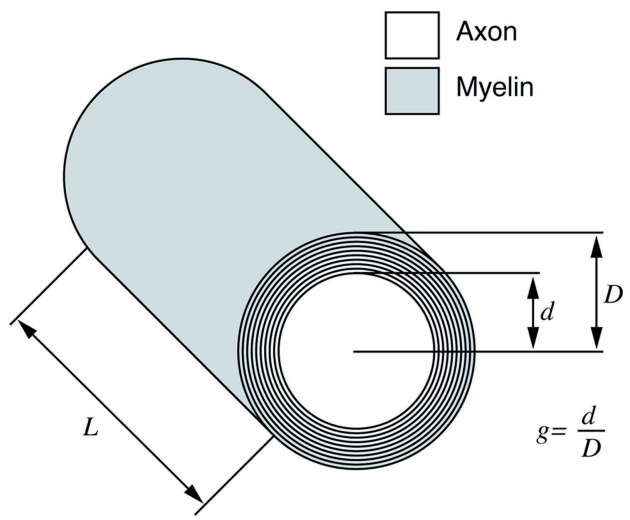

FIGURE 4 | Schematic representation of an axon and its myelin sheath illustrating *g* ratio.

in the *g* ratio could change the proportion of tissue occupied by axons and myelin, respectively.

G RATIO AND CONDUCTION VELOCITY

By assuming that geometrically similar fibres should possess similar membrane properties, Rushton (1951) derived a formula showing

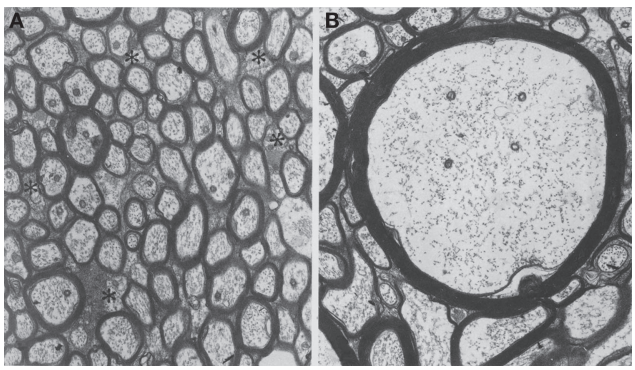

FIGURE 5 | Electron micrograms of axons in the monkey corpus callosum. Note wide variations in the axonal diameter and myelin thickness. **(A)** Asterisks indicate small unmyelinated axons. **(B)** A large myelinated axon in the midbody of the corpus callosum. Same magnification (8,500×) for both micrographs. Reprinted from LaMantia and Rakic (1990).

that, for a constant *g* ratio, the conduction velocity *v* and fibre length *l* should be proportional to the fibre diameter *D*:

$$v \propto l \propto Dg\sqrt{-\log(g)}$$

where $g = d/D$.

Rushton observed that this expression has a maximum for $g = e^{-1/2} \approx 0.6$. A fibre with a *g* ratio of 0.6 will have the maximum conduction velocity for its length. Note that the theory applies to a generic (peripheral and central) myelinated axon. The optimal *g*-ratio was obtained from purely theoretical considerations, namely by maximising conduction velocity in the equation. In **Figure 6**, we plot the variation of fibre length as a function of fibre diameter and axonal diameter. The continuous line shows the values of *D* and *d* that, according to Rushton, have the "optimal" *g* ratio of 0.6 thus maximising conduction velocity.

Rushton noticed, however, that (peripheral) fibres of large diameter have also a larger *g* ratio, that is, they have a relatively thinner myelin sheath. In a later work, Berthold et al. (1983) presented an analysis of the variability and development of myelination in the peripheral nervous system of the cat. Again, they observed that axonal diameter plotted against fibre diameter produced a non-linear curve, where the larger fibres have also increasingly larger axons. A similar observation has been made recently by Chatzopoulou in the central nervous system, namely in the mouse optic nerve (Chatzopoulou et al., 2008; **Figure 7**).

Interestingly, when the cross-sectional area of the myelin sheath was plotted against axonal diameter, the relationship became linear, as if the volume of the myelin sheath depended on the surface of the axonal fibre (Berthold et al., 1983). Based on this observation, here we model this relationship between axonal diameter and fibre diameter as

$$d = B\frac{\pi}{4}(D^2 - d^2)$$

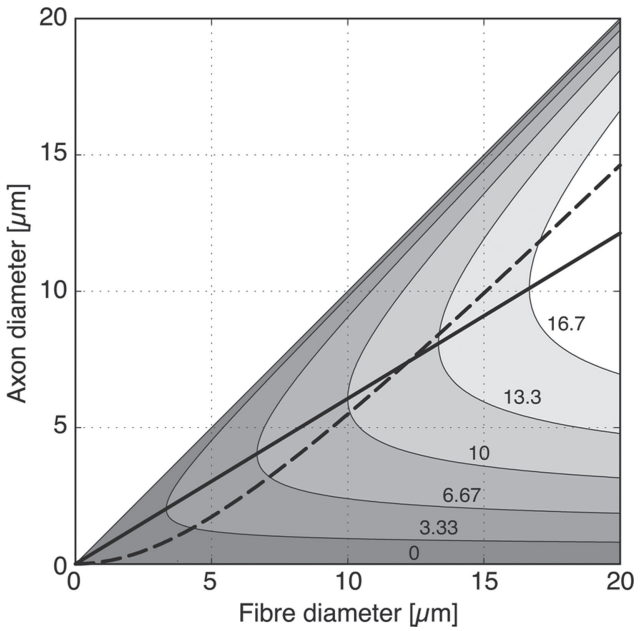

FIGURE 6 | Relationship between axon diameter and fibre diameter (axon diameter + thickness of the myelin sheath) in fibres of different length. After Rushton (1951), conduction velocity is maximum when the proportion of axon diameter to fibre diameter, i.e. *g* ratio, is close to 0.6 (solid line). If the axon diameter is taken to be proportional to the *cross-section* of the myelin sheath, the relationship between axon diameter and fibre diameter is given by the discontinuous curve. Note that *g* ratio expressed in the latter way is higher than 0.6 in large-diameter fibres, which would be more typical for longer (>13 cm) fibres. Grey levels: fibre length from 0 to 20 cm.

where B is a constant of proportionality. Solving the quadratic equation for d we obtain two roots, only one giving positive diameters:

$$d = \frac{1}{2B}\left(\sqrt{(1+4D^2B^2)} - 1\right)$$

We estimated the value of Bertehold's proportionality constant to be $B = 0.1\ \mu m^{-1}$, from the slope of the scatter plots in Figures 7d and 7e in Berthold et al. (1983). The discontinuous curve in **Figure 6** shows the values of D and d for which the axonal diameter is proportional to the myelin cross-section. This curve crosses the line showing the optimal *g* ratio when the fibres have a diameter of

$$D = \frac{\sqrt{e}}{B(e-1)}$$

for which the axonal diameter is

$$d = \frac{1}{B(e-1)}$$

For our estimated value of B, this indicates that fibres with a diameter larger than 9.6 μm will have a relatively thinner myelin sheath; and brains with increasingly larger proportions of such large diameter fibres will have progressively lower concentration of myelin.

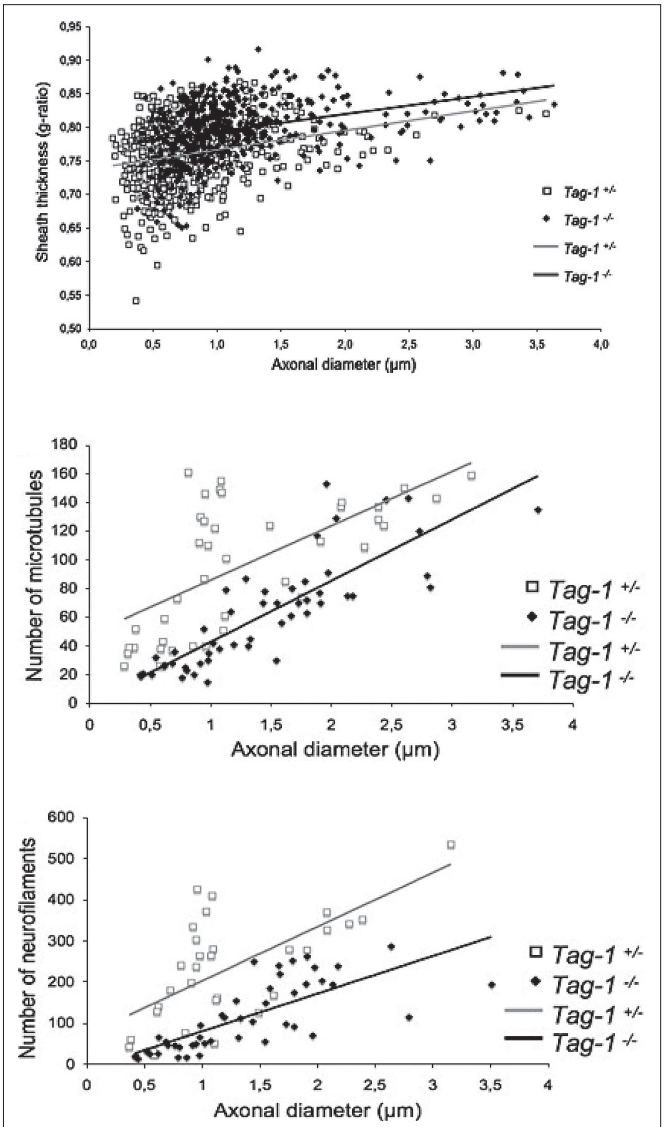

FIGURE 7 | *G* ratio (top), number of microtubules (middle) and number of neurofilaments (bottom) according to axonal diameter in the optic nerve of adult Tag-1$^{+/-}$ and Tag-1$^{-/-}$ mice (TAG-1 is a cell-adhesion molecule). Reprinted from Chatzopoulou et al. (2008).

AXON AND ITS CYTOSKELETON

What determines axonal diameter? Axon consists mainly of neurofilaments (NF) and microtubules (MT), the former outnumbering the latter 5–10 times (Lee and Cleveland, 1996). Neurofilaments support the cylindrical structure of an axon and, as such, protect the core from compressive stress, securing its unobstructed state (Kumar et al., 2002). Axonal diameter is influenced both by the number of NF and their spacing (Hoffman et al., 1984, 1987; Kumar et al., 2002). The former is regulated by NF synthesis (gene expression) and the amount of NF undergoing (slow) axonal transport (Hoffman et al., 1987, 1984); not surprisingly, the number of NF, but also that of MT, varies as a function of axon diameter (Chatzopoulou et al., 2008; **Figure 7**). The latter depends on the phosphorylation of NF sidearms (e.g. Garcia et al.,

2003; Kumar et al., 2002). This process appears to be regulated by a protein synthesized by oligodendrocytes, namely myelin-associated glycoprotein; this "outside-in" signalling pathway provides a cellular mechanism for the coupling between myelination and axonal calibre (Yin et al., 1998; Garcia et al., 2003). Theoretically, sex differences in *g* ratio could emerge either through differences in the rate of synthesis and/or axonal transport of NF, or the rate of phosphorylation of the NF sidearms.

CONCLUSIONS AND IMPLICATIONS

The initial work from our laboratory has suggested a disproportionate growth of axon and myelin sheath, respectively, during male adolescence (Perrin et al., 2008). This phenomenon turned our attention to possible sex differences in *g* ratio. Both the experimental work reviewed above and the model predict lower concentration of myelin in WM regions occupied by large-diameter fibres; we have observed, indirectly, this phenomenon in the CST at the level of internal capsule (Herve et al., 2009). It is likely that the male brain contains, overall, a larger proportion of large-diameter fibres. What might be the neurobiology underlying such a sex difference?

As pointed out above, axonal diameter depends primarily on the number of NF and their configuration. Although the number of MT also increases with the axonal diameter, directionality of this relationship is not clear. The link between testosterone and cellular processes relevant vis-à-vis cytoskeleton are rather tentative at present. Initial *in vitro* studies suggest that testosterone up-regulates α- and β-tubulin, the building blocks of MT (Butler et al., 2001). Furthermore, neuropathy is one of the key symptoms of Kennedy's disease (or X-linked spinal and bulbar muscular atrophy), a condition caused by an expanded polyglutamine (polyQ) stretch in the androgen receptor (Brooks and Fischbeck, 1995; Hsiao et al., 1999; La Spada et al., 1991). This effect might be mediated by a PolyQ-AR induced inhibition of kinesin-mediated axonal transport (Morfini et al., 2006) or a decrease in the expression of dynactin 1, a motor for retrograde axonal transport (Katsuno et al., 2006); slow axonal transport is essential for moving elements of cytoskeleton (Barry et al., 2007).

Is the rate of axonal transport different in small- and large-diameter fibres? This appears to be the case. Murayama et al. (2006) used MRI to measured the rate of *in vivo* transport of Mn^{++} ions from the eye to the lateral geniculate nucleus of the monkey and found that a faster transport in the magno-cellular that parvocellular pathway. The Mn^{++} transport depends, at least in part, on kinesin-based processes underlying (active) axonal transport (Bearer et al., 2007). In general, axonal cytoskeleton (i.e. NF and MT) and motor proteins are essential contributors to a large number of cellular processes, such as cell metabolism (e.g. transport of mitochondria and glycolytic enzymes) and neurotransmission (e.g. transport of synaptic-vesicle precursors), as well as growth and cell survival (e.g. retro-grade transport of growth factors). Thus, any sex differences in axonal transport may have a number of downstream effects.

Finally, the model presented above suggests that the *g* ratio will increase in large fibres beyond the optimal value of 0.6. We speculate that the presumed testosterone-induced changes in axonal diameter during male adolescence increase the probability of suboptimal *g* ratio in large-diameter fibres and, in turn, decrease conduction velocity in these fibres. Such a disturbance may interfere with the timing of inter-regional communication and contribute to the emergence of a "disconnection" syndrome hypothesized to underlie disorders such as schizophrenia (Friston and Frith, 1995; Goldberg et al., 1997).

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Myelination and isochronicity in neural networks

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Our brain contains a multiplicity of neuronal networks. In many of these, information sent from presynaptic neurons travels through a variety of pathways of different distances, yet arrives at the postsynaptic cells at the same time. Such isochronicity is achieved either by changes in the conduction velocity of axons or by lengthening the axonal path to compensate for fast conduction. To regulate the conduction velocity, a change in the extent of myelination has recently been proposed in thalamocortical and other pathways. This is in addition to a change in the axonal diameter, a previously identified, more accepted mechanism. Thus, myelination is not a simple means of insulation or acceleration of impulse conduction, but it is rather an exquisite way of actively regulating the timing of communication among various neuronal connections with different length.

Keywords: isochronicity, thalamocortical pathway, conduction velocity, axon diameter

INTRODUCTION

The timing when a neuron receives its incoming input has a great influence on how the input is processed and how it affects the postsynaptic neurons. On the other hand, an increase in the body size and the amount of information processed, as a result of evolution, inevitably required the expansion of the brain (Finarelli and Flynn, 2009; Jerison, 1955, 1961, 1973; Laughlin and Sejnowski, 2003). This resulted in situations where the excitation of a presynaptic neuron needs to arrive within a fixed window of time at target neurons located at multiple remote sites at variable distances. Such apparently paradoxical transmissions, in fact, take place in various regions of the brain. Some representative examples of such isochronicity can be observed in olivocerebellar connections (Lang and Rosenbluth, 2003; Sugihara et al., 1993), transcallosal connections in the visual cortex (Innocenti, 1995, 2009; Innocenti et al., 1994), amygdalocortical pathways (Pelletier and Pare, 2002), and still others that will be discussed later on. Intriguingly enough, these connections do not necessarily adopt the same strategy to accomplish isochronicity. Recently, we found that the thalamocortical pathway also exhibits the isochronic property, but with a novel mechanism, which involves differential myelination along the axon (Salami et al., 2003). Apparently this is an exquisite way of producing isochronicity and may apply to other systems. In this brief review, we will first survey how this is achieved in the thalamocortical pathway, and then discuss other isochronic pathways for comparison.

ISOCHRONICITY IN THE THALAMOCORTICAL PATHWAY

Neurons in the thalamus send axons to a wide area of the somatosensory cortex through different trajectories of various travelling lengths. Nevertheless, we reported that action potentials in the thalamic cells arrive almost simultaneously at each target cortical cell in layer IV. By using thalamocortical slices in which connection from thalamus to cortex were kept intact, EPSPs were recorded from layer IV cells in response to the stimulation of multiple sites such as thalamus, white matter (WM), and intracortical sites between WM and layer IV. This allowed us to measure the conduction velocity of each part of the axon while action potentials were travelling down the thalamocortical fibers. We found that the isochronicity in this system is achieved by changing the conduction velocity (CV) within the individual axons. The CV decreases significantly upon entering the gray matter by up to 10-fold (Salami et al., 2003). Originating from the thalamus, the axons of relay cells run in a straight path through the striatum up to the WM, at which point each axon diverges widely. Some ascend directly into the cortex up to layer IV, while others run in the subcortical WM for variable distances before ascending into the cortex. Although the total distances travelled vary across pathways, the distance of the intracortical regions is almost the same. That is, all the projections target the same layer IV in a cortex that has uniform thickness. The total conduction time primarily depends on the time spent within the cortex (gray matter). Thus, the strategy of making the CV of longer and variable parts by far faster than that of shorter and constant parts is a way of eliminating the variability in traversed distances (**Figure 1**).

Myelination is involved in this process in two-fold. First, since CV is proportional to the square root of the axon diameter in unmyelinated axons, to have 10 times larger CV, axon diameter needs to be 100 times larger, which is most unlikely. This led us to predict that thalamocortical fibers would be myelinated. Thus, secondly, we suspected that the extent of myelin might cause the observed difference of CV. Histological staining of the myelin revealed that the difference of myelination between the intracortical and extracortical (WM) regions played an important role in the generation of CV difference. Developmentally, CV difference could be seen after the end of the 2nd postnatal week. At this time there is practically no myelination observed in the grey matter, while thalamus to white matter portions are already heavily myelinated (Salami et al., 2003). It appeared that such differential myelination continued in

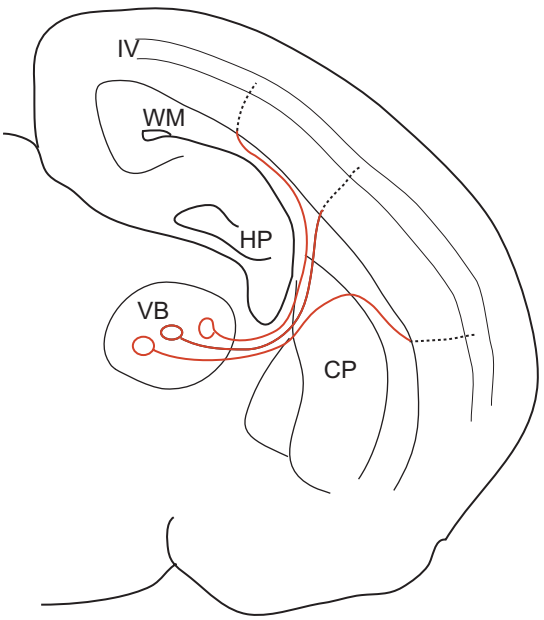

FIGURE 1 | A schematic illustration of the thalamocortical pathway, showing regional differences in CV. By having a 10-fold faster CV from the VB to the WM (red lines), most of the conduction time is spent on the intracortical regions (dotted lines), whose length is generally constant due to the homogeneous structure of the cortex. Thus, isochronicity of conduction time from VB to layer IV cells is achieved. VB: ventrobasal nucleus of thalamus, WM: white matter, HP: hippocampus, CP: caudate putamen, IV: layer 4 [Cited from Salami et al., PNAS 100, 6174–6179 (2003)].

the adulthood. Since this was only a light microscopic observation, EM-level confirmation remains to be done, however.

This intra-axonal, segmental difference is well-suited to influence the difference in CV. Thus, we hypothesized that difference of myelination plays a major role in creating isochronicity. In other words, myelination is not merely insulation among neighboring cells, but is a method of regulating the timing of postsynaptic activations. A change in the CV along a given axon has been reported in other regions of the brain. The axons of retinal ganglion cells increase their CV from the optic nerve to the optic tract at the point of the optic chiasm, by increasing both their diameter and myelination (Baker and Stryker, 1990). Similarly in the peripheral nervous system, Aδ fibers supplying mechanoreceptors exhibit serious reduction in the CV upon entering the spinal cord (Traub and Mendell, 1988). In addition to sensory neurons, motoneurons of the ventral horn increase their sheath thickness and axon diameter upon exiting the spinal cord (Fraher, 1976, 1978). Our results indicate that these changes in CV and the associated anatomical changes may well play a role in the regulation of activation timing.

ISOCHRONICITY IN OTHER REGIONS IN THE BRAIN

Isochronicity has been reported in other regions of the brain. In rats, axons of the inferior olive innervate widely distributed regions of the cerebellar cortex. The conduction time to the different parts of the cerebellum is relatively invariant despite differences in path length. The isochronicity in the olivocerebellar projection is the result of a differential CV. An earlier study has shown that longer fibers conduct faster than shorter ones due to differential axon diameters rather than differential myelination (Sugihara et al., 1993). A recent study challenged this view, claiming that myelination plays a primary role in generating a uniform olivocerebellar conduction time (Lang and Rosenbluth, 2003). Interestingly, it has been questioned whether this invariance holds for larger animals, such as cats (Aggelopoulos et al., 1995), although even in this study the latency of olivocerebellar projections varied only by several milliseconds.

Isochronic property of transcallosal connections is one of the most intensively studied within the framework of the computational properties of axons by Innocenti and his group (Innocenti, 1995, 2009). Based on the simulation of action potential propagation determined by axonal diameter, these authors provided a detailed description of strategies for callosal connections to achieve synchronous activation of their targets (Innocenti et al., 1994).

In the cortex, layer V pyramidal neurons project to various subcortical regions as well as to the contralateral side. One recent study showed that layer V pyramidal neurons in the ventral temporal lobe innervate diverse regions such as the caudate putamen, parietal cortex, amygdala, and thalamic nuclei on the ipsilateral side with isochronic spike delivery based on the differential CVs in each fiber branch (Chomiak et al., 2008). By combining the actual measurement of the axonal inner and outer diameters, with theoretical predictions based on partial myelination, the observation of Chomiak et al. on isochronicity appears to be best supported by partial or differential myelination. It may be worth mentioning that the same neurons send axons to the contralateral side through the corpus callosum, but the CVs were significantly slow and not isochronic with ipsilateral connections.

The lateral amygdala is believed to play an important role in establishing fear memory in cooperation with the perirhinal cortex through Hebbian synaptic interactions, where the timing of synaptic input is important. The perirhinal cortex is an elongated structure as compared to the rather small nucleus of the lateral amygdala. Electrophysiological experiments have revealed that the lateral amygdala is isochronically connected with a large portion of perirhinal cortex (Pelletier and Pare, 2002), but the mechanism of this isochronic connection is yet to be elucidated.

ISOCHRONICITY IN THE DEVELOPING BRAIN

Although brain and body sizes increase during development, the conduction times of certain connections remain constant. One study in humans has revealed that the somatosensory and motor conduction times remain constant even after 2 years of age, despite substantial increases in the axon length that occur with body growth (Eyre et al., 1991). Experiments in animals investigated the rubrospinal tract in cats (Song et al., 1995), and revealed that the conduction time from the red nucleus to L1 reached the minimum adult level at P30, when the spinal cord is only half the adult length. CV increased linearly from E59 to P30 (from 1–34 m/s). Since myelination of the rubrospinal tract was believed to have begun before E59, the adult level conduction time was achieved mainly by myelination until P30, then thereafter, axon diameter was increased so as to keep the constant conduction time as the axon length increased. Thus, these authors proposed that compensatory interaction of

myelination and axon diameter led to the isochronic conduction during development.

In other systems, however, myelination is unlikely to be involved in isochronicity during development. The locus coeruleus (LC) in the dorsal pons sends axons widely throughout the brain including the frontal cortex, but latencies remain constant throughout life (Nakamura et al., 1987), even when the size of the brain increases to more than 2 times its original size. Since axons of the LC consist of C fibers lacking myelin, mechanism(s) other than myelination should account for the associated increase in the CV.

ISOCHRONICITY AND ACTIVITY-DEPENDENT MYELINATION

One implication from these studies is that each pathway has its own conduction time, which might be determined by its function. Myelination, axon diameter, and other factors such as the structure, or the number of spacing of Ranvier nodes, and ion channel composition and/or its density, all of these would affect the CV, and conduction time may be employed to achieve its characteristic value. On the other hand, since the environment around a given neuron, or tissue, or whole individual continuously changes, a regulatory mechanism that changes in an input- or activity-dependent manner is desirable to adapt to the changing environment.

Myelination indeed changes in an experience- or input-dependent manner. In dark-reared mice, the number of myelinated axons in the optic nerve decreased (Gyllensten and Malmfors, 1963). Similarly, premature eye opening in rabbit reduced the expression of myelin (Tauber et al., 1980). A diffusion tensor imaging study revealed that extensive piano practicing in childhood results in a thicker white matter, which is believed to be due to a change in myelination (Bengtsson et al., 2005). Several lines of evidence have identified how action potentials regulate myelination. A specific pattern of neural activity was shown to lower the expression of L1-CAM (Itoh et al., 1995) that is necessary for myelin induction by oligodendrocytes (Barbin et al., 2004), as well as by Schwann cells (Stevens et al., 1998). In addition, ATP released from axon terminals as a result of neural activities facilitates the differentiation of oligodendrocytes through adenosine and P1 receptors. For astrocytes, ATP causes the release of leukemia inhibitory factor (LIF), which then stimulates myelination by oligodendrocytes (Ishibashi et al., 2006). A classical view on myelin thickness is that the thickness of myelin sheath is closely related with the axon diameter. Theoretically, optimal or fastest CV is provided when *g*-ratio is 0.65, where *g*-ratio is defined as the axon diameter divided by total fiber diameter. Although it is generally true that axons conforming to this *g*-ratio is widely observed, it is also true that there are still others with large variation in *g*-ratio around the mean, and considerable differences are seen between fibers (Berthold et al., 1983). In fact, optic nerve axons have uniform diameters, but exhibits five different CVs. These are ascribed to as many differences in myelination (Freeman, 1978). Similarly, as mentioned before, multiple CVs along the same fiber were reported from other studies (Baker and Stryker, 1990; Traub and Mendell, 1988). If as we discussed earlier, each pathway has its own conduction time, and if myelination changes in an input-dependent manner to adapt to environment, it seems reasonable to propose that the difference of the extent of myelination may be the result of adaptation whereby myelination influences its own conduction time.

FUNCTIONAL SIGNIFICANCE OF ISOCHRONICITY

What is the functional significance of such isochronicity of action potential conduction? The issue of isochronicity has actually a long history and has been demonstrated also in invertebrates. Among the oldest of these examples are the classical studies on the squid giant axons. Conduction time of the giant axons between the stellate ganglia and the mantle musculature is such that activation of the whole mantle takes place synchronously, allowing effective escape to occur. This is achieved by faster CV for longer axons, by changing axon diameters (Young, 1939) exclusively. That is because invertebrates do not have myelination with which the CV could be enormously (~100 times) increased. In other words, vertebrates are endowed with an additional mechanism in regulating CV. It appears quite reasonable to think that vertebrates would exploit these strategies independently. In electric organs of a certain kind of fish, synchronous activation of electrocytes to produce larger electrical potentials is attained by two mechanisms; first, same as squid axon, faster CV for longer pathways, and secondly, longer circuitous route for cells closer to the target (Bennett, 1970). The avian auditory system is another similar example where circuitous routes are used to compensate for shorter distance. The conduction time between the cochlear nucleus and its ipsi- and contralateral nucleus laminaris is matched by lengthening the path to the ipsilateral nucleus, to create a delay line for detecting the differential timing of sound inputs to both ears for sound localization. In these cases, the advantage of the isochronous conduction is obvious.

By analogy, in those connections we have discussed above, such as olivocerebellar, amygdalo-perirhinal, and corticofugal from ventral temporal cortex, isochronous activations of target cells should have clear temporal advantages. However, at this point, how isochronicity is advantageous does not seem obvious in these systems. One interesting view is that the olivocerebellar system serves as an intrinsic timing device that is essential for motor co-ordination (Llinas, 1988). This view is based on the observation that neurons in the inferior olive are connected by gap junctions and thus exhibit oscillatory activity. This is supposed to propagate to a wide territory of the cerebellar cortex, a process for which isochronicity is essential.

A similar situation might apply to the thalamocortical pathway. Thalamic cells also show oscillatory activities intrinsically (Contreras et al., 1996) that could cause synchronization in the functionally related target cortical cells. Isochronous or synchronous activation of group of cells distributed in distant cortical locations with zero phase-lag have been shown in visual cortex (Gray et al., 1989), and callosally connected areas (Engel et al., 1991). These findings attracted attention as relevant to the problem of connecting features responsible for object recognition in the spatially fractured nature of sensory representation over the cortical mantle (Llinas et al., 2002; Singer, 1999) and providing the perception of unity (von der Malsburg and Buhmann, 1992). For such binding to work correctly, isochronous activation of related cells in the global cortical area would be a necessary condition.

Isochronous activation of thalamocortical as well as transcallosal connections provides a physiological and anatomical explanation for these observations. Synchronous activities may also be appropriate for producing synfire chains (Abeles, 1991). Another possible explanation for the resultant isochronicity might be, as we discussed before, that each connection has its own characteristic conduction time for information to be correctly processed in the network. Under such conditions, if postsynaptic target neurons are widely distributed, isochronous conduction will result. This is in some sense consistent with isochronicity in the developing brain as we described earlier. During development, once a pathway with a characteristic conduction time becomes functional, the specific conduction time presumably needs to be kept constant in spite of the increasing distances subsequent to growth of the body. The corpus callosum displays a significant variety in terms of myelination (Aboitiz et al., 1992). In rats, the fraction of myelinated fibers is zeroe at birth, then it gradually increases to 53% at 300 days post-conception (Seggie and Berry, 1972). Similarly in humans, none of the callosal fibers are myelinated at birth, and in adults 30% of the fibers remain unmyelinated. In addition, analyses of fiber composition revealed a wide variety of fiber diameters and extent of myelination depending on the target area. Callosal regions connecting prefrontal and temporoparietal association areas consist of small caliber with low myelinated fibers, whereas regions connecting primary and secondary sensorimotor areas include highly myelinated, large-caliber fibers (Aboitiz et al., 2003). Consequently, the conduction time between two hemispheres varies from 30 ms via myelinated axons to as long as 300 ms via unmyelinated ones (Fields, 2008). Since callosal fibers connect a variety of cortical regions with various functions, conduction times for each functions are likely to be diverse. The extent of myelination, as well as axon diameter might help regulate the conduction time to its optimal value for communicating between hemispheres. This leads to another interesting question; namely, how the conduction time of each pathway is determined in the network. Overall, our understanding regarding the time devoted to each step in neural processing is still, unfortunately, severely limited.

CONCLUSION

Isochronic propagation of information in spite of variable neuronal distances eliminates the limitation of connectional distances, and is permissive for the expansion of the brain. This phenomenon is seen widely in various brain regions, and at several phylogenetic levels, as well as during development. Isochronicity is achieved mainly by differential CVs, either within individual axons or among axons. It is suggested that the vertebrates obtained another option in generating differential CVs, that is, changing myelination, in addition to regulating axon diameters.

