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Title: Delayed myelination and neurodevelopment in male seizure prone (FAST) versus seizure resistant (SLOW) rats

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Summary

Objective: Aberrant myelination and developmental delay have been reported in epilepsy. However, it is unclear whether these are linked to intrinsic mechanisms that support a predisposition towards seizures and the development of epilepsy. Thus, we compared rates of myelination and neurodevelopment in male rats selectively bred for enhanced susceptibility to kindling epileptogenesis (FAST) with male rats bred for resistance (SLOW).

Methods: Myelin-specific gene expression was compared in the brainstem, cerebellum and cerebral hemisphere of FAST and SLOW rats on postnatal days (PND) 5, 11, 17, 23 and 90 to determine strain-specific myelination rates. Myelin protein levels were also compared at PND 5 and 23 in the brainstem. Relative rates of neurodevelopment were evaluated between PND5-21 using physical growth landmarks and neuromotor tests including righting reflex, cliff avoidance, negative geotaxis and locomotor activity.

Results: Myelin-specific mRNA expression was significantly down regulated in FAST rats on PND5 and 11 in all three brain structures, indicating relatively delayed myelination. Likewise, corresponding protein levels were significantly lower in FAST brainstem on PND5. Developmental delay was evident in the FAST strain such that only 9% of FAST pups, compared to 81% of SLOW, had open eyes by PND13, locomotor activity was significantly reduced between PND12-16 and neuromotor task acquisition was delayed between PND5-10.

Significance: Relative delays in myelination and neurodevelopment co-occurred in the seizure-prone FAST strain in the absence of seizures. These findings suggest these symptoms are not seizure-induced and may be mechanistically linked to an underlying pathophysiology supporting a predisposition towards developing epilepsy.

Keywords: developmental delay, seizure susceptibility, epilepsy, neurodevelopmental disorders

Introduction

Myelination in brain occurs predominantly during early postnatal development and forms a compact myelin sheath around axons to insulate and secure neural communication. Any deviation from the

'normal' myelination can ultimately affect myelin integrity and thereby appropriate functioning of the central nervous system. Accordingly, aberrant myelination indicative of altered structural connectivity have been associated with various clinical phenotypes including behavioural impairments[1] and developmental delay[2]. The prevalence of co-occurring delays in myelination and development suggests a functional relationship yet the nature of that relationship is unclear, with one study demonstrating a positive correlation between the two [2] while another reported no correlation in children with idiopathic developmental delay [3]. Abnormal myelination and developmental delay have been observed in children with chronic epilepsy [4; 5] and neurodevelopmental disorders [6-9] that often comorbid with epilepsy.

Clinical studies designed to examine rates of myelination and neurodevelopment in children with epilepsy are often confounded by the influence of existing recurrent seizures [10], making it difficult to determine cause and effect. In this study, we compared rates of myelination and neurodevelopment in a rat strain that has been selectively breed to have enhanced vulnerability to kindling epileptogenesis (FAST) versus resistant (SLOW) male rats [11]. FAST rats also display a behavioural phenotype reminiscent of neurodevelopmental disorders that comorbid with epilepsy, namely ASD and ADHD [12]. Furthermore, and of relevance to this study, our recent MRI and DTI studies demonstrated white matter structural and volume differences in the corpus callosum and the cerebellum between FAST and SLOW adult rats [13; 14]. In light of this finding, and the fact that investigation into myelination in developmentally delayed children has largely focused on the cortex or subcortical regions such as the brainstem [15] and the cerebellum [16], in this study myelination was compared in these structures by way of myelin-specific gene and protein expression at various postnatal developmental time points. The four principle myelin components examined are involved in both formation and structural integrity of the myelin sheath, namely Myelin Basic Protein (MBP), Proteolipid Protein (PLP), Myelin Associated Glycoprotein (MAG) and Myelin Oligodendrocyte Glycoprotein (MOG). Rates of neurodevelopment were evaluated by comparing the acquisition of physical landmarks, appropriate reflexes and locomotor activity. Given FAST rats are highly seizure prone and exhibit behavioural profiles resonant with neurodevelopmental disorders comorbid with epilepsy, we hypothesized that, relative to SLOW rats, FAST rats would exhibit delays in both myelination and neurodevelopment.

Materials and Methods

Animals

The FAST and SLOW rat strains were derived from parental populations of Wistar and Long Evans Hooded rats using a selective breeding process based on fast and slow rates of amygdala kindling [11]. All experimental procedures used in this study adhered to the Australian Code of Practice and were approved by the Florey Animal Ethics and the University of Melbourne Ethics Committees. Rats were housed at 21±1°C in Double Decker ™ acrylic cages (Tecniplast®, Buguggiate (VA), Italy) under a 12-hour light/dark cycle (Lights on between 7 a.m. – 7 p.m.) with *ad libitum* access to food and water. Male rats were selected for this study given the higher prevalence of epilepsy [17], ASD [18] and ADHD [19] in males.

Tissue collection cohort: 20 adult male rats (10 SLOW and 10 FAST) were housed individually with 2 females from the same strain. Females (20 SLOW and 20 FAST) were monitored for signs of pregnancy and then singly housed. To determine the time and date of birth, females were checked for litters 3 times a day (7am, 12pm and 6pm). All pups were sexed on PND3 to establish the number of male pups available. In total, 80 (40 SLOW and 40 FAST) male pups were assigned to one of 5 developmental time points (PND5, 11, 17, 23 and 90) on the day of tissue collection. All pups within each time point (n=8) were taken from separate litters.

Postnatal development cohort: 16 adult male rats (8 SLOW and 8 FAST) were individually housed with a female from the same strain. Females were monitored for signs of pregnancy to calculate gestational length and then singly housed and checked for litters as per above. Birth times for resultant litters were matched between strains with maximum birth time difference of three hours and pups were sexed on PND 3. A total of 22 (11 SLOW and 11 FAST) male pups from eight different litters (4 SLOW and 4 FAST) were selected for developmental tests with no more than four brothers.

Rate of myelination

Tissue collection and preparation: In rats, myelination occurs majorly until PND 90 [20] and rapid myelin formation starts from PND 10-12 days that peaks around PND 20 [21]. Therefore, five time points were selected: one before peak time (PND 5), three during peak/rapid myelination (PND 11, 17 and 23) and one during the end period of myelination (PND 90). FAST (n=8) and SLOW (n=8) male rats were live decapitated on each of PND5, 11, 17, 23 or 90 and brain regions including the left and right cerebral hemispheres, cerebellum and brainstem were extracted. The brainstem and

cerebellum were cut through the midline into two equal parts, one for mRNA and the other for protein extraction, and then snap frozen in liquid nitrogen.

