Development

Myelination of Axons Corresponds with Faster Transmission Speed in the Prefrontal Cortex of Developing Male Rats

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Visual Abstract

Significance Statement

Neural processing improves during childhood and adolescent development, but the specific factors contributing to these developmental changes are largely unknown. The present study shows that between two and six weeks of age in male rats, axons in the prefrontal cortex undergo microstructural and electrophysiological changes that speed up neural transmission. These axonal changes could contribute to some of the developmental improvements in behavioral control and cognitive abilities dependent on the prefrontal cortex.
Myelination of prefrontal circuits during adolescence is thought to lead to enhanced cognitive processing and improved behavioral control. However, while standard neuroimaging techniques commonly used in human and animal studies can measure large white matter bundles and residual conduction speed, they cannot directly measure myelination of individual axons or how fast electrical signals travel along these axons. Here we focused on a specific population of prefrontal axons to directly measure conduction velocity and myelin microstructure in developing male rats. An in vitro electrophysiological approach enabled us to isolate monosynaptic projections from the anterior branches of the corpus callosum (corpus callosum-forceps minor, CCFM) to the anterior cingulate subregion of the medial prefrontal cortex (Cg1) and to measure the speed and direction of action potentials propagating along these axons. We found that a large number of axons projecting from the CCFM to neurons in Layer V of Cg1 are ensheathed with myelin between pre-adolescence [postnatal day (PD)15] and mid-adolescence (PD43). This robust increase in axonal myelination is accompanied by a near doubling of transmission speed. As there was no age difference in the diameter of these axons, myelin is likely the driving force behind faster transmission of electrical signals in older animals. These developmental changes in axonal microstructure and physiology may extend to other axonal populations as well, and could underlie some of the improvements in cognitive processing between childhood and adolescence.

Key words: anterior cingulate; conduction velocity; forceps minor; g-ratio; myelin whole-cell patch clamp

Introduction

Cognitive abilities and behavioral control improve significantly during childhood and adolescent development (Casey et al., 2000; Lenroot and Giedd, 2006). To understand how these functions improve, we must first identify the factors underlying enhanced neural processing in the medial prefrontal cortex (mPFC) of the maturing brain. This is a brain region that integrates information from multiple sources to process complex processes including affective perception of pain (Fuchs et al., 2014), modulation of stress responses (Law et al., 2009), behavioral control (Takenouchi et al., 1999), attention (Rushworth et al., 2003; Kaping et al., 2011), and working memory (Seamans et al., 1995). Physical changes to axonal pathways projecting into the mPFC could contribute to improved prefrontal functions in adulthood (Chugani et al., 1987; Paus et al., 1999; Sowell et al., 1999). Here, we sought to determine when and how prefrontal axons change in developing male rats.

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Notably, myelin ensheathment is complex and can vary in length and thickness along the same axon depending on where it resides within the brain (Tomassy et al., 2014). It is therefore important to examine myelin microstructure along different portions of axons that reside in white versus gray matter regions of the brain.

The present study investigated how prefrontal axons change physically and functionally during development. To accomplish this goal, we focused on an axonal population that extends from the anterior branches of the corpus callosum (corpus callosum-forceps minor, CC<sub>FM</sub>) into the dorsal mPFC (anterior cingulate cortex, Cg1). The Cg1 is not fully developed by adolescence (Benes, 1989; Cunningham et al., 2002, 2008) and has been shown to be critical for attention (Koike et al., 2016), spatial memory (Wartman et al., 2014), and decision-making (Kennenley et al., 2006). We also found myelinated axons in this region are sensitive to alcohol (Vargus et al., 2014). Thus, the location of the Cg1 respective to the CC<sub>FM</sub> provides a unique experimental preparation to isolate, measure, and analyze different physical and electrophysiological properties of these axons in developing animals. We used a two-pronged approach to study these axons in preadolescent and adolescent male rats. Neurotransmission speed of individual axons was assessed by slice electrophysiology and myelin ensheatment was assessed using microstructural histology. This strategy enabled us to detect age-dependent changes in myelin and faster neural processing within individual axons that could contribute to improved neural processing in a brain region critically important for executive functions and behavioral control.