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Linking white and grey matter in schizophrenia: oligodendrocyte and neuron pathology in the prefrontal cortex

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Neuronal circuitry relies to a large extent on the presence of functional myelin produced in the brain by oligodendrocytes. Schizophrenia has been proposed to arise partly from altered brain connectivity. Brain imaging and neuropathologic studies have revealed changes in white matter and reduction in myelin content in patients with schizophrenia. In particular, alterations in the directionality and alignment of axons have been documented in schizophrenia. Moreover, the expression levels of several myelin-related genes are decreased in postmortem brains obtained from patients with schizophrenia. These findings have led to the formulation of the oligodendrocyte/myelin dysfunction hypothesis of schizophrenia. In this review, we present a brief overview of the neuropathologic findings obtained on white matter and oligodendrocyte status observed in schizophrenia patients, and relate these changes to the processes of brain maturation and myelination. We also review recent data on oligodendrocyte/myelin genes, and present some recent mouse models of myelin deficiencies. The use of transgenic and mutant animal models offers a unique opportunity to analyze oligodendrocyte and neuronal changes that may have a clinical impact. Lastly, we present some recent morphological findings supporting possible causal involvement of white and grey matter abnormalities, in the aim of determining the morphologic characteristics of the circuits whose alteration leads to the cortical dysfunction that possibly underlies the pathogenesis of schizophrenia.

Keywords: myelin, myelin-related genes, development, anterior cingulate cortex, cingulum bundle

WHITE MATTER AND COGNITIVE FUNCTION

The role of white matter in neural circuit integrity may be appreciated in terms of supporting neural functioning. Most neurons in the brain necessitate adequate myelination of their axons in order to maintain functional processing at all levels of neural systems, from autonomic processes and sensorimotor integration, to mood and thought. The importance of myelination for cognitive functioning becomes apparent in diseases that are known to be caused or affected by deficiencies in myelin, where patients show deficits in intellectual, social and emotional functioning (Dwork et al., 2007; Schmahmann et al., 2008). Leukodystrophies and leukoencephalopathies, diseases characterized by progressive degeneration of the white matter, if diagnosed in late adolescence or early adulthood can present with psychotic symptoms sometimes indistinguishable from those of schizophrenia (Davis et al., 2003; Denier et al., 2007; Walterfang et al., 2005). Likewise, patients with multiple sclerosis who display cognitive and psychiatric symptoms frequently have white matter lesions in the frontal and temporal lobes, which are the brain regions most implicated in schizophrenia (Davis et al., 2003).

Schizophrenia is a severe psychiatric illness that affects close to 1% of the population worldwide. The diagnosis is generally established at first onset of the symptoms, which occurs in most cases in early adulthood. The disease is characterized by a number of mental abnormalities that result in a distortion of perception and expression of reality. There are prominent sensory symptoms, most frequently taking the form of auditory and visual hallucinations, although such sensations can affect any sensory modality. In addition to hallucinations, the patients may experience paranoid delusions, present with disorganized thoughts and speech, and a variable degree of social and occupational dysfunction. There is a considerable degree of inheritability of the disease and prenatal causes, such as insult to the brain during embryonic development, have also been considered to play a key role in the expression of the disease at a later time (see Fallon et al., 2003).

Schizophrenia has been shown to exhibit myelin deficiencies and changes in white matter volume in the brain (Davis et al., 2003; Dwork et al., 2007; Karoutzou et al., 2008; Segal et al., 2007b; Sokolov, 2007; Walterfang et al., 2006). The myelin hypothesis in schizophrenia was first presented by Hakak et al. (2001) after their pivotal finding of altered expression of myelin-related genes in human postmortem tissue (Hakak et al., 2001). Myelin-related gene expression levels have matched the observations made on white matter abnormalities by diffusion tensor imaging (DTI), and were later confirmed in several other studies. Since the first suggestions of a myelin-related pathophysiology underlying schizophrenia, there have been numerous and extensive reports and reviews on the myelin hypothesis (Davis et al., 2003; Dwork et al., 2007; Karoutzou et al., 2008; Segal et al., 2007b; Sokolov, 2007; Walterfang et al., 2006).

Substantial deficits in myelination occur in schizophrenia, which is interesting to consider in light of previous hypotheses that the disease results from abnormal brain development (Lewis and Levitt,

2002; Rapoport et al., 2005; Weinberger, 1986, 1987) and altered neuronal circuitry (Selemon and Goldman-Rakic, 1999), particularly in the prefrontal cortex (PFC). Neuropathologic findings in both white matter and grey matter suggest that myelin alterations in the anterior cingulate cortex (ACC) may underlie some of the behavioral deficits related to prefrontal dysfunction. We discuss some of the white matter findings and relate these to grey matter pathologies in schizophrenia, elucidating a possible impact that white matter abnormalities have on neuronal morphology and function.

IMAGING AND NEUROPATHOLOGIC FINDINGS IN SCHIZOPHRENIA

IMAGING STUDIES

Fractional anisotropy (FA), a measure of the directionality of water movement within the spaces in-between axons, provides an indication of white matter tract directionality and, by measuring the strength of the direction vector of water diffusion, possibly of tract integrity or coherence. A major advantage of this approach is that it can be used to study changes in schizophrenia *in vivo*, allowing investigation of different stages of the disease. *In vivo* DTI studies have revealed decreased FA in several major white matter tracts in schizophrenia (Buchsbaum et al., 1998, 2006; Kubicki et al., 2007; Lim et al., 1999; Shergill et al., 2007), including the cingulum (Kubicki et al., 2003; Wang et al., 2004). In addition, positron emission tomography (PET) imaging has demonstrated increased relative metabolic rates in white matter in schizophrenia, which may represent white matter inefficiency or defects resulting in increased metabolic needs (Buchsbaum et al., 2007), in contrast to findings in the grey matter which have shown decreases in regional cerebral blood flow in the ACC (Tamminga et al., 1992).

Although previous DTI studies have shown decreases in FA in the cingulum bundle as well as in the overlying cingulate gyrus in patients with schizophrenia (Fujiwara et al., 2007; Kubicki et al., 2003; Kumra et al., 2005; Sun et al., 2003; Wang et al., 2004; White et al., 2008), the findings have been somewhat inconsistent, due in large part to small subject samples and different methods of identifying particular brain regions of interest (Kubicki et al., 2007). Segal and collaborators recently investigated the volume and FA in the cingulate gyrus in a large group of subjects with chronic schizophrenia along with a group of patients with recent-onset schizophrenia and healthy control subjects matched for age and sex (Segal, 2008; Segal et al., 2007a). The anterior cingulate gyrus was traced and segmented into axial portions allowing detection of localized changes. Volume was calculated for the anterior cingulate gyrus, and average FA values were calculated for each segment looking separately at grey and white matter. A significant decrease in the overall grey matter volume was found in the anterior cingulate gyrus in persons with schizophrenia. In both grey and white matter, persons with recent-onset schizophrenia had the highest FA in several regions, and persons with chronic schizophrenia had the lowest (**Figure 1**). These results demonstrate both white and grey matter abnormalities in the cingulate gyrus in schizophrenia (Segal, 2008), which may reflect abnormalities in neuron spacing or columnar organization.

MRI studies of the grey matter have revealed regionally reduced cortical volumes in schizophrenia (Honea et al., 2005, 2008; Nesvag et al., 2008; Okugawa et al., 2007), including the ACC (Baiano et al., 2007; Wang et al., 2007). Magnetic transfer imaging (MTI) assessment of macromolecular structural integrity enables separate analysis of white and grey matter, which may help to elucidate early neuropathological changes. Foong et al. (2001) found MTI changes in the grey matter of frontal and temporal areas, while white matter abnormalities were observed only in temporal areas.

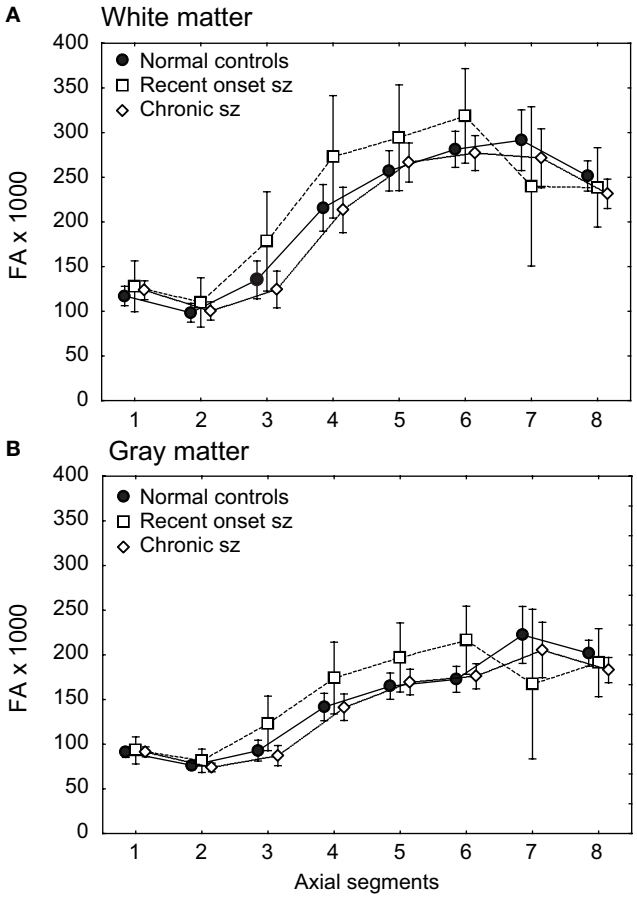

FIGURE 1 | Human DTI along rostrocaudal axial segments of the entire cingulate gyrus, spanning from area 32 (segment 1) to area 23 (segment 8). (A) FA values showing a significant region × diagnosis effect in the cingulate white matter, with patients with recent-onset schizophrenia displaying the highest FA in most segments, followed by control subjects and then patients with chronic schizophrenia (ANCOVA $F_{14,567} = 2.42$, $p = 0.003$). **(B)** The same pattern was noted in cingulate grey matter, also with a significant region × diagnosis effect (ANCOVA $F_{14,567} = 3.01$, $p < 0.001$). Vertical bars indicate 95% confidence intervals and values are computed for the age covariate at its mean. Normal controls ($n = 38$), recent-onset schizophrenia ($n = 6$), chronic schizophrenia ($n = 41$); sz, schizophrenia; FA, fractional anisotropy.

OLIGODENDROCYTE AND MYELIN STUDIES

In attempts to localize and identify a cellular correlate of the white matter changes observed by brain imaging *in vivo*, oligodendrocytes have come to be an important focus of investigation. Analyses of the number, densities and distribution patterns of oligodendrocytes can be performed in both the white and grey matter. Stark et al. (2004) found decreased oligodendrocyte densities in cingulate area 24 but not in the adjacent paracingulate area 32, and Hof et al. (2003) found

decreased densities of oligodendrocytes in the prefrontal area 9 of the superior frontal gyrus in subjects with schizophrenia. In contrast, in a subsequent study, Hof and coworkers evaluated the degree of oligodendrocyte clustering in the anterior cingulum bundle, but found no differences using postmortem tissue from chronic schizophrenics versus age-matched controls (Segal et al., 2009). These results suggest that more subtle oligodendrocyte or myelin anomalies may underlie the structural deficits observed by brain imaging in the cingulum bundle in schizophrenia. On the ultrastructural level, electron microscopy studies of oligodendrocytes in the PFC have demonstrated apoptotic oligodendrocytes, irregularities of mitochondria in oligodendrocytes and damaged myelin in area 10 in schizophrenic brains (Uranova et al., 2001, 2004).

GREY MATTER AND NEURON STUDIES

Postmortem studies, assessing the gyrification index, have found reductions in cortical folding in schizophrenia (Kulynych et al., 1997). Studies on changes in neuronal densities in different cortical regions in schizophrenia have been conflicting, and no definite pattern of neuronal density alterations has yet been established. These differences in observation may in part be due to differences in methodological approaches and procedures and the cortical regions studied. Some postmortem studies of the ACC and dorsolateral PFC (DLPFC) have suggested a decrease in neuronal density. Benes reported a lower neuronal density (mainly in layer II) in areas 24 and 10, primarily of small interneurons (Benes et al., 1991), which suggested an alteration in intrinsic neuronal circuits (Benes, 2000). Other investigators have shown increased neuronal density in areas 9 and 46, without increased absolute numbers of neurons in patients with schizophrenia (Selemon et al., 1995, 1998). This implied that cortical volume in select cortical regions is reduced in schizophrenia, possibly because of reduced neuropil. Goldman-Rakic and coworkers proposed that an altered brain connectivity plays a critical role in the development of schizophrenia (Selemon and Goldman-Rakic, 1999). In other studies on subcortical regions, observations have been made of reductions in the size and total neuron numbers, but not in neuronal density, in the putamen and the amygdala (Kreczmanski et al., 2007).

Cytoarchitectural studies have analyzed neuronal arrangements in terms of interneuronal distances, or mean cell spacing (Casanova et al., 2005, 2008) showing that mean cell spacing was reduced in area 9 in schizophrenic patients, which would imply a higher neuronal density. Rajkowska and coworkers found that in area 9, there was a downward shift in neuronal sizes, accompanied by increases in the density of "small neurons" in layer II, interpreted as GABAergic interneurons, while there was a decrease in the density of "very large neurons" in layer III, presumably pyramidal neurons, in patients with schizophrenia. Concomitant morphological studies at the single neuron level have demonstrated impoverished dendritic structures of pyramidal neurons (Broadbelt et al., 2002) and loss of dendritic spines in schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000; Sweet et al., 2009), as well as in non-human primate models (Selemon et al., 2007).

Another interesting finding is an anomalous distribution of the so-called interstitial white matter neurons in schizophrenia. These interstitial neurons have been suggested to be remnants of subplate neurons that normally undergo programmed cell death during brain maturation (Chun and Shatz, 1989). However, in certain species including human, these white matter interstitial neurons are to some degree normally found in healthy adults (Kostovic and Rakic, 1980). The interstitial white matter neurons have been found to be increased in prefrontal white matter (Akbarian et al., 1996; Anderson et al., 1996) and temporal white matter (Rioux et al., 2003) in subjects with schizophrenia, supporting further the presence of a neurodevelopmental abnormality in schizophrenia (Weinberger, 1986, 1987) (See **Table 1** for a summary of imaging, and grey and white matter studies in schizophrenia.).

THE INVOLVEMENT OF ACC IN SCHIZOPHRENIA

THE CINGULATE GYRUS AND THE CINGULUM BUNDLE

The ACC has been studied in many neuropathologic investigations of schizophrenia. From a topographical viewpoint, the ACC consist of Brodmann area 24, and includes the subgenual area 25, and according to some authors also the paracingulate prefrontal area 32. In the human, area 24 can be subdivided along its rostrocaudal and dorsoventral extent, through which it shows gradients in cytoarchitecture as well as topography in its afferent and efferent projections (Ongür et al., 2003; Palomero-Gallagher et al., 2008; Vogt et al., 1995), and area 32 extends dorsocaudally as a dorsal strip (32′) overlying area 24 (**Figure 2**). The cingulum bundle is the major coherent white matter tract of the cingulate gyrus, radiating superiorly from the corpus callosum to the cingulate cortex. The ACC receives processed multimodal sensory information from insular, temporal, parietal association cortices, and emotional information from the amygdala and the orbitofrontal cortex (Jones and Powell, 1970). The multimodal sensory input enables the ACC to respond to stimuli with motivational significance, activating motor and visceromotor responses, including vocalizations. For details on cingulate circuitry, see Beckmann et al. (2009), Iversen (1984), Kunishio and Haber (1994), Van Hoesen et al. (1993), Vogt and Pandya (1987) and Vogt et al. (1987).

THE ROLE OF ACC IN BEHAVIOR WITH IMPLICATIONS FOR SCHIZOPHRENIA

The first observation that the ACC had a role in emotional and visceromotor behavior was from non-human primate studies. Electrical stimulation of the ACC in monkeys generates changes in blood pressure, heart rate, respiratory rate, and agitation and vocalizations (Devinsky et al., 1995; Jürgens et al., 1967; Neafsey, 1990; Smith, 1945). Primate lesion studies have shown aggressiveness, emotional blunting, and impaired infant-mother interactions, further indicating that the ACC has an important role in emotional and social functions (Devinsky et al., 1995; Glees et al., 1950; Mirsky et al., 1957). However, these early lesion studies commonly involved more than just area 24 or area 32 and often included the parts of the OFC, and later confirmation studies have shown various effects in monkeys (Devinsky et al., 1995; Hadland et al., 2003). In the human, different observations have been made from tumors, strokes, seizures and electrical stimulation studies involving the ACC, but these have been quite variable (Devinski and Luciano, 1993; Devinsky et al., 1995). For example, surgical interventions of the ACC have focused on management of pain, chronic depression and obsessive-compulsive behavior (Devinsky et al., 1995). It is likely that the social aspects of the ACC that have been observed are related to its connections with the OFC. For an overview of the

Table 1 | Examples of neuropathological observations on the human cerebral cortex and underlying white matter in schizophrenia.

Parameter	Method	Observations in schizophrenia	References
IMAGING STUDIES			
White matter fractional anisotropy	DTI	Decreased FA in the cingulum bundle	Kubicki et al. (2003)
		Decreased FA in the cingulum bundle	Sun et al. (2003)
		Decreased FA in the cingulum bundle	Wang et al. (2004)
		Decreased FA in the frontal WM	Kumra et al. (2005)
Myelin water fraction	MRI	Reduced myelin water fraction in frontal WM	Flynn et al. (2003)
White matter metabolism	PET	Increased in the cingulum bundle	Buchsbaum et al. (2007)
Gyrification index	MRI	Reduction in cortical folding in frontal regions	Kulynych et al. (1997)
Sulcal patterning	MRI	Shallower sulcal depth in the parietal operculum	Csernansky et al. (2008)
Cortical volume	MRI	Reduced volume of frontal lobes	Andreasen et al. (1986)
		Reduced volume of the ACC	Baiano et al. (2007)
		Reduced volume of the ACC	Koo et al. (2008)
		Cortical thinning of prefrontal and temporal cortices	Nesvag et al. (2008)
		Cortical thinning of ACC, temporal and parietal cortices	Narr et al. (2005)
		Progressive grey matter loss starting in the parietal cortex and progressing towards temporal cortex and DLPFC	Thompson et al. (2001)
Grey matter metabolism	rCBF	Decreased rCBF in ACC	Tamminga et al. (1992)
	PET	Decreased glucose metabolic rates in the ACC	Haznedar et al. (2004)
Macromolecular structure integrity	MTI	Alterations in frontotemporal GM and temporal WM	Foong et al. (2000)
OLIGODENDROCYTE AND MYELIN STUDIES			
Oligodendrocyte density in white matter	Stereology	Decreased density in the WM of SFG	Hof et al. (2003)
		Unaltered density in the cingulum bundle	Segal et al. (2009)
Oligodendrocyte density in grey matter	Stereology	Decreased density in area 24 but not in area 32	Stark et al., (2004)
		Decreased density in the SFG	Hof et al. (2003)
Oligodendrocyte morphology	EM	Apoptotic oligodendrocytes in area 10	Uranova et al. (2001)
Myelin sheaths	EM	Damaged myelin in area 10	Uranova et al. (2001)
Gene expression of myelin-related genes	Microarrays association analysis	Decreased expression of myelin-associated glycoprotein (MAG), myelin and lymphocyte protein (MAL), 2′,3′-cyclic nucleotide 3′-phosphodiestase (CNP), gelsolin, transferrin and HER3 (neuregulin receptor) in the DLPFC	Hakak et al. (2001)
	Association analysis	Association of 10 single nucleotide polymorphisms from six myelin-related genes	Jungerius et al. (2008)
Protein expression of myelin-related genes		Decreased expression of CNP in GM of anterior PFC	Flynn et al. (2003)
GREY MATTER AND NEURON STUDIES			
Capillary lengths	Stereology	No differences in are 24 and area 9	Kreczmanski et al. (2005)
Neuronal density	2D morphometric analysis*	Decreased in area 24 and area 10	Benes et al. (1991)
	3D morphometric analysis*	Increases in area 9 and area 46	Selemon et al. (1995, 1998)
Interstitial white matter neurons	2D analysis	Increased neurons in prefrontal white matter	Akbarian et al. (1996) and Anderson et al. (1996)
Neuronal distribution	Stereology	Decreased mean cell spacing in area 9	Casanova et al. (2005, 2008)
Neural soma size	3D analysis*	Smaller mean neuronal somas in area 9	Rajkowska et al. (1998)
Neuronal integrity	Golgi stains	Decreased number of dendrites in area 32	Broadbelt et al. (2002)
		Decreased dendritic spine density in DLPFC	Glantz and Lewis (2000), Kolluri et al. (2005) and Sweet et al. (2009)
Synaptic proteins	Synaptophysin	Alterations in synaptic protein expression	Glantz and Lewis (1997) and Eastwood and Harrison (1995)

This table is not a comprehensive summary of all neuropathologic findings in schizophrenia. Rather, it gives examples of some of the latest morphological observations in which the myelin hypothesis may have an impact, in relation to some of the classical neuropathologic findings.
WM, white matter; GM, grey matter; ACC, anterior cingulate cortex; DLPFC, dorsolateral prefrontal cortex; SFG, superior frontal gyrus; FA, fractional anisotropy; DTI, diffusion tensor imaging; MTI, magnetic transfer imaging; MRI, magnetic resonance imaging; rCBF, regional cerebral blood flow; PET, positron emission tomography; EM, electron microscopy
**Biased to tissue orientation and limited sampling.*

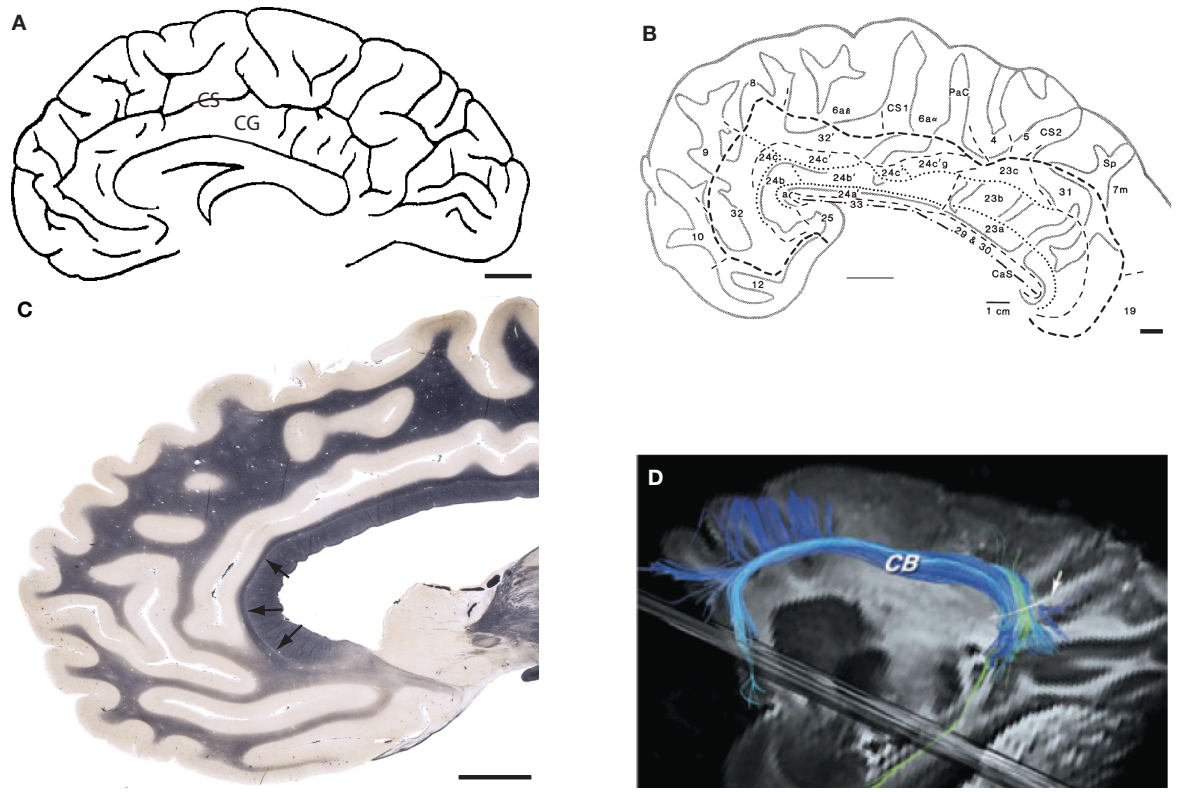

FIGURE 2 | The cingulate cortex and cingulum bundle. (A) Medial surface view of the human brain, depicting the cingulate gyrus (CG) and the cingulate sulcus (CS). **(B)** Human flat map of the cingulate cortex and surrounding areas. Note that the anterior cingulate is composed of the subgenual area 25, the paracingulate area 32, and six cytoarchitecturally different subregions of area 24 (24a,b,c,a′,b′,c′, respectively). **(C)** Human sagittal myelin stain showing the cingulate cortex and the cingulum bundle (arrows) as a thin fiber tract overlying the corpus callosum. **(D)** The cingulum bundle in the rhesus monkey, as visualized with DTI. Panels **(A,B)** modified from Vogt et al. (1995), and panel **(D)** from Schmahmann et al. (2007), with permissions. Scale bars 1 cm.

ACC in social function, see Amodio and Frith (2006), Bush et al. (2000), Rudebeck et al. (2008) and Rushworth et al. (2007a,b). It is noteworthy that both the sensory integration and social processing modalities are pertinent to the presumed ACC dysfunction in schizophrenia. It is however important to keep in mind that social and emotional functions are separate entities though they commonly interact.

BRAIN MATURATION AND MYELINATION

MYELINATION SEQUENCES

During ontogeny, the cognitive development of children and young adults depends closely on the progressive myelination of cortical axons (Casey et al., 2005; Fuster, 2002; Gibson and Petersen, 1991; Paus, 2005). As first shown by Flechsig in 1901, and later by Yakoklev in human postmortem myelin preparations (Flechsig, 1901; Yakovlev and Lecours, 1967), the regions that are myelinated first include the spinal cord and brainstem, and then myelination continues dorsally towards the frontal cortex, with proximal pathways myelinating prior to distal pathways, sensory pathways prior to motor pathways, and downstream projection pathways prior to association pathways (Volpe, 2000) and prefrontal regions myelinating the last (Lenroot and Giedd, 2006). Although initiated prenatally in humans, most tracts and regions become myelinated during the first year of life, and myelination continues into the second and third decade of life in humans (**Figure 3**). These early reports have been confirmed and further refined with modern brain imaging techniques (Ballesteros et al., 1993; Knickmeyer et al., 2008; Lenroot and Giedd, 2006; Miller et al., 2003; Mukherjee and McKinstry, 2006; Mukherjee et al., 2001, 2002; Paus et al., 2001; Sowell et al., 2003; Volpe, 2000). In addition, the number of oligodendrocytes drastically increases after birth through maturity (O'Kusky and Colonnier, 1982). It is this increase in oligodendrocytes and myelination that accounts for the large increase in white matter volume observed during the first years of life (Knickmeyer et al., 2008; Lenroot and Giedd, 2006).

The PFC is the last region of the brain to mature (Fuster, 2002). The volume of prefrontal white matter increases through childhood and early adolescence, and is not complete until early adulthood (Paus et al., 2001). As such, myelination *per se* can be used as an index of cortical maturation (Fuster, 2002). In the human cingulum bundle, the onset of myelination is around gestational week 38 and is fully myelinated at 1 year of age in humans (Gilles et al., 1983). It should be kept in mind however that when adulthood is reached, the cortical areas 24, 25 and 32 are poorly myelinated (Ongür et al., 2003), although the underlying cingulum bundle is highly myelinated.

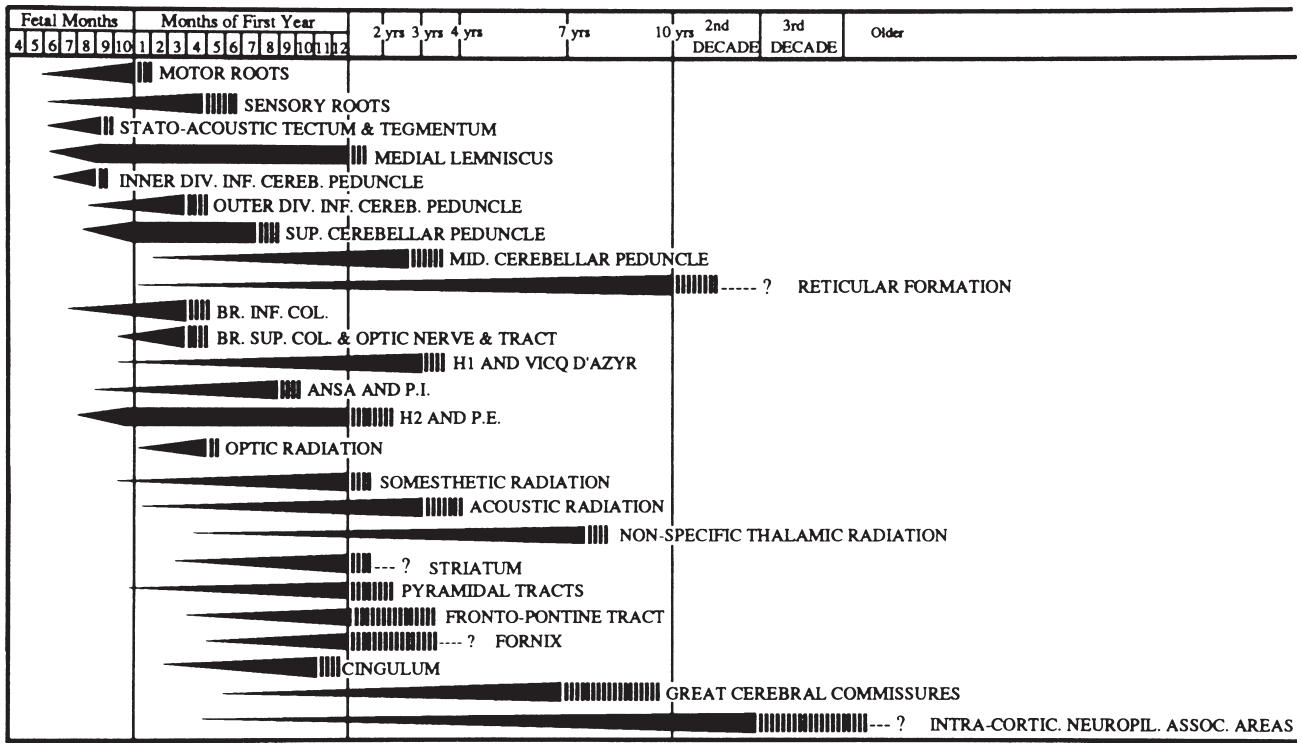

FIGURE 3 | Myelination during brain maturation in the human. From Yakovlev and Lecours (1967), with permission.

This brain maturation process of myelination and white matter volume expansion occurs simultaneously with a grey matter volume reduction (Pfefferbaum et al., 1994), and an increase in synaptogenesis which is followed by synaptic pruning and elimination (Huttenlocher, 1979). For example, Pfefferbaum showed with MRI that the volume of cortical grey matter decreases starting at 5 years of age in the human, while the white matter volume continues to increase through the third decade of life (Pfefferbaum et al., 1994). This has been confirmed and extended to include an analysis of the progression of white and grey matter changes through the complete human lifespan, in which frontal and parietal grey matter volumes peak at around 10–12 years of age and temporal grey peaks at 16–18 years of age (Thompson et al., 2005), and the white matter volume does not start to decline until after the age of 50 (**Figure 4**) (Sowell et al., 2003). The classic work of Huttenlocher (1979) showed that in the human medial PFC, the peak synaptic density occurs at 3–4 years of age, and starts to decline at mid-to-late adolescence. The pruning of axonal connections during brain development and maturation may be necessary for adequate formation of appropriate neuronal circuits. Thus, there is an interplay between progressive and regressive events that occur during brain maturation (Gogtay et al., 2004; Lenroot and Giedd, 2006; Sowell et al., 2003, 2004; Toga et al., 2006). In summary, the overall brain development and maturation occurs at several levels: (i) axonal, with wiring and myelination; (ii) dendritic, with arborization and spine formation; (iii) synaptic, with synaptogenesis and pruning; (iv) neuronal, with postnatal overshoot of neurons and programmed cell death; and (v) glial, with oligodendrocyte, astrocyte, and microglia maturation.