Quantitative PCR: Total RNA was isolated from brainstem, cerebellum and left cerebral hemisphere of FAST (n=8) and SLOW (n=8) rats on PND5, 11, 17, 23 or 90 using the QIAGEN RNeasy Lipid mini-kit (Melbourne, Australia) as per the manufacturer's instructions. RNA concentration and purity was determined using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific; Wilmington, U.S.A.). cDNA was synthesized from 1µg of RNA using reverse transcription with RT² HT First Strand Kit (QIAGEN; Melbourne, Australia). Quantitative PCR reactions were set up in a 384 well plate format using the EpMotion 5070 liquid handling robot (Eppendorf; Melbourne, Australia). cDNA was amplified using ViiA™ 7 Real-Time PCR System (Life technologies; Melbourne, Australia), SYBR® Select Master Mix (Life technologies; Melbourne, Australia) containing SYBR® GreenER™ dye, AmpliTaq® DNA Polymerase, dNTPs with dUTP/dTTP blend, heat-labile uracil-DNA glycosylase (UDG) and ROX passive reference dye. QIAGEN RT^2 qPCR Primer Assays (Melbourne, Australia) were used for genes of interest including MBP, PLP, MAG, MOG and two housekeeping genes (Gapdh, Sdha, Actb, Ubc, Tbp). Details of RT^2 qPCR Primer Assays are listed in Supplementary Table 1. Relative rates of myelination between the two strains were measured by comparing transcript levels of these myelin proteins across developmental time points. Relative transcript expression was determined using $\Delta\Delta C_T$ method [22]. C_T values of MBP, PLP, MAG and MOG were normalized to one of the housekeeping genes and then FAST samples were normalized to SLOW rat samples.

Western Blotting: Given that expression of myelin genes was observed to be different during early postnatal development that normalized by PND 23, protein expression was evaluated at PND5 and 23. Protein lysates were prepared from the remaining half of the brainstem isolated from SLOW (n=8) and FAST (n=5) rat pups on PND5 and 23. Tissue was homogenized in RIPA buffer (150 mM sodium chloride, 50 mM trisaminomethane pH 7.5, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.5% NP-40), 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Sigma-Aldrich®, Melbourne, Australia) at 1mL per 100 mg tissue followed by sonication. Lysates were spun at 17,000rpm at 4°C for 20 minutes to remove insoluble tissue debris and clear supernatant was stored at -80°C. Protein concentration was measured using colorimetric detection method based BCA Protein Assay Kit (Thermo Scientific Pierce, Melbourne, Australia). 10µg of protein was run on 4-12% Bis-Tris polyacrylamide gels (NuPAGE® Novex®, Melbourne, Australia)

for protein separation. Proteins were transferred to Odyssey® PVDF (Polyvinylidene fluoride) membrane (Millennium Science, Melbourne, Australia) via wet transfer method using Bio-Rad Mini Trans-Blot® Cell transfer system (Bio-Rad, Melbourne, Australia). PVDF membranes were blocked in Odyssey® Tris-Buffered Saline blocking buffer (Millennium Science, Melbourne, Australia). Each membrane was cut into two halves. One half was probed with primary antibodies against rat MBP (MAB386) at 1:2000 dilution or mouse PLP (MAB388) at 1:1000 or mouse MOG (MAB5680) (Merck Millipore, Melbourne, Australia) at 1:1000 dilution for overnight at 4°Cand the other half was probed with a mouse alpha-Tubulin (05-829; Merck Millipore, Melbourne, Australia) antibody as a loading control at 1:5000 dilution for overnight at 4°C. According to the primary antibody host, blots were probed with fluorescently tagged secondary antibodies i.e. IRDye® 800CW Goat anti-Mouse (H+L) (925-32210; Millennium Science, Melbourne, Australia) or IRDye® 800CW Goat anti-Rat (H+L) (925-32219; Millennium Science, Melbourne, Australia) at 1:20,000 dilution for 1 hour at room temperature and proteins were detected using Odyssey® Clx infrared imaging system (Millennium Science, Melbourne, Australia). Protein band intensities were quantified using the Image J software, with MBP, PLP and MOG protein levels normalized to alpha-tubulin levels.

Neurodevelopment

From PND5-21 all pups were weighed and examined daily for eye opening (both eyes fully open). Neurobehavioral testing was also conducted daily between PND5 and 21. As crawling and walking develops around PND10-12 with locomotion maturation at PND15 [23], locomotor activity was assessed daily from PND11 to 21. Testing took place between 7.30 -10.00 am and tests were conducted in the following order: eye opening, body weight, righting reflex, cliff avoidance, negative geotaxis, wire hanging and locomotor activity. All pups were removed from the home cage and placed in a small holding cage during testing period. Separation from the mother was kept to less than 30 minutes to minimize stress. Tests were video recorded and examined by a single investigator blinded to the rat strain.

Righting reflex: When placed in supine position rat pups reflexively try to right themselves. It is associated with sensory-motor coordination and subcortical maturation [24]. Pups were placed in a supine position on a flat surface, held in this position for 5 seconds and then released. The time taken by each pup to move from supine to quadrupedal position was recorded. Daily trials were limited to 60 seconds.

Cliff avoidance: This reflex involves retraction when placed on the edge of a platform. It is associated with neuromotor development [25]. Pups were placed on the edge of a platform with their forepaws and nose extending over the edge. The avoidance response was scored as 0, 1 and 2 according to the time required to fully retract the body and have all four paws on the platform (0 = no response i.e. when pup was unable to turn back, 1 = slow response more than 5 seconds, 2 = immediate response within 5 seconds).

Negative geotaxis: it is associated with cerebellar integration [24]. When placed in head-down position rats try to orient themselves into a head-up direction. Pups were placed facing downwards in the center of a 45° inclined board (43cm length x 28cm wide) with a friction surface. The time required to make a full 180° rotation was recorded up to a maximum of 60 seconds. The trial was not included in the analysis if the pup fell down the board during the trial or moved downwards instead of turning upwards.