Materials and Methods

Animals
Male Wistar rats were ordered from Charles River (preadolescent rats were shipped with nursing moms). Separate animals were used for the electrophysiological and myelin microstructure histological experiments. For the electrophysiological experiments, “preadolescent” animals were postnatal day (PD)8–PD15 and “adolescent” animals were PD40–PD58. In these experiments, 21 total cells were recorded from five preadolescent rats and 13 total cells were recorded from four adolescent rats. For the myelin microstructure histological experiments, brains were processed from four preadolescent (PD15) and four adolescent (PD43) animals. All animals were kept on a 12/12 h light/dark cycle (lights on at 8 A.M.), with food and water available ad libitum. Weaning of pups does not occur until PD21; thus, all preadolescent animals were housed with nursing moms and three to four other pups, whereas adolescent animals were housed two to three per cage in this study. All procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Experiment 1: in vitro slice electrophysiology
Preparation of brain tissue slices for electrophysiology
Following CO<sub>2</sub> euthanasia, brains were rapidly removed and placed into ice-cold cutting solution, which contained 89.1 mM sucrose, 13.88 mM glucose, 87.27 mM NaCl, 2.48 mM KCl, 1.25 mM sodium phosphate monobasic monohydrate, 25 mM sodium bicarbonate, 7 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, and 0.37 mM CaCl<sub>2</sub>. Coronal slices were cut at 300-μm thickness with a vibratome (Leica VT1200 S with Vibracheck). The slices were then incubated in artificial CSF (aCSF) at 33°C for 45 min. aCSF contained 127 mM NaCl, 25 mM sodium bicarbonate, 25 mM glucose, 2.5 mM KCl, and 1.25 mM sodium phosphate monobasic monohydrate. The cutting solution and aCSF were bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> gas.

Stimulation and recordings for electrophysiology
Slices were transferred to a recording chamber and continuously perfused with aCSF. During recording, neurons were located using an Olympus BX51W1 microscope with 4× and 60× objectives. Cells in the Cg1 were then patched under the whole-cell voltage-clamp mode (the amplifier was HEKA EPC10/2 USB with Patch master for data acquisition). Micropipettes had a bath resistance between 3 and 11 MΩ (pipette puller Narishige PC-10), with an internal solution containing 130 mM KGlut, 10 mM KCl, 10 mM HEPES, 1 mM EGTA, 3 mM MgATP, and 0.5 mM NaGTP combined with Alexa Fluor 594 fluorescent dye to visualize the patched cells (Fig. 1B). It is important to note that while Alexa Fluor 594 dye has been shown to alter AMPA-receptor-mediated EPSCs at +40 mV, these effects were not evident at lower voltage (~60 mV, Maroteaux and Liu, 2016). It is therefore unlikely that this dye had a measurable effect on our results, as our cells were held at ~80 mV (described below). A concentric bipolar stimulating electrode was used to excite fibers in the dorsal-medial region of the CC<sub>FM</sub> (Analog Stimulus Isolator Model 2200 from A-M Systems). For all animals, the boundary between the CC<sub>FM</sub> white matter and the beginning of the gray matter (Cg1) could easily be discerned under 60× magnification because fiber tracts were visible in CC<sub>FM</sub>, whereas no fiber tracts were visible in cortical tissue. The stimulation electrode was placed in the CC<sub>FM</sub> and it remained there through the duration of the experiment, whereas the recording electrode was moved from one cortical neuron to another within the Cg1. EPSCs were recorded in the patched cells within the cortical gray matter under voltage clamp at ~80 mV. We held the cells at this voltage to increase EPSC amplitudes so that EPSCs with small amplitudes could be detected reliably. Initially, a stimulation ramp protocol was run to determine how the EPSC profile changed with increasing stimulation voltage, and then the cell was repeatedly stimulated with a constant intensity to establish a stable response. A low intensity stimulation paradigm (10–30 μA, 0.2 ms) was used to generate a minimal amplitude EPSC. This strategy was used for two reasons. First, in order for a cell to respond to such a low stimulation, the CC<sub>FM</sub> axon stimulated must have a monosynaptic connection to the cell. This assumes that if there were a cell intermediate to the stimulated axon and the recorded cell, the low intensity stimulation of the axon would generate an EPSC in this intermediate cell, but this would not be sufficient to elicit an action potential that would then stimulate an EPSC in the patched cell (illustrated in Fig. 1D). Second, the axon terminal must have
multiple synapse sites on the postsynaptic cell to generate a detectable change in current, i.e., the sum of the EPSCs delivered must be detectable above noise. This strategy therefore isolated the single axon that has the strongest connection to the recorded cell. Threshold was determined by lowering the stimulation intensity to the minimum level at which EPSCs could still be evoked, accompanied by frequent failures. The EPSC onset was measured as the time point where the slope of the current changed from baseline following the EPSC artifact.
Electrophysiological measurements and analyses