BRAIN MATURATION AND SCHIZOPHRENIA

It is possible that the regionally specific remodeling of grey and white matter that takes place into the third decade of life underlies some of the structural and functional changes that leads to the development of psychiatric disorders such as schizophrenia. The fact that the PFC matures last and that myelination is not complete until late adolescence may be significant, as the timing coincides with the typical onset of symptoms in schizophrenia. This suggests that a dysfunctional myelination process could underlie the pathogenesis of schizophrenia. Also several other psychiatric diseases, such as anxiety, mood, and personality disorders, first manifest themselves during early adulthood, possibly reflecting aberrations in brain maturation mechanisms (Paus et al., 2008). In fact, Paus and others discusses that "an exaggeration of typical adolescent changes…has occurred in patients with schizophrenia" (Keshavan et al., 1994). In fact, several of the observed neuropathologic findings in schizophrenia, such as reductions in frontal grey matter volumes (Baiano et al., 2007; Sporn et al., 2003), reductions in prefrontal metabolism (Andreasen et al., 1992), and reductions in plasma membrane phospholipid levels (Pettegrew et al., 1991) are "consistent with an exaggeration of the changes that occur in typical development" (Paus et al., 2008). Imaging work by Thompson and coworkers have also related brain maturation with the development of schizophrenia (Gogtay et al., 2008; Thompson et al., 2001).

However, it is interesting to note the lack of neurological comorbities in schizophrenia in comparison with other more typical white matter diseases. In dysmyelinating and hypomyelinating diseases such as the leukoencephalopathies, the effects of a myelin

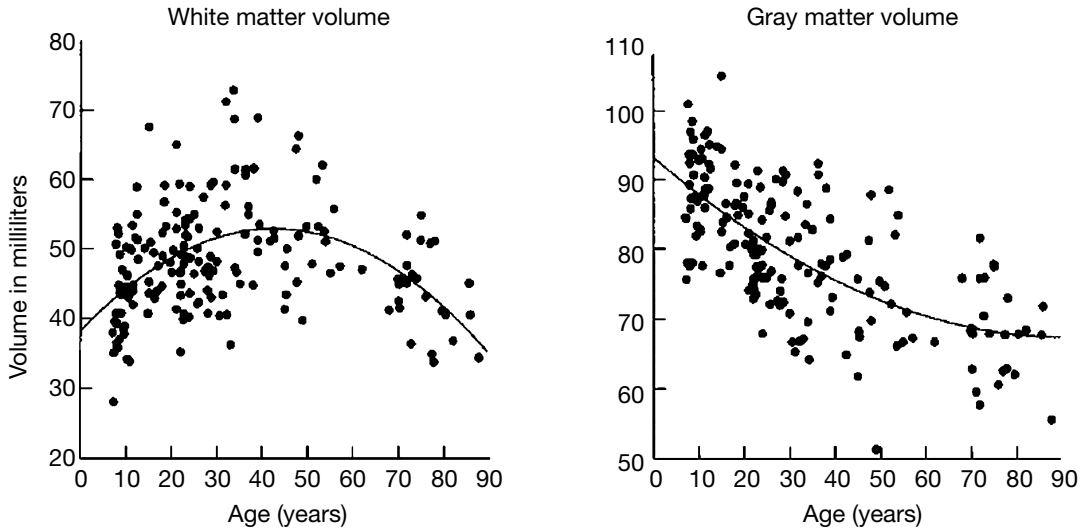

FIGURE 4 | Grey matter and white matter volumes in healthy subjects assessed with MRI. From Sowell et al. (2003), with permission.

deficiency may be striking and fatal (see reviews by Lyon et al., 2006; Schmahmann and Pandya, 2007; Schmahmann et al., 2007, 2008; Walterfang et al., 2005). If the myelin hypothesis holds true, and myelin deficiencies prove to be one of the central causes of the development of schizophrenia, one might argue and question why classic schizophrenia patients show so few neurologic symptoms. Several other white matter abnormalities often generate disturbances at the neuron level, such as seizures and/or psychomotor developmental delays. Why patients with schizophrenia do not particularly exhibit similar neurologic comorbidities, such as seizures or sensorimotor deficits, is unknown. It may be that only specific pathways become myelin-deficient, such as the late developing and poorly myelinated regions of the PFC, leading to the generation of behavioral symptoms seen in schizophrenia. Since the diverse circuits in the brain do not mature at the same time, if there is a developmental insult, this may affect only a certain population of neurons undergoing myelination, and result in a pathway-specific deficiency.

GENETICS

GENETIC ASSOCIATION OF OLIGODENDROCYTE AND MYELIN-RELATED GENES IN SCHIZOPHRENIA

In a groundbreaking study using gene microarray analysis to examine gene expression levels in postmortem samples from schizophrenia patients (Hakak et al., 2001), it was found that the expression of six myelin-related genes predominantly expressed in oligodendrocytes, including the myelin-associated glycoprotein (MAG), myelin and lymphocyte protein (MAL), 2′,3′-cyclic nucleotide 3′-phosphodiestase (CNP), gelsolin, transferrin and HER3 (ErbB3) was significantly decreased in the DLPFC in postmortem schizophrenic brains. The decreased expression of oligodendrocyte-related gene products was later confirmed and extended to other brain areas, implying that there is a pathology of oligodendrocytes underlying schizophrenia (Dracheva et al., 2006; Hakak et al., 2001; Haroutunian et al., 2006, 2007; Katsel et al., 2005a,b, 2008; McCullumsmith et al., 2007; Tkachev et al., 2003). Genetic linkage studies have also implicated myelin-related loci in schizophrenia (Bailer et al., 2000; Levinson et al., 1998) although linkage studies are now considered somewhat controversial in complex psychiatric disorders. This molecular pathology, showing a reduced myelin-related gene expression, has been shown in the DLPFC, hippocampus, superior temporal cortex, and the cingulate gyrus (Katsel et al., 2005b; McCullumsmith et al., 2007; Sugai et al., 2004). These results from gene expression studies led to genetic association studies, to clarify whether reduced expression of oligodendrocyte and myelin genes in schizophrenia represents an early event in the etiology of the disorder, or merely result from treatment with no direct causative relation to the disorder. Much evidence, including whole genome association studies, have identified myelin- and oligodendrocyte-related genes as susceptibility genes for schizophrenia.

One of the most promising schizophrenia-related genes is neuregulin 1 (*NRG1*) gene (Stefansson et al., 2002, 2003; Williams et al., 2003). NRG1 and the NRG1-receptor ERBB4 are involved in several aspects of nervous system development including oligodendrocyte development (Calaora et al., 2001; Corfas et al., 2004; Sussman et al., 2005). Several lines of studies support genetic association of *NRG1* with schizophrenia (Munafo et al., 2006), and associated endophenotypes (Bramon et al., 2008; Mata et al., 2009). A genetic locus–locus interactive analyses between *NRG1* and *ERBB4* genes provided evidence for a significant interaction between the *NRG1* Icelandic schizophrenia risk haplotype and *ERBB4* (Norton et al., 2006), suggesting that *NRG1* may mediate its effects on schizophrenia susceptibility through functional interaction with *ERBB4*. Given the emerging role of NRG1 and ERRB4 in oligodendrocyte development, it is possible that alterations in *NRG1* and *ERBB4* affect oligodendrocytes, leading to schizophrenia.

Reticulin 4 (*RTN4*, also known as NOGO) is a myelin-associated protein that inhibits the outgrowth of neurites and nerve terminals. Novak et al. (2002) reported over-expression of *RTN4* in the brains of people with schizophrenia and also evidence for genetic association between a marker in the 3′UTR of the gene. Several groups

have subsequently failed to replicate these genetic findings (Chen et al., 2004; Covault et al., 2004; Gregorio et al., 2005; Xiong et al., 2005), however, a moderately large study (Woo and Crowell, 2005) demonstrated modest evidence for association (Chen et al., 2004). Interestingly, three rare non-synonymous variants have recently been reported in the RTN4 receptor in schizophrenia cases but not in controls (Sinibaldi et al., 2004).

Additional genes showing reduced expression have been analyzed in genetic association studies. These genes include *OLIG2* and *CNP1*. Olig2 is a basic helix-loop-helix (bHLH) oligodendrocyte transcription factor that, together with Olig1 is sufficient and necessary for the formation of oligodendrocytes (Ross et al., 2003; Sauvageot and Stiles, 2002). Association analysis revealed strong evidence for association for this gene. Of six informative single nucleotide polymorphisms (SNPs) analyzed, four showed genetic association (Georgieva et al., 2006), which has been further confirmed in Chinese (Huang et al., 2008), but not Japanese (Usui et al., 2006), cohorts. *CNP1*, encodes CNPase, which is important for process formation of oligodendrocytes (Hakak et al., 2001). The *CNP1* gene maps to a region in which there is a previously reported significant linkage to schizophrenia in a single large pedigree. Significant association of a functional SNP was observed, and interestingly, this SNP is shown to be associated with low CNPase expression using allelic expression analysis in human brain (Peirce et al., 2006). This association was replicated in Caucasian (Voineskos et al., 2008), but not in Asian, cohorts (Tang et al., 2007; Usui et al., 2006). There have also been reports of genetic association between schizophrenia and myelin-oligodendrocyte glycoprotein (MOG; (Liu et al., 2005), proteolipid protein 1 (Qin et al., 2005), claudin 5 (Sun et al., 2004) and gelsolin (Xi et al., 2004). The gene encoding QKI, the quaking homologue KH domain RNA binding, is located in 6q25-27, and this region had been shown to be a susceptibility locus for schizophrenia as identified in a large pedigree from northern Sweden (Lindholm et al., 2001). Some evidence of genetic association was reported in this population (Aberg et al., 2006a,b), but this was not observed in a Chinese sample (Huang et al., 2009). Another gene reported to be associated with schizophrenia is *MAG*. MAG is a MAG that plays important roles in myelination. Support for a role for MAG in schizophrenia susceptibility has been reported in both family based and case control studies in Han Chinese populations, but still controversial.

Finally, *PTPRZ1*, a gene encoding receptor protein tyrosine phosphatase beta (RPTPβ) is a new and promising candidate gene for schizophrenia (Buxbaum et al., 2008). RPTPβ is expressed in oligodendrocytes, and appears to modulate ERBB4 signaling. Association analysis of *PTPRZ1* showed highly significant association of this gene to schizophrenia in this first study, however, this association was not replicated in a Japanese cohort (Ito et al., 2008).

MOUSE MODELS OF WHITE MATTER DYSFUNCTION

Transgenic mouse models may serve as vehicles for studying the morphological and anatomical abnormalities that may result from a genetic defect affecting myelination. Some recent mouse models of white matter dysfunction have emerged during the last few years, which may serve as putative animal models for schizophrenia. The evidence described above are beginning to provide enough construct validity for mice with disruption of oligodendrocyte and myelin-associated genes as animal models for schizophrenia, and several knockout mice for oligodendrocyte and myelin-related genes have been investigated.

For example, CNPase knockout mice show no obvious delay in myelination and oligodendrocyte development, but develop ataxia and motor deficits at 4 months and die (Lappe-Siefke et al., 2003). A detailed histological analysis found axonal loss in these mice, a feature observed in schizophrenia. As NRG1 regulates oligodendrocyte development through ERB receptors on oligodendrocytes, Corfas and colleagues generated a mouse expressing a dominant negative ERB receptor in oligodendrocytes, and found oligodendrocyte and myelin abnormalities in this line. These mice showed reduced locomotion and social dysfunction, with increased dopamine signaling and hypersensitivity to amphetamine, reflecting aspects of the disorder (Roy et al., 2007). In the same way, mice deficient in Rtn4r have been studied, and altered locomotor activity (Hsu et al., 2007) and reduced working memory function (Budel et al., 2008) were observed. The transmembrane protease Bace1 is a key molecule that regulates NRG1 signaling and myelination (Hu et al., 2006). Savonenko et al. (2008) reported that Bace1-null mice show schizophrenia-related phenotypes in multiple behavioral domains, including deficits in prepulse inhibition and novelty-induced hyperactivity, hypersensitivity to a glutamatergic psychostimulant, cognitive impairments, and deficits in social recognition. Fgfr2 is expressed in oligodendrocytes and involved in the formation of myelin membranes and Kaga et al. (2006) generated conditional knockout mice of this gene and found that conditional knockout mice are hyperactive and that dopamine receptor antagonist abolished this abnormality.

The MAG knockout model is another relevant mouse model of myelin deficits, in light of the studies that found decreased expression of MAG in schizophrenia (Hakak et al., 2001; McCullumsmith et al., 2007; Tkachev et al., 2003). MAG is known to interact with neuronal membranes where it helps maintain the periaxonal space of myelin sheaths (Li et al., 1994), is involved in initiation of myelination (Montag et al., 1994), and has been shown to inhibit neurite outgrowth and impair axonal regeneration (Quarles, 2009). This has led to the hypothesis that MAG promotes maturation, maintenance and survival of myelinated neurons (Quarles, 2009). MAG knockout mice may therefore have disruptions in normal myelinated tract development that are reflected in altered anisotropy or fiber length density. Several studies have described developmental abnormalities in the MAG knockout model but have not demonstrated a dysfunctional phenotype (Li et al., 1994; Loers et al., 2004; Weiss et al., 2000, 2001). Behavioral studies of these mice showed fairly subtle abnormalities. Mice missing the *Mag* gene are less proficient than wild-type mice in maintaining balance on a rotating cylinder and display hyperactivity and impaired hindlimb reflex extension (Pan et al., 2005). However, the mutant mice showed no differences in spatial learning and memory or in swimming speed, as demonstrated in a Morris water maze (Montag et al., 1994).

Another mouse model recently used in research on schizophrenia is the QKI model or "Quaking" mutant (Haroutunian et al., 2006; Lauriat et al., 2008). Qk^{v} is an autosomal recessive mutation in mice that leads to severe dysmyelination of the CNS due to defects in oligodendrocyte maturation and RNA metabolism of myelin

components, and all isoforms of QKI (QKI5, 6, 7) are deleted in the mice with this mutation. The "quaking" mice show reduced number of myelin lamellae, lack of myelin sheath compaction, and abnormalities in the structure of nodal regions. In addition to that, alterations of dopamine system parameters, including increased dopamine metabolism and increased dopamine D_2 receptor binding, have been observed (Nikulina et al., 1995). Homozygous mice that survive to adulthood exhibit a characteristic tremor or "quaking" (Sidman et al., 1964), with abnormal composition and structure of myelin (Baumann and Pham-Dinh, 2001). As a homozygote, this mouse has traditionally been used in epilepsy research in virtue of its myelin and conduction abnormalities, whereas the heterozygote has a milder form of white matter dysfunction, and has been used as a putative schizophrenia model (Aberg et al., 2006a,b). The *QKI* gene product is an mRNA binding protein involved in determination of glial fate and oligodendrocyte differentiation (Ebersole et al., 1996; Larocque and Richard, 2005) and has been implicated in schizophrenia in several studies (Chenard and Richard, 2008; McCullumsmith et al., 2007; McInnes and Lauriat, 2006), in addition to the genetic studies cited above.

Mice treated with cuprizone, a drug that induces demyelination, demonstrated altered behavior including hyperactivity, sensorimotor gating anomalies, and memory alterations (Franco-Pons et al., 2007). Interestingly, these defects lasted after the discontinuation of cuprizone treatment, suggesting developmental insults to oligodendrocytes and myelin might contribute to schizophrenia. Zhang et al. found that the atypical antipsychotic, quetiapine, promoted the differentiation of oligodendrocyte lineage cells and prevented cortical demyelination and the concomitant spatial working memory impairment induced by cuprizone (Xu et al., 2009; Zhang et al., 2008).

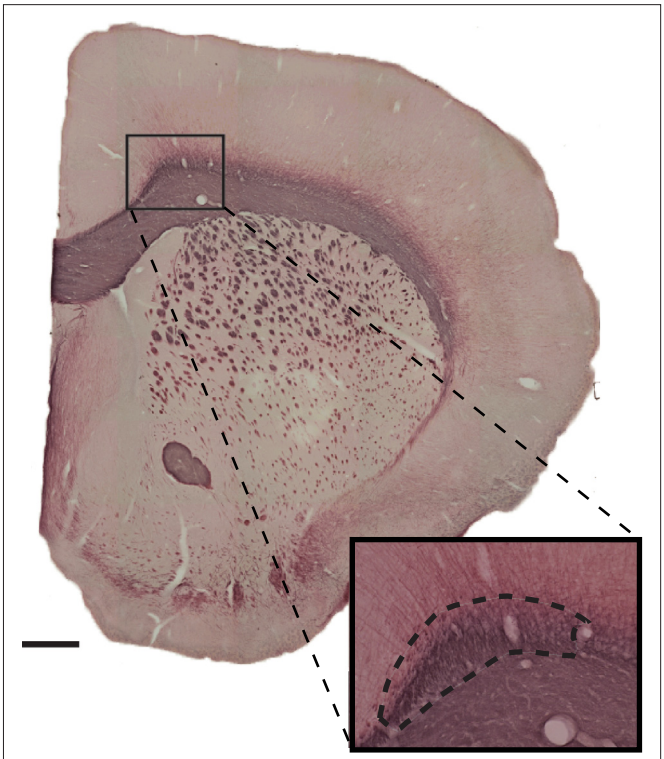

FIGURE 5 | Low magnification photomicrograph of a myelin Black Gold stain of a wild-type mouse. Inset depicts the outlined cingulum bundle for analysis of fiber length density. Scale bar 50 μm.

RECENT MORPHOLOGICAL FINDINGS FROM TRANSGENIC MICE

To date, two mouse models have been investigated for morphological alterations: the MAG model and the QKI model. Hof and coworkers examined two measures of white matter integrity in the MAG knockout model (Höistad et al., 2008; Segal, 2008). The cingulum bundle was examined using both DTI to examine white matter coherence as well as histological techniques to measure myelinated fiber length density. Diffusion anisotropy imaging was performed in adult MAG knockout mice, measuring the FA in a region of the cingulum bundle. At matched histological levels, using sections stained for myelin with Black Gold (Schmued and Slikker, 1999), myelinated fiber length density, defined as fiber length per unit of white matter volume was evaluated (**Figure 5**). The MAG knockout model displayed no alterations in either FA or fiber length density in the cingulum bundle (Segal, 2008). Thus, the effects of dysmyelination in the MAG model may be very subtle and may require ultrastructural studies to pinpoint the precise neuropathologic alterations.

We also performed morphological analysis of regional overall changes in cytoarchitecture in the ACC of the MAG and QKI mouse models (Höistad et al., 2008). Using stereologic methods, the number, density and spatial distribution patterns of neurons and oligodendrocytes were investigated. The effects of dysmyelination on neuron and oligodendrocyte numbers and densities in the ACC in these models revealed slight decreases in the overall volume of the ACC. Both the MAG and the QKI mouse models displayed lower total neuron numbers, but no difference in estimates of neuronal density, and differences in oligodendrocytes in the ACC were observed only as a trend in the QKI model. Thus, the QKI model may prove to be a more valuable model of myelin deficiencies than the MAG model, especially considering the absence of changes in the FA and fiber length density in this model.

Furthermore, we analyzed the dendritic structure of pyramidal neurons in these mouse models to assess whether disrupted myelination of axonal pathways that provide inputs to the neocortex severely affect the dendritic integrity of target neurons, resulting in dendritic attrition, loss of dendritic spines, and alterations in spine morphology. This permits an evaluation of the effect of abnormal myelination on the structure and function of pyramidal neurons in select regions relevant to schizophrenia, for example the medial PFC. The hypothesis we are investigating is elucidating potential morphological effects on target neurons, as a consequence of myelin deficiencies in the afferent axonal tracts (Segal et al., 2007b). Single pyramidal neurons were injected with a fluorescent dye and then analyzed morphometrically.

In the MAG model, analyses of pyramidal neurons in layers II and III in the PFC have shown that in young mice (3 months) the basal dendrites showed a reduced level of dendritic branching compared to their wild-type littermates (Segal et al., 2007b), while less remarkable effects were observed on the apical branches. This may suggest that the dendritic tree of the MAG mice is undergoing a selective pathology that may be related to alterations in

specific axonal pathways influencing principally the basal dendrites, and as such possibly of thalamic origin. These data imply that a disturbance in the organization of myelin, due to impaired expression of MAG, may result in alterations in morphology of layers II and III pyramidal cells, particularly with respect to basal dendritic integrity. Such alterations may lead to abnormalities of specific white matter tracts and affect the prefrontal circuits. Preliminary observations of spine densities in young MAG mice have so far not revealed differences between knockout and control mice (Segal et al., 2007b). However spine pathology may be more prominent in aged mice as a function of aging *per se*.

In the QKI model, analyses of pyramidal neurons in layers II and III in the ACC of old mice showed shorter dendritic lengths of both apical and basal dendrites (Höistad et al., 2008). The apical dendrites displayed shorter dendritic lengths distal from the soma, fewer numbers of branch radial intersections, and fewer higher order branches, whereas no differences were observed in the basal dendrites (**Figures 6A–D**). Preliminary observations of spine densities in the QKI mouse has suggested that dendritic spines are in fact more numerous in QKI mice compared to control littermates (**Figures 6E–H**). This may reflect compensatory mechanisms similar to sprouting. These observations are in line with our stereologic findings, which demonstrate that the QKI mice exhibit lower total neuron numbers and lower volumes of the ACC than control mice.

The preliminary evidence presented in **Figure 6** on the QKI model, suggests possible support for the viability of the hypothesis that myelin deficiencies may have morphological effects on target neurons, although the extent of these effects are not fully analyzed. We are aware of the fact that the relationship between disrupted myelin and changes in spine densities may not appear as a direct or causal one. It remains plausible that deficits in myelination may cause significant alterations in connectivity of select components of the afferent systems to cortical neurons, and as a result, a partial differentiation of those targets, which may alter the spine densities and spine morphologies. Comparably, detrimental changes in spine densities have been found during aging and in stress conditions (Duan et al., 2003; Radley et al., 2008).The possibility that myelin and oligodendrocyte changes impact on the integrity of the pyramidal neuron dendritic tree fits well within the general context of the effects of white matter disruption in the brain.

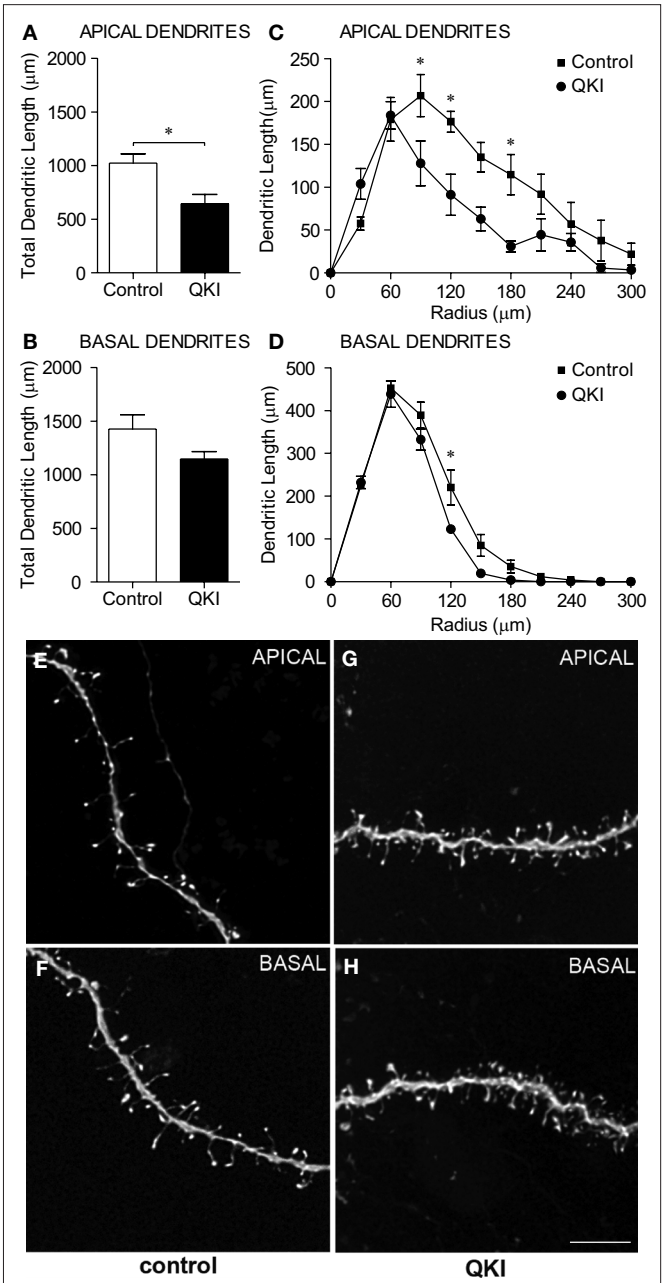

FIGURE 6 | Dendritic arbors and spines in the control and QKI mouse model. (A,B) Arbor analysis showing total dendritic lengths in apical and basal dendrites, $*p < 0.05$, Student's *t*-test. **(C,D)** Dendritic lengths in apical and basal dendrites, as a function of the radial distance from the cell soma, $*p < 0.05$, two-way ANOVA with Bonferroni's *post hoc* test (Höistad et al., 2008). **(E–H)** Dendritic segments of Lucifer yellow-filled neurons in the medial PFC were scanned at high resolution on a confocal laser scanning microscope. 3-Dimentionally reconstructed dendritic segments, 50–100 µm from the cell soma, show hyperspiny dendrites on both the apical and basal branches in the QKI mouse. Scale bar 5 µm.

FUTURE DIRECTIONS

Evidence from very different lines of research supports the premise that dysfunction of oligodendrocytes is a critical factor in the development of schizophrenia. The precise role oligodendrocytes hold in the cascade of malfunctions that results in the constellation of deficits seen in the disease is still unknown. Layers II and III pyramidal neurons in the ACC may be the targets of axonal pathways affected in schizophrenia. Quantitative information on neuronal integrity in mouse models is important for understanding downstream effects of myelin genetic abnormalities, and to assess the validity of models in the context of observable neuropathologic changes in human brains. These studies need to be extended to additional models reflecting the genetic complexity of schizophrenia, and electron microscopy studies should be used further to assess structural aberrations in oligodendrocytes and myelin sheaths, as well as immunogold approaches to study synaptic integrity by visualizing pre- and postsynaptic proteins. Correlative morphology and density analyses of dendritic spines will help clarify plastic changes in responses to myelin challenges.

The data obtained in transgenic mice will offer critical correlates to neuropathologic features that can be analyzed in postmortem human materials. Combined analysis of human specimen and relevant mouse models offers a unique opportunity to investigate myelin deficits that have a clinical impact. As a result of such combined approaches, a model of schizophrenia with characterized molecular defects that can be used for developing therapeutic approaches will hopefully emerge.

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Regulation of myelin genes implicated in psychiatric disorders by functional activity in axons

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Myelination is a highly dynamic process that continues well into adulthood in humans. Several recent gene expression studies have found abnormal expression of genes involved in myelination in the prefrontal cortex of brains from patients with schizophrenia and other psychiatric illnesses. Defects in myelination could contribute to the pathophysiology of psychiatric illness by impairing information processing as a consequence of altered impulse conduction velocity and synchrony between cortical regions carrying out higher level cognitive functions. Myelination can be altered by impulse activity in axons and by environmental experience. Psychiatric illness is treated by psychotherapy, behavioral modification, and drugs affecting neurotransmission, raising the possibility that myelinating glia may not only contribute to such disorders, but that activity-dependent effects on myelinating glia could provide one of the cellular mechanisms contributing to the therapeutic effects of these treatments. This review examines evidence showing that genes and gene networks important for myelination can be regulated by functional activity in axons.

Keywords: oligodendrocyte, axon, activity, schizophrenia, depression, white matter, ATP, LIF

INTRODUCTION

The establishment and development of psychiatric disorders are likely to involve aberrant regulation and expression of many genes, together with multiple environmental factors, ultimately leading to illness. In recent years researchers have begun to focus on the potential role of white matter and oligodendrocytes in the pathophysiology of psychiatric disorders (for a recent review see Dwork et al., 2007). Myelination can be viewed as a highly dynamic process which can be altered by impulse activity in axons (Demerens et al., 1996; Stevens et al., 1998) and by environmental factors. It is becoming clear that myelination continues into adulthood and may contribute to plasticity of cognitive function, learning and memory (Fields, 2005, 2008). Perturbations in the molecular processes leading to axon myelination will consequently result in axon dysfunction and abnormal electrical conduction, therefore impairing the transfer of information across brain regions. It is likely that axon health and dysfunction contribute to the pathophysiology of a number of psychiatric disorders, and axon survival is dependent on the close association of axons with myelinating glia (Nave and Trapp, 2008). The guiding hypothesis for this review is that in addition to the well appreciated synaptic dysfunction in psychiatric disorders, oligodendrocytes also play a major role, and that myelination by oligodendrocytes well into adulthood may be regulated by the firing of action potentials in axons. This type of regulation may be analogous to activity-dependent changes in neurons and synaptic connectivity as a consequence of environmental stimuli (for a recent review see Fields et al., 2005).

There are several mechanisms by which oligodendrocytes could sense functional activity in axons (**Figure 1**). Oligodendrocytes at various stages of development have ion channels, purinergic and other membrane receptors that allow myelinating glia to detect impulse activity through the activity-dependent release of molecules from axons (**Figures 1B,D,E**). Thus activity-dependent regulation of oligodendrocytes could contribute to cellular mechanisms promoting recovery through environmental interventions and other non-drug treatments of psychiatric illnesses. Drug treatments for neuropsychiatric illnesses may also act in part through effects on myelinating glia. Oligodendrocytes have neurotransmitter receptors for glutamate, serotonin, and dopamine, making it likely that antipsychotic drugs acting through these neurotransmitter systems would also have actions on myelinating glia that may be detrimental or beneficial in psychiatric disorders. Finally, synaptic communication between axons and immature myelinating glia (oligodendrocyte progenitor cells), have been described recently in white matter (Karadottir et al., 2008; Kukley et al., 2007; Lin et al., 2005), providing a rapid means of direct communication between axons and myelinating glia.

Myelination is a complex biological process that involves an intricate regulatory network among many different cell types in the nervous system (Rosenberg et al., 2007). Many of the genes revealed in genomic studies of mental illness that are crucial to the normal functioning of the myelination program and myelin-maintenance are themselves candidates for regulation by electrical activity in axons. Many of these genes relate to oligodendroglia function; however some of these genes are expressed in astrocytes and some in neurons where they may have independent effects or act indirectly on myelinating glia.