Wire Hang test: The wire hang test is used to examine neuromuscular strength [26]. The front paws of each pup were placed on a horizontal rod (2 mm thick, 50 cm long) secured 40 cm above a padded surface by their forelimbs. The time (seconds) until pup fell was recorded with maximum time of 60 seconds.

Locomotor activity: It examines locomotor and neuromuscular development [23]. Each pup was placed in the middle of a photocell cage (43 cm × 43 cm × 31 cm, l × w × h, ENV-520, Med Associates Inc., St. Albans, VT, USA) equipped with an automated infrared transmitter and receiver system used to detect the three dimensional position of the pup. For each pup a spatiotemporal map was then created to reflect minute by minute movement patterns and the total distance (cm) travelled within 5 minutes was calculated using Activity Monitor 4.0 Software (Med Associates Inc.; U.S.A).

Statistical analysis

Statistical analysis of qPCR and western blots were performed using Mann-Whitney U two tailed test to identify strain difference between four myelin proteins expression (MBP, PLP, MAG and MOG) at each development time point. All physical growth and neurodevelopmental behavior datasets were analyzed using 2-way Analysis of Variance (ANOVA) for repeated measures with strain and PND as the two independent variables. Strain x PND pairwise comparisons were assessed using Bonferroni post-hoc analysis. Statistical significance was defined as $p<0.05$. Data were expressed as mean ± SEM.

Results

Gestational length of FAST and SLOW mothers

The gestational length of FAST and SLOW mothers was within the average range found in rodents. No significant difference was observed in gestation period of FAST (21.3±0.6) and SLOW (21.5±0.6) mothers ($U = 98.5$, $p = 0.5$, Mann Whitney unpaired two tailed test).

Delayed myelination in FAST versus SLOW rats

Cerebral hemisphere: mRNA expression of MBP, PLP and MOG was significantly reduced in the left cerebral hemisphere of FAST versus SLOW male pups at PND5 (MBP $U = 2$, $p = 0.001$, PLP $U = 5$, $p = 0.003$ and MOG $U = 5$, $p = 0.001$, Mann Whitney unpaired two tailed test, Figure 1A, B & D respectively) and PND 11 (MBP $U = 4$, $p = 0.002$, PLP $U = 9$, $p = 0.02$ and MOG $U = 6$, $p = 0.005$, Mann Whitney unpaired two tailed test). MAG in FAST pups was significantly reduced at only PND11 ($U = 10$, $p = 0.02$, Mann Whitney unpaired two tailed test, Figure 1C). FAST and SLOW rats showed similar expression levels from PND17 onward for each of the myelin transcripts examined.

Cerebellum: MBP and PLP were lower in FAST cerebellum at PND5 (MBP $U = 9$, $p = 0.02$; PLP $U = 11$, $p = 0.03$, Mann Whitney unpaired two tailed test, Figure 2A&B respectively) and PND11 (MBP $U = 7$, $p = 0.007$; PLP $U = 7$, $p = 0.007$, Mann Whitney unpaired two tailed test) and became similar from PND17 onward. However, no significant difference was observed in the mRNA expression of MAG or MOG (Figure 2 C&D respectively) at any time point.

Brainstem: Transcript levels for all four myelin proteins were significantly lower in FAST brainstem at PND5 (MBP $U = 0$, $p = 0.001$; PLP $U = 4$, $p = 0.002$; MAG $U = 4$, $p = 0.002$; MOG $U = 3$, $p = 0.001$, Mann Whitney unpaired two tailed test, Figure 3) and PND11 (MBP $U = 0$, $p < 0.001$; PLP $U = 1$, $p < 0.001$; MAG $U = 9$, $p = 0.02$ and MOG $U = 12$, $p = 0.04$, Mann Whitney unpaired two tailed test). As in cerebellum and cerebral hemisphere, expression levels for all four transcripts were similar between the strains from PND17 onward.

To establish whether myelin protein levels were in concordance with relative mRNA expression differences identified between these strains, western blot analysis was performed using the brainstem of PND5 and PND23 FAST and SLOW male pups. These time points were chosen given the largest consistent strain expression differences occurred at PND5 whereas similar expression levels for all four transcripts were documented at PND23. Western blot results indicated that FAST pups had significantly lower levels of the two main isoforms of MBP i.e. 21.5 kDa ($U = 6$, $p = 0.04$, Mann

Whitney unpaired two tailed test, Figure 4A, B) and 18.5 kDa (U = 4, p = 0.02, Mann Whitney unpaired two tailed test), PLP (U = *0.5*, p = 0.005, Mann Whitney unpaired two tailed test, Figure 4C, D) and MOG (U = 3 , p = 0.01, Mann Whitney unpaired two tailed test, Figure 4E, F) at PND5 but no significant difference in any of these proteins was evident at PND 23. MAG protein levels could not be quantified as the expression was too low to detect at both time points given it comprises only approximately 1.0% of total myelin protein constituent [27].

Developmental delay in FAST pups

Despite that body weight was similar in FAST and SLOW male pups between PND5-21 (Figure 5A), eye opening indicated significant effects of strain ($F_{(1, 70)}$ = 23.23, p = 0.0001, ANOVA) and time ($F_{(6, 70)}$ = 107.48, p = 0.0001, ANOVA). Post-hoc analysis revealed a significant difference between FAST and SLOW pups at PND13 (t(6) = 8.5, p = 0.0001, Bonferroni) with only 9% of FAST pups opening their eyes compared to 81% of SLOW pups (Figure 5B). All pups of both strains had opened their eyes by PND 15.

Righting Reflex: A significant effect of strain ($F_{(1, 170)}$ = 50.62, p < 0.001, ANOVA) and time ($F_{(16, 170)}$ = 35.89, p < 0.001, ANOVA) was observed for righting reflex acquisition. While the reflex execution improved in both strains as development progressed, FAST pups took significantly more time than SLOW pups to successfully complete the response between PND5 and 11 (PND5 t(16) = 4.191, p = 0.001, PND6 t(16) = 5.48, p < 0.001, PND7 t(16) = 3.224, p = 0.03, PND8 t(16) = 6.769, p < 0.001, PND10 t(16) = 4.835, p < 0.001, Bonferroni; Figure 5C).