Images of the patched cells were taken under 4× magnification to determine the distance between the stimulation site and recorded cell (transmission distance). Two different methods were used to determine the response latency. The first method used the time difference between the start of the stimulation artifact (Fig. 1C, arrow) and the detection of the post-artifact current change that fell below the baseline current of the cell. The second method determined the latency between the start of the artifact and the peak of the resulting EPSC. This method was only used to calculate the response latency jitter for each cell, as the EPSC peak does not necessarily reflect the most accurate measure of EPSC onset. Jitter is the variability in response latency observed throughout various stimulations. A response latency jitter value higher than 0.2 ms indicates that the response is synaptically driven, or orthodromic (Pelletier and Paré, 2002), as neurotransmission time can vary between trials. Jitter for each series of stimulations was calculated as the standard deviation of the response latencies using ≥10 individual traces.

Experiment 2: histology

Processing of brain tissue for analysis of myelin

Pre-adolescent (PD15) and adolescent (PD43) rats were perfused with 1% paraformaldehyde/1.25% glutaraldehyde in 0.12 M phosphate buffer, pH 7.4 with 0.2 mM CaCl₂ added. After 4 d postfixation, a vibratome was used perfused with 1% paraformaldehyde/1.25% glutaraldehyde. Processing of brain tissue for analysis of myelin was calculated as the standard deviation of the response latencies using ≥10 individual traces.

Statistical analyses

All statistical analyses were performed using R statistical software package (R Core Team, 2014). Student’s t tests were used to assess the difference in conduction velocity, jitter, forceps minor area, and myelinated axon number between the two age groups. To analyze g-ratio, the average g-ratio was calculated for each region in each rat by analyzing 100 axons for Cg1 and 100 axons for CC_Fm in each animal. Groups were compared by Student’s t test using the average g-ratio of each rat. Pearson correlation analyses were used to test for relationships between latency and transmission distance, velocity and distance, and between g-ratio and axon diameter. A two-sample Kolmogorov–Smirnov test was used to compare axon diameter distributions between groups. Statistical significance was defined as p ≤ 0.05 using two-tailed tests.

Results

Conduction velocity increased from pre-adolescence to adolescence in CC_Fm axons that synapse onto cells in Layer V of the Cg1

We evaluated how age impacted the speed of neurotransmission in CC_Fm fibers with monosynaptic connections to cells in the Cg1. Conduction velocity (calculation includes synaptic delay) nearly doubled in these axons from pre-adolescence (PD8–PD15) to adolescence (PD40–PD58, 0.11 ± 0.01 vs 0.18 ± 0.01 m/s, respectively, f(24,68) = 6.47, p = 0.000001; Fig. 1G). Importantly, jitter was above 0.2 ms for all cells (range: 0.26–7.91), indicating that the stimulation was orthodromic and traveled down the axon to the terminals in Layer V of Cg1 (Fig. 1E).
There was a positive relationship between conduction velocity and transmission distance in both pre-adolescent and adolescent groups.

To determine whether conduction velocity remains constant across axons or changes depending on how far away the postsynaptic cell is from the stimulation, we first analyzed the relationship between the time to EPSC onset ("response latency") and the distance between the stimulation site and the recorded cell ("transmission distance"). These variables were not significantly correlated...
in either age group (pre-adolescents: \( r = 0.24, p = 0.30 \); adolescents: \( r = 0.44, p = 0.14 \); Fig. 1F). However, when we examined the relationship between conduction velocity and transmission distance, there was a significant, positive correlation in both pre-adolescents (\( r = 0.61, p = 0.004 \)) and adolescents (\( r = 0.69, p = 0.0085 \)). Thus, transmission speed was fastest in the axons that projected to cortical cells further away from the CCFM (Fig. 1H).