TRANSCRIPTIONAL REGULATION OF MYELIN GENES IN OLIGODENDROCYTES

Regulating transcription of structural components of myelin, such as PLP1, MBP, MAG, MOG, and CNP is clearly critical in the process of oligodendrocyte development and the subsequent correct myelination of specific axons. Several of these major components

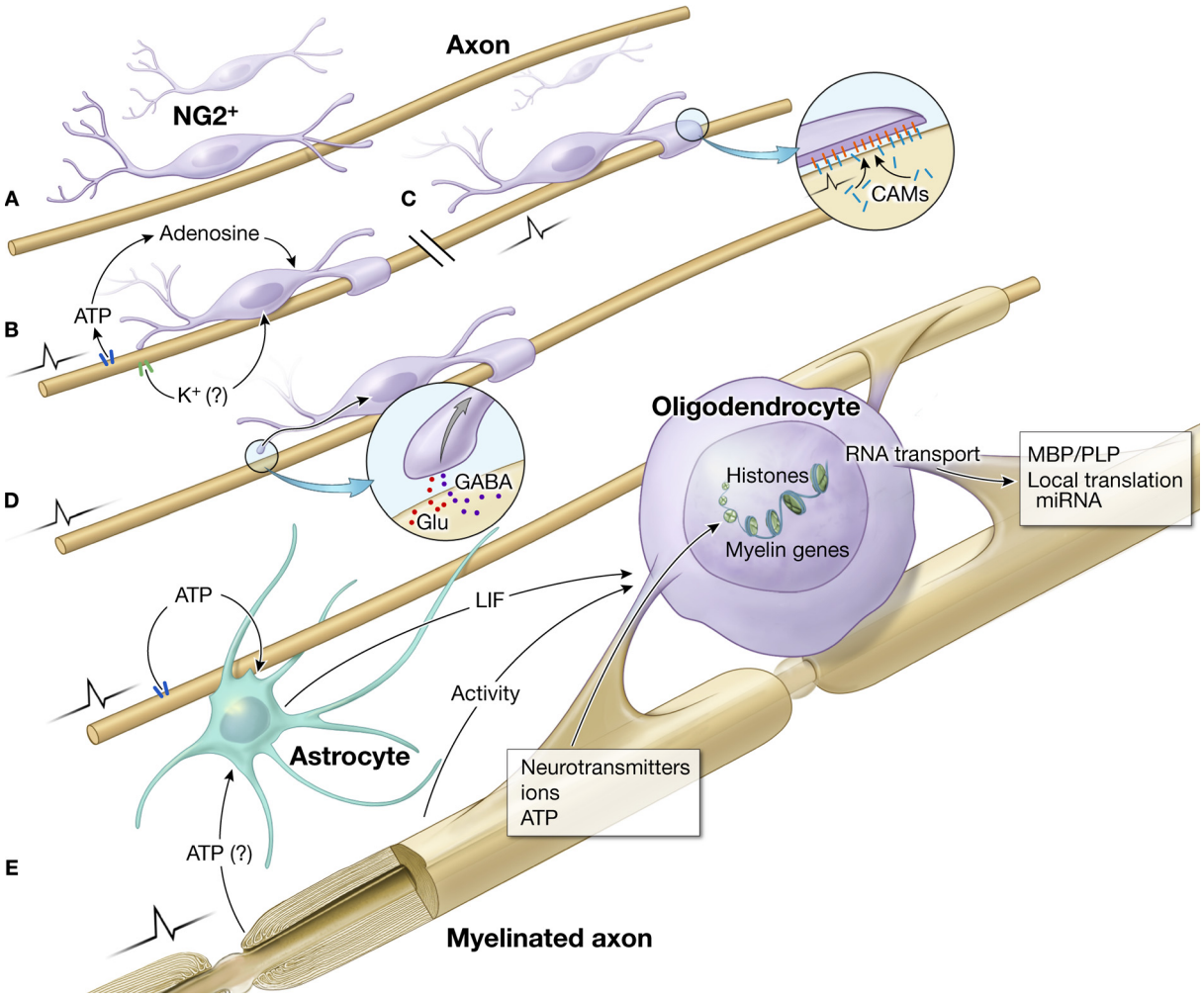

FIGURE 1 | Impulse activity in axons regulates oligodendrocyte development and myelination at several stages and via different signals. **(A)** Immature OPCs ($NG2^+$ cells) in white matter on an electrically silent unmyelinated axon. Such cells persist in significant numbers in the adult brain. **(B)** Electrical activity causes ATP release from axons, which generates adenosine that stimulates differentiation of NG2 cells to a mature oligodendrocyte, and promotes myelination (Stevens et al., 2002). K^+ is released from electrically active axons. Blocking K^+ channels in oligodendrocytes in culture has been shown to regulate oligodendrocyte proliferation and lineage progression (Ghiani et al., 1999). **(C)** Electrical activity can also alter the expression of cell adhesion molecules on the axon that are involved in initiating myelination (Itoh et al., 1995, 1997). This has been shown to regulate myelination by Schwann cells in the PNS, but the same molecule (L1-CAM) is involved in myelination by oligodendrocytes (Barbin et al., 2004). **(D)** The release of the neurotransmitters Glu (glutamate) or GABA from synapses formed on NG2 cells (Kukley et al., 2007), could provide another mechanism to regulate myelination in response to functional activity. **(E)** After NG2 cells differentiate into oligodendrocytes, ATP released from axons firing action potentials stimulates the synthesis and release of the cytokine LIF from astrocytes, which promotes myelination (Ishibashi et al., 2006). Myelination during development and postnatally may be regulated by several other unidentified activity-dependent signaling molecules affecting development of oligodendrocytes and myelin formation. Electrical activity in axons, via the release of neurotransmitters, ions and ATP may influence gene expression in oligodendrocytes by histone modification, RNA transport, local translation and regulate mRNA stability and translation by miRNAs.

of myelin have been shown to be regulated by action potential firing or by alterations in intracellular calcium or cAMP (Atkins et al., 1997, 1999; Gao et al., 2004; Studzinski et al., 1999); both of these second messengers can be regulated by neural impulse activity. Phosphorylation of MBP is regulated by MAP kinase in response to action potential firing during long-term potentiation (LTP) in the hippocampus (Atkins et al., 1999), a cellular model of memory, and by direct electrical stimulation of white matter in hippocampus. The functional significance of MBP phosphorylation in oligodendrocyte development and myelination is unclear. Additionally the level of mRNA and protein for MBP and PLP in an oligodendrocyte cell line are sensitive to increased intracellular calcium (Studzinski et al., 1999) and myelin associated glycoprotein (MAG) is sensitive to levels of cAMP (Gao et al., 2004). Therefore several of the major myelin genes can be regulated by electrical activity or mechanisms by which second messenger signal transduction can be modified. Many of the major components of myelin are deregulated in psychiatric disorders, particularly in schizophrenia (Hakak et al., 2001).

In order for myelination to proceed, a complex network of transcriptional repression and activation must be activated (Wegner,

2008). Several of the transcription factors required for repression and activation of myelin genes have been found to be abnormally expressed in the brains of patients with psychiatric disorders and in particular those with schizophrenia (Katsel et al., 2005).

Of particular interest is the transcription factor SOX10 (Tkachev et al., 2003) which is required for the expression of two of the major components of myelin, the proteins MBP and PLP1 (Stolt et al., 2002). Recently it has been shown that in Schwann cells, the myelinating glia of the peripheral nervous system, Sox10 is a component of a calcium-sensitive transcriptional complex (Kao et al., 2009). Calcium is the primary second messenger communicating action potential firing to intracellular responses (Eshete and Fields, 2001), and signaling to the nucleus to regulate gene transcription (Fields et al., 2005). The Sox10/NFAT complex is critical for Schwann cell development and activates several genes known to be regulators of myelination in the peripheral nervous system, such as KROX20 (Kao et al., 2009). Interestingly the SOX10 gene is located in a major susceptibility locus for schizophrenia and reduced expression of this gene was found to be correlated with an increase in the methylation state of the allele found in schizophrenia patients (Iwamoto et al., 2005).

An increase in the methylation of chromatin is indicative of a transcriptionally inactive state and it will be interesting to see if other transcription factors and genes required for oligodendrocyte function are regulated in this manner. It is well established that epigenetic modification of chromatin structure by DNA methylation is a critical event in the transcriptional regulation of gene expression (Kouzarides, 2007). In neurons DNA methylation can be affected by membrane depolarization and electrical activity in a Gadd45b-dependent manner (Ma et al., 2009). It is unknown if epigenetic regulation in oligodendrocytes can be regulated through a similar activity-dependent mechanism. However, the alteration of chromatin structure by histone deactylases (HDACs) is also thought to play a major role in the repression of myelin gene transcriptional inhibitors such as Tcf4 and Id4 (He et al., 2007a). However repression of the negative regulators of myelin gene transcription is in itself not sufficient to allow immature oligodendrocytes ($NG2^+$ cells) to progress to more mature developmental stage and begin the process of myelination (He et al., 2007b; **Figure 2**).

External signals generated by electrical activity in axons, such as ATP or glutamate release from axons, can cause changes in intracellular calcium levels in oligodendrocytes and therefore these axonally derived signals may play a role in the epigenetic regulation of the transcriptional apparatus required for lineage progression and myelination by oligodendrocytes and in the process of remyelination (**Figure 1E**). Clearly a perturbation of this type of epigenetic regulation in psychiatric disorders, perhaps by soluble axon-derived signals such as ATP or glutamate, would provide a link with environmental cues as axon firing patterns reflect environmental stimuli.

REGULATION OF MYELIN GENES BEYOND TRANSCRIPTION

The complexity of mRNA expression and metabolism and the localization of specific mRNAs to subcellular compartments in oligodendrocytes will all contribute to the eventual pool of mRNA available for the translational machinery. RNA transport, splicing and stability mechanisms are tightly regulated by intracellular signal transduction in many cell types in the nervous system.

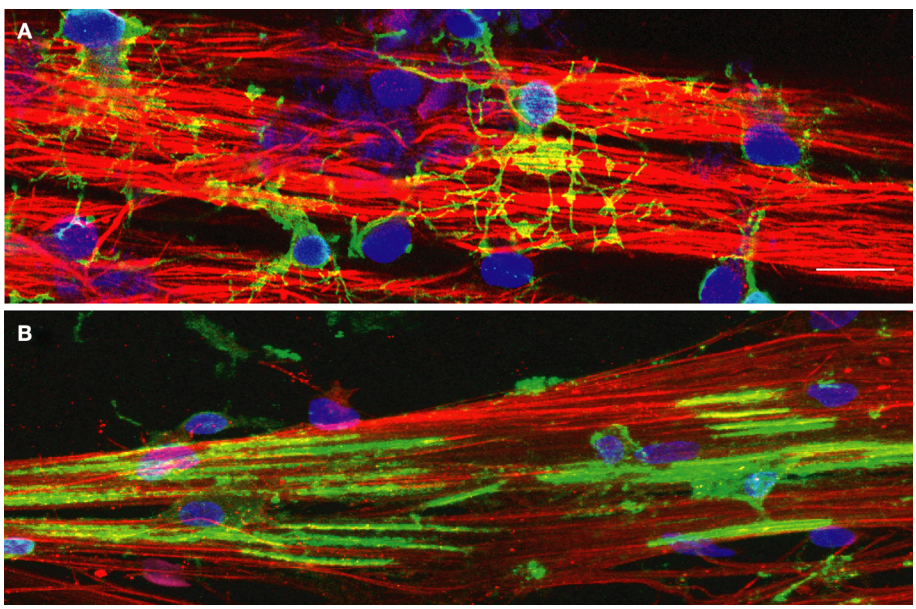

FIGURE 2 | $NG2^+$ cells and MBP^+ oligodendrocytes grown on DRG axons in culture. DRG axons grown in culture for 3 weeks were immunostained for neurofilament (red) to identify axons and DAPI (blue) to label cell nuclei. **(A)** Immature oligodendrocyte progenitor cells (OPCs) were plated onto DRG axons and immunostained for the immature OPC marker NG2 (green) after 2 days in co-culture. $NG2^+$ positive OPCs have differentiated into multipolar cells with many processes contacting multiple axons in order to initiate the myelination process. **(B)** OPCs grown in co-culture with DRG axons for 7 days were immunostained with myelin basic protein (MBP), a component of the myelin sheath. Immature OPCs have differentiated into a myelination phenotype with expression of MBP and the formation of multiple segments of compact myelin associated with the axons. Scale bar = 15 μm.

Interestingly, many of the myelin genes are alternatively spliced during development and several of these mRNAs are targets for transport by specific RNA binding proteins (McInnes and Lauriat, 2006). Importantly, a broad spectrum of RNA binding proteins has been found to be deregulated in schizophrenia (Katsel et al., 2005), including quaking (QKI) and hnRNAPA2. Deregulation of such a large group of RNA binding proteins will have many downstream consequences for RNA metabolism and localization in oligodendrocytes. For example, Aberg et al. (2006) demonstrated that deregulation of several genes expressed in oligodendrocytes and associated with schizophrenia were associated with alternative expression of QKI splice variants. Therefore disruption in the expression of the QKI gene has downstream consequences for oligodendrocyte development and myelination and this may lead to a predisposition to psychiatric illness.

It is also known that hnRNPA2 is a carrier protein for MBP mRNA in oligodendrocytes (Ainger et al., 1997); it is thought that hnRNPA2-MBP complexes are transported into oligodendrocyte processes on microtubules (Carson and Barbarese, 2005), however the axonal signal for this mechanism is not thought to be a soluble factor released from the axon, but instead a cell adhesion molecule expressed on the axon (White et al., 2008). Nonetheless it is attractive to hypothesize that RNA metabolism beyond transcriptional control may also be subject to regulation by extracellular signaling cues derived from electrically active axons. There is evidence in the literature that in neuronal processes RNA transport is regulated by electrical activity (Willis et al., 2007). A similar type of regulation could in theory control RNA transport in the polarized process of oligodendrocytes in contact with individual axons. This type of regulation may provide precise control, in response to signals derived from electrically active axons, of the available mRNA pool of myelin genes in individual processes of developing and mature oligodendrocytes.

Another potential mechanism by which post-transcriptional regulation of oligodendrocyte-related genes may be accomplished is to regulate mRNA homeostasis by the binding of specific micro RNAs (miRNAs) to myelin gene mRNAs. Micro RNAs are regulators of translation and RNA stability (Kosik and Krichevsky, 2005); this is achieved by miRNA binding to the UTR of target RNAs and directly influencing the amount of mRNA available to the translational machinery. The study of the regulation of miRNAs in the brain is still at a relatively early stage, however the functional targets of several miRNAs have been described in neurons (Fiore et al., 2008) and oligodendrocytes (Lau et al., 2008). Interestingly, a very recent study has suggested the involvement of a miRNA (miR-219) in NMDA signaling (Kocerha et al., 2009), whereby blocking NMDA receptor function by regulating a CaMKII subunit that signals downstream of this receptor, a similar effect was seen by treating mice with antipsychotic medication. It is interesting to note that mature oligodendrocytes also express NMDA receptors on myelinating processes (for a recent review of glutamate signaling in white matter see Bakiri et al., 2009) but the role of these receptors is currently unknown. Additionally Beveridge et al. (2008) reported that levels of another miRNA (miR-181b) are elevated in schizophrenia; targets of this miRNA are downregulated in the same brain region as the miRNA is upregulated in. Of the miRNAs known to function in neurons, several have been shown to be regulated by electrical activity in neuronal processes (for example Wayman et al., 2008). It is tempting to speculate that miRNA regulation of translation in oligodendrocytes may operate in a similar manner in response to soluble factors released from electrically active axons, such as BDNF, glutamate, ATP and GABA. Given the very early stage of determining functions for miRNAs in oligodendrocyte biology, further work is required to show the specific miRNAs that may target many of the genes implicated in oligodendrocyte function and are implicated in psychiatric disorders.

REGULATION OF GENES IN ASTROCYTES

In addition to oligodendrocyte-related genes, other non-neuronal genes such as glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed in astrocytes, have been implicated in psychiatric disorders (Martins-de-Souza et al., 2008; Torrey et al., 2000). Astrocytes can have an important influence on development of oligodendrocytes by secretion of trophic factors and cytokines. GFAP is an integral component of the astrocyte cytoskeleton and altered expression of GFAP can have many effects on astrocyte biology (Haydon, 2001). Therefore GFAP dysregulation and by implication astrocytic dysfunction could have profound effects on axon health, neuro-transmission, and neuron-glia signaling, and perhaps most importantly indirectly in the process of myelination. Additionally, GFAP expression is thought to be regulated by DNA methylation (Song and Ghosh, 2004) and recent evidence suggests that activity in neuronal circuits can regulate the expression of GFAP in hippocampal cell culture (Cohen and Fields, 2008). Therefore extracellular factors generated by neuronal activity have been shown to regulate GFAP expression and astrocyte differentiation and this could occur through the regulation of chromatin structure. Astrocytes have also been shown to have a direct role in regulating myelination and oligodendcrocyte development. Studies in cell culture have shown that astrocytes promote myelination in response to electrical stimulation of axons via the release of a cytokine Leukemia Inhibitory Factor (LIF), in response to ATP released from electrically active axons (Ishibashi et al., 2006). Taken together it is becoming clear that astrocyte function, which can be regulated by neuronal activity, can have profound effects on the myelination process and may contribute to disease progression in psychiatric disorders (**Figure 1E**).

REGULATION OF GENES IN AXONS

Another mechanism by which electrical activity could influence gene expression in oligodendrocytes is by the expression of cell surface signaling molecules such as specific cell adhesion receptors in axons (**Figure 1C**). This general type of mechanism has already been shown to regulate myelination in the peripheral nervous system (Stevens et al., 1998). In these studies, different cell adhesion molecules on axons, NCAM, N-Cadherin, and L1-CAM were regulated by specific frequencies of electrical stimulation (Itoh et al., 1995, 1997). L1-CAM is required for initiation of myelination by Schwann cells (Wood et al., 1990) and downregulating the L1 gene by electrical stimulation at the appropriate frequency inhibited myelin formation in cell culture (Stevens et al., 1998). L1-CAM is also required for myelination by oligodendrocytes (Barbin et al., 2004), suggesting that specific patterns of impulse activity could affect myelination in the brain and spinal cord.

The expression of specific proteins and protein complexes at sites of axon-glia contact could provide a direct link between axon signaling and the regulation of gene expression in oligodendrocytes (**Figure 1C**). In support of this idea it has been demonstrated that extracellular stimuli can regulate the localization of specific mRNAs in axons (Willis et al., 2007), and it has been demonstrated that the expression of cell surface receptors can be modulated by electrical activity in axons (Itoh et al., 1995). Therefore, taken together, regulation of myelination by an activity-dependent signaling cascade, originating in axons, may allow direct coupling of neuronal activity and oligodendrocyte intracellular signaling.

In an interesting recent study by White et al. (2008) regulation of localized MBP translation in oligodendrocytes has been shown to be dependent upon the neuronal adhesion molecule L1 binding to oligodendrocytes resulting in Fyn-kinase activation and translation of MBP mRNA in oligodendrocyte processes in contact with the axon. This study potentially links electrical activity in axons via L1 expression activation of Fyn-kinase, resulting in increased translation of MBP in oligodendrocyte processes. Perturbation of any part of this signaling mechanism could result in defective myelin. This type of axon-glia interaction may be a common regulatory mechanism by which myelin deposition is targeted to the correct axon site in response to the expression of a cell surface receptor by impulse activity in the axon.

ACTIVITY DEPENDENT REGULATION OF AXON-DERIVED DIFFUSIBLE MOLECULES

Importantly, it has been demonstrated that diffusible molecules released from axons firing action potentials can be detected by myelinating glia, with subsequent control of glial development and myelination. Cell culture studies have shown that action potentials induced by electrical stimulation release ATP from axons, which activates P2 receptors on myelinating glia of the peripheral nervous system (Schwann cells). This regulates proliferation and differentiation of Schwann cells in accordance with functional activity in axons, by stimulating calcium influx, activation of CaMKII, MAPK, and the transcription factors c-fos, Krox24, and CREB (Stevens and Fields, 2000). P2 receptors are also present on oligodendrocyte at early stages of development (Fields and Burnstock, 2006). Adenosine, derived from ATP released from electrically active axons, acts on immature oligodendrocytes to promote differentiation and myelination (Stevens et al., 2002; **Figure 1B**). Other molecules released by electrically active axons that could in theory influence myelinating glia include potassium (K^+) and neurotransmitters. Several studies have demonstrated that blockade of K^+ channels or membrane depolarization with veratridine inhibits oligodendrocyte cell proliferation and differentiation (Ghiani et al., 1999). Growth factors, such as BDNF, which are known regulators of oligodendrocyte differentiation (Van't Veer et al., 2009), can be secreted or regulated in an activity-dependent manner and by environmental experience, providing another potential general mechanism for activity-dependent regulation of myelination. Interactions between BDNF and serotonin in mood disorders have been reported (Martinowich and Lu, 2008), and the BDNF gene has been associated with increased risk for a number of neuropsychiatric disorders (Martinowich et al., 2007).

REGULATION OF GENE EXPRESSION AND PHARMACOLOGICAL INTERVENTION IN PSYCHIATRIC DISORDERS

Drug treatments for psychiatric disorders that correct deregulation of genes involved in myelination and oligodendrocyte dysfunction are an appealing possibility. In this regard pharmacological regulation of the activity of specific histone deacetylases (HDACs) is an interesting avenue of investigation, although therapeutic intervention of the modulation of HDAC activity in mouse models of demyelination has shown mixed results. Therefore it may be important to identify cell-type specific regulators of chromatin structure as it relates to oligodendrocyte function, thus targeting any drug treatments more specifically to limit off-target effects on other cell populations in the brain. Regulation of gene expression by medication beyond transcriptional regulation may provide a more specific mechanism to target myelin genes in oligodendrocytes. In this regard a very interesting piece of preliminary data comes from the study by Aberg et al. (2006) who show that the level of QKI mRNA can be influenced by medication used to treat schizophrenic patients. Modulation of QKI levels would have effects on the mRNA levels and cellular localization of many of the myelin genes and therefore oligodendrocyte function in the process of myelination. This research is at an early stage; however it opens up new routes that may be used to correct the defects seen in myelin gene expression in oligodendrocytes.

A recent study (Roy et al., 2007) implicates erb-signaling in oligodendrocytes in the functioning of dopaminergic neurons. Erb4 and its ligand neuregulin have been linked genetically to several psychiatric disorders (Corfas et al., 2004). This finding is perhaps of great clinical relevance as many antipsychotic medications work through modulation of dopamine. It is currently unclear exactly how dopamine function is disrupted by oligodendrocyte dysfunction; however this finding provides yet another link between oligodendrocyte biology and the correct functioning of neurons.

The extent to which dopamine levels could lead to mental illness in part through effects on myelinating glia, and whether antipsychotic treatments have therapeutic action in part through effects on oligodendrocytes are two intriguing questions of current investigation. There is evidence that dopamine can influence oligodendrocyte development and function. Both D2 and D3 dopamine receptors are expressed in oligodendrocytes or oligodendrocyte progenitors (Bongarzone et al., 1998; Howard et al., 1998). Quetiapine, a D2 receptor agonist used as an antipsychotic drug, increases synthesis of myelin basic protein and facilitates myelination in rat embryonic cortical cultures (Xiao et al., 2008). The D2/D3 agonist quinpirole also increases the number of oligodendrocyte progenitor cells (OPCs) and it decreases the number of mature oligodendrocytes in primary cell culture. Dopamine also can be toxic to oligodendrocyte progenitors by inducing superoxide generation and lowering glutathione levels (Hemdan and Almazan, 2008). Agonists for dopamine D2 and D3 receptors have been shown to provide significant protection of oligodendrocytes against oxidative injury (Rosin et al., 2005). On the contrary, haloperidol, a typical antipsychotic drug blocking D2 activity reduces myelin proteins in mice treated for 30 days (Narayan et al., 2007).

Schizophrenia and depression can also involve imbalances in the neurotransmitter serotonin, and several drug treatments act through regulating serotonin levels, for example the serotonin reuptake inhibitor fluoxetine (PROZAC). Serotonin receptors are expressed in Schwann cells (Gaietta et al., 2003), the myelinating glia of the PNS, and the human polymavirus, JC virus, which causes multifocal leukoencaphalopathy, binds the 5HT2a serotonin receptor (Elphick et al., 2004) on oligodendrocyte progenitor cells (Schaumburg et al., 2008). The serotonin antagonists, metoclopramidine, chlorpromazine, clozapine, and serotonin itself all significantly inhibit viral infection (Elphick et al., 2004), which indicates that medications affecting serotonin levels could influence oligodendrocytes. Indeed, the antidepressant drug, fluoxetine, increases cell proliferation of precursors in cell culture that can give rise to astrocytes, neurons, or oligodendrocytes (Zusso et al., 2004). Serotonin injection into the CNS of dogs has long been known to cause severe demyelination (Saakov et al., 1977), and serotonin reduces the number of oligodendrocytes in adult mouse brain (Moller, 2007).

Other neurotransmitters can regulate different steps of oligodendrogliogenesis through such ion channels and receptors as the delayed K^+ rectifier, the AMPA/kainate, dopamine or muscarinic receptors (review see Belachew et al., 1999). This suggests the possibility for activity-dependent regulation of oligodendrocyte differentiation and myelination, and raises the possibility of medications acting on neurotransmitters or the excitation of specific circuits could influence oligodendrocytes.

CONCLUSIONS AND FUTURE WORK

Evidence from multiple areas of research including human brain imaging studies and large scale mRNA profiling analyses strongly indicate that defects in myelin and abnormal expression of myelin genes, and their regulators, are common in many psychiatric disorders. It is plausible that perturbations in axon conductance brought about by defects in myelin are responsible for many of the cognitive impairments seen in psychiatric disorders. It is also clear that oligodendrocyte development and myelination and the process of remyelination involve a complex network of intracellular modification and extracellular signaling cues. It is apparent that activity in axons can not only regulate gene expression in the axon, but may also in turn regulate gene expression in oligodendrocytes. Therefore the identification of signaling cues generated by axons firing action potentials, which may be responsible for regulating gene expression in oligodendrocytes, is likely to be an important area for future research.

The situation is complex as both positive and negative regulators of oligodendrocyte function will be involved, and growth factors, cytokines, and neurotransmitters can have pronounced dose-dependent effects that may stimulate or inhibit oligodendrocyte development. Research thus far has identified three molecular mechanisms regulating myelination by action potentials in axons: (1) adenosine derived from ATP released from electrically active axons stimulates differentiation of OPCs (Stevens et al., 2002); (2) after oligodendrocytes mature, ATP released from axons acts on astrocytes to stimulate the synthesis and release of LIF which promotes myelination (Ishibashi et al., 2006); (3) action potentials at appropriate frequencies down regulate the cell adhesion molecule L1-CAM to inhibit myelination (Stevens et al., 1998).

Many other soluble factors released from axons firing action potentials, in addition to ATP and adenosine, are potential candidates for regulating myelination, such as GABA, glutamate, and nitric oxide; growth factors, such as BDNF, or enzymes that are able to modify protein complexes expressed on the surface of oligodendrocyte processes, are also likely to be involved in activity-dependent myelination. Changes in specific complexes of proteins expressed on axons in response to firing patterns, notably cell adhesion molecules and other membrane receptors, is another general mechanism regulating oligodendrocyte development and myelination to neural impulse activity. It is likely that a combination of all these intercellular communication pathways, and multiple cell types, are at work regulating myelinating glia in the mammalian brain in accordance with functional activity in individual axons in neural circuits mediating cognitive function.

Researchers are only beginning to develop an understanding of how electrical activity can regulate myelin formation. Even less is known about axon-derived soluble factors and their possible role in regulating other aspects of oligodendrocyte biology, such as cell polarity and vesicle trafficking. It is still unclear if the myelin abnormalities seen in psychiatric disorders are the cause of axon dysfunction or a consequence of it, but there is strong evidence to suggest both processes are at work. Effects of drugs that are used in treating psychiatric illnesses on myelinating glia, suggest that some of the beneficial action could be mediated through effects on oligodendrocytes, and conversely, that the cellular changes in white matter seen in schizophrenia, chronic depression, and other psychiatric illnesses could be induced in part by the treatments. Only a much clearer understanding of the complex relationship between myelinating oligodendrocytes and axons (**Figures 1 and 2**) will allow novel therapies to be developed in the treatment of myelin dysfunction and psychiatric disease.

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Cortical white matter: beyond the pale remarks, main conclusions and discussion

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INTRODUCTION

This Special Topic "Cortical white matter: beyond the pale" includes 10 articles from 32 authors. These articles in this offer a summary of some of the current thinking regarding myelin, and the associated cellular populations in the white matter. The articles range from the micro–level of ultrastructure and molecular factors, to populational organization, and cognitive effects. All the articles devote at least some discussion to myelin in psychiatric conditions, raising the prospect of new paradigms of investigation and treatment. In this article the main conclusions, and some of what the host editors (Kathleen Rockland and Javier DeFelipe) consider the most interesting remarks, have been extracted from each of the individual articles. These commentaries are not necessarily directly derived from the original work of the authors, and may be the result of the collective work of several different laboratories. This is followed by a section dedicated to more general comments and a discussion of the issues raised. The authors who have participated in this article are listed in alphabetical order.

A commentary on

Neurons in the white matter of the adult human neocortex by Suarez-Sola, M., Gonzalez-Delgado, F. J., Pueyo-Morlans, M., Medina-Bolivar, O. C., Hernandez-Acosta, N. C., Gonzalez-Gomez, M., and Meyer, G. (2009). *Front. Neuroanat.* 3:7. doi: 10.3389/neuro.05.007.2009.

REMARKS AND MAIN CONCLUSIONS

1. In the human brain, the white matter (WM) underlying the cerebral neocortex is highly developed and occupies a much larger volume than in other mammals. Although the dominant components of the WM are the complex fiber tracts, their ensheathing myelin and supporting glia, there are also large numbers of neurons dispersed among the fibers, termed the "interstitial neurons" (IN). They are prominent in the primate WM, and poorly developed in the rodent. The species differences may reflect a direct correlation between the size of the cortical gray matter, the amount of WM connecting the neocortex, and the number of IN.

2. In human, the border between gray and WM is relatively sharply defined at the bottom of the sulci and along the flanks of the gyri, but more difficult to delimit at the crowns or apices of the gyri, where radial fiber fascicles intermingle with radial rows of layer VIb neurons and IN seem to be continuous with neurons of layer VIb.

3. The highest density of IN is in the WM immediately subjacent to the gray matter, in the zone that contains the association or "U" fibers of the cortical convolutions, and then gradually decreases with increasing distance from the gray matter. Very few neurons lie among the long fiber tracts in the deep WM, such as internal capsule, superior and inferior longitudinal fasciculi, or corpus callosum. However, there is no sharp boundary between the superficial WM, rich in IN, and the deep WM, where IN are sparse. There may also be regional differences in the density of IN, with lowest numbers in the visual cortex, and higher numbers in the frontal and prefrontal cortex.

4. The IN display a variety of morphologies ranging from pyramidal-like to bipolar and multipolar. They can be classified into the two main neuronal categories also present in the gray matter, namely excitatory glutamatergic cells and inhibitory GABAergic neurons.

5. IN of the cortical WM are often referred to as "subplate" cells. During development, the subplate is a transient cell compartment just below the future layers VI-II, or "cortical plate". Birthdating studies in rodents and carnivores revealed that subplate neurons are generated at the same time as Cajal-Retzius cells in the marginal zone (or future layer I), and prior to the birth of cortical plate neurons.

6. Subplate cells perform multiple developmental functions: they extend pioneer fibers into the internal capsule and direct thalamo-cortical pathfinding, serve as transient synaptic targets for thalamocortical fibers, and provide a substantial glutamatergic input into the maturing cortical plate, helping in the establishment of ocular dominance columns in the primary visual cortex. As the cortical plate matures, many subplate neurons degenerate and undergo programmed cell death. The survivors continue into adult life as IN of the WM.

7. Subplate neurons are morphologically and neurochemically heterogeneous. The GABAergic subpopulations may express a variety of peptides such as neuropepide Y, somatostatin and/or cholecystokinin, or contain nitric oxide synthase. It

is not known if developmental cell death affects specific cell classes within the subplate, or whether all subpopulations are equally reduced.