Cliff Avoidance: Response to cliff avoidance improved with age in both strains beginning from null (0 score) or slow (1 score) to quick response (2 score) between PND5-14 (Figure 5D). A 2-way ANOVA revealed significant effects of time ($F_{(16, 170)}$ = 48.11, p < 0.001, ANOVA) and strain ($F_{(1, 170)}$ = 129.03, p < 0.001, ANOVA). FAST pups performance was significantly slower than SLOW pups between PND5 to 10 (PND5 t(16) = 4.098, p = 0.001, PND6 t(16) = 6.44, p < 0.001, PND7 t(16) = 10.54, p < 0.001, PND8 t(16) = 7.611, p < 0.001, PND9 t(16) = 7.611, p < 0.001, PND10 t(16) = 5.854, p < 0.001, Bonferroni).

Negative Geotaxis: Irrespective of strain, the response time to negative geotaxis decreased gradually between PND5-10 (Figure 5E). However, two factorial analysis revealed significant main effects of strain ($F_{(1, 170)}$ = 171.38, p < 0.001, ANOVA) and time ($F_{(16, 170)}$ = 72.29, p < 0.001, ANOVA). The relatively delayed acquisition times in FAST versus SLOW pups was observed between PND5-10 (PND5 t(16) = 10.28, p < 0.001, PND6 t(16) = 10.82, p < 0.001, PND7 t(16) = 10.55, p < 0.001,

PND8 t(16) = 11.31, $p < 0.001$, PND9 t(16) = 4.678, $p < 0.001$, PND10 t(16) = 3.55, $p = 0.009$, Bonferroni).

Wire Hanging: The ability to grip and hang on the rod did not develop prior to PND12 in either strain (Figure 5F). After PND12, increase in hang time was observed in both strains with main effect of time ($F_{(16, 170)} = 32.34$, $p < 0.001$, ANOVA) but no effect of strain ($F_{(1, 170)} = 1.94$, p = 0.17, ANOVA).

Locomotor activity: Significant main effects of time ($F_{(10, 110)} = 64.89$, $p < 0.001$, ANOVA) and strain ($F_{(1, 110)} = 80.70$, $p < 0.001$, ANOVA) were identified when distance travelled was compared in FAST and SLOW pups. Locomotor activity increased in both strains as development progressed. However, mean distances covered by FAST pups were consistently lower than those of SLOW pups, a difference that reached significance on PND15 (t(10) = 6.013, $p < 0.001$, Bonferroni) and PND16 (t(10) = 6.705, $p < 0.001$, Bonferroni; Figure 5G).

Discussion

This study compared rates of myelination alongside neurodevelopment in seizure prone FAST versus resistant SLOW rats. Relative to SLOW pups, FAST pups showed temporally-related delays in both aspects of development (Figure 6). Delayed myelination has often been reported in children exhibiting developmental delay [2], epilepsy [4] and comorbid disorders like ASD and ADHD [1; 9]. Indeed, delayed white matter growth has been documented in a mouse model of fragile X syndrome [28], a disorder characterized by seizures and autistic symptoms including developmental delay [29]. Importantly however, these and similar studies have not been designed to determine whether the delay is secondary to the presence of seizures or whether it contributes to the pathogenesis associated with seizure vulnerability. Evidence for the latter includes the fact that children born preterm often display disrupted myelination [30] and are at higher risk of developing epilepsy [31]. Moreover, a study using a rat model of malformations in cortical development (MCD), which are linked to both epilepsy and developmental delay in humans, concluded that aberrant white matter development may be the underlying pathology supporting enhanced seizure vulnerability [32]. The FAST/SLOW model, wherein no spontaneous seizures occur, precludes any involvement of seizure

sequelae in relative rates of myelination or functional development and thereby identifies any observed deviations as inherent to the vulnerable or resistant state.

Developmental delay is one predictor of intractable childhood epilepsy [33]. In this study, we confirmed a significant delay in FAST versus SLOW pup development such that, despite similar weight gain, FAST pups exhibited delayed eye opening and relatively poor performance in several neuromotor tasks prior to PND14. While documented eye opening begins in most rat strains by the end of second postnatal week [34], this process began on PND11 in SLOW pups and not until PND13 in FAST pups indicating that, as with their seizure profiles in both the kindling and chemoconvulsant models, these strains lie at opposing ends of the 'normal' spectrum. While complete acquisition of some tasks appeared to coincide with eye opening there is unlikely to be a direct functional association given that only the righting reflex is considered to have any visual-cue involvement and task performances were greatly improved in both strains prior to eye opening. Interestingly, locomotor activity levels proved lower in FAST pups throughout the pre-weaning period despite weight and neuromuscular (grip strength) equivalencies. This finding is in contrast to the many experiments demonstrating relative hyperactivity in FAST versus SLOW rats from young adulthood onward [12-14]. Important to the clinical relevance of this finding is the fact that a PND20 rat matches a human age of only 8 months, a time point when the corresponding locomotor activity would not yet be measurable in humans.

The architectural importance of MBP, PLP, MOG and MAG to the development of myelin sheath, and thereby appropriate neural communication, is clear in humans and several animal models. For instance, triple (*plp-/-*, *mbp-/-*, *mag-/-*) mutant mice exhibit hypomyelination and high frequency seizure onset by 3 months of age [35]. In humans, comparative whole transcriptome screening of focal cortical dysplasias (neocortical malformations with strong epileptogenic potential) to nondysplastic temporal neocortex from epilepsy patients reported reduced expression of myelin-associated transcripts including *mbp*, *mog* and *mag* [36]. Moreover, experimental studies on myelin-deficient (md) rats have demonstrated that lack of myelin results in regional axolemmal abnormalities such as axolemmal voltage-gated sodium channels being more diffusely distributed at higher densities. It is suggested that this ion flux increase across axolemma might lead to corresponding increased extracellular potassium levels, spontaneous activity due to random opening of the channels, low threshold for excitation and ephatic activation of neighboring axons[37]. Indeed hypomyelination

during postnatal development of FAST compared to SLOW rats might contribute towards their fast kindling tendency. Abnormal myelination has also been linked to conditions associated with heightened seizure susceptibility including developmental delay, ASD and ADHD. Of relevance, given the comorbid ASD/ADHD features manifest in the FAST strain [12], aberrant myelination has been proposed as a pathophysiological factor for persistent ADHD symptoms and poor cognitive performance in adult ADHD patients [9]. Moreover, abnormal callosal myelin development in children with autism [7] and in the BTBR mouse model of ASD [38], behavioral impairments have been associated with myelin protein down regulation and abnormalities in myelin formation [39]. These studies, and the fact that naïve FAST rats live seizure-free, suggest the delay in myelination observed in FAST versus SLOW pups is mechanistically linked to their ADHD/ASD-like behaviors and seizure vulnerability. Certainly any formative compromise to the insulating capacity of the myelin sheath is likely to negatively impact cognitive development and facilitate seizure recruitment. Consequently, these findings provide evidence for the rapid network recruitment that is endemic to FAST rats and strongly inhibited in SLOW rats [40].