### The number of myelinated axons increased from PD15 to PD43 in the Cg1 and CCFM

To address whether developmental increases in the speed of neurotransmission between the CCFM and Cg1 corresponds to increased myelination in these regions, we first assessed if there were age-dependent changes in the cross-sectional area of the CCFM (myelinated portion). The size of CCFM was more than three times larger in adolescent compared to pre-adolescent rats (0.70 ± 0.13 vs 2.39 ± 0.32 mm², respectively, \( t_{(3.95)} = 5.65, p = 0.005 \); Fig. 2A,B). The developmental increase in the size of the CCFM size may be due in part to a near 5-fold increase in the density of myelinated axons in this structure (28.64 × 10³ ± 2.69 × 10³ axons per mm² in pre-adolescent animals vs 125.15 × 10³ ± 12.26 × 10³ axons per mm² in adolescent animals, \( t_{(3.29)} = 8.88, p = 0.002 \); Fig. 2D). The change in the number of myelinated axons was more substantial in the Cg1, with a 90-fold increase in density from pre-adolescence to adolescence (0.33 × 10² ± 0.07 × 10² axons per mm² vs 30.34 × 10³ ± 2.44 × 10³ axons per mm², respectively, \( t_{(3.00)} = 14.17, p = 0.0008 \); Fig. 2C).

No measurable changes in relative myelin thickness were detected in the CCFM between PD15 and PD43

We next measured the average g-ratio of myelinated axons in the CCFM of both age groups to determine if there were developmental increases in the relative thickness of myelin on these axons. G-ratios were not different between the two age groups, indicating that the relative thickness of myelin sheaths was not a factor contributing to the larger size of the CCFM in older animals (0.73 ± 0.01 vs 0.74 ± 0.02, respectively, \( t_{(5.43)} = 0.78, p = 0.47 \); Fig. 3B). Based on the combined findings above (a developmental increase in the number of myelinated axons without an overall decrease in g-ratos), we assume that these CCFM axons are undergoing de novo myelination and the process from initiation of wrapping to completion of a myelin segment occurs quite rapidly.

### The size (diameter) of myelinated axons in the CCFM did not change with development

To address whether developmental increases in the speed of neurotransmission corresponds to increased axon diameter, we measured average myelinated axon diameter and assessed the distribution of diameters in CCFM between PD15 and PD43. Average axon diameter did not change between the two age groups (0.89 ± 0.10 vs 0.89 ± 0.07 μm, respectively, \( t_{(75.01)} = -0.02, p = 0.98 \)). Two-sample Kolmogorov–Smirnov test showed no significant changes between the distributions (\( p = 0.17 \)). Thus, both the average diameter of axons and the range of axon diameters did not increase from pre-adolescence (range: 0.3–2.3 μm) to adolescence (range: 0.4–2.1 μm; Fig. 3C).
Discussion

The brain undergoes a series of maturational processes during childhood and adolescence that is thought to drive enhanced cognitive function in adulthood. To gain a better understanding of these developmental factors at the circuit level, we used histological and electrophysiological approaches to test for changes in axonal myelination and neurotransmission speed in the developing mPFC of male rats. Between two and six weeks of age, a large number of axons are being myelinated in this anterior region of the brain, and the addition of myelin segments along the axon appeared to move anterogradely from the corpus callosum out toward the axonal terminals in Layer V of the adjacent cortex (Fig. 4). These data complement previous studies investigating white matter changes in the developing brain of rodents (Kim and Juraska, 1997; Markham et al., 2007; Calabrese and Johnson, 2013; Downes and Mullins, 2014; Mengler et al., 2014; Willing and Juraska, 2015), and provide new evidence suggesting that increases in prefrontal white matter could be partially due to de novo myelination of axons rather than the thickening of myelin sheaths on previously-myelinated axons. Correspondent with myelination of prefrontal axons, was a significant increase in the speed at which electrical signals travel down these axons. By isolating monosynaptic projections, we gained insight into how electrical information travels from the anterior branches of the corpus callosum to the mPFC. The robust morphologic changes and improvements in transmission speed in individual prefrontal axons that were observed in the present study provide a means by which cognitive processing could improve between childhood and adolescence (Casey et al., 2000; Lenroot and Giedd, 2006).