8. Although human subplate neurons are heterogeneous, a useful marker of the glutamatergic component is the putative transcription factor T-brain-1 (Tbr1). The chronology of Tbr1 expression in human fetuses can be traced to the early cortical plate at 10 GW, which is strongly Tbr1+. From 14 to 25 GW, large numbers of Tbr1+ neurons are continuously added to the subplate compartment, which increases in width concurrent with the growth of the cortical plate, although the highest density is always at the border between cortical plate and subplate. In perinatal brains, Tbr1-immunoreactivity changes from a nuclear to a cytoplasmic staining that is widely expressed in neurons in the cortical gray and white matter, and thus no longer useful as a marker molecule of the subplate. In the absence of molecules specific for the human subplate it is difficult to ascertain how many subplate cells survive as IN.
9. Altogether, these data show that the IN of the human WM are not identical to the early-born subplate neurons described in rodents and cat. Rather, the cell populations in the maturing WM seem to be complemented by newly arriving neurons generated at much later stages of corticogenesis. A possible explanation for the discrepancy across species may be the extraordinary increase in cortical connectivity during evolution, which leads to an increase in size and complexity of the WM in the primate brain. In parallel to the increase of the WM compartment, a continuous supply of IN may be required during the whole period of corticogenesis. This implies that primate IN are not just incidental remnants of early-born neurons, but rather seem to belong to a distinct neuronal system that is intimately connected to the WM and may carry out activities pertinent to this location.
10. The GABAergic interneurons of the cortical gray matter are highly diverse, and many attempts have been undertaken to classify them according to a variety of morphological and neurochemical properties. A recent inventory of mouse cortical interneurons has led to the identification of 13 cell classes based on the combined expression of the calcium-binding proteins calbindin (CB), calretinin (CR) and parvalbumin (PV), and neuropetides, such as vasointestinal polypeptide, NPY, cholecystokinin, somatostatin and choline acetyltransferase. Of the three calcium-binding proteins present in the cortical gray matter, only CB and CR are expressed in IN. PV+ cells are the largest group of cortical interneurons which includes basket cells and chandelier cells. PV is not found in the adult human WM, and the few deep PV+ neurons occasionally found below the gray matter are more likely to represent displaced layer VI neurons. CB+ IN have been reported in the WM; they are concentrated in the superficial WM, often aligned along the gray/WM border and most numerous in the apex regions.
11. CR is abundant in gray-matter interneurons mostly of supragranular layers, but its presence in the WM has not attracted much attention. This is surprising insofar as CR+ IN are the most prominent cell population in the superficial and deep adult human WM.
12. The distribution and relative prominence of interneurons expressing calcium-binding proteins are species and area-dependent. CR+ cells seem to be more prominent in primate than in rodent cortex, not only in the gray matter but also in the WM. In the mouse, CR+ interneurons derive from the caudal ganglionic eminence and migrate tangentially all over the cortex. By contrast, primate interneurons have a double origin, with early-born cells migrating from ganglionic eminences, and later-born cells deriving from the subventricular zone (SVZ) of the cortical wall. In particular, CR+ cells are very prominent in the SVZ and deep WM during late human fetal development.
13. Nitric oxide (NO) is a gaseous messenger molecule synthetized by several isoforms of the enzyme nitric oxide synthase (NOS). In the brain, two NOS forms are constitutively expressed, nNOS in neurons, and eNOS in endothelial cells. Nitrergic, i.e. NO-producing neurons, can be visualized by NADPH-diaphorase histochemistry, as well as by immunohistochemistry using anti-nNOS antibodies. There are two main cell classes: Type 1 NADPH-d neurons are intensely stained in a Golgi-like fashion, displaying medium size to large somata and long varicose processes. Most type 1 neurons are in the superficial WM, whereas type 2 NADPH-d neurons are restricted to the cortical gray matter. Type 2 neurons are only lightly stained and have small somata and short processes. Both types express GABA, and about 4% of type 1 neurons co-express CB. Type 1 neurons can also express neuropeptide Y (NPY) and somatostatin. Although most GABAergic neurons are interneurons with local axons, some NADPH-d/nNOS+ neurons in the WM of rat, cat and monkey project over long distances to distant, functionally unrelated cortical areas.
14. One of the most interesting features of the type 1 NADPH-d neurons is their close association with blood vessels. Their axonal plexuses form a dense network around microvessels, and their long processes may contact distant arterioles and capillaries. Since NO is a potent vasodilator, NOS-containing neurons are thought to be involved in the coupling of metabolic changes related to neuronal function with local increases in blood flow. Due to their strategic location just below the cortical gray matter, NOS+ IN may be contacted by corticopetal fibers and, in response, act on neighboring microvessels. On the other hand, NPY is a powerful vasoconstrictor able to antagonize the vasodilating effect of NO that co-localizes with NOS in a subset of IN. Somatostatin and NPY are expressed in IN of the superficial WM. They act directly on smooth muscle cells of cortical arterioles, and may thus constrict cortical microvessels in an activity-dependent manner. The NOS/NPY+ IN thus form part of the neural system involved in the coupling of cortical microvessels to neuronal activity.
15. The NOS+ IN in the cortical WM are an important component of the vasoactive pathways which also include subcortical cholinergic and serotoninergic systems. In fact, most NOS-expressing IN are cholinoceptive, meaning that they receive cholinergic fibers from the nucleus basalis. Since the axons of NOS+ IN may spread over considerable distances into the cortical gray matter, a single IN may coordinate local blood flow in neighboring and distant cortical areas in response to corticopetal and corticofugal activation.

16. The subcortical WM and its resident IN have been associated with a variety of neurological and psychiatric disorders. Alterations of somatostatin, NPY and/or NADPH-d+ IN were observed in Alzheimer disease. However, schizophrenia is the disease which seems to show the most dramatic abnormalities of the WM. Diffusion tensor imaging revealed disturbances of myelin function and distribution, alterations of connectivity and integrity of fiber tracts such as the cingulate bundle and uncinate fasciculus. A higher incidence of changes occurrs in the frontal lobes, middle temporal structures including hippocampus and amygdala, and superior temporal gyrus, as well as in subcortical centers.
17. Schizophrenia also affects the IN in diverse ways. In the frontal lobe of schizophrenic patients, the IN density was decreased in the superficial WM, but increased in the deeper WM. NADPH-d+ IN show the same maldistribution as microtubule associated protein 2 (MAP2)+ cells in general. While some studies reported an increase in IN density in inferior parietal and dorsolateral prefrontal areas in deficit syndrome patients, others observed no change neither in superficial nor in deep WM. The conflicting reports on the changes of IN density in schizophrenia were summarized by Eastwood and Harrison, who observed a density increase in the superficial WM and no change in deeper compartments.
18. A special and rather minor subclass of IN expresses the extracellular matrix molecule Reelin, which is important for brain development and adult neuronal plasticity. In the adult cortical gray matter, Reelin is expressed by a subgroup of GABAergic interneurons; in the WM, very few scattered Reelin+ cells can be visualized using immunohistochemistry. Conversely, Reelin mRNA has been reported to be abundant in IN in the superior temporal cortex, and to be significantly reduced in schizophrenic patients, in keeping with the finding that alterations of Reelin expression are a putative vulnerability factor in schizophrenia and mood disorders.
19. Most discussions of IN changes in psychoses are based on the view that IN are remnants of the early-born subplate population. The early generation of IN in rodents and carnivores, and their maldistribution in schizophrenic patients, have led to the hypothesis that a migration defect of the subplate during embryonic or early fetal development underlies the pathogenesis of schizophrenia. As stated above, the developmental history of the subplate is very different in nonprimate mammals and in primates including human. An important task for future research would be a molecular taxonomy of all neuronal populations in the WM, similar to the work done on interneurons in the gray matter. It would be particularly important to differentiate between early-born components, probably related to transient roles of the subplate, and later-appearing resident cells, which may not be important for development but rather involved in activities proper to the adult WM. The recent discovery of subplate-specific molecules in mice is a useful step in this direction, and it is hoped that similar work will shed light on the origins, categories and functional roles of human IN.

GENERAL COMMENTS AND DISCUSSION

DeFELIPE

Comment on points 4 and 13:
Are there studies that address the issue of synaptic connections of IN? studies on what is the proportion of these neurons that project over long distances?

ROCKLAND

Response to DeFelipe's comment:
Some articles in the next Special Topic (Cortical GABAergic neurons: stretching it) will talk about synaptic inputs, but I believe less is known about the output. I do not believe we have numbers yet about proportion. In part because there are multiple target structures, retrograde tracers are only partly useful in addressing this question.

ROCKLAND

Comment on points 2 and 5, and in general:
It may be worth remarking that "layer VIB" is used here to denote deep layer VI. This is not to be confused with "layer VIB" in rodents, sometimes also called "layer VII" and which constitutes a separate population. Questions of nomenclature are likely to be taken up in a subsequent Special Topic, as referenced above.

As these authors remark (points 6 and 7), the subplate neurons perform multiple functions developmentally, and themselves constitute a heterogeneous group. It is interesting to consider this in conjunction with Friedlander and Torres-Reveron, who emphasize the multiple and changing roles of WM neurons over a lifetime.

HÖISTAD AND HOF

Comment on interstital neurons, the nomenclature and species differences:
Suárez-Sola et al. discuss the so-called "interstitial neurons" (IN) of the WM of the adult human cortex, which are located directly subjacent to the cortical gray matter and are found in high numbers especially in the prefrontal cortex. Initially they were regarded as remnants from the subplate during neurodevelopment, however in primates, IN rather seem to belong to a distinct neuronal system that carries out activities pertinent to the subcortical WM.

Interestingly, some species have in adult stage large numbers of WM neurons, in particular large-brained mammals such as artiodactyls and cetaceans (Hof et al., 1999), while they are only rudimentary in the brain of non-primate mammals. As the authors indicate, such species differences may reflect a correlation between the size of the cortical gray matter, the amount of WM interconnecting the neocortex, and the number of IN. The higher number of IN in large-brained animals may possibly support one of the proposed functions of the IN, which is coordination of activity among neocortical regions.

These comparative differences may also possibly be indicative of species-specific importance, similar to other specialized neuron types (such as spindle "von Economo" neurons in the anterior cingulate and frontoinsular cortices, Betz cells in M1, or Meynert cells in V1). However, the IN are a very heterogenous population of neurons, some pyramidal-like and covered with spines, thus presumably glutamatergic, while others are multipolar or bipolar and express GABA and a variety of neuronal markers (such as

CB, CR, neuropeptide Y, somatostatin, and NOS). One of the proposed functions of the IN involves the coordinating of regulation of blood flow. Particularly in view of the presence of NO in some IN (as visualized by the presence of NADPH-d/nNOS+), and their close association with blood vessels, the IN have been suggested to be involved in the regulation of blood flow and neurovascular coupling. In addition, the expression of NPY, a powerful vasoconstrictor, could potentially antagonize the vasodilating effect of NO. Interestingly, comparable functions have been reported for select classes of neocortical interneurons characterized by their content of neuropeptides and particular morphologies (Cauli et al., 2004). If the IN have a role in blood flow regulation, it remains to be clarified however why are there fewer IN in visual cortex but higher numbers in frontal cortex, and also why they appear only rudimentary in smaller mammals such as rodents.

In the human, abnormalities of the IN in the frontal lobe have been observed in schizophrenia, although reports from different investigators are conflicting (summarized by Eastwood and Harrison, 2005). As the authors indicate, it is important to differentiate between early-born IN (probably related to transient roles of the subplate) and later-appearing resident cells, which may not be important for development but rather involved in activities proper to the adult WM.

KOSTOVIC

Authors and commentators discussed the significance of the prominence of subplate zone and neurons, and of the large numbers of interstitial neurons in the primate brain and particularly in human. The answer may be in the obvious fact that subplate prominence and number of interstitial neurons is related to the enormous increase of cortico-cortical connectivity in human (Kostovic and Rakic, 1990). The increase of complexity of cortico-cortical connections requires a prolonged existence of the subplate zone as well as prolonged developmental function of subplate neurons (Kostovic and Judas, 2006). This is also consistent with the increased number of gyri and gyral white matter in human and primates (remark 2).

In respond to Rockland comments about synaptic inputs, I also refer to the next special topic "Cortical GABAergic Projection Neurons" and in particular to the article "Subplate cells: amplifiers of neuronal activity in the developing cerebral cortex" of Luhmann, Kilb and Hanganu-Opatz. Without endogenous activity of the subplate neurons, cortex can not properly develop. Here we emphasize prolonged coexistence of subplate circuitry with the gradual formation of adult-like thalamo-cortical and cortico-cortical circuitry (Kostovic and Judas, 2006). Thus, the subplate zone should always be defined as a synaptic layer.

The glutamatergic component of subplate zone factor Tbr1 (remark 8) is recognized as a useful marker. There are several other specific markers found in the subplate zone, such as *CplX3, CTGF, Nurr-1/Nr 4a2, Mox D1, CTGF* and *F-spondin* (Ayoub and Kostovic, 2009).

Involvement of white matter neurons in neurological and psychiatric disorders (remarks 16 and 17) may have a developmental interpretation: selective vulnerability of the subplate zone and neurons during development. This may be a crucial factor in pathogenesis of several neurological mental and cognitive disorders (Kostovic and Judas, 2006).

MEYER

Response to the comments by Höistad and Hof regarding the number of interstitial neurons (IN) in different species. They argue that large brains have in general large amounts of cortical gray matter, accompanied by large numbers of cortical association fibers and thus an increased volume of cortical white matter, requiring large numbers of IN. The question is why the visual cortex has relatively few IN. I think that the connections of the primary visual cortex – Brodmann´s area 17- are well defined and highly specific. On the one hand, area 17 itself is a narrow koniocortex, and the proportion of intracortically projecting neurons higher than in other cytoarchitectonic areas. On the other hand, callosal fibers are sparse if not absent in most of area 17, and they only increase toward the 17/18 border, the representation of the vertical meridian. Area 17 is thus lacking a substantial component of the cortical white matter tracts, and in fact, macroscopic observation of the human striate area shows that the amount of white matter is smaller than in the nearby occipital association areas. By contrast, prefrontal areas have many and varied fiber connections which contribute to the huge volume of the underlying white matter. A possible additional function of IN may be that they serve as guideposts for distinct fiber fascicles, perhaps by establishing synaptic contacts. The primary visual cortex may thus not be the best model for studying IN, the more so since also during development subplate neurons below area 17 play important roles in specific visual functions, such as the establishment of ocular dominance columns. Studies on subplate neurons in primate cortex development should also include frontal and parietal association areas where IN are known to be very numerous in the adult.

A commentary on

The changing roles of neurons in the cortical subplate by Friedlander, M. J., and Torres-Reveron, J. (2009). *Front. Neuroanat.* 3:15. doi: 10.3389/neuro.05.015.2009.

REMARKS AND MAIN CONCLUSIONS

1. We propose that certain types of neurons can undergo a temporal re-specification of function over the lifespan. Specifically, we suggest that the population of cortical subplate neurons does so although it is not known whether individual neurons in that cohort of cells change their function or if the surviving subplate cells represent a subpopulation that has different functions at different stages of the life cycle.
2. Although we propose a long term type of multi-tasking over the lifespan, there may be other types of neuronal multitasking operating over shorter time scales. For example, within minutes, as information is being processed, neuromodulators and recent bouts of activity could unmask emergent functional properties such as regulation of gene expression leading to differential functions of individual neurons within the neuronal circuit within which the cell is embedded. Recording of electrical activity from large populations of interacting neurons will be required, while following individual neurons' activity profiles for extended periods, with tetrode arrays to directly test these ideas. Current technology can apply *in vivo* optical monitoring of the dynamics of the structure of dendrites, spines and axons

although this approach is generally limited to superficial cortical layers. New advances in imaging technology will be required for similar tracking of deep cells such as subplate neurons during development. It will also be of interest to evaluate whether changes in neuronal function occur over the course of aging. For example, the neocortex shrinks during normal aging due primarily to atrophy of cells and the neuropil. Future studies of individual cellular and neuronal network function during aging may reveal other examples of serial neuronal multi-tasking.

3. Differential expression of genes plays a major role in neuronal development and functional differentiation not only from early embryonic stages but also into senescence. These changes can be programmed to occur at defined stages or can be triggered by local signals, by environmental inputs or in a neuronal activity-regulated manner. Such temporally modulated regulation of gene expression can play a role in target recognition and path-finding, synaptogenesis, refinement of synaptic connections. In addition, other influences such as sensory or motor activity, cognition, stress, infectious agents and traumatic events can alter gene expression patterns in the brain throughout life.
4. After differentiation to a particular phenotype, little change is thought to occur in each neuron's fundamental properties such as their anatomical projections, location, position, chemical neurotransmitter, and the functions of the cell within the framework of the particular network where it resides.
5. Pleiotropy (the ability of a single gene to influence multiple phenotypic traits) is well established. Neurons can express pleiotropic genes or respond to pleiotropic gene products at different times throughout an organism's life, potentially increasing information processing ability longitudinally and responding to stimuli and stressors. Like genes, whole neurons could increase their information processing contribution combinatorially by serving different functions over the course of the lifespan – a pleiotropy of cellular function in the temporal domain. The neurons of the cortical subplate are one candidate population of cells that may behave in this manner.
6. The subplate cells emerge from the ventricular zone under the cerebral cortex, migrating below the marginal zone to the cortical preplate that is then split by the differentiating neurons of the cortical plate, some neurons taking up residence in the marginal zone and others settling below the cortical plate in the subplate (SP). The cortical plate neurons form most of the cortical layers (layers 2–6) while the marginal zone neurons become layer 1 and the SP neurons become interstitial cells of the cortical white matter as well as clustering at the bottom of the cortical plate just below layer 6.
7. SP cells are among the first cortical neurons to differentiate into a neuronal phenotype; they express microtubule associated protein-2 and neuropeptides before the cortical plate neurons, they receive synaptic inputs and generate action potentials through embryonic development. These cells also serve as pioneers issuing axons into the internal capsule where they serve an important role by innervating the thalamus and providing a scaffold for the innervation of the cortex by the thalamocortical axons.
8. The SP neurons are also transiently innervated by the ingrowing thalamocortical axons before the eventual thalamocortical target neurons within cortical layer 4 settle in their ultimate positions in the cortical plate to receive their innervation. Layer 4 neurons receive innervation by both SP neurons and thalamic axons during this period followed by removal of inputs from the SP cells through a competitive process. If the SP cells are lesioned, the thalamic axons fail to innervate their correct target areas and cortical columnar organization does not develop normally. Thus, these neurons contribute to establishing functional cortical architecture during development. After performing those functions, most of these cells die.
9. The SP neurons appear to play an important but fleeting role in orchestrating early cortical development. However, although most of these cells die soon after the innervation of the cortical plate by thalamic axons and the retraction of the SP neurons' axons that innervate layer 4, many of them (10–20%) survive. These cells remain throughout development into adulthood as a compressed band along the bottom of layer 6 (cortical layer 6b or cortical layer 7 or subgriseal cells) and as dispersed interstitial neurons scattered in the white matter. It is a matter of considerable interest to know the fate of this group of surviving cells – are they quiescent, do they serve a role in guidance in the postnatal brain as they did prenatally or do they take on an entirely new function? If they change their function and/or connectivity, this suggests a form of temporal pleiotropy for these cells. As these SP cells are greatly reduced in number during development, it is possible that they serve no major functional role after this period. However, this seems unlikely and there are other examples of numerically small neuronal types that contribute in important ways through processes such as numerical expansion of target innervation by axonal and synaptic divergence; strategically positioned or particularly strong synaptic outputs (and/or potent neuromodulatory outputs). Thus, the fact that many of these cells are lost during development should not exclude the possibility that the remaining population of these cells, although relatively small in sheer number, may play some additional important role in cortical information processing. In order to evaluate such a hypothesis, it is necessary to evaluate directly the anatomical and electrophysiological properties of this reduced cohort after their initial role in cortical development and after the elimination of the majority of the cells have occurred.
10. SP cells have been shown to be particularly susceptible to or play a role in the pathogenesis of disorders including early neonatal hypoxic-ischemic injury, trisomies, microcephaly and seizures. Although their potential role in such diseases has been studied, there are few studies of the functional properties of the surviving subplate neuronal population in the normal brain, likely due to their sparseness and location, which make such studies difficult. These surviving cells express

markers typical of neurons including MAP-2 and NeuN and many express the synthetic enzyme for the production of nitric oxide (NO), nitric oxide synthase that can be visualized as NADPH diaphorase (NADPHd) activity. Not only the somata and dendrites are positive for NADPHd but that there is also considerable staining of fine processes and varicosities, suggesting the possibility that these cells may provide a diffusible signal (NO) in the white matter that could play a role in plasticity and/or pathogenesis.

11. Retrogradely transported tracers applied to the surface of the cortex (layer 1) backfill surviving WM and SP neurons' somata, indicating that their axons reach the cortical surface. This projection as well as the presence of boutons on their axons in other cortical layers have been demonstrated for individual surviving white matter and subplate neurons, where their axonal arborizations are visualized by intracellular single cell filling. These neurons also issue axon collaterals within the white matter and deep layer 6, providing the neuroanatomical substrate for them to play a role in a local functional neuronal network.
12. Surviving SP neurons generate action potentials; they receive both excitatory and inhibitory synaptic inputs; and they respond to sustained membrane depolarization with minimal spike frequency adaptation. Thus, these cells retain a neuronal phenotype, they receive synaptic inputs from other neurons and they innervate the various cortical layers. We have also recently found that these cells provide glutamatergic excitatory synaptic inputs to neurons in cortical layer 6.
13. Interestingly, GABAergic white matter neurons with projection axons have been identified in primates and we have seen a subset of GABAergic WM and SP neurons in rat using immunohistochemistry, although we have yet to record from an identified surviving presynaptic GABAergic neuron. In addition to having fast glutamatergic excitatory synaptic output, these cells also stain positively for various neuromodulators including substance P, CCK, somatostatin and nitric oxide synthase (NOS). The diversity of secreted chemicals that these surviving cells contain together with their capacity to maintain protracted non-decrementing trains of action potentials in response to a sustained depolarizing drive may afford these surviving neurons the capacity to provide strong neuromodulatory effects to cells in the overlying cortex.
14. That these cells remain as neurons but also have the capacity to play different roles at different stages of development is suggested by several factors. These include the persistence of intrinsic electrophysiological and synaptic properties, survival of the glutamatergic phenotype, receipt of excitatory and inhibitory synaptic inputs from other sources after the loss of their thalamocortical inputs and the re-arrangement of their axonal outputs from transiently innervating layer 4 to innervating all cortical layers. Much of the information about the properties of these surviving cells must, by necessity be obtained from *in vitro* brain slice preparations so there is little known about their properties within the circuitry of the intact brain. While their basic electrophysiological properties can be studied in the brain slice preparation, features such as how they process sensory information or identifying the sources of their synaptic inputs from distant sites are difficult to determine *in vivo,* since the cells are sparse (WM interstitial cells) or compressed in a thin sheet (the subplate cells at the bottom of layer 6 or subgriseal cells). Thus, although we now know somewhat more about the intrinsic and local synaptic properties of these cells in the postnatal brain, their precise function within the mature cortical network must remain somewhat speculative.
15. The surviving group of SP neurons may function as a sort of cortical gatekeeper, modulating information flow into and out of modules of overlying cortex to other cortical sites. Neurons of the nucleus reticularis thalami (NRT) proximal to thalamic nuclei carry out a similar function as a scattered cohort of GABAergic neurons that are embedded within the internal capsule and receive collateral excitatory innervation from thalamocortical axons as well as from corticothalamic axons and provide connectivity to each other within the NRT. They innervate thalamic neurons in inhibitory feedback projection from the thalamus and provide an inhibitory feed-forward projection from layer 6 of the cortex, as well. The NRT cells can modify the information processing state and the relay of information from the sensory periphery to the cortex by modulating membrane potential. The cohort of surviving white matter and subplate neurons may perform a related function in the cortex.
16. The dendritic arborizations of the WM and SP neurons within cortical layer 6 position them strategically to receive synaptic input that is otherwise destined for layer 4 from collaterals of thalamocortical axons that also arborize in layer 6. In addition, they could also receive synaptic input from the axons of cells in cortical layer 2 and 3 that send axon collaterals to layer 6 and even into the white matter. That is, these neurons could also receive a copy of information that has been processed within the cortical columnar structure and is being relayed to other cortical areas.
17. Although their somata are located within the white matter, many of the interstitial white matter neurons also have dendrites located in layer 6 where they are also positioned to potentially receive similar synaptic inputs. A difference between these neurons and the NRT cells [see point 15] is that most of the surviving subplate and many white matter neurons are glutamatergic vs. GABAergic. However, it is interesting to note that a substantial fraction of the subplate neurons are GABAergic, although we apparently have only recorded from the glutamatergic ones in our paired recordings since in all cases, the postsynaptic response was excitatory. It is not clear why our recordings should select only the glutamatergic neurons in the SP but because our results are so far limited to that sub-population, the multi-tasking behavior of these cells might be limited to certain subsets.
18. The excitatory synaptic output of the SP and WM neurons to the cortical layers above could provide either feed-forward (for the thalamocortical inputs) and/or feedback (for the cortical efferents) information. Since the surviving SP neurons are mostly excitatory and they innervate neighboring SP cells in addition to the overlying cortical neurons, they

could act as an amplification network for important signals through activating of a local network of neighboring like-type cells as well as a subset of postsynaptic targets in the overlying cortex. Recurrent excitation in such an arrangement could however, create network instability or seizures but depending on the types of cells that are targeted (e.g. glutamatergic excitatory vs. GABAergic inhibitory neurons), the properties of such a circuit may allow for selective amplification and contrast enhancement through feedback inhibition. The neuromodulatory chemicals in the SP and WM neurons such as NOS and various neuropeptides such as substance P further enhance the potential of these cells' output functions through signal gating or selective amplification that could be useful for attention, sensory learning by enhancing signal to noise ratios or changing activation thresholds and synaptic integration properties of neurons within the cortical network.

19. *Summary.* There still remain considerable issues to be resolved regarding the role of these intriguing SP neurons within the mature neocortex. For example, how do the surviving cells avoid elimination during development? Which cells provide the synaptic inputs to these neurons? What are the functional properties of these neurons *in vivo*? What role do the many neuromodulators released by these cells play in information processing? Do these surviving white matter and subplate neurons retain the capacity to re-enable early developmental processes in the adult cortex after injury or disease? The answers to many of these questions must await experiments where these cells are studied in the adult brain *in vivo* with selective targeting techniques. However, it is clear that subplate neurons perform important functions in the cortex during early development and that a substantial number of these cells organizes into a different functional network in the postnatal brain that could contribute to cortical function in other ways. Whether such longitudinal pleiotropy of neuronal phenotype applies throughout the lifespan to the aging brain and/or to other neuronal populations remains to be evaluated. If so, this would dramatically enhance the capacity of neuronal networks throughout the lifespan.

GENERAL COMMENTS AND DISCUSSION

ROCKLAND

As the authors point out (point 9 and elsewhere), the terminology for subgriseal and white matter neurons is not standardized. Even in rodent, the same neuron population can be called "layer 6B," "layer 7," or subgriseal. This important issue is likely to be more extensively addressed in the following Special Topic, on GABAergic cortical projection neurons.

ROCKLAND

The possibility of pleiotropy of cellular function in the temporal domain – serial lifespan related and/or short-term (activity related?) neuronal multi-plexing – is provocative (points 2, 5, and elsewhere). In further investigations of this point, the sparseness of this population may actually be advantageous, if cell-type specific markers can be functionally exploited.

ROCKLAND

The authors very legitimately note that strength is not always in numbers (point 15), and that this relatively sparse population may still be exerting a significant influence.

KOSTOVIC

It is interesting that subplate neurons form a well delineated cyto-architectonical layer in rodent brain-a compressed band along the bottom of layer 6 (remark 6). This cyto- architectonical correlate of the subplate feature may be explained by major differences in organization of white matter between rodents and primates: simplified corona radiata and absence of gyri in rodents. However, this also indicates a more uniform population of subplate neurons and a restricted developmental origin in rodents. The article makes very good points about the changing role and different function of subplate neurons over the course of the lifespan (remark 5), and the involvement of the subplate in the generation of action potentials. These factors will elaborate the explanation of the developmental roles, as will be presented in the next Specific topic "Cortical GABAergic Projection Neurons"

A commentary on

Individual differences in distinct components of attention are linked to anatomical variations in distinct white matter tracts by Niogi, S., Mukherjee, P., Ghajar, J., and McCandliss, B. D. (2010). *Front. Neuroanat.* 4:2. doi: 10.3389/neuro.05.002.2010.

REMARKS AND MAIN CONCLUSIONS

1. White matter tracts provide the anatomical connectivity essential for normal cognitive functioning that requires the integration of neural computation across spatially separated cortical regions such as attention and executive function abilities. This has potentially strong implications for understanding how variations in structural properties of white matter tracts from one person to another may systematically influence individual variations in efficiency across a wide range of cognitive domains, even within healthy individuals exhibiting no signs of neural or cognitive dysfunction. In support of this notion of a dimensional structure-function relationship between white matter tract microstructure and cognitive abilities, an increasing number of diffusion tensor imaging (DTI) studies have shown that individual differences in white matter microstructure are systematically linked to individual differences in cognitive domains including reading and phonological processing, numeracy and mathematical abilities, executive attention, visual attention, alerting, and memory. Additionally, it has been demonstrated that reaction time measures are sensitive to detecting variations in efficiency of cognitive domains.
2. Moreover, a growing body of evidence suggests that DTI assessments taken from different white matter tracts may correlate specifically with performance in different cognitive domains. As an example, DTI measures from two distinct white matter tracts in the same population of subjects have been found to correlate with performance in two distinct cognitive domains. We showed that within the same population correlations exist between reading ability and frac-

tional anisotropy (FA) in a left superior-inferior fiber tract, but these measures were unrelated to a similar structure-function correlation between short term memory and FA in a frontal association tract. Similarly, double dissociation findings within a single population of normal healthy adults demonstrating structural and functional specificity were found for the relationships between attention and FA in the anterior corona radiata (ACR) versus long term memory formation and the uncinate fasiculus (UF). Such findings serve to establish that specific, separable structure-function associations can be found across different neural networks, and variations in white matter tract properties are often closely linked to variations within these distinct cognitive domains. Such individual differences studies have, however, yet to examine whether such structure-function distinctions might also hold true for components within a specific cognitive domain.