Despite the common co-occurrence of delays in myelination and neurodevelopment in epilepsy and its comorbid conditions, the nature or even existence of a biological relationship between these phenotypic features remains unclear [2; 3]. The theory that a common biological experience underlies co-expression of symptoms is perhaps most supported in Angelman Syndrome (AS), a neurodevelopmental disorder characterized by several neurological problems including developmental delay, movement disorders and epilepsy. Aberrant myelination, thinned corpus callosum [41] have been reported in AS patients. Moreover, a study revealed disruption in the expression of cortical myelin proteins in a mouse model of AS and showed correction of the myelin defects when pregnant mothers were fed a higher fat diet [42]. Given the role of fat in myelination, the clinical efficacy of the ketogenic diet and FAST rats naturally exhibit lower plasma non-esterified fatty acids (NEFA) levels [43], despite maintenance on an identical diet, future investigation into the role of fat metabolism in strain-specific behavioral and seizure profiles would be relevant to epilepsy, ADHD and ASD. Use of the FAST/SLOW model in these ways will provide better understanding of the nature of the trifectic relationship between developmental delay, delayed myelination and seizure predisposition.

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Conflict of Interest

None of the authors has any conflict of interest with respect to the research, authorship, finance and/or publication of this article. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Key Points

- Delayed myelin-specific mRNA expression during myelination in male FAST versus SLOW rats.
- Delayed eye opening in male FAST versus SLOW rats.
- Delayed neuromotor task acquisition in male FAST versus SLOW rats.

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Figure legends

Figure 1: Relative mRNA expression of myelin proteins in cerebral hemisphere.

Comparative analysis between SLOW and FAST rats of mRNA expression of myelin proteins at postnatal age 5, 11, 17, 23 and 90; (**A**) MBP, (**B**) PLP, (**C**) MAG, (**D**) MOG; Mann Whitney two-tailed T-Test * $p<0.05$, ** $p<0.01$, *** $p<0.001$; MBP myelin basic protein, PLP proteolipid protein, MOG myelin oligodendrocyte glycoprotein, MAG myelin associated glycoprotein.

Figure 2: Relative mRNA expression of myelin proteins in cerebellum.

Comparative analysis between SLOW and FAST rats of mRNA expression of myelin proteins at postnatal age 5, 11, 17, 23 and 90; (**A**) MBP, (**B**) PLP, (**C**) MAG, (**D**) MOG; Mann Whitney two-tailed T-Test * $p<0.05$, ** $p<0.01$; MBP myelin basic protein, PLP proteolipid protein, MOG myelin oligodendrocyte glycoprotein, MAG myelin associated glycoprotein.

Figure 3: Relative mRNA expression of myelin proteins in brainstem.

Comparative analysis between SLOW and FAST rats of mRNA expression of myelin proteins at postnatal age 5, 11, 17, 23 and 90; (**A**) MBP; (**B**) PLP; (**C**) MAG; (**D**) MOG; Mann Whitney two-tailed T-Test * $p<0.05$, ** $p<0.01$, *** $p<0.001$; MBP myelin basic protein, PLP proteolipid protein, MOG myelin oligodendrocyte glycoprotein, MAG myelin associated glycoprotein.

Figure 4: Comparison of myelin protein expression between male SLOW and FAST rats.

Brainstem expression of myelin proteins MBP (**A**), PLP (**C**) and MOG (**E**) in FAST and SLOW rats at PND 5 and PND 23. Representative Western blot of MBP (21.5 and 18.5 kDa) (**B**), PLP (~26 kDa) (**D**), MOG (28 kDa) (**F**) protein normalized to α-tubulin (50 kDa). Mann Whitney two-tailed T-Test * $p<0.05$, ** $p<0.01$. MBP myelin basic protein, PLP proteolipid protein, MOG myelin oligodendrocyte glycoprotein, MAG myelin associated glycoprotein, PND postnatal day.

Figure 5: Developmental delay in male FAST compared to SLOW rats.

(**A**) Mean body weight of pups; (**B**) Percentage of pups opened their eyes; (**C**) Time response for righting on a surface; (**D**) Score on latency to withdraw from a cliff; (**E**) Time taken to turn 180° angle upwards on 45° angled slant board; (**F**) Latency to fall from hanging wire; (**G**) Total distance moved in 5 minutes duration; Values represent means with SEM; 2-Way ANOVA repeated measures *$p<0.05$, **$p<0.01$, ***$p<0.001$