Myelination of prefrontal axons appears to be a key factor underlying the developmental increases in neural processing. There was a significant increase in the number of myelinated axons in the CCFM and Cg1 and the change in the size of the CCFM in older animals is presumably due to de novo myelination of axons rather than thickening of myelin sheaths on previously-myelinated axons. Consistent with this interpretation, we did not find an age-dependent increase in the relative thickness of myelin (decrease in g-ratio). In older animals, electrical signals traveled twice as fast along axonal projections from the CCFM to neurons in Layer V of the Cg1. Two different mechanisms could account for increased trans-
mission speed: (1) larger diameter of the axons or (2) myelination of the axons (Hursch, 1939; Waxman, 1980; Hartline and Colman, 2007). As there was no age difference in the mean diameter or range of diameters in myelinated axons we can rule out increased diameter as a mechanism for increased axonal speed. Instead, myelination appears to be responsible for the developmental increase in axonal signal transmission speed.

To the best of our knowledge, this is the first demonstration of increased conduction velocity in this specific fiber population (CCFM → Cg1), providing insight into one mechanism by which neural processing is enhanced during development. Perhaps similar increases in conduction velocity are occurring in axons within the posterior branches of the corpus callosum (splenium) and other regions that are being myelinated during this time (Kim and Juraska, 1997; Downes and Mullins, 2014; Mengler et al., 2014).

The data herein allow us to make inferences about how the myelination process takes place in the juvenile prefrontal cortex. Myelin ensheathment of prefrontal axons during this developmental period seems to move from the more lateral portion in white matter to the more medial portion in gray matter. In our sampled region, we observed that >28,000 axons/mm² were myelinated in the CCFM of PD15 animals, but only 334 axons/mm² were myelinated in the Cg1 at this age. By PD43, the number of myelinated axons increased dramatically in the Cg1, reaching over 30,000 axons/mm². Based on our electrophysiological evidence in monosynaptic axonal projections and macroscopic visualization of these axonal projections in embedded slices, we assume the microstructural myelin parameters obtained from the CCFM versus the Cg1 serve as representative examples of white versus gray matter segments of same group of axons. Presumably, oligodendrocytes are adding myelin sheath segments along these prefrontal axons moving from the lateral portions in the white matter toward the medial portions residing in the gray matter, i.e., moving anterogradely down the axon toward the terminals. Research in zebrafish indicates that the process of myelination of axons occurs quickly (Czopka et al., 2013). Oligodendrocytes initiate and complete myelination of a single segment along an axon within just 5 hours (Czopka et al., 2013). The rapid myelination processes could also explain why we did not detect age differences in g-ratios despite the robust increase in de novo myelination during this time. Presumably, the short period of time between the initiation and completion of myelin ensheathment of an axonal segment prevented us from capturing axons at a point when the new myelin sheaths were still thin. One benefit of rapid myelination may be the ability of the axon to quickly reach its optimal g-ratio (~0.77 for axons in the central nervous system; Chomiak and Hu, 2009).

Several studies have demonstrated that variations in either axonal diameter or in myelin ensheathment can generate variability in transmission speed. This variability serves to synchronize the arrival of the signals to the same destination despite differences in fiber length, i.e., isochronicity (Baker and Stryker, 1990; Sugihara et al., 1993; Salami et al., 2003; Lang and Rosenbluth, 2003). We found that conduction velocity was higher in the axons that had to travel further away from CCFM to reach their target cells in Layer V of the Cg1. This suggests a possibility of isochronicity of incoming neural signals to these postsynaptic cells. Moreover, this capability may already be in place by PD15 because the positive relationship between transmission distance and velocity was similar in both age groups. The fact that there was a negligible number of myelinated axons found in the Cg1 of younger animals argues against differential myelination as a mechanism underlying this relationship. Perhaps variation in axonal diameters plays a role, but this remains to be determined.