3. Attention is a complex cognitive domain that has been extensively investigated by employing cognitive paradigms attempting to isolate functional components as well as by employing neural studies to investigate their associated brain systems. Although many competing theories have proposed a number of potential components of attention, one highly influential approach has proposed that three broad functional distinctions can be made that account for a multitude of findings across cognitive studies, neuropsychological investigations, and neuroimaging studies. Posner and Petersen proposed that investigating attention in terms of three separable component functional processes – alerting, orienting, and executive function – would help integrate a host of cognitive and neural investigations. Over the last two decades, these components of attention have been linked to separable brain networks via functional neuroimaging, electrophysiology, and lesion studies.
4. The efficiency of the individual components of the attention system proposed by Posner and Petersen can be separately measured using the Attention Network Test (ANT). The ANT is a reaction time task that measures the latency to decide whether a specific arrow symbol points leftward or rightward (the imperative stimulus). The ANT is designed to examine how such response times are impacted by different components of attention. To manipulate the alerting component of attention, the imperative stimulus which otherwise appears after a random time interval is preceded by a visual warning cue to alert the subject that a stimulus is about to appear. To manipulate the orienting aspects of attention, the location of the imperative stimulus which is otherwise unpredictably either above or below the central fixation cross is revealed in advance by a spatial cue that orients the subject's spatial attention to the correct location. The executive component of attention is manipulated by introducing or removing conflicting irrelevant information; the imperative stimulus is presented along with "flanking" arrows on either side that either point in the same direction or in the incongruent (conflicting) direction. Examining how these three classes of manipulations (alerting, orienting, and conflict) impact response times provides an assay for the efficiency of each of these three components of attention. Given the simplicity and sensitivity of the ANT to three largely independent components of attention, it has been used in children and adults in normal populations, and in cohorts with neuropsychiatric disorders such as ADHD, schizophrenia, and borderline personality disorder.
5. By approaching the human attention system as one comprised of several separable networks, one can create paradigms in which they operate in a fairly independent fashion in order to examine them individually. Alternatively, different paradigms may prove useful in studying the ways in which these systems interact.
6. Functional and neuropsychological studies have associated performance in conflict tasks (executive control) to the frontal cortex, and more specifically to a network including the anterior cingulate gyrus and lateral prefrontal cortex. The conflict component of attention mediates inhibitory control, resolution of conflicting stimuli impacting decision making, and, in a broader sense, can be considered necessary for decision planning and decision making. This network likely includes white matter tracts that serve to connect these regions with other structures.
7. The frontal lobes contain numerous complex connections with different parts of the brain, many of which pass through the thalamus. As such, it is likely that tracts from the thalamus extending to the frontal lobe and anterior cingulated gyrus, such as the ACR, may be part of the executive attention network associated with the conflict component of the ANT. Indeed, a recent study demonstrated that white matter integrity along the left ACR correlated significantly with conflict performance from the ANT in a group of normal adults and also in a cohort of adults with mild traumatic brain injury.
8. The alerting component of attention is proposed to be responsible for activating the required cognitive systems to make the person ready to respond to a task. This form of phasic alerting is modulated by thalamic, frontal, and parietal regions. Although the reticular activating system is known to be necessary for tonic alertness, it also plays a critical role in phasic alerting. A likely white matter pathway that connects the proposed regions critically involves the internal capsule. The internal capsule is made up of an anterior limb (ALIC), and posterior limb (PLIC) and the bend between the two limbs referred to as the genu. The ALIC and PLIC directly relay motor and sensory information with ascending and descending fibers between the cerebral cortex and the pyramids of the medulla.
9. The orienting network selects spatial and sensory information. Commonly, this is tested with visual cues indicating the location of an impending target (as in the ANT). The visual orienting system has been associated with brain areas such as the superior and inferior parietal lobes, frontal eye fields, and subcortical areas including the superior colliculus and reticular nuclei in the thalamus. In considering white matter tracts likely to modulate the efficiency of the orienting network, the optic radiations relay visual information via neurons from the lateral geniculate nucleus of the thalamus to the visual cortex. Such geniculocortical circuitry has been implicated in attention studies. Additionally, the orienting system must relay and compare spatial infor-

mation from both visual fields which requires connectivity between hemispheres. Lesions studies of the splenium of the corpus callosum, fMRI studies of interhemipsheric transfer, and studies examining callosal thickness in ADHD suggest that commissural fibers, particularly the splenium of the corpus callosum may play a large modulatory role in the function of the visual spatial orienting network.

10. Prior neuroimaging studies focused primarily on functional activations using fMRI or EEG to isolate the anatomic substrates for the attention networks. These studies have focused on the functional activations (i.e. gray matter) involved in the attention components. It remains unclear what specific white matter pathways modulate each component of attention. As has been demonstrated in several other domains reviewed above, individual differences in white matter microstructure within tracts associated with particular attention networks may closely correlate with variations in efficiency of these attentional processes.
11. One technique to quantify white matter integrity is DTI. The principle governing DTI is that water diffuses more readily along the orientation of axonal fibers than across the fibers due to hindrance from structural elements such as the axolemma and the myelin sheath. The degree of directionality is termed anisotropy. Anisotropy can be measured as the variation in the eigenvalues of the diffusion tensor. Fractional anisotropy (FA), a normalized measure of anisotropy, has been shown to be sensitive to microstructural changes in white matter integrity and organization. Increasing numbers of DTI studies that correlate FA with cognitive function indicate that such measurements can be used to account for a wide range of cognitive skill.
12. *The Conflict Network*. The correlation analysis together with the multiple regression dissociation analysis provides evidence that microstructural integrity of the ACR modulates executive attention. The finding of a frontal tract associated with executive attention is strongly supported by previous literature. Functional imaging studies have provided strong consistent support for the notion that the frontal lobes, particularly the anterior cingulate gyrus, is associated with attentional control during the Stroop Task and the conflict portion of the Attention Network Task. Additionally, neuropsychological data clearly highlight the central role of the frontal lobes in executive attention. Previous DTI studies have also implicated frontal white matter tracts with attention performance. For example, integrity of white matter tracts associated with brain activity impact executive attention. Furthermore, we demonstrated that a closely related frontal executive skill, short term memory, also demonstrated strong correlations with ACR in children. Finally, in a recent study of 43 patients suffering from mild traumatic brain injury, the left ACR integrity assessed by FA was shown to correlate with conflict scores on the ANT. It is interesting to note that in the current study, significant correlation between conflict scores and FA did not reach significance in the right ACR, but appeared only as a non-significant trend. Given this trend, and no significant finding of a direct effect of laterality it is possible that both left and right ACR regions play a role in conflict, and the specific contributions of right ACR may be apparent in studies with greater power.
13. *The Orienting Network*. Regarding the orienting network, the correlation analysis together with the multiple regression dissociation analysis demonstrates that microstructural integrity of the splenium of the corpus callosum modulates the efficiency of the orienting component of attention. This finding is consistent with previous findings that demonstrate that the visual-spatial attentional orienting system is dependent on a functional network that includes left and right posterior parietal regions, and is linked to a larger neural network including frontal eye fields, and subcortical areas including the superior colliculus and reticular nuclei in the thalamus. We propose that the splenium of the corpus callosum includes white matter tracts involved in this overall network, and that this region provides a large convenient homogenous white matter region of interest that can be used to assess individual differences in this network. Our more specific findings implicating the splenium of the corpus callosum as part of the network that modulates orienting performance is consistent with Noudoost and coworkers' report of a case study that demonstrates the role of interhemispheric connections in making an integral visual map across hemifields that can be used for visual spatial attention. Furthermore, splenium of the corpus callosum lesions commonly cause visual spatial neglect, a deficit that specifically impacts orienting functions of attention. Such lesions can also cause other forms of disconnection syndromes that may be linked to spatial orienting functions as in the case or hemialexia.
14. *The Alerting Network*. The correlation analysis together with the multiple regression dissociation analysis provides evidence that the PLIC modulates individual differences in the alerting component of attention. This relationship is generally consistent with the previous literature suggesting a role of the PLIC for this component of attention. For example, Sturm and coworkers presented PET evidence from normal volunteers that suggested alerting functions involve a vast network of regions, including inferior parietal-thalamic networks, brainstem structures, and frontal regions, largely lateralized to the right hemisphere. Fimm and coworkers investigated alerting related impairments of attention in 15 patients with acute circumscribed vascular lesions confined to the basal ganglia, internal capsule, and thalamus. In his study, five out of seven patients showed evidence of lesions to the PLIC of the internal capsule. Fimm and coworkers suggested that thalamo-parietal projections transversing through the PLIC via the superior peduncle of the thalamus could lead to a disconnection of functionally relevant structures that impair attention.
15. Next, we turn consideration to the separability hypothesis regarding the three attentional networks. In evaluating these specificity findings, it is important to consider that the ANT was specifically designed to stress the potential conditions under which the three attention networks could be shown to be separable in their operations and individual difference

patterns. More recently, several investigations have begun to explore other conditions under which these networks might interact and influence one another in important ways. To the degree to which the ANT exposes these networks to be anatomically and functionally separable, it is possible that white matter tract integrity is an important *independent modulator* of function, such that individual differences in white matter tract microstructure in one network might be functionally associated with one component of attention relative to the other components of attention. This hypothesis was addressed by a series of multiple regressions, in which the variance in function for each established white matter tract ROI was assessed for the key proposed associated function, while controlling for the influence of the other component attentional functions.

16. Results of this multiple regression analysis demonstrated three key findings: the ACR is a unique modulator (relative to the other two ROIs) of the conflict network function, the PLIC is a unique modulator of the alerting network, and the splenium is a unique modulator of the orienting network. This correlational triple dissociation can be interpreted similarly to the "gold-standard" double-dissociation tests in neuropsychology commonly required when attempting to establish specificity of structure-function relationships. Such findings are critical in lesion studies in which the mere presence of any form of brain damage can cause widespread, non-specific difficulties in any performance assessment of cognitive function, and establishing specificity of functional and structural loss is critical. Similarly, in linking individual differences between white matter properties and cognitive performance, establishing similarly high levels of specificity between particular regions and particular functions is critical.
17. In addition to showing functional specificity, the correlational dissociation analysis shows that within each functional network, a gradient of individual differences in function positively correlates with a gradient of individual differences in white matter tract microstructure. Furthermore, since this study focused on normal healthy adults, rather than reflecting the impact of neural damage on related loss of function, the results may be more relevant to understanding normal population variations in white matter tract microstructure and how such differences are linked to efficiency of components of attention. As such, these findings may provide the basis of relating dimensional differences in structure-function relationships that exist within normal populations with more extreme ranges of variation of structural damage and related loss of function.
18. In conclusion, combining these observations with previous fMRI and neuropsychological studies suggests the components of attention are comprise of segregated functional networks and that individual differences in white matter tract microstructural integrity might modulate these functionally specific neural networks. Thus, understanding individual differences in white matter tract microstructural integrity may prove an important complement to fMRI studies of functional organization of cortical function.

GENERAL COMMENTS AND DISCUSSION

HÖISTAD AND HOF

In regard to point 12, a recent fMRI study (Fan et al., 2008) using the ANT and examining physiological response of several brain regions in terms of interactions between conflict processing and activity of the anterior rostral cingulate cortex, and the effective connectivity between it and other cortical domains using psychophysiological interaction analysis and dynamic causal modeling showed a significant integration of the anterior cingulate with the caudal cingulate zone of the ACC and the lateral prefrontal, primary, and supplementary motor areas above and beyond the main effect of conflict and baseline connectivity. The intrinsic connectivity from the anterior to the caudal cingulate cortex was modulated by the context of conflict, indicating that conflict processing is associated with the effective contribution of the rostral cingulate to the neuronal activity of the caudal cingulate cortex, as well as other cortical regions.

A commentary on

The effects of normal aging on myelinated nerve fibers in monkey central nervous system by Peters, A. (2009). *Front. Neuroanat.* 3:11. doi: 10.3389/neuro.05.011.2009.

REMARKS AND MAIN CONCLUSIONS

1. There are two types of nerve fibers in the central nervous system, myelinated and unmyelinated ones. The myelinated nerve fibers are axons of neurons that are ensheathed by internodal lengths of myelin formed by oligodendrocytes. Developmentally, the internodal lengths of myelin are produced at the ends of processes of oligodendrocytes and each internode is generated by a spiral wrapping of a paired sheet of oligodendrocytic plasma membrane. Initially the successive turns of the spiral of paired membrane sheets are separated by cytoplasm, but eventually the cytoplasm is extruded from between the turns. As a result, mature, compact myelin is formed. At the ends of each internodal length of myelin are regions called paranodes, and here the turns of the spiral wraps of myelin membrane successively terminate, the innermost one terminating first. As the turns of myelin terminate, the sheath gradually becomes thinner, and eventually ends at the nodes of Ranvier, which separate the successive internodal lengths of myelin. At the nodes the axon is bare, but possesses a dense undercoating.
2. An oligodendrocyte forms several internodal lengths of myelin, each one on a different axon, and in general the larger the diameter of the axon, the thicker is its myelin sheath and the longer its internodes and its paranodes. And since there seems to be some limit to the amount of myelin an individual oligodendrocyte can produce and maintain, oligodendrocytes that myelinate small diameter axons form more internodal lengths of myelin than those that myelinate larger diameter axons.
3. Myelin contains lipoproteins, so that in unfixed brains the myelin sheaths have a white sheen. Consequently, tracts of the central nervous system that contain mostly myelinated nerve fibers and few neurons are referred to as white matter. In contrast, gray matter contains the cell bodies and dendrites of neurons and fewer myelinated nerve fibers.

4. The first hint that there are age-related changes in myelinated nerve fibers came from the observation that in old humans and monkeys there is a decrease in the intensity of haemotoxylin staining of white matter. The underlying reason for this increased staining pallor is still not clear, but it is now know that there are a number of age-related alterations of myelinated nerve fibers in the primate central nervous system, such as a loss of some myelinated nerve fibers and alterations in the morphology and composition of myelin sheaths, that could account for the decrease in staining intensity.
5. Magnetic resonance imaging (MRI) studies of both human and monkey brains have shown there is a loss of white matter from the cerebral hemispheres with age.
6. Over the life span of the monkey the average number of myelinated nerve fibers lost from the optic nerve and from the anterior commissure is about 45%, while from the fornix and the splenium of the corpus callosum, the loss is about 25%. In all four structures the correlations between the decreasing numbers of myelinated nerve fibers and increasing age are significant.
7. In contrast, there is no measurable loss of myelinated nerve fibers from the visual cortex, but the inability to detect a loss may be due to the relatively sparse numbers of myelinated nerve fibers present in cortex, because a few myelinated nerve fibers with degenerating axons have been seen in cortex. Indeed myelinated nerve fibers with degenerating axons, as indicated in the electron microscope by the presence of dense axoplasm with a loss identifiable organelles, or the presence of empty myelin sheaths, have been encountered in all of the parts of the aging monkey brain that we have examined, suggesting that myelinated nerve fiber loss is ubiquitous. And based on earlier studies of Wallerian nerve fiber degeneration, there is little doubt that once an axon degenerates, breakdown and degeneration of its myelin sheath inexorably follows.
8. Myelinated nerve fiber loss from white matter in pathways must result in some disconnection between various parts of the central nervous system. But interestingly, although there are no significant correlations between the extent of myelinated nerve fiber loss from the splenium of the corpus callosum and the cognitive decline shown by monkeys, there are correlations between cognitive decline and myelinated nerve fiber loss from the anterior commissure and the fornix. In this context, it is interesting that cutting the splenium of the corpus callosum, which is the principal fiber pathway connecting the occipital cortices, has little effect on cognition. The anterior commissure provides the interhemispheric connection between the entire temporal lobe, as well as parts of the orbitofrontal cortex, prepiriform cortex and the amygdalas, and numerous studies have shown that the anterior commissure provides a pathway whereby visual information can reach the opposite hemisphere and contribute to behavioral responses, such as two-choice discrimination. The fornix, on the other hand, carries the main output from the hippocampus, and studies of the effects of lesioning the fornix in both monkeys and humans have revealed the role of the fornix in memory and have described amnesia as a major consequence of making such lesions.
9. It might be assumed that since myelinated nerve fibers are lost from white matter with age, that there must be a concomitant loss of the neurons from which the nerve fibers arise. For the optic nerve, this may be the case, since retinal ganglion cells are subject to damage from ocular changes and systemic disease that occurs frequently in the elderly. But for the other central nervous system pathways, in which the myelinated nerve fibers arise from cortical neurons, a different reason has to be sought, because, as stated above, recent studies have shown that in normal aging few neurons are lost from the cerebral cortices of either monkeys or humans. Freeman and coworkers have recently shown that in normally aging humans, cortical neuron numbers are preserved even when there is cortical atrophy. To account for the age-related loss of myelinated nerve fibers from white matter, we have suggested that only the portion of the axonal plexus of a pyramidal cell that enters the white matter, degenerates by a dying back process, leaving the more extensive local axonal plexus in the cortex intact. This scenario would account both for the loss of some myelinated nerve fibers from white matter and for the failure to detect myelinated nerve fiber loss from the cerebral cortex itself.
10. Obviously, in normal aging some myelin sheaths degenerate as a consequence of their axons degenerating, but in other cases myelin sheaths degenerate even though the axon is intact. In the latter category there are two kinds of myelin sheath alterations. The most common age-related degenerative alteration is an accumulation of dark cytoplasm in pockets that are produced by a splitting of the major dense line The location of the dense cytoplasm in splits of the major dense line implies that the cytoplasm must be derived from the parent oligodendrocyte, because the major dense line of the myelin sheath is produced by apposition of the cytoplasmic faces of the plasma membrane of the oligodendrocyte forming the myelin sheath.
11. Proof of the fact that the accumulation of dense cytoplasm in normal aging is a degenerative change comes from studies of cuprizone toxicity, which leads to oligodendrocyte death. This results in the formation of dense cytoplasm in the cytoplasmic process on the inner face of the myelin sheath.
12. Another, but less common myelin alteration associated with aging is the formation of myelin balloons. These balloons can be as large as 10 μm in diameter, so that even by light microscopy the larger balloons are visible as holes in the neuropil of the aging cortex. Electron microscopic analyses show that these holes are really localized fluid-filled cavities that are accommodated by splits in the intraperiod line of the affected sheaths, and since the intraperiod line is produced by apposition of the outer faces of the cytoplasmic membrane of the oligodendrocyte, the fluid-filled sacs are potentially in contact with the extracellular space.
13. There is evidence that the formation of balloons is a degenerative process, since myelin balloons can be produced by cuprizone and tetraethyl tin toxicity, and by chronic copper poisoning. Balloons can also occur in early phases of Wallerian degeneration and in severe diabetes.

14. When the percentage of myelinated nerve fibers showing either the presence of dense cytoplasm or of balloons is examined, it is found that the frequency of such profiles increases significantly with age. More importantly, there are significant correlations between cognitive declines and the frequency of profiles of degenerating sheaths in cortical area 46, splenium of the corpus callosum, anterior commissure, and fornix. An exception is primary visual cortex, in which there is no correlation between cognitive decline and myelin sheath degeneration. This may be because primary visual cortex has little role in cognition. It is presumed that the correlations between myelin degeneration and cognition are due to the degeneration resulting in a slow down in conduction velocity. This would adversely affect the timing in neuronal circuits.
15. Duce and coworkers have identified a number of genes that might produce cytotoxicity in white matter. These genes range from ones that can affect life span, to ones that can affect the reorganization of glial cytoskeleton. Others can produce oxidative and proteolytic injury, and yet others are cell cycle inhibitors. But these authors focus particular attention on a gene called Klotho, a multifunctional gene that is known to defend against oxidative stress, and suggest that with a decrease in the activity of Klotho there is a loss of this protection, which may result in the death of oligodendrocytes.
16. There are other age-related alterations in myelin sheaths, which indicate that myelin continues to form with age. The first is an increase in the overall thickness of normal myelin sheaths with age. However, the increase in thickness of sheaths is not uniform. The mean increase in the numbers of lamellae is largely because thick sheaths, with more than 10 lamellae, become more common in old monkeys.
17. Another change that is considered to indicate the continued formation of myelin is the formation of sheaths that contain redundant myelin, so that the sheaths are too large for their enclosed axons. When such sheaths are cross-sectioned and examined by electron microscopy the axon is seen to be located at one end of an excessively large sheath that loops off into the surrounding neuropil.
18. When the frequencies of various kinds of profiles of myelinated nerve fibers are quantified in the vertical bundles of nerve fibers in the cerebral cortex, it becomes evident that the frequency of profiles of paranodes increases with age.
19. We have not been able to identify demyelinated nerve fibers in the monkey brain, but this should not be a surprise, since such demyelinated nerve fibers would be expected to resemble unmyelinated nerve fibers. However, in support of the fact that demyelination is taking place, we have seen fragments of degenerating myelin within the cytoplasm of both microglia, and more commonly within astrocytes in the brains of aging monkeys. Also some of the amorphous phagocytosed material within the cytoplasm of astrocytes in the cerebral cortex of old monkeys labels for antibodies to myelin basic protein.
20. In monkey cerebral cortex stained with Perl's reaction for ferric iron the processes of some oligodendrocytes in old monkeys show swellings along their lengths, and when these swellings are examined in the electron microscope it is seen that they contain dense inclusions. Most probably the material is produced by degeneration of some components of the myelin sheaths that belong to the oligodendrocytes, and it is tempting to suggest that the material is related to the dense cytoplasm that accumulates between the lamellae of some sheaths in old monkeys.
21. It is also common in old monkeys to find oligodendrocytes in pairs, rows and groups, suggesting that oligodendrocytes may be proliferating with age, and when comparisons are made between the numbers of oligodendrocytes in young and old primary visual cortices it is evident that there is an increase in the numbers of oligodendrocytes with age. In contrast, there are no changes in the frequency of either astrocytes or microglial cells with age.
22. What is the origin of the increased numbers of oligodendrocytes that are generated, and why are they necessary? The formation of groups and rows of oligodendrocytes during aging could be taken to suggest that oligodendrocytes are dividing, but the prevailing view is that mature oligodendrocytes do not divide, and in a study of the generation of new cells in the adult dentate gyrus of the hippocampus in old monkeys using BrdU labeling no labeled oligodendrocytes were found. It is more likely that new oligodendrocytes originate from the oligodendroglial precursor cells which express NG2 chondroitin sulfate. These cells are scattered throughout the central nervous system, and in adult rodents they account for about 5% of all neuroglial cells.
23. Moreover, Rivers and coworkers have recently shown that in adult mice many of the newly generated oligodendrocytes that arise from the oligodendrocytic precursor cells during adulthood are involved in myelination. They calculate that about 20% of all oligodendrocytes in the adult corpus callosum are generated during adulthood and that many of these cells form myelin. In contrast, the same group calculates that only about 5% of the adult-born oligodendrocytes in the cerebral cortex appear to be involved in the elaboration of myelin sheaths.
24. Unfortunately there is no information about the rate of turnover of oligodendrocytes in the adult monkey, but there is no reason to doubt that it is significantly different from in rodents.
25. *A synthesis*. It is proposed that the following scenario can explain the available data on the effects of age on myelinated nerve fibers in the central nervous system of the monkey.
    a. During aging some neurons lose their long projecting myelinated axons that enter white matter, while retaining their local plexuses so that the parent neuron does not die. The consequence of this is that, as has been demonstrated, some myelinated nerve fibers are lost from white matter, even though there is no significant loss of neurons from the cerebral cortex. For other neurons the effects of aging are less severe, since their axons remain intact, even though some of the internodal lengths of myelin that ensheath them degenerate.

b. The process of demyelination probably begins as an oligodendrocyte shows stress and starts to accumulate dense inclusions in swellings of its processes and in its perikaryon, as well as in spaces between the lamellae of the myelin sheaths for which the oligodendrocyte is responsible. Ultimately the oligodendrocyte dies, which results in the degeneration and loss of the internodal lengths of myelin belonging to that oligodendrocyte. Oligodendrocyte precursor cells are then activated and generate new oligodendrocytes that repair the damage by remyelinating the bare lengths of axons.
c. In the process of remyelination, several new oligodendrocytes are involved in the replacement of the original internode of myelin. These oligodendrocytes produce shorter internodal lengths than the original one, and the new sheaths are thinner.
d. Thus, when profiles of sectioned myelin sheaths in older monkeys are examined, it is found there is an increase in the number of profiles of paranodes, and this is accompanied by an increase in the total number of oligodendrocytes. This breakdown of myelin sheaths, together with the formation of shorter internodal lengths of myelin and the consequent increase in the number of nodes of Ranvier, would result in a slowing down of the rate of conduction along affected myelinated nerve fibers. Consequently the timing in neuronal circuits would be affected and contribute to cognitive impairment that occurs with increasing age.

GENERAL COMMENTS AND DISCUSSION

DeFELIPE

Comment on point 16:
Is it possible that thin myelinated axons are more vulnerable with age and, therefore, thicker axons may seem to be more common or are there quantitative studies regarding this subject?

ROCKLAND

I wonder if there is any "intercommunication" or shared signal among the different axons that are myelinated by any given oligodendrocyte? Or, similarly, between those oligodendrocytes that myelinate a given segment of axon (point 2)?

ROCKLAND

Peters notes several conditions that could be assumed to result in circuitry-significant changes in conduction velocity. In this regard, see Kimura and Itami.

HÖISTAD AND HOF

There is a loss of white matter (WM) from the cerebral hemispheres with age. Peters presents some of the observed ultrastructural morphological changes that may occur in WM during aging, including myelin balloons, redundant myelin, split sheaths and sheaths with dense cytoplasm. He discusses evidence that redundant myelin may be due to uncontrolled production of myelin with age, while thicker myelin sheaths may be evidence for the continued formation of myelin with aging. Some questions that arise are whether the myelin changes observed in aging are different in WM versus the grey matter, and if age-related changes in the grey matter can be area or layer specific. In addition, how are normal aging alterations in myelin different from those observed in disease, for example in schizophrenia (Uranova et al., 2001). Is there a commonality between normal aging and alterations seen in disease, which could lead to comparable alterations of neuronal communications and result in specific cognitive and behavioral changes?

A commentary on

Oligodendrocyte development and the onset of myelination in the human fetal brain by Jakovcevski, I., Filipovic, R., Mo, Z., Rakic, S., and Zecevic, N. (2009). *Front. Neuroanat.* 3:5. doi: 10.3389/neuro.05.005.2009.

REMARKS AND MAIN CONCLUSIONS

1. The origin and differentiation of oligodendrocytes have been extensively studied in animal models, and are especially well documented in rodents, thanks to advances in various molecular biology techniques that have provided the means of genetic mapping of cell lineages in the developing mouse brain. Along the neural tube oligodendrocytes are produced ventrally under the influence of the morphogen Sonic Hedgehog (Shh), and migrate at the progenitor stage to dorsal regions. It has been shown, however, that after the initial wave of ventrally derived oligodendrocytes, they are completely replaced by a dorsally derived population. In the ventral diencephalon and telencephalon, Shh influences the expression of oligodendrocyte lineage genes, Olig1 and Olig2, which are basic helix-loop-helix (bHLH) transcription factors. The question, however, remains to which extent could observations obtained on these animal models relate to human brain development.
2. Early oligodendrocyte progenitor cells are characterized by their expression of platelet derived growth factor receptor alpha (PDGFRα) and NG2 proteoglycans, and by a typical morphology with few ramified processes. These cells are still mitotic, so we consider them "progenitors". Later along the oligodendrocyte lineage, cells are not considered to be proliferative any more. Thus in this review, we use the term "precursors" for these cell types. We detected the first PDGFRα expressing (PDGFRα$^+$) cells in the forebrain of 10-gw old fetus, but they appear in higher numbers only around 15 gw, when they are most numerous in the ganglionic eminences and in the cortical VZ/SVZ. By midgestation (19–22 gw) oligodendrocyte precursor cells invade more dorsal areas of the telencephalic wall, including the cortical plate. During the whole period of our observation (from the onset of neurogenesis, at 5 gw, until the beginning of the third trimester, 24 gw) early oligodendrocyte progenitors were most dense in the cortical SVZ, consistent with their origin from this secondary proliferative zone in the human cortex.
3. In the next stage in oligodendrocyte development, late oligodendrocyte precursor cells are characterized by O4 immunoreactivity, whereas pre-myelinating oligodendrocytes are reactive to O1 antibody. O4 and O1 antibodies were raised against glycoproteins in the oligodendrocyte membrane and are shown to specifically label cultured late oligodendrocyte precursor cells. Staining of tissue sections with these antibo-

dies is often hampered by the instability of the glycan epitopes to fixation. Earlier study of rat brain sections has shown that, indeed, it is not fixation but freezing that disperses the antigens and hampers immunostainings. In our studies, however, we have been able to detect O4 and O1 expressing cells in human brains when we omitted detergent from the blocking solution.

4. In the human forebrain at midgestation (20–22 gw), we reported that $O4^+$ and $O1^+$ cells are especially dense in the subplate layer, immediately below the cortical plate. This distribution of late oligodendrocyte precursors raises a possibility that transient subplate layer is important for maturation of oligodendrocytes. Considering the importance of a transient subplate layer which receives the thalamic afferents essential for proper wiring of cortical neurons, it is tempting to speculate that oligodendrocytes come to the subplate to obtain yet unknown signals necessary for their maturation and for myelination of the axons in correct sequence.
5. Maturation of oligodendrocytes is marked by the expression of myelin proteins, and the two major myelin proteins, myelin basic protein (MBP) and proteolipid protein (PLP), are the first to be expressed at detectable levels. MBP was detected very early in human embryonic brains (around 5 gw), but this expression was attributed to Golli/MBP splice variants. In the forebrain, the first MBP^+ cells of typical oligodendrocyte morphology were found at 18 gw, around the middle of intrauterine development. MBP^+ cells are scattered through the intermediate zone, the future white matter, and increase in numbers with progression of development. Our evidence supports the ventral to dorsal progression of oligodendrogenesis, also reported in rodents. Indeed, a ventro-dorsal gradient in the extent of myelination and in oligodendrocyte precursor cells density was seen in the human fetal forebrain.
6. In the rodent forebrain oligodendrocytes are initially derived from ventrally positioned ganglionic eminences (GE), whereas later they originate from the dorsal cortical SVZ. In human fetal forebrains at midgestation a subpopulation of cortical oligodendrocyte progenitor cells was expressing Dlx2 and Nkx2.1, transcription factors specific for ventrally derived cells in rodents. However, as these transcription factors show a strong signal in the human proliferative zones of both GE and cortex, the origin of human Dlx2- and Nkx2.1-expressing oligodendrocyte progenitor cells could not be simply determined. Another large population of oligodendrocyte progenitor cells at this stage did not express Dlx2 and Nkx2.1 transcription factors, and could represent a population of dorsally derived oligodendrocytes in the human brain. Yet a third population of cells co-labeled with oligodendrocyte progenitor markers (PDGFRα, NG2, Olig1), stem cell marker nestin, and markers of cells from hematopoietic lineage (CD34, CD68) was present in what appears to be a stream of cells migrating between the GE and the cortical SVZ. These combined findings suggest multiple origins of human cortical oligodendrocytes and have broad implications for normal brain development as well as white matter pathologies.
7. It is possible that oligodendrocytes derived from various sources have different roles, or myelinate different axonal pathways. This becomes especially important and demands further investigation in the light of reports that implicate oligodendrocytes in other functions in addition to "traditional" myelin formation, including synaptic regulation and signaling at the nodes of Ranvier.
8. Studies of the origin of oligodendrocytes in rodents have shown that the ventral morphogen Shh is essential for early specification of oligodendrocyte progenitors in the neural tube. Shh activates a cascade of transcription factors including Pax10, Dlx2, Nkx2, Olig1 and Olig2. Olig2 was described in neural progenitors that give rise to both motor neurons and oligodendrocytes in the ventral spinal cord region. Olig genes belong to a bHLH group of transcription factors that are necessary and sufficient for generation of oligodendrocytes and for myelination in mice. In the human fetal brain we demonstrated the expression of Olig2 in the GE and the medial cerebral cortex starting as early as 5 gw, before the onset of either neurogenesis or oligodendrogenesis. Our study also revealed that various cell types later in development express both Olig1 and Olig2. Most notably, Olig2 is expressed in all MBP^+ cells in the human fetal forebrain and spinal cord at midgestation, and in around 50% of early oligodendrocyte progenitors in the SVZ. At the same time and at the same location, cortical SVZ, a subpopulation of $MAP2^+$ neuronal progenitors is also $Olig2^+$. In contrast, mature neurons in the cortical plate labelled with either NeuN or GABA antibodies, do not co-express Olig2. Taken together these observations suggest the existence of a common progenitor cell for oligodendrocytes and at least some neuronal classes in the human forebrain.
9. Genetic mapping and heterologous transplantation fate mapping studies in mice have shown that the domain within medial ganglionic eminence gives rise to cortical interneurons. There is, however a controversy about the necessity of Olig2 for cortical interneuron fate determination. When Olig2 was ablated from cortical progenitors no change in interneuronal populations was observed. One possible explanation for this finding could be that other bHLH genes, like Olig1, compensate for it. In our study we found Olig1 co-localized in vimentin-expressing cortical radial glia cells, a cell type demonstrated to be a multiple neural progenitor. We have no evidence that in human brain Olig1 is co-expressed in neuronal populations, but that does not preclude the possibility since by the time neurons acquire their fate, this gene may get, down-regulated. This inability to draw conclusions about temporal developmental events is an intrinsic limitation of all studies on fixed human tissue, and is the most important reason why we resorted to different *in vitro* systems.
10. *In vitro*, Olig2 plays a role in self-renewal of mouse neurosphere cultures, and in differentiation of neurons and oligodendrocytes in appropriate condition media. In cultures with growth factors (FGF2 and EGF), Olig2 was expressed in almost all progenitors derived from mouse GE or cortex, whereas dorsal transcription factors, Pax6 and Emx1,

were downregulated. In contrast, in cryosections of human lateral ganglionic eminence at 15–20 gw, Olig2 and Pax6 were co-expressed in the same progenitor cells. This finding was extended to cell cultures from cortical VZ/SVZ. Co-expression of these two transcription factors in dividing progenitor cells is consistent with an increased complexity of human neural progenitor cells in comparison to other mammals.