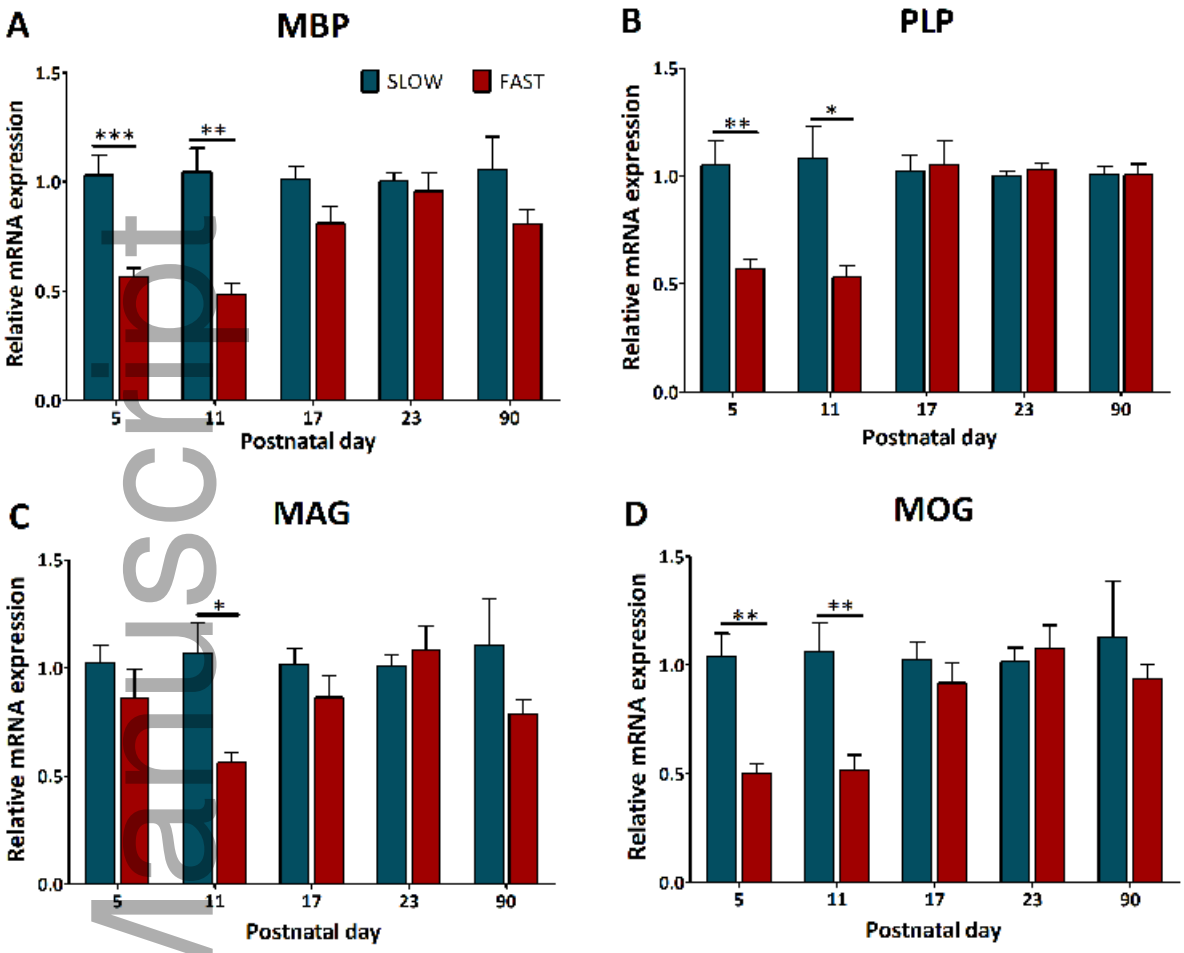
Figure 6: Schematic view of neurodevelopment and myelination between the two strains.

FAST compared to SLOW rats displayed developmental delay and delayed myelination during same time period window (PND 5-16) indicating a temporal relation between delayed development and myelination.

Supplementary Material

Supplementary Table 1. RT2 qPCR Primer assays for myelin and housekeeping genes.

The RefSeq accession number refers to the sequence used to design the primer assay.



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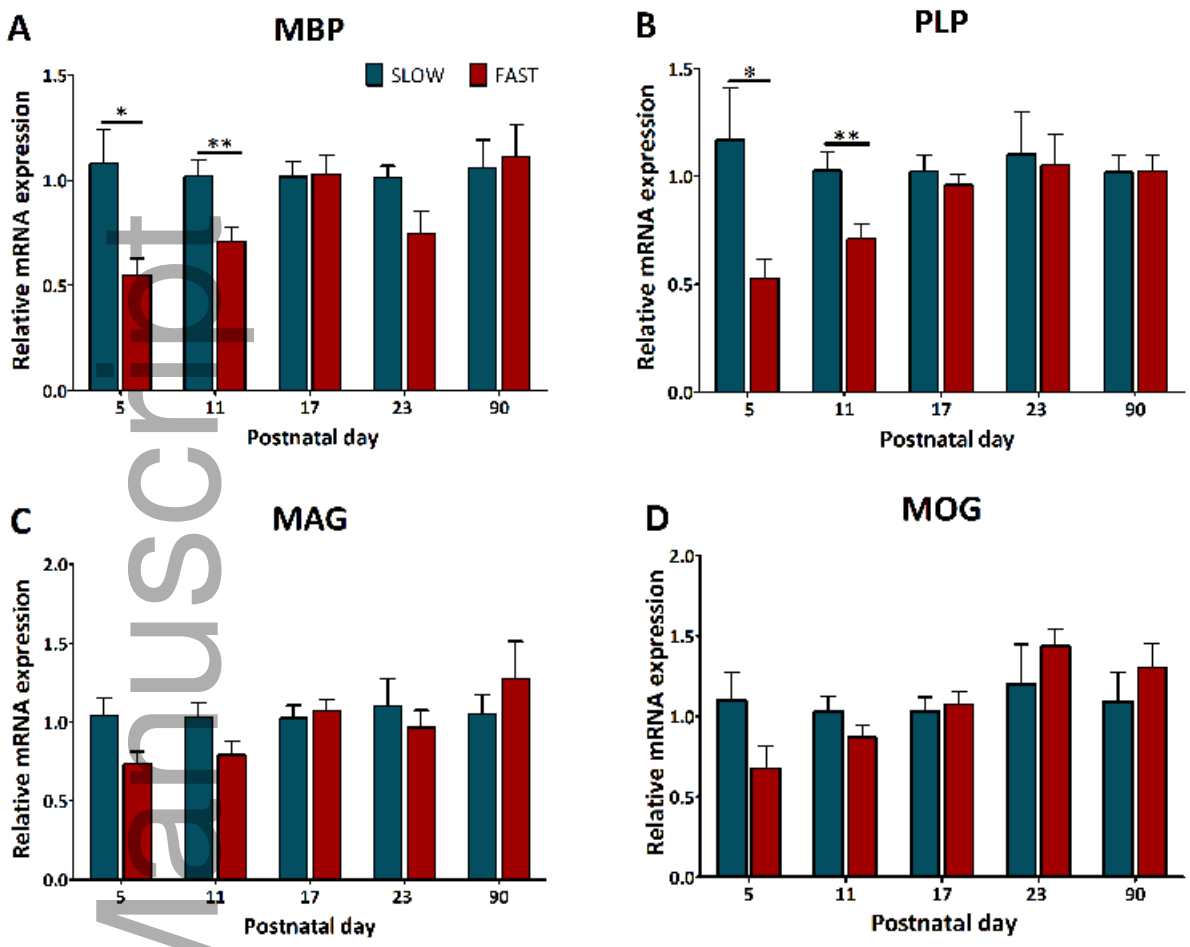