Developmental improvements in prefrontal function could be due to a number of factors, including the higher axonal transmission speed observed in the current study. There is also documented evidence for increased connectivity between prefrontal and limbic regions (Cunningham et al., 2002), changes in excitatory and inhibitory neurotransmission (Jackson et al., 2001; Floresco and Tse, 2007; Grace et al., 2007), all of which could lead to enhanced neural processing and communication. We noted a trend of an increase in jitter in the older group, which could signify other developmental changes. Higher jitter is often associated with multiple axonal inputs synapsing onto the same cell, especially if these inputs are polysynaptic. However, the stimulation paradigm used for these experiments isolated monosynaptic connections. Therefore, increased variability in neurotransmission time could be due to more monosynaptic connections projecting to the same cell in older animals or to other factors such as calcium availability, neurotransmitter release, opening of the channels, etc. Additional research would be required to distinguish between these different possibilities.

Several limitations of the present study should be considered. First, because we focused on male rats in this initial study, it is unknown whether females show similar developmental changes in prefrontal axons. Second, without a more extensive timeline, we were not able to determine if the increase in myelination of these prefrontal axons between PD15 and PD43 is gradual or sudden. A diffusion tensor imaging study in male rodents shows that FA sharply increases between PD12 and PD18 in the cingulum—an axonal bundle that is adjacent to the corpus callosum (Calabrese and Johnson, 2013). These imaging data suggest that there is a rapid increase in myelination or axonal alignment of cingulum axons. As the CCFM region sampled in our study may contain some of the anterior axons of the cingulum, it will be important in future studies to refine the timeline and determine how rapidly CCFM axons are being myelinated, and if this developmental trajectory differs with sex. Third, as with many developmental studies we must consider the confounding variables of studying these two age groups. Animals under PD21 such as the pre-adolescent animals in our study are usually housed in larger groups with nursing moms, whereas animals older than PD21 such as the adolescent animals in our study have already been...
separated from their mothers and were housed in pairs or triads. Moreover, the animals herein were shipped from the vendor, and were therefore exposed to this stressor during different developmental time points and with or without nursing moms. Housing conditions and stress hormones are known to impact myelin (Chari et al., 2006; Makinodan et al., 2012). Differential sensitivity to these experiences could have modulated some of the age-related differences in myelin measures, although it is unlikely to fully account for the 5- and 90-fold changes in myelinated axon number observed in the present study. Fourth, our electrophysiological design isolated only part of the axonal pathway projecting to the cells in Layer V. As such, the origin of these axons is unknown and we can only infer that isochronicity was evident before adolescence based on the relationship between transmission distance and speed in younger animals. Future studies using tract tracing techniques could help determine the origin of these axons and would allow for more direct investigation of isochronicity, similar to what has been observed in other brain regions (Baker and Stryker, 1990; Sugihara et al., 1993). Finally, we were not able to measure diameters of unmyelinated axons, as our experimental design only allowed us to visualize myelinated axons. It should be noted that the average and range of diameters of myelinated axons in the CCpm did not change with age despite a substantial increase in the number of myelinated axons, similar to what has been shown in the CC splenium (Kim and Juraska, 1997). It is therefore reasonable to assume that axonal diameters may be similar in unmyelinated and myelinated axons. In conclusion, the data altogether provide insight into the microstructural changes that occur within prefrontal axons during adolescence and how these developmental changes may affect the speed of electrical signals coming into this brain region. The observed developmental increase in conduction velocity, coupled with a lack of significant change in axonal diameter across development, suggests myelination may be the key contributor to this change in axonal speed. These findings highlight the critical role myelin may be playing in brain maturation just before and during adolescence. Future studies in rodents could allow for direct comparison between the microstructural methods used in the current study with macrostructural imaging techniques such as diffusion tensor imaging (DTI). This would help improve our interpretation of imaging data obtained in humans. This study also provides an important foundation for future studies investigating how toxic substances or environmental disruptions could interrupt normal development and lead to impaired function of these prefrontal circuits.

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