11. We reported that in the VZ/SVZ of the fetal forebrain, cells occasionally co-express radial glia cells markers and oligodendrocyte lineage markers, suggesting lineage relationship. Radial glia are multipotent progenitor cells in rodents, generating projection neurons and a subpopulation of forebrain oligodendrocytes. Similar to these findings, radial glia cells are also neuronal and oligodendrocyte progenitor cells in human fetal brain.
12. Dorsal transcription factor Pax6 has been reported to be important for generation of neurons in rodents, and also in humans. Pax6 however may also have a role in oligodendrogenesis. Other transcription factors, such as Olig1 and Olig2 may be necessary to specify radial glia as oligodendrocyte progenitors. Indeed we have observed that Olig1 and Olig2 can be co-expressed with the radial glia marker vimentin along the ventricular zone at 15 gw. It would be important to determine whether a majority or only a specific subtype of human oligodendrocytes is generated from radial glia cells and whether radial glia are the only early oligodendrocyte progenitors in the cortical VZ/SVZ. Alternatively radial glia cells could generate oligodendrocytes only at a specific developmental stage or at a particular brain region, such as the cortical VZ/SVZ.
13. Formation of myelin during primary myelination and its restoration in remyelination, involves proliferation, migration and differentiation of oligodendrocyte progenitors into myelin-forming oligodendrocytes. Growth factors, cytokine members of the interleukin (IL) superfamily, chemokines, such as growth-related oncogene alpha (GRO-α) also referred to as CXCL1, and other mediators influence oligodendrocyte progenitor cells' proliferation and/or differentiation, and are also very important for remyelination. Moreover, it has been demonstrated that this chemokine is upregulated during experimental autoimmune encephalomyelitis (EAE) and around lesions in multiple sclerosis. We studied the distribution of chemokine CXCL1 and its receptor CXCR2 in human developing forebrain, and reported that it is highly expressed in cortical VZ/SVZ in accord with an earlier study in rodents. We demonstrated that in contrast to rodents where CXCL1 directly induces oligodendrocyte proliferation, in human fetal brain CXCL1 has an indirect effect, acting through astrocyte secretion of IL-6, to increase oligodendrocyte proliferation.
14. In the absence of microglia or astrocytes, MBP^+ oligodendrocytes had less branched processes and their number was reduced as well. Similar to findings on proliferation, the differentiation of oligodendrocyte progenitors was decreased by neutralization of IL-6, but not of CXCL1 secreted from astrocytes. We propose that a novel interaction between astrocytes and oligodendrocytes, the one that includes CXCL1/IL-6 signaling pathway, enhances development of human fetal oligodendrocytes.
15. During remyelination in multiple sclerosis, basic processes of oligodendrocyte development including proliferation, migration and differentiation of oligodendrocyte progenitors are recapitulated. For that reason it is of extreme importance to understand these processes during normal oligodendrogenesis. We describe here that constitutive expression of CXCL1 has physiological relevance, whereas exogenous CXCL1 might be relevant in pathological conditions, such as developmental disorders of white matter, or in multiple sclerosis.
16. A wide variety of signals that may be involved in initiation of myelination of CNS axons has been reported. Although mouse oligodendrocytes *in vitro* have the capacity to form myelin-like membranes in the absence of neurons, co-culture with neurons significantly increases MBP gene expression in these cultures, suggesting that axons are necessary for efficient myelination.
17. *In vivo* experiments in the rat optic nerve demonstrated that oligodendrocyte proliferation and subsequent myelination depend on the electrical activity in axons. Other studies have confirmed that ion channel activation or electrical activity in axons can regulate myelination, suggesting that oligodendrocytes preferentially myelinate axons which fire action potentials.
18. Myelination in the human brain progresses over several decades, which is much longer than a complete lifespan of the commonly studied animals. Recently emerged data suggest that myelination may play an important role in data processing by neurons. On the one hand, it is now clear that human white matter changes with experience, i.e. that myelinating glia respond to environmental cues, as shown by magnetic resonance imaging of neglected children. On the other hand, myelin thickness influences conduction velocity, which is in turn instrumental in regulation of synchronous firing of action potentials and proper function of the brain.
19. Several myelin proteins, including Nogo-A, MAG and MOG cause the growth-cone collapse that prevents axons from reaching their targets. This feature of myelin is now appreciated as an important regulatory mechanism to suppress sprouting and formation of abnormal connections in development.
20. Before myelination is initiated, oligodendrocyte precursors transform first to pre-myelinating oligodendrocytes and then into mature myelin-producing cells. During this process, myelin proteins, MBP and PLP, shift their expression from cell bodies to processes that form myelin sheaths. In humans there is a clear dissociation between the time of oligodendrocyte differentiation and the beginning of myelination in the fetal forebrain.
21. We demonstrated that a prominent increase in myelination coincided with the down-regulation of a polysialic acid conjugated form of the neural cell adhesion molecule (PSA-NCAM). This finding is consistent with the notion that a PSA-mediated signaling mechanism might be one of the regulators of primary myelination in the human fetal brain.

It is important to stress that PSA-NCAM is not the only cell adhesion molecule to be implicated as an axonal inhibitor of primary myelination. Many other molecules, for example neural adhesion molecule L1, have been shown to be downregulated from the axonal surface upon the onset of myelination. This suggests that adhesion molecules are an important signal during the initial contact between axon and oligodendrocyte, but their downregulation is needed for myelination to proceed. Other types of molecules that are inhibitory for myelination comprise oligodendrocyte myelin proteins and their receptors.

22. *Rodent vs. Human*. Since most studies of mammalian brain development are done on rodents, we find it useful to understand how various time-points in rodent brain development correlate with much longer human brain development.
    a. In the literature there are just a few attempts to relate processes during rodent brain development with those in humans. Previous attempt to draw parallels between development of experimental animals and humans resulted in designation of Carnegie stages. This classification is based on somatic morphology of embryos and it presumes that brain development is linearly predictable from somatic development, and that all brain regions develop at equivalent rates across species. Other attempts were done to make rat/primate comparisons based on neuroanatomy, comparing rat embryonic days 11–21 with human weeks 4–16. However, most of these comparisons do not take into account disparities in relative sizes of human brain regions.
    b. Studies of human brain development from our laboratory suggest that processes like neurogenesis and oligodendrogliogenesis progress at a pace in the human brain, so every process has to be compared separately. Whereas neocortical neurogenesis does begin at 5 gw, corresponding to mouse embryonic day 10, the ending of neurogenesis is not that clear, since at the last time-point of our study, 24-gw neurons are still being born out of cells isolated from the cortical VZ/SVZ. Cortical oligodendrogenesis begins around 10 gw in humans, but it progresses well into adulthood in humans. MBP expression peaks in the mouse cortical and subcortical white matter at P20, when primary myelination is completed.
    c. Human primary forebrain myelination takes decades, compared to weeks in rodents. The most important reason for this prolonged development is that the much larger human brain has entire neocortical regions which rodent brains completely lack, whereas some of the key regions of rodent brains are in human relatively underdeveloped (e.g. olfactory bulbs).
    d. Another interesting issue concerns the overall number of oligodendrocytes in human versus rodent brain. Although numbers of oligodendrocytes needed to myelinate large human brains are much higher than in rodents, it is not as clear if the densities and proportions of oligodendrocytes are different. In mouse cerebral and cerebellar cortex, for example, the density of oligodendrocytes was estimated to be $12.5 \times 10^3/mm^3$, which makes for approximately 5% of all cells detected by the nuclear staining. Comparable stereologic studies of the human prefrontal cortex suggest very similar densities for oligodendroglia in humans.

GENERAL COMMENTS AND DISCUSSION

DeFELIPE

Comment on point 23.3:
It is not clear for me what is the relationship between the prolonged time of myelination and the presence of more specialized neocortical regions in humans compare to rodents, and to the fact that some regions of rodent brains are in human relatively underdeveloped.

ROCKLAND

The theme of heterogeneity and cellular diversity re-appears, in the context of multiple generative sources of oligodendrocytes (point 7). As the authors comment, the implications of this fact are poorly understood, but could include important issues of species specialization (point 22).

KOSTOVIC

In this paper, correlation between oligodendrocyte development and other neurogeneic events is best documented for the subplate zone (remark 4). Dense accumulation of oligodendrocyte precursors in the zone of waiting thalamic afferents is important for later myelination of thalamo-cortical axons. Laminar specificity of glia distribution seams to be related to other neurogenetic events. Accordingly, oligodendrogliogenesis in the human brain is prolonged compared to rodent brain (remark 22).

A commentary on
Growth of the human corpus callosum: modular and laminar morphogenetic zones by Jovanov-Milosevic, N., Culjat, M., and Kostovic, I. (2009). *Front. Neuroanat.* 3:6. doi: 10.3389/neuro.05.006.2009.

REMARKS AND MAIN CONCLUSIONS

1. To accomplish complex tasks, mammals require coordinated brain activity, based on precise and efficient connections between the two hemispheres. These connections consist of axons that traverse the telencephalic midline, principally in three commissural tracts: the corpus callosum, the hippocampal commissure and the anterior commissure. Among these, the corpus callosum is the most voluminous fiber tract, and in the human species reaches its maximum complexity and size relative to brain volume.
2. Anatomical studies in experimental rodents demonstrated that the majority of contralaterally projecting (callosal) neurons are located in cortical layers II/III and layer V, while in the primate brain fibers of the corpus callosum predominantly originate from layer III pyramidal neurons of the neocortex.
3. Axons of the callosal neurons elongate to the intermediate zone, then navigate medially through a well-defined pathway along the medial wall of the ipsilateral ventricle, cross the midline, grow further into the contralateral hemisphere towards the target region and area (usually homotopic), and finally enter the appropriate cortical layer to establish functional connections. Members of the Netrin,

Slit, Semaphorin, Ephrin and Wnt families of guidance molecules and their receptors, coordinate this extremely demanding navigation.

4. Several well-known developmental mechanisms, such as guidance by pioneering axons, guidance by pre-existing axonal tracts and guidance by cellular structures have been ascribed to various morphogenetic zones involved in the complicated pathfinding during the formation of commissures in a mammalian brain. However, these different morphogenetic zones have some principal properties in common: (1) strategic location (2) sequential appearance and dissolution, i.e. particular developmental window (3) modular or laminar appearance (4) versatile expression of guidance cues and (5) abundance of extracellular matrix (ECM). Disturbances in finely tuned expression of guidance and ECM molecules in the morphogenetic zones might cause structural or functional anomalies ranging from subtle cognitive impairment to severe developmental abnormalities, including dysgenesis and agenesis of the corpus callosum and other commissures.
5. As soon as they arise from the soma of future cortical neurons, the predetermined cortical efferents grow toward the intermediate zone guided by gradients of different guidance molecules. When they reach the intermediate zone, the axonal populations have to decide to grow medially around the ipsilateral ventricle as future callosal axons, or to grow laterally toward the internal capsule as long subcortically projecting axons, in both cases avoiding proliferative zones which express repelling cues, e.g., semaphorin3A (SEMA3A). Data regarding this decision point in human callosal formation are still missing, but exuberant axonal bifurcations have been reported in mice.
6. The early growth of future callosal axons in humans and the subsequent morphogenesis of the callosum were firstly demonstrated by histological methods and described in the classical embryological studies. Prior to corpus callosum formation, at 11 postconceptional weeks (PCW), a new structure designated as *massa commissuralis* is rapidly formed after the fusion of median groove banks above the septal area in the so called "*commissural plate* of Hochstetter". The first axons, named pioneering, approach and penetrate the massa commissuralis at the mediosagittal plane after 11 PCW. Therefore, the medially positioned massa commisuralis is probably the first midline structure that expresses specific molecules and morphogenes for guidance and nurture of pioneering callosal axons in humans.
7. Correlation of *in vitro* MRI and histological analysis of the developing human cerebrum revealed that the commissural plate, as well as other transient fetal zones, can be visualized on T1-weighted images already at 10 PCW. The commissural plate is then at the onset of its development and visibility on MRI scans as a thickened dorsal part of the telencephalon impar. The callosal fibers that penetrate massa commissuralis and form the *callosal plate*, can be demonstrated by histological methods at 12–13 PCW, while the earliest stage of their visualization by DTI is at 14–15 PCW. Some reports have suggested that the first axons are the ones that will form the rostrum, the genu and the body, while others suggest that the callosum will grow in both anterior and posterior directions, with a more prominent anterior growth.
8. With the formation of the callosal plate, several morphogenetic zones appear along the midline and subsequently develop into transient cellular structures: the midline sling, the glial wedge and the glia of indusium griseum (indusial glia). These developmental structures were first recognized and described in morphological studies of experimental animal models. Later they were further explored in mice by modern molecular methods, and for each structure a critical role in the morphogenesis of the corpus callosum has been established. Recently, histological and correlated histological/MRI studies demonstrated that these transient structures are also present during the period of early development of the human forebrain midline, indicating conservation of developmental mechanisms and structures during mammalian evolution. These morphogenetic structures situated in strategic locations have overlapping developmental windows between 13 and 20 PCW.
9. The midline sling was originally described as a thin concave lamina consisting of migrating glia-like cells that tightly underline the ventral surface of the developing corpus callosum. It was later proven that, at least in mice, a substantial portion of cells that forms the sling are neurons. Neuronal nuclear antigen (NeuN), CR and glial fibrillary acidic protein (GFAP) have been demonstrated in the human midline sling, indicating that it also consists of neuronal and glial cells. The origin of the cells that compose the midline sling in humans is complex and not clearly understood.
10. The list of guidance molecules, transcription factors and morphogens involved in corpus callosum formation in mice and humans, which are expressed either in the midline structures or by growing callosal axons, is continuously expanding. However, evidence for expression of these molecules in the human brain midline is still very scanty.
11. Studies in experimental rodents have demonstrated that ECM molecules such as laminin, fibronectin, proteoglycan NG2, heparan and chondroitin sulfate proteoglycans play an important role in the guidance of commissural axons. So far, in the human brain only tenascin-C was convincingly shown to be present dorsally and ventrally to the corpus callosum until 20 PCW. ECM molecules rather than just constituting a structural scaffold, interact with guidance cues and can critically modulate axonal guidance function.
12. Taken together, studies on human brain development have confirmed that developmental mechanisms governing the early formation of the forebrain midline and corpus callosum are indeed very similar to, if not the same as those described in experimental rodents. However, the human fetal brain is significantly larger than the brain of prenatal rodents and the distances the growing commissural axons have to cover are notably longer in every segment of their complex journey. Therefore, it is reasonable that commissural connections in

humans require a longer period of contemporaneous persistence of supporting midline structures and probably additional morphogenetic zones.

13. There are only a few histological studies describing the later stages of corpus callosum development in humans. In fetuses at 18–20 PCW, it is a well-developed fibrillar structure, which in coronal planes, runs transversally toward the midline and after crossing curves along the roof and lateral wall of the lateral cerebral ventricle. In the midsagittal plane, the outlines of the major callosal parts (genu, body and splenium) are clearly visible and assume the same shape and position as in the adult brain, with the exception of being much smaller in their rostrocaudal extent and thickness. DTI studies of the fetal brain at 19 PCW show the callosal radiation with its typical shape, similar to the one seen in neonates, with axons from all parts of the callosum forming a mohawk-shaped structure. At this stage the callosal cross-sectional area is only 5% of the size seen in a 5-year-old infant. At the neonatal stage, just before apparent myelinization of the callosum starts, it is half the size.
14. Beside the early midline structures, a morphogenetic role in formation of the human corpus callosum during the second half of gestation has been described for two additional zones: callosal septa and subcallosal zone (SCZ). These two midline structures jointly form "grooves" in which callosal bundles are laid down during the second half of gestation.
15. Postmortem analysis of the corpus callosum (age range from the 18 PCW to adult), immunostained for GFAP, NeuN and chondroitin sulfate proteoglycan (CS-56), in addition to classical histological methods, revealed the existence of modular cellular structures; namely, the callosal septa, which are most prominent during the second half of gestation.
16. During the developmental window of 18–34 PCW, the number of callosal septa is individually variable. Usually 15–20 thicker and longer septa and numerous smaller septa are unevenly distributed along the anteroposterior axis. In the genu and the anterior part of the callosal body, the septa are more numerous and more regularly spaced. In the rest of the callosal body, their number declines, while in the splenium it increases again, but the septa are still less numerous and less prominent there, in comparison with the anterior portion of the callosum. At the cellular level, the callosal septa contain: GFAP reactive meshwork, NeuN positive neurons, CS-56 immunoreactive ECM and expression of the guidance molecule SEMA3A in cells and ECM. At midgestation, SEMA3A is expressed in septa of the anterior third of the callosum and above the fornix. It is important to note that chondroitin sulfate proteoglycan in the ECM plays an important role in the cellular localization of SEMA3A, and their interaction can modulate the biological activity of this guidance molecule.
17. The callosal septa have not been shown by conventional MRI, yet the abundance of ECM and proteoglycans in septa, as well as their radial orientation between callosal bundles, should influence the MRI signal in diffusion-weighted imaging of the human fetal brain.
18. The ventral part of the callosal septa is in continuation with the SCZ, which like a thin lamina occupies the median and paramedian territories situated between the developing corpus callosum (dorsally) and the fornix bundles (ventrally). The cellular composition of the SCZ (revealed by acetylcholinesterase histochemistry and Golgi staining) is characterized by area-specific neuron-like cells with long and wavy processes, large glia-like cells, maturing neurons, migratory-like neurons and radial glial cells, with a difference in distribution between the medial (nucleus septohippocampalis) and lateral portion (allocortical counterpart of the SVZ).
19. During the early postnatal period, the callosal septa become thinner and shorter, lose their neuronal and chondroitin sulfate proteoglycan content. At the same time, the number of cells in the SCZ decreases.
20. The corpus callosum of adult primates consists of "segments" which contain topographically segregated callosal fibers for a given cortical area. Thus, callosal septa with their topographic arrangement, shape and content most likely provide a basis for topographically ordered commissural projections from one hemisphere to another, or at least maintain the topographical relationship during development. Confirmation of this suggestion can be found in the fact that the basic topography and terminal field patterns of callosal projections in monkey brain are established already by E133, well before birth.
21. In addition, tractography studies in humans showed an evident dorsoventral fiber distribution in the adult human brain, with earlier developed medial cortical areas sending their fibers dorsally through the corpus callosum, while later developed laterodorsal cortical regions send them ventrally. In this respect, callosal septa and SCZ with their abundance of ECM and guidance molecules along the ventral aspect of the corpus callosum are strategically located for influencing the growth of callosal axons.
22. One of the principal mechanisms of cortical development is an overproduction of axons, axonal branches and synapses, the so-called developmental exuberance, which is followed by a subsequent selection and refinement based on adequate target region recognition and activity of the functional connections. The corpus callosum is a pivotal example for such developmental exuberance, since the number of callosal axons in monkeys at E165, exceeds the number of callosal axons present in the adult by at least 3.5 times. Thus, the later morphogenesis of the corpus callosum is even more complex due to the processes of retraction of exuberant callosal fibers. In the primate brain, this continues during the first three postnatal months. In the human brain, decrease in size of midsagittal cross-sectional area was observed after 32 PCW and this lasts to the second postnatal month. This suggests the beginning of a retraction of exuberant callosal axons, concomitantly with the resolution of waiting compartments and the resolution of the majority of the callosal septa. The remnants of septa that continue for some time after birth may help in the process of withdrawal of exuberant callosal axons, since these structures and processes temporally overlap.
23. In addition, the septa may also provide corridors for migration of later-born neurons, since thick callosal bundles on the roof of the lateral ventricle represent a structural barrier. The

groups of neurons originating from the SVZ may use glial fibers in the callosal septa to "climb", while guidance molecules and ECM facilitate migration towards the cortex.

24. *Concluding remarks.*
    a. Recent progress in gene targeting methods, advances in axonal tracing, and high-resolution MRI techniques have revealed the morphogenetic zones and their role in guiding callosal axons across the midline.
    b. Although, the corpus callosum at midgestation assumes a shape and position not essentially different from that in the adult brain, it is still far from its definitive rostrocaudal extent and thickness.
    c. In the developing brain, all histogenetic events (neurogenesis, gliogenesis, migration, cell differentiation, axonal extension and synaptogenesis) proceed within laminar or modular compartments or zones. These do not have an equivalent in the adult brain. Therefore, different structures and processes have to be interpreted in a precisely defined spatial and temporal context. In that respect, it is important to note that in humans the complex event of interhemispheric integration through the corpus callosum continues throughout gestation and well after birth. The second half of gestation and the early neonatal period are important for the multiple-fold increase of callosal axonal number, selection of functional axons, withdrawal of exuberant axons and targeting of the region, area, layer and cells of the cortical plate.
    d. Insight into the organization of the morphogenetic zones involved in development of cortico-cortical pathways is a prerequisite for the studies of preterm and term-born infants in normal development and pathological conditions.
    e. Considering the clinical significance of the corpus callosum (over 50 different syndromes display dysgenesis of the corpus callosum and over 40 genes are linked to these anomalies, its importance for cognitive functions and its frequent lesioning in the perinatal period, we would strongly encourage long-term studies of the formation of commissural pathways in humans.

GENERAL COMMENTS AND DISCUSSION

DeFELIPE

General comment:

I was wandering if there are gender differences in the growth of the human corpus callosum.

ROCKLAND

I recently learned that agenesis of the corpus callosum is accompanied by vertical fingerlike structures (Probst's bundles) in the interhemispheric walls. Can the authors (or Others) comment on a possible relation to the developmentally important septa?

ROCKLAND

Comment on point 2:

I would say that callosal connections between the association areas, in particular, often involve layer 5 as well as 3, in macaque. This raises another possibly useful species difference, as I believe callosal connections in rodents originate more equally from layers 3 and 5?

A commentary on

Could sex differences in white matter be explained by g ratio? by Paus, T., and Toro, R. (2009). *Front. Neuroanat.* 3:14. doi: 10.3389/neuro.05.014.2009.

REMARKS AND MAIN CONCLUSIONS

1. *In vivo* studies of the human brain have revealed the presence of striking sex differences in the volume and microstructure of white matter. In adults, the overall volume of white matter (WM) is higher in men than women; in most studies, this is true even after accounting for sex differences in brain size. On the other hand, sex differences in the "density" (the term "density" or "concentration" refers to the probability of classifying a voxel as belonging to a specific type of tissue without making any inference about possible underlying neurobiological processes) and FA of WM constituting various fibre tracts appear to vary across the tracts, being higher in women than men in some WM pathways and vice versa in others.
2. Many of these sex differences in WM emerge during adolescence. We and others have shown a steep increase in the WM volume during adolescence in boys but not girls.
3. The total volume of white matter is determined by the number of axons, their calibre and the thickness of myelin sheath produced by the oligodendrocytes. Given the known elimination of axons in the early post-natal period, age-related increases in the volume of white matter during brain development in childhood and adolescence can be accounted for by increases in axonal calibre and/or thickness of the myelin sheath.

   In the same sample of typically developing adolescents, we have observed the following three phenomena: (1) WM volume increases steeply during adolescence in boys but not girls; (2) magnetization-transfer ratio (MTR) decreases with age in boys while it does not change (or increases slightly) in girls; and (3) WM density in the cortico-spinal tract (CST) decreases with age in adolescent boys but increases in girls. We have proposed that these phenomena may be best explained by a disproportionate growth of the axon compared with the growth of its myelin sheath and, as such, by changes in g ratio (axon diameter/fibre diameter; see below). We have speculated that a subtle shift in g ratio in WM of the male brain during adolescence would affect the proportion of WM tissue occupied by axons and myelin, thus explaining the emergence of sex differences in WM density and MTR described above.
4. G ratio refers to the ratio between axon diameter d and fibre diameter D; fibre diameter is the sum of the axon diameter (or calibre) and the thickness of the myelin sheath.
5. In the monkey corpus callosum (CC), unmyelinated axons, accounting for about 30% of all axons in the genu and less than 10% elsewhere in the CC, have a small diameter (~0.1 μm), whereas the diameter of myelinated axons varies between 0.1 and 2.5 μm, with the largest axons (2.5 μm) found in the CC midbody. The largest axons in the human brain are found in the internal capsule and, most likely, correspond to the cortico-spinal neurons; the size of these axons

increases from birth (~1 μm), through childhood (12 μm at 7 years of age) into adulthood (24 μm). These numbers correspond to the fibre diameter and, as such, reflect both the radial growth of the cortico-spinal axons and their increased myelination with age. We have suggested that changes in the WM density in the corticospinal tract observed in our studies in relation to plasma levels of testosterone reflect changes in the diameter of these axons.

6. In general, axons of large diameter have a thick myelin sheath. It turns out that the g ratio is relatively stable at ~0.6. This observation, together with the small diameter of unmyelinated axons, suggests possible coupling between myelination and axon diameter. But the range of g ratio varies considerably across axons of the same fibre tract, as a function of age, mouse strain or disease.
7. Importantly, g ratio appears to increase as a function of axon diameter, indicating a relatively thinner myelin sheath in large axons.
8. By assuming that geometrically similar fibres should possess similar membrane properties, Rushton derived a formula showing that, for a constant g ratio, the conduction velocity v and fibre length l should be proportional to the fibre diameter.
9. What determines axonal diameter? Axon consists mainly of neurofilaments (NF) and microtubules (MT), the former outnumbering the latter 5–10 times. Neurofilaments support the cylindrical structure of an axon and, as such, protect the core from compressive stress, securing its unobstructed state. Axonal diameter is influenced both by the number of NF and their spacing. The former is regulated by NF synthesis (gene expression) and the amount of NF undergoing (slow) axonal transport; not surprisingly, the number of NF, but also that of MT, varies as a function of axon diameter. The latter depends on the phosphorylation of NF side arms. This process appears to be regulated by a protein synthesized by oligodendrocytes, namely myelin-associated glycoprotein; this "outside-in" signalling pathway provides a cellular mechanism for the coupling between myelination and axonal calibre. Theoretically, sex differences in g ratio could emerge either through differences in the rate of synthesis and/or axonal transport of NF, or the rate of phosphorylation of the NF side arms.
10. *Conclusions and implications*.
    a. The initial work from our laboratory has suggested a disproportionate growth of axon and myelin sheath, respectively, during male adolescence. This phenomenon turned our attention to possible sex differences in g ratio. Both the experimental work reviewed above and the model predict lower concentration of myelin in WM regions occupied by large-diameter fibres; we have observed, indirectly, this phenomenon in the cortico-spinal tract at the level of internal capsule. It is likely that the male brain contains, overall, a larger proportion of large-diameter fibres. What might be the neurobiology underlying such a sex difference?
    b. Although the number of MT also increases with the axonal diameter, directionality of this relationship is not clear. The link between testosterone and cellular processes relevant vis-à-vis cytoskeleton are rather tentative at present. Initial *in vitro* studies suggest that testosterone up-regulates α-and β-tubulin, the building blocks of microtubules. Furthermore, neuropathy is one of the key symptoms of Kennedy's disease (or X-linked spinal and bulbar muscular atrophy), a condition caused by an expanded polyglutamine (polyQ) stretch in the androgen receptor. This effect might be mediated by a PolyQ-AR induced inhibition of kinesin-mediated axonal transport or a decrease in the expression of dynactin 1, a motor for retrograde axonal transport; slow axonal transport is essential for moving elements of cytoskeleton.
    c. Is the rate of axonal transport different in small-and large-diameter fibres? This appears to be the case. Murayama and coworkers used MRI to measured the rate of *in vivo* transport of Mn^{2+} ions from the eye to the lateral geniculate nucleus of the monkey and found that a faster transport in the magno-cellular that parvocellular pathway. The Mn^{2+} transport depends, at least in part, on kinesin-based processes underlying (active) axonal transport.
    d. In general, axonal cytoskeleton (i.e., NF and MT) and motor proteins are essential contributors to a large number of cellular processes, such as cell metabolism (e.g. transport of mitochondria and glycolytic enzymes) and neurotransmission (e.g. transport of synaptic-vesicle precursors), as well as growth and cell survival (e.g. retro-grade transport of growth factors). Thus, any sex differences in axonal transport may have a number of downstream effects.
    e. The model presented in this article suggests that the g ratio will increase in large fibres beyond the optimal value of 0.6. We speculate that the presumed testosterone induced changes in axonal diameter during male adolescence increase the probability of suboptimal g ratio in large-diameter fibres and, in turn, decrease conduction velocity in these fibres. Such a disturbance may interfere with the timing of inter-regional communication and contribute to the emergence of a "disconnection" syndrome hypothesized to underlie disorders such as schizophrenia.

GENERAL COMMENTS AND DISCUSSION

DeFELIPE

Comment on point 3:

The authors claim that the total volume of white matter is determined by the number of axons, their calibre and the thickness of myelin sheath. However numerous neurons are present in the white matter. Do the authors think that the presence of these neurons has little impact on the volume of white matter?

HÖISTAD AND HOF

Paus and Toro observed differential increases in WM volume in boys compared to girls during adolescence (i.e., after 12 years of age). They propose that there is a disproportionate growth of axons compared to the growth of myelin sheaths during male adolescence (Perrin et al., 2008). It remains to be determined whether, or how, sex hormones play a role in this development.

Inherent to the issues of axon and myelin growth, is the issue of identifying a possible coupling between myelination and axon diameter. For example, several thin axons are unmyelinated, whereas

large diameter axons are heavily myelinated. As such, what determines axon diameter, and what are the signals required for the axon to get myelinated? Paus and Toro discuss the link between axon diameter and neurofilament, the main axonal cytoskeletal protein. It turns out that neurofilament phosphorylation, which determines neurofilament spacing, may in fact be regulated by MAG (myelin associated glycoprotein) expressed in oligodendrocytes (Yin et al., 1998). These authors have shown that MAG modulates caliber, neurofilament spacing, and neurofilament phosphorylation of myelinated axons, and that absence of MAG results in axonal atrophy and Wallerian degeneration of myelinated fibers. Thus, MAG may provide one of the mechanisms that are responsible for the coupling between myelination and axonal caliber. Interestingly, it has been found by Hof and coworkers that MAG deficient mice do not exhibit any changes in FA of the cingulum bundle as measured by DTI, nor do they exhibit any changes in fiber length densities of the cingulum bundle as assessed by stereologic analysis (Segal, 2008). This may point to the fact that not all pathways are equally affected in the MAG knockout mouse. It is equally possible that sex hormones exert a direct or indirect effect on myelination or axon growth through mechanisms unknown as yet, which represent interesting targets for investigations considering the gender-dependent differences in white matter that occur during adolescence.

A commentary on

Myelination and isochronicity in neural networks by Kimura, F., and Itami, C. (2009). *Front. Neuroanat.* 3:12. doi: 10.3389/neuro.05.012.2009.