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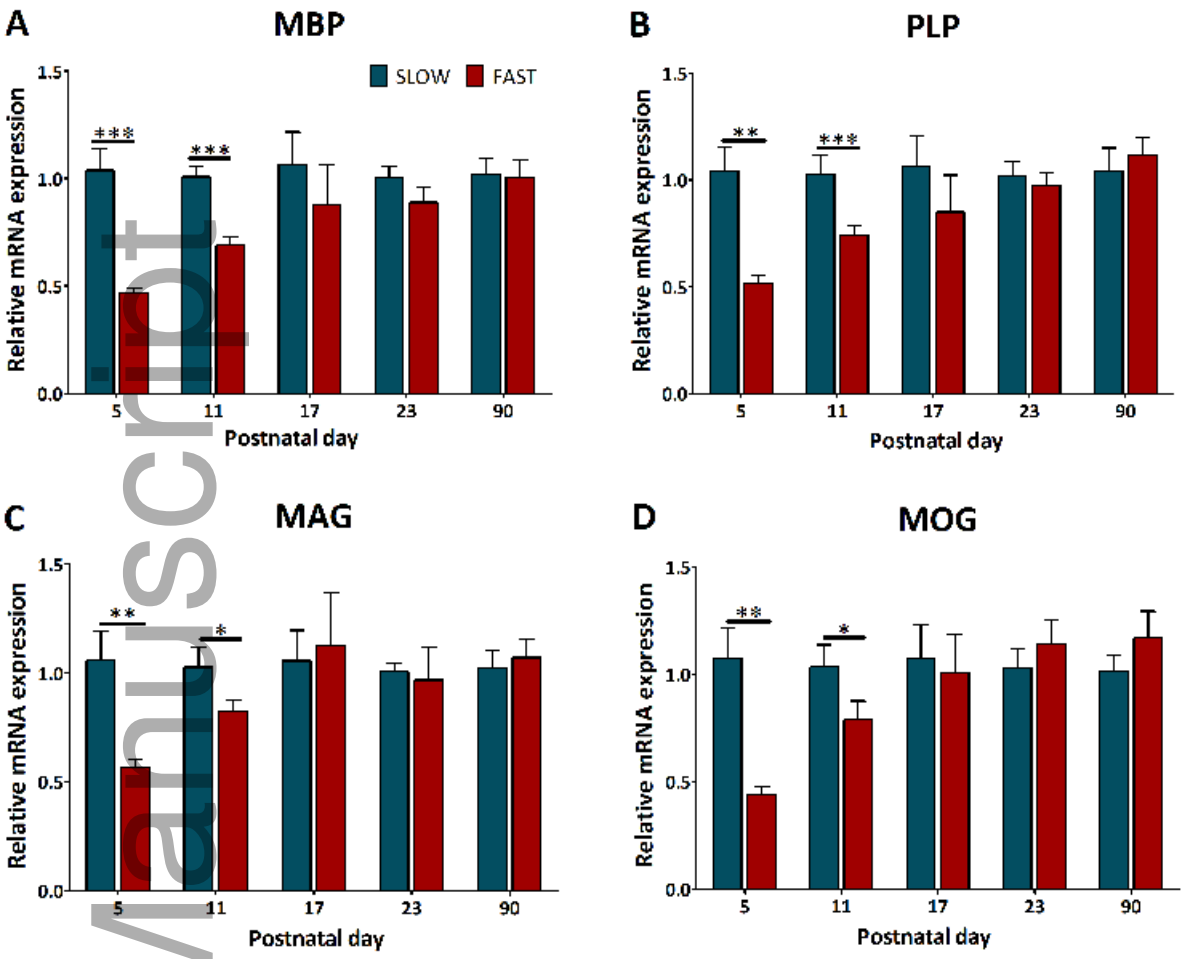

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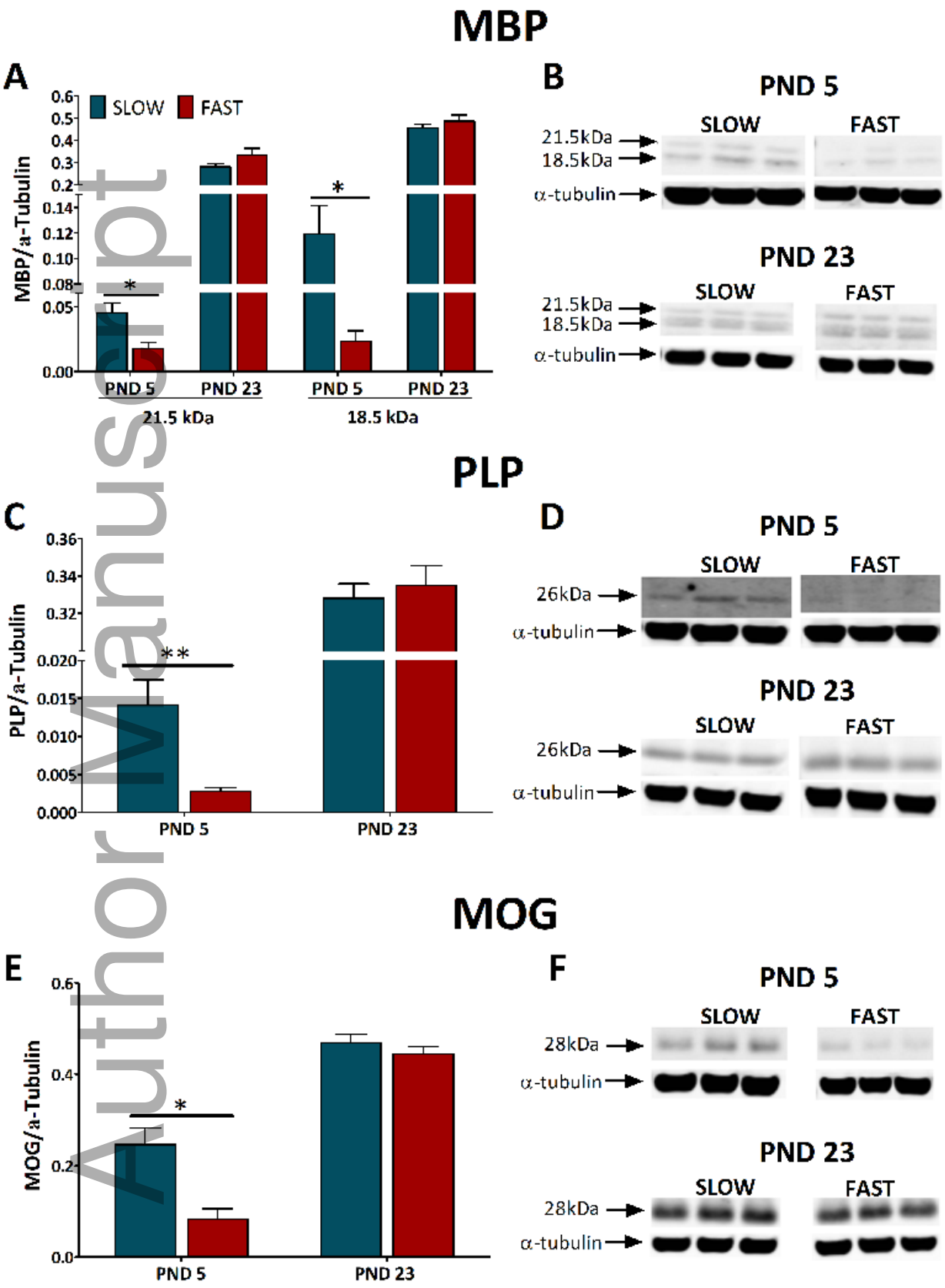

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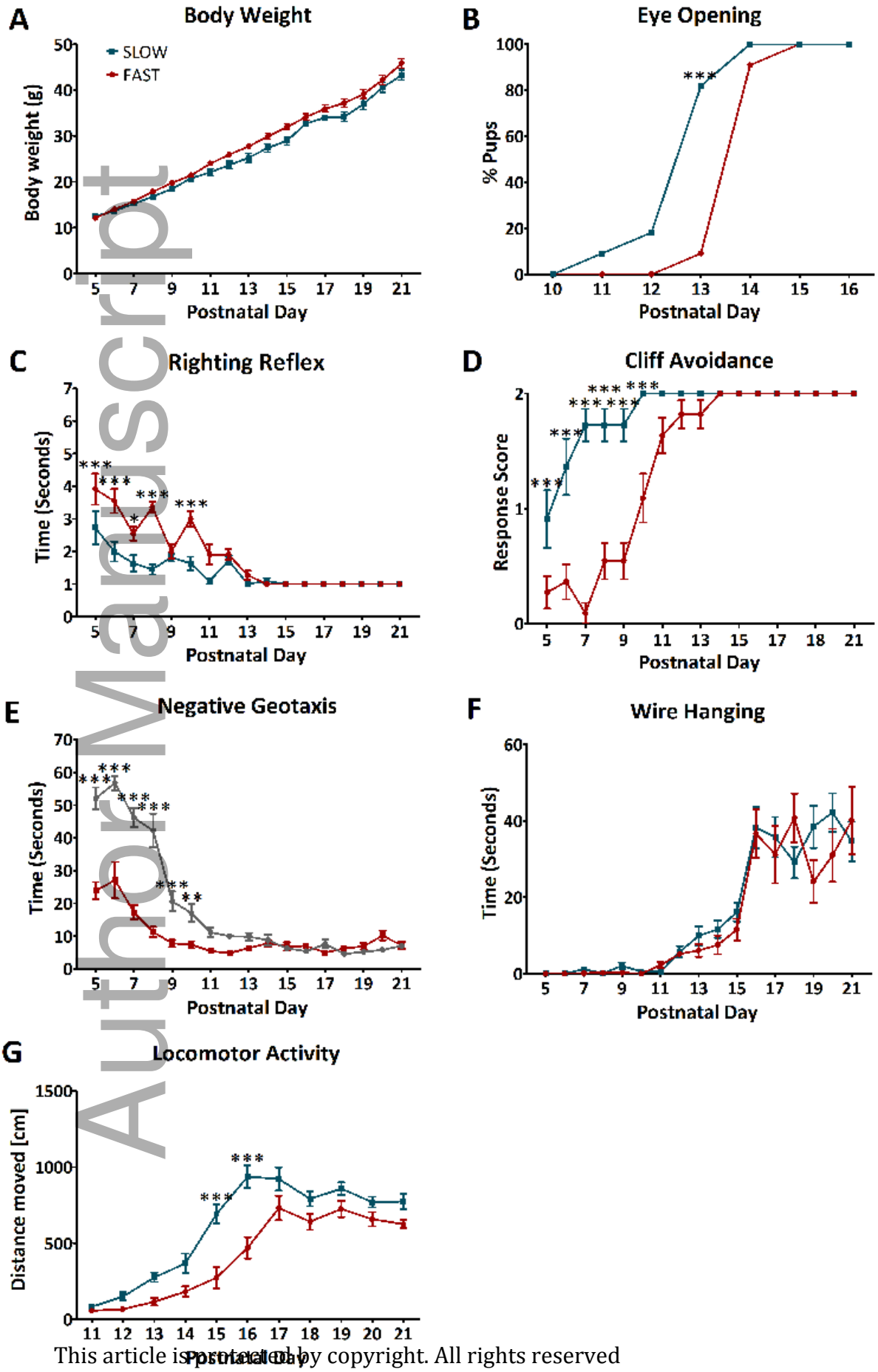

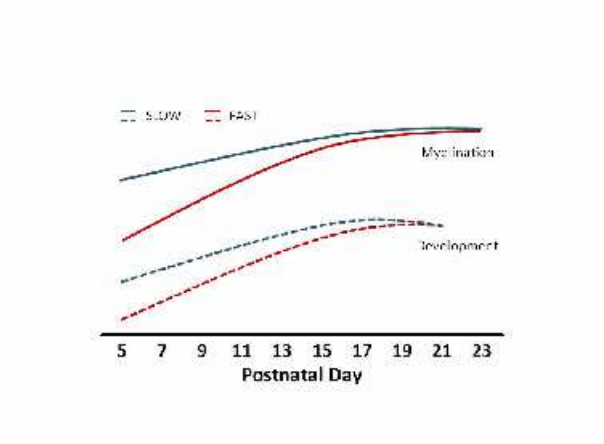

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