REMARKS AND MAIN CONCLUSIONS

1. The timing when a neuron receives its incoming input has a great influence on how the input is processed and how it affects the postsynaptic neurons. On the other hand, an increase in the body size and the amount of information processed, as a result of evolution, inevitably required the expansion of the brain. This resulted in situations where the excitation of a presynaptic neuron needs to arrive within a fixed window of time at target neurons located at multiple remote sites at variable distances. Such apparently paradoxical transmissions, in fact, take place in various regions of the brain. Some representative examples of such isochronicity can be observed in olivocerebellar connections, transcallosal connections in the visual cortex and amygdalo-cortical pathways.
2. Intriguingly enough, these connections do not necessarily adopt the same strategy to accomplish isochronicity. Recently, we found that the thalamocortical pathway also exhibits the isochronic property, but with a novel mechanism, which involves differential myelination along the axon. Apparently this is an exquisite way of producing isochronicity and may apply to other systems.
3. Neurons in the thalamus send axons to a wide area of the somatosensory cortex through different trajectories of various travelling lengths. Nevertheless, we reported that action potentials in the thalamic cells arrive almost simultaneously at each target cortical cell in layer IV. We found that the isochronicity in this system is achieved by changing the conduction velocity (CV) within the individual axons. The CV decreases significantly upon entering the gray matter by up to 10-fold. Originating from the thalamus, the axons of relay cells run in a straight path through the striatum up to the WM, at which point each axon diverges widely. Some ascend directly into the cortex up to layer IV, while others run in the subcortical WM for variable distances before ascending into the cortex. Although the total distances travelled vary across pathways, the distance of the intracortical regions is almost the same. That is, all the projections target the same layer IV in a cortex that has uniform thickness. The total conduction time primarily depends on the time spent within the cortex (gray matter). Thus, the strategy of making the CV of longer and variable parts by far faster than that of shorter and constant parts is a way of eliminating the variability in traversed distances.
4. Myelination is involved in this process in two-fold. First, since CV is proportional to the square root of the axon diameter in unmyelinated axons, to have 10 times larger CV, axon diameter needs to be 100 times larger, which is most unlikely. This led us to predict that thalamocortical fibers would be myelinated. Thus, secondly, we suspected that the extent of myelin might cause the observed difference of CV. Histological staining of the myelin revealed that the difference of myelination between the intracortical and extracortical (WM) regions played an important role in the generation of CV difference.
5. Isochronic property of transcallosal connections is one of the most intensively studied within the framework of the computational properties of axons by Innocenti and coworkers. Based on the simulation of action potential propagation determined by axonal diameter, these authors provided a detailed description of strategies for callosal connections to achieve synchronous activation of their targets.
6. In the cortex, layer V pyramidal neurons project to various subcortical regions as well as to the contralateral side. Chomiak and coworkers showed that layer V pyramidal neurons in the ventral temporal lobe innervate diverse regions such as the caudate putamen, parietal cortex, amygdala, and thalamic nuclei on the ipsilateral side with isochronic spike delivery based on the differential CVs in each fiber branch. By combining the actual measurement of the axonal inner and outer diameters, with theoretical predictions based on partial myelination, the observation of Chomiak and coworkers on isochronicity appears to be best supported by partial or differential myelination. It may be worth mentioning that the same neurons send axons to the contralateral side through the corpus callosum, but the CVs were significantly slow and not isochronic with ipsilateral connections.
7. One implication from these studies reviewed here is that each pathway has its own conduction time, which might be determined by its function. Myelination, axon diameter, and other factors such as the structure, or the number of spacing of Ranvier nodes, and ion channel composition and/or its density, all of these would affect the CV, and conduction time may be employed to achieve its characteristic value. On the other hand, since the environment around a given neuron, or

tissue, or whole individual continuously changes, a regulatory mechanism that changes in an input- or activity-dependent manner is desirable to adapt to the changing environment.

8. Myelination indeed changes in an experience- or input-dependent manner. For example, the DTI. study of Bengtsson and coworkers revealed that extensive piano practicing in childhood results in a thicker white matter, which is believed to be due to a change in myelination.
9. Several lines of evidence have identified how action potentials regulate myelination. A specific pattern of neural activity was shown to lower the expression of L1-CAM that is necessary for myelin induction by oligodendrocytes, as well as by Schwann cells. In addition, ATP released from axon terminals as a result of neural activities facilitates the differentiation of oligodendrocytes through adenosine and P1 receptors. For astrocytes, ATP causes the release of leukemia inhibitory factor (LIF), which then stimulates myelination by oligodendrocytes.
10. The corpus callosum displays a significant variety in terms of myelination. For example, in humans, none of the callosal fibers are myelinated at birth, and in adults 30% of the fibers remain unmyelinated. In addition, analyses of fiber composition revealed a wide variety of fiber diameters and extent of myelination depending on the target area. Callosal regions connecting prefrontal and temporoparietal association areas consist of small caliber with low myelinated fibers, whereas regions connecting primary and secondary sensorimotor areas include highly myelinated, large-caliber fibers. Consequently, the conduction time between two hemispheres varies from 30 ms via myelinated axons to as long as 300 ms via unmyelinated ones. Since callosal fibers connect a variety of cortical regions with various functions, conduction times for each functions are likely to be diverse. The extent of myelination, as well as axon diameter might help regulate the conduction time to its optimal value for communicating between hemispheres. This leads to another interesting question; namely, how the conduction time of each pathway is determined in the network.

GENERAL COMMENTS AND DISCUSSION

DeFELIPE

Comment on points 7 and 10:
If each pathway has its own conduction time and isochronous activation of thalamocortical and cortico-cortical connections are thought to be crucial in the binding mechanisms of sensory perception, how can both arguments could be related to explain the global binding?

ROCKLAND

Concerning the statement of differential myelination along the axon (point 2), light microscopic analyses of individual axons have remarked what appears to be changes in axon caliber (Innocenti et al., 1994; Rockland and Drash, 1996). Since it has been difficult to investigate identified axons along their full length, data on this point are largely lacking. However, the changes and specializations along the trajectory of a single axon are likely to be a source of interesting and potentially important future research.

A commentary on
Linking white and grey matter in schizophrenia: Oligodendrocyte and neuron pathology in the prefrontal cortex by Höistad, M., Segal, D., Takahashi, N., Sakurai, T., Buxbaum, J. D., and Hof P. R. (2009). *Front. Neuroanat.* 3:9. doi: 10.3389/neuro.05.009.2009.

REMARKS AND MAIN CONCLUSIONS

1. Most neurons in the brain necessitate adequate myelination of their axons in order to maintain functional processing at all levels of neural systems, from autonomic processes and sensorimotor integration, to mood and thought.
2. The importance of myelination for cognitive functioning becomes apparent in diseases that are known to be caused or affected by deficiencies in myelin, where patients show deficits in intellectual, social and emotional functioning.
3. Leukodystrophies and leukoencephalopathies, diseases characterized by progressive degeneration of the white matter, if diagnosed in late adolescence or early adulthood can present with psychotic symptoms sometimes indistinguishable from those of schizophrenia. Likewise, patients with multiple sclerosis who display cognitive and psychiatric symptoms frequently have white matter lesions in the frontal and temporal lobes, which are the brain regions most implicated in schizophrenia.
4. Schizophrenia is a severe psychiatric illness that affects close to 1% of the population worldwide. The diagnosis is generally established at first onset of the symptoms, which occurs in most cases in early adulthood. The disease is characterized by a number of mental abnormalities that result in a distortion of perception and expression of reality. There are prominent sensory symptoms, most frequently taking the form of auditory and visual hallucinations, although such sensations can affect any sensory modality. In addition to hallucinations, the patients may experience paranoid delusions, present with disorganized thoughts and speech, and a variable degree of social and occupational dysfunction. There is a considerable degree of inheritability of the disease and prenatal causes, such as insult to the brain during embryonic development, have also been considered to play a key role in the expression of the disease at a later time.
5. Schizophrenia has been shown to exhibit myelin deficiencies and changes in white matter volume in the brain. The myelin hypothesis in schizophrenia was first presented by Hakak and coworkers after their pivotal finding of altered expression of myelin-related genes in human postmortem tissue. Myelin-related gene expression levels have matched the observations made on white matter abnormalities by DTI, and were later confirmed in several other studies. Since the first suggestions of a myelin-related pathophysiology underlying schizophrenia, there have been numerous and extensive reports and reviews on the myelin hypothesis.
6. Substantial deficits in myelination occur in schizophrenia, which is interesting to consider in light of previous hypotheses that the disease results from abnormal brain development and altered neuronal circuitry, particularly in the prefrontal cortex (PFC). Neuropathologic findings in both white matter

and grey matter suggest that myelin alterations in the anterior cingulate cortex (ACC) may underlie some of the behavioral deficits related to prefrontal dysfunction.

7. Fractional anisotropy (FA), a measure of the directionality of water movement within the spaces in-between axons, provides an indication of white matter tract directionality and, by measuring the strength of the direction vector of water diffusion, possibly of tract integrity or coherence. A major advantage of this approach is that it can be used to study changes in schizophrenia *in vivo*, allowing investigation of different stages of the disease. *In vivo* DTI studies have revealed decreased FA in several major white matter tracts in schizophrenia, including the cingulum. In addition, positron emission tomography (PET) imaging has demonstrated increased relative metabolic rates in white matter in schizophrenia, which may represent white matter inefficiency or defects resulting in increased metabolic needs, in contrast to findings in the grey matter which have shown decreases in regional cerebral blood flow in the ACC.
8. Although previous DTI studies have shown decreases in FA in the cingulum bundle as well as in the overlying cingulate gyrus in patients with schizophrenia, the findings have been somewhat inconsistent, due in large part to small subject samples and different methods of identifying particular brain regions of interest.
9. MRI studies of the grey matter have revealed regionally reduced cortical volumes in schizophrenia, including the ACC
10. In attempts to localize and identify a cellular correlate of the white matter changes observed by brain imaging *in vivo*, oligodendrocytes have come to be an important focus of investigation. Analyses of the number, densities and distribution patterns of oligodendrocytes can be performed in both the white and grey matter. Stark and coworkers found decreased oligodendrocyte densities in cingulate area 24 but not in the adjacent paracingulate area 32, and Hof and coworkers found decreased densities of oligodendrocytes in the prefrontal area 9 of the superior frontal gyrus in subjects with schizophrenia. In contrast, in a subsequent study, Hof and coworkers evaluated the degree of oligodendrocyte clustering in the anterior cingulum bundle, but found no differences using postmortem tissue from chronic schizophrenics versus age-matched controls. On the ultrastructural level, electron microscopy studies of oligodendrocytes in the PFC have demonstrated apoptotic oligodendrocytes, irregularities of mitochondria in oligodendrocytes and damaged myelin in area 10 in schizophrenic brains.
11. Postmortem studies, assessing the gyrification index, have found reductions in cortical folding in schizophrenia. Studies on changes in neuronal densities in different cortical regions in schizophrenia have been conflicting, and no definite pattern of neuronal density alterations has yet been established.
12. Cytoarchitectural studies have analyzed neuronal arrangements in terms of interneuronal distances, or mean cell spacing showing that mean cell spacing was reduced in area 9 in schizophrenic patients, which would imply a higher neuronal density. Rajkowska and coworkers found that in area 9, there was a downward shift in neuronal sizes, accompanied by increases in the density of "small neurons" in layer II, interpreted as GABAergic interneurons, while there was a decrease in the density of "very large neurons" in layer III, presumably pyramidal neurons, in patients with schizophrenia. Concomitant morphological studies at the single neuron level have demonstrated impoverished dendritic structures of pyramidal neuronsand loss of dendritic spines in schizophrenia, as well as in non-human primate models.
13. Another interesting finding is an anomalous distribution of the so-called interstitial white matter neurons in schizophrenia. These interstitial neurons have been suggested to be remnants of subplate neurons that normally undergo programmed cell death during brain maturation. However, in certain species including human, these white matter interstitial neurons are to some degree normally found in healthy adults. The interstitial white matter neurons have been found to be increased in prefrontal white matter and temporal white matter in subjects with schizophrenia, supporting further the presence of a neurodevelopmental abnormality in schizophrenia.
14. It is possible that the regionally specific remodeling of grey and white matter that takes place into the third decade of life underlies some of the structural and functional changes that leads to the development of psychiatric disorders such as schizophrenia. The fact that the PFC matures last and that myelination is not complete until late adolescence may be significant, as the timing coincides with the typical onset of symptoms in schizophrenia. This suggests that a dysfunctional myelination process could underlie the pathogenesis of schizophrenia. However, it is interesting to note the lack of neurological comorbidities in schizophrenia in comparison with other more typical white matter diseases. In dysmyelinating and hypomyelinating diseases such as the leukoencephalopathies, the effects of a myelin deficiency may be striking and fatal.
15. If the myelin hypothesis holds true, and myelin deficiencies prove to be one of the central causes of the development of schizophrenia, one might argue and question why classic schizophrenia patients show so few neurologic symptoms. Several other white matter abnormalities often generate disturbances at the neuron level, such as seizures and/or psychomotor developmental delays. Why patients with schizophrenia do not particularly exhibit similar neurologic co-morbidities, such as seizures or sensorimotor deficits, is unknown. It may be that only specific pathways become myelin-deficient, such as the late developing and poorly myelinated regions of the PFC, leading to the generation of behavioral symptoms seen in schizophrenia. Since the diverse circuits in the brain do not mature at the same time, if there is a developmental insult, this may affect only a certain population of neurons undergoing myelination, and result in a pathway-specific deficiency.
16. In a groundbreaking study using gene microarray analysis to examine gene expression levels in postmortem samples from schizophrenia patients Hakak and coworkers found that the expression of six myelin-related genes predominantly expressed in oligodendrocytes, was significantly decreased in the DLPFC in postmortem schizophrenic brains. The decreased expression of oligodendrocyte-related gene products was later confirmed and extended to other brain areas, implying that there

is a pathology of oligodendrocytes underlying schizophrenia. Genetic linkage studies have also implicated myelin-related loci in schizophrenia although linkage studies are now considered somewhat controversial in complex psychiatric disorders.

17. Much evidence, including whole genome association studies, have identified myelin- and oligodendrocyte-related genes as susceptibility genes for schizophrenia. One of the most promising schizophrenia-related genes is neuregulin 1 (*NRG1*) gene. NRG1 and the NRG1-receptor ERBB4 are involved in several aspects of nervous system development including oligodendrocyte development Given the emerging role of NRG1 and ERRB4 in oligodendrocyte development, it is possible that alterations in *NRG1* and *ERBB4* affect oligodendrocytes, leading to schizophrenia.
18. Transgenic mouse models may serve as vehicles for studying the morphological and anatomical abnormalities that may result from a genetic defect affecting myelination. Some recent mouse models of white matter dysfunction have emerged during the last few years, which may serve as putative animal models for schizophrenia.
19. Evidence from very different lines of research supports the premise that dysfunction of oligodendrocytes is a critical factor in the development of schizophrenia. The precise role oligodendrocytes hold in the cascade of malfunctions that results in the constellation of deficits seen in the disease is still unknown.
20. *Future directions*
    a. Layers II and III pyramidal neurons in the ACC may be the targets of axonal pathways affected in schizophrenia. Quantitative information on neuronal integrity in mouse models is important for understanding downstream effects of myelin genetic abnormalities, and to assess the validity of models in the context of observable neuropathologic changes in human brains. These studies need to be extended to additional models reflecting the genetic complexity of schizophrenia, and electron microscopy studies should be used further to assess structural aberrations in oligodendrocytes and myelin sheaths, as well as immunogold approaches to study synaptic integrity by visualizing pre- and postsynaptic proteins.
    b. Correlative morphology and density analyses of dendritic spines will help clarify plastic changes in responses to myelin challenges. The data obtained in transgenic mice will offer critical correlates to neuropathologic features that can be analyzed in postmortem human materials. Combined analysis of human specimen and relevant mouse models offers a unique opportunity to investigate myelin deficits that have a clinical impact. As a result of such combined approaches, a model of schizophrenia with characterized molecular defects that can be used for developing therapeutic approaches will hopefully emerge.

GENERAL COMMENTS AND DISCUSSION

DeFELIPE

General comment:

The authors state that schizophrenia has been proposed to arise partly from altered brain connectivity. Could the authors, or whoever is interested, comment from his/her own experience on what particular connections are affected? How specific are these changes? Are GABAergic neurons affected or is it thought that mostly pyramidal neurons are vulnerable?

ROCKLAND'S

Response to DeFelipe's comment:

To the extent that long-distance connections are involved, this will be hard to determine in humans (as long as DiI or comparable post-mortem tracer remains refractory.) Also, it is hard to know what level to search: presynaptic arbors or synapses? Postsynaptic targets? Receptors, so this will involve a concerted ongoing effort. One related recent report is Rinaldi et al. (2008).

DeFELIPE

Comment on point 20.1:

It is not clear to me why only layers II and III pyramidal neurons in the ACC may be the targets of axonal pathways affected in schizophrenia. Are not affected pyramidal neurons in other cortical areas and layers?

HÖISTAD AND HOF

There is a plethora of unsuspected pathologic alterations in schizophrenia. Many studies have demonstrated changes in pyramidal neuron populations in various cortical regions (hippocampus, cingulate, dorsolateral prefrontal, primary auditory cortices to name a few). Similarly GABAergic neurons have been shown to be affected, and to present abnormal distribution in frontal and temporal regions alike. Further hinting to a developmental problem is the fact that neurons do not migrate to their appropriate location, or tend to agglomerate in the white matter under the cortex. As such it is not only the ACC that is affected. It may be that some domains of cortex are more vulnerable to an underlying developmental pathology that is revealed postpuberty and may affect different regions in different patients. Assessing the true variability in distribution of changes and their severity among patients with schizophrenia is a daunting task to undertake, but is the necessary neuropathologic index ultimately required to understand the disease process fully, in the context of genetic influences, inferences from animal models and *in vitro* models, and *in vivo* functional imaging. This issue speaks to the need of rigorous, extensive, quantitative analyses of postmortem human materials to enhance our understanding of the disease.

A commentary on

Regulation of myelin genes implicated in psychiatric disorders by functional activity in axons by Lee, P. R., and Fields, R. (2009). *Front. Neuroanat.* 3:4. doi: 10.3389/neuro.05.004.2009.

REMARKS AND MAIN CONCLUSIONS

1. The establishment and development of psychiatric disorders are likely to involve aberrant regulation and expression of many genes, together with multiple environmental factors, ultimately leading to illness. In recent years researchers have begun to focus on the potential role of white matter and oligodendrocytes in the pathophysiology of psychiatric disorders.
2. Myelination can be viewed as a highly dynamic process which can be altered by impulse activity in axons and by environmental factors. It is becoming clear that myelination continues into

adulthood and may contribute to plasticity of cognitive function, learning and memory. Perturbations in the molecular processes leading to axon myelination will consequently result in axon dysfunction and abnormal electrical conduction, therefore impairing the transfer of information across brain regions.

3. There are several mechanisms by which oligodendrocytes could sense functional activity in axons. Oligodendrocytes at various stages of development have ion channels, purinergic and other membrane receptors that allow myelinating glia to detect impulse activity through the activity-dependent release of molecules from axons. Thus activity-dependent regulation of oligodendrocytes could contribute to cellular mechanisms promoting recovery through environmental interventions and other non-drug treatments of psychiatric illnesses.
4. Drug treatments for neuropsychiatric illnesses may also act in part through effects on myelinating glia. Oligodendrocytes have neurotransmitter receptors for glutamate, serotonin, and dopamine, making it likely that antipsychotic drugs acting through these neurotransmitter systems would also have actions on myelinating glia that may be detrimental or beneficial in psychiatric disorders. Finally, synaptic communication between axons and immature myelinating glia (oligodendrocyte progenitor cells), have been described recently in white matter, providing a rapid means of direct communication between axons and myelinating glia.
5. Myelination is a complex biological process that involves an intricate regulatory network among many different cell types in the nervous system. Many of the genes revealed in genomic studies of mental illness that are crucial to the normal functioning of the myelination program and myelin-maintenance are themselves candidates for regulation by electrical activity in axons. Many of these genes relate to oligodendroglia function; however some of these genes are expressed in astrocytes and some in neurons where they may have independent effects or act indirectly on myelinating glia.
6. Regulating transcription of structural components of myelin, such as PLP1, MBP, MAG, MOG, and CNP is clearly critical in the process of oligodendrocyte development and the subsequent correct myelination of specific axons. Several of these major components of myelin have been shown to be regulated by action potential firing or by alterations in intracellular calcium or cAMP; both of these second messengers can be regulated by neural impulse activity.
7. Several of the major myelin genes can be regulated by electrical activity or mechanisms by which second messenger signal transduction can be modified. Many of the major components of myelin are deregulated in psychiatric disorders, particularly in schizophrenia.
8. In order for myelination to proceed, a complex network of transcriptional repression and activation must be activated. Several of the transcription factors required for repression and activation of myelin genes have been found to be abnormally expressed in the brains of patients with psychiatric disorders and in particular those with schizophrenia.
9. Of particular interest is the transcription factor SOX10 which is required for the expression of two of the major components of myelin, the proteins MBP and PLP1. Recently it has been shown that in Schwann cells, Sox10 is a component of a calcium-sensitive transcriptional complex. Calcium is the primary second messenger communicating action potential firing to intracellular responses, and signaling to the nucleus to regulate gene transcription. The Sox10/NFAT complex is critical for Schwann cell development and activates several genes known to be regulators of myelination in the peripheral nervous system. Interestingly the SOX10 gene is located in a major susceptibility locus for schizophrenia and reduced expression of this gene was found to be correlated with an increase in the methylation state of the allele found in schizophrenia patients.
10. External signals generated by electrical activity in axons, such as ATP or glutamate release from axons, can cause changes in intracellular calcium levels in oligodendrocytes and therefore these axonally derived signals may play a role in the epigenetic regulation of the transcriptional apparatus required for lineage progression and myelination by oligodendrocytes and in the process of remyelination. Clearly a perturbation of this type of epigenetic regulation in psychiatric disorders, perhaps by soluble axon-derived signals such as ATP or glutamate, would provide a link with environmental cues as axon firing patterns reflect environmental stimuli.
11. The complexity of mRNA expression and metabolism and the localization of specific mRNAs to subcellular compartments in oligodendrocytes will all contribute to the eventual pool of mRNA available for the translational machinery. RNA transport, splicing and stability mechanisms are tightly regulated by intracellular signal transduction in many cell types in the nervous system. Interestingly, many of the myelin genes are alternatively spliced during development and several of these mRNAs are targets for transport by specific RNA binding proteins. Importantly, a broad spectrum of RNA binding proteins has been found to be deregulated in schizophrenia, including quaking and hnRNAPA2. Deregulation of such a large group of RNA binding proteins will have many downstream consequences for RNA metabolism and localization in oligodendrocytes.
12. Another potential mechanism by which post-transcriptional regulation of oligodendrocyte-related genes may be accomplished is to regulate mRNA homeostasis by the binding of specific micro RNAs (miRNAs) to myelin gene mRNAs. Micro RNAs are regulators of translation and RNA stability; this is achieved by miRNA binding to the UTR of target RNAs and directly influencing the amount of mRNA available to the translational machinery. The study of the regulation of miRNAs in the brain is still at a relatively early stage, however the functional targets of several miRNAs have been described in neurons and oligodendrocytes.
13. In addition to oligodendrocyte-related genes, other non-neuronal genes such as glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed in astrocytes, have been implicated in psychiatric disorders. Astrocytes can have an important influence on development of oligodendrocytes by secretion of trophic factors and cytokines. GFAP is an integral component of the astrocyte cytoskeleton and altered expression of GFAP can have many effects on astrocyte

biology. Therefore GFAP dysregulation and by implication astrocytic dysfunction could have profound effects on axon health, neuro-transmission, and neuron-glia signaling, and perhaps most importantly indirectly in the process of myelination.

14. Extracellular factors generated by neuronal activity have been shown to regulate GFAP expression and astrocyte differentiation and this could occur through the regulation of chromatin structure. Astrocytes have also been shown to have a direct role in regulating myelination and oligodendrocyte development. Studies in cell culture have shown that astrocytes promote myelination in response to electrical stimulation of axons via the release of a cytokine Leukemia Inhibitory Factor, in response to ATP released from electrically active axons. Taken together it is becoming clear that astrocyte function, which can be regulated by neuronal activity, can have profound effects on the myelination process and may contribute to disease progression in psychiatric disorders.
15. Another mechanism by which electrical activity could influence gene expression in oligodendrocytes is by the expression of cell surface signaling molecules such as specific cell adhesion receptors in axons. This general type of mechanism has already been shown to regulate myelination in the peripheral nervous system.
16. The expression of specific proteins and protein complexes at sites of axon-glia contact could provide a direct link between axon signaling and the regulation of gene expression in oligodendrocytes. In support of this idea it has been demonstrated that extracellular stimuli can regulate the localization of specific mRNAs in axons, and it has been demonstrated that the expression of cell surface receptors can be modulated by electrical activity in axons. Therefore, taken together, regulation of myelination by an activity-dependent signaling cascade, originating in axons, may allow direct coupling of neuronal activity and oligodendrocyte intracellular signaling.
17. It has been demonstrated that diffusible molecules released from axons firing action potentials can be detected by myelinating glia, with subsequent control of glial development and myelination.
18. Adenosine, derived from ATP released from electrically active axons, acts on immature oligodendrocytes to promote differentiation and myelination. Other molecules released by electrically active axons that could in theory influence myelinating glia include potassium and neurotransmitters.
19. Growth factors, such as BDNF, which are known regulators of oligodendrocyte differentiation, can be secreted or regulated in an activity-dependent manner and by environmental experience, providing another potential general mechanism for activity-dependent regulation of myelination. Interactions between BDNF and serotonin in mood disorders have been reported, and the BDNF gene has been associated with increased risk for a number of neuropsychiatric disorders.
20. Drug treatments for psychiatric disorders that correct deregulation of genes involved in myelination and oligodendrocyte dysfunction are an appealing possibility. In this regard pharmacological regulation of the activity of specific histone deacetylases (HDACs) is an interesting avenue of investigation, although therapeutic intervention of the modulation of HDAC activity in mouse models of demyelination has shown mixed results.
21. It may be important to identify cell-type specific regulators of chromatin structure as it relates to oligodendrocyte function, thus targeting any drug treatments more specifically to limit off-target effects on other cell populations in the brain. Regulation of gene expression by medication beyond transcriptional regulation may provide a more specific mechanism to target myelin genes in oligodendrocytes.
22. The extent to which dopamine levels could lead to mental illness in part through effects on myelinating glia, and whether antipsychotic treatments have therapeutic action in part through effects on oligodendrocytes are two intriguing questions of current investigation.
23. Dopamine also can be toxic to oligodendrocyte progenitors by inducing superoxide generation and lowering glutathione levels. Agonists for dopamine D2 and D3 receptors have been shown to provide significant protection of oligodendrocytes against oxidative injury. On the contrary, haloperidol, a typical antipsychotic drug blocking D2 activity reduces myelin proteins in mice treated for 30 days.
24. Schizophrenia and depression can also involve imbalances in the neurotransmitter serotonin, and several drug treatments act through regulating serotonin levels, for example the serotonin reuptake inhibitor fluoxetine (PROZAC). Serotonin receptors are expressed in Schwann cells, and the human polymavirus, JC virus, which causes multifocal leukoencaphalopathy, binds the 5HT2a serotonin receptor on oligodendrocyte progenitor cells.
25. The serotonin antagonists, metoclopramidine, chlorpromazine, clozapine, and serotonin itself all significantly inhibit viral infection, which indicates that medications affecting serotonin levels could influence oligodendrocytes. Indeed, the antidepressant drug, fluoxetine, increases cell proliferation of precursors in cell culture that can give rise to astrocytes, neurons, or oligodendrocytes.
26. Other neurotransmitters can regulate different steps of oligodendrogliogenesis through such ion channels and receptors as the delayed potassium rectifier, the AMPA/kainate, dopamine or muscarinic receptors. This suggests the possibility for activity-dependent regulation of oligodendrocyte differentiation and myelination, and raises the possibility of medications acting on neurotransmitters or the excitation of specific circuits could influence oligodendrocytes.

GENERAL COMMENTS AND DISCUSSION

ROCKLAND

This article presents the myelin environment as an attractive paradigm for investigating coupling of neuronal activity and intracellular signaling, in this case by oligodendrocytes. Another application, emphasized by this and several of the other articles as well, is in the psychiatric domain. Because of its distinctiveness

and quasi-isolation, white matter may offer some advantages over the more usual gray matter assays in investigating effects, possibly leading to new modes of treatment.

HÖISTAD AND HOF

Lee and Fields review data indicating that myelination can be altered by activity in axons. Their paper illustrates the global interplay and communication that exists between axons, oligodendrocytes, and astrocytes. Three important aspects are discussed: how activity in axons is sensed by oligodendrocytes and astrocytes, the regulation of myelin and myelin-associated genes, and the relevance for psychiatric disorders.

The activity in axons needs to be sensed by the surrounding oligodendrocytes and astrocytes. For example, this can be done through axon-derived diffusible signals including ATP, adenosine, K^+, glutamate, and GABA (Lee and Fields, 2009 Figure 1). [The issue of "diffusing signals" adheres to the concept of volume transmission, originally presented by Fuxe and Agnati (see Agnati et al., 2000; Fuxe et al., 2007) and discussed by Fields (2004)]. It has been shown that oligodendrocytes and astrocytes have receptors and ion channels that enable them to sense the activity in their surrounding. For example, oligodendrocytes have receptors for glutamate, serotonin, and dopamine, as well as purinergic receptors (Fields and Burnstock, 2006). Astrocytes have also been shown to have transmitter receptors (see, e.g., Magistretti et al., 1983; Hosli and Hosli, 1993; Porter and McCarthy, 1997). In addition, synaptic contacts between immature oligodendrocytes and axons have been found, as reviewed by Lee and Fields (Kukley et al., 2007; Karadottir et al., 2008). Astrocytes may also influence oligodendrocyte development by secretion of trophic factors and cytokines. For instance, extracellular factors generated by neuronal activity can regulate GFAP expression in astrocytes. It is possible that GFAP in astrocytes indirectly affects oligodendrocytes and myelination, axons, and neuron-glia interactions. In axons, cell adhesion molecules such as NCAM may influence axon-glia interactions. For example, myelin deposition is targeted to the correct axon site in response to a cell surface receptor expression induced by activity in the axon.

The regulation of myelin or myelin-associated genes can be made either in oligodendrocytes themselves, or via astrocytes and axons. For example, in oligodendrocytes, an important RNA-binding protein, QKI, has been shown to be downregulated in schizophrenia (Katsel et al., 2005; Aberg et al., 2006), which may have downstream consequences on oligodendrocyte development and myelination. It has also been found that the level of QKI mRNA can in fact be influenced by medication used to treat schizophrenia (Aberg et al., 2006). As several antipsychotic medications have their effects through dopamine (as well as serotonin) receptors, Lee and Fields discuss the evidence that dopamine can influence oligodendrocyte development. Although the authors state that "it is unclear exactly how dopamine function is disrupted by oligodendrocyte dysfunction", it should be kept in mind that the axons of the mesolimbic and mesocortical monoamine neurons, which have been classically implicated in the pathophysiology of schizophrenia, are mainly unmyelinated (as observed by Fuxe, 1965a,b; Descarries and Mechawar, 2000). Hence, any potential effects that oligodendrocyte and myelin dysfunction would have on monoamine axons are likely indirect, or rather affect other axons in the circuitry of psychiatric disorders.

FIELDS

Hoistad and Hof raise an interesting question of what is the pathophysiology of schizophrenia. The literature suggests that the underlying cause of this disorder may be less certain and more complex than the comment suggests. In addition to dopamine, serotonin, and norepinephrine, other neurotransmitter systems, including glutamate, D-serine, and GABA are involved. Anatomical evidence also implicates involvement beyond the mesolimbic and mesocortical pathways. White matter differences have been detected in patients with schizophrenia in widespread brain regions, including prefrontal, hippocampal, temporal, uncinate fasciculus, fornix, cingulate fasciculus, anterior cingulum, superior cerebellar peduncle, and caudate (Fields, 2008, supplemental table). Most would agree that schizophrenia is a complex disorder and it is likely a group of disorders rather than a single disease.

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