Visualization of Myelin During Development Using Black Gold II

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Abstract

Our goal was to create a histological atlas of myelin in the developing mouse brain for use in the study of disorders associated with abnormal myelination. The problem has been that myelin stains tend to be unreliable, of poor quality and can be difficult to use. Black-Gold II is a halogen gold phosphate complex with improved myelin staining properties relative to earlier Black-Gold and gold chloride methods (Schmued et al., *Brain Res*, 2008,1229: 210-7). In this study we used Black Gold II staining to track the development of myelin from the perinatal period through late adolescence in the C57BL6J mouse.

All procedures followed the policies of the Society for Neuroscience for the use of animals in neuroscience research and the project was approved by the WSU IACUC. Male and female C57BL6J mice were removed from their litters on day 5, 10, 15, 20, 25, 28, or 60 post-partum and their brains were removed and fixed in 3% buffered formalin for at least 10 days prior to thin-sectioning at 40 microns. Sections were obtained in both the coronal and sagittal planes. Tissues were mounted on gelatin-subbed slides and stored at 4 c until stained. Animals from each age group were counterbalanced in each staining run to ensure that staining variables were kept to a minimum. The staining protocol followed that of Schmued et al. (2008). After staining and coverslipping, the slides were photographed and the resulting images were analyzed using NIH image-J software to determine the density of myelin staining.

Myelin was visible at all ages, albeit faintly in ages younger than day 15 postpartum. Myelin staining appears to peak by day 28 in C57BL6J male mice. These data demonstrate that the Black-Gold II stain can be used effectively to study postnatal myelination in mice and should prove to be a useful tool for the study of developmental disorders associated with abnormal myelination.

Introduction

While working on a project involving early brain development, we found that while there are a number of very good atlases available for the study of neuron development no atlas existed for evaluating myelination during development. So we began work on a developmental atlas of myelination in the mouse brain. We tried a number of different stains (e.g., luxol fast blue, gold chloride and chromic acid-hematoxylin) and found the stains were generally unreliable, produced poor quality slides with considerable background staining and were difficult to use. For example, the traditional gold chloride method required block staining that only allowed the visualization of structures at the surface of the tissue block as the stain only penetrated 1-2 mm into the block (see Figure 1). After further pilot studies, we determined that a modification of the gold chloride method called Black-Gold II produced the most consistent staining and it could be used to stain thin-sections allowing for sequential analysis of the tissue. Black-Gold II is a halogen gold phosphate complex with improved myelin staining properties relative to earlier Black-Gold and gold chloride methods (Schmued et al., *Brain Res*, 2008,1229: 210-7). In this study we used Black-Gold II staining to track the development of myelin from the perinatal period through late adolescence in the C57BL6J mouse.

Methods

Subjects. All procedures followed the policies of the Society for Neuroscience for the use of animals in neuroscience research and the project was approved by the WSU IACUC. Twenty-eight male and female C57BL6J mice were housed in mixed sex litters until weaning on day 25 and then by gender in groups of 3-4 until testing. The animals were maintained on a 12 hr (7:00 P.M. to 7:00 A.M.) light cycle with *ad libitum* access to food and water.

Apparatus. A Vibratome Ultrapro 5000 crytostat equipped with a 5040 microtome (St. Louis, MO) was used to section the tissues. Slides were photographed for image analysis using a Pathscan Enabler V digital slide scanner (Meyer Instruments, Houston, TX, USA).

Procedure. Subjects were removed from their litters on day 5, 10, 15, 20, 25, 28, or 60 post-partum and their brains were removed and fixed in 3% buffered formalin for at least 10 days prior to thin-sectioning at 40 microns. Sections were obtained in both the coronal and sagittal planes. Tissues were mounted on gelatin-subbed slides and stored at 4 ºC until stained. Animals from each age group were included in each staining run to ensure that staining variables were kept to a minimum. The Black-Gold II (Histo-Chem, Jefferson, AR) staining protocol followed that of Schmued et al. (2008). After staining and coverslipping, the slides were photographed and the resulting images were analyzed using NIH image-J software to determine the density of myelin staining.

Results

A summary of the results is presented in Table 1. As the mice aged, Black-Gold II staining for myelin increased in intensity in the corpus callosum ($F(6,21)= 45.231$, $p < .0001$), area S1BF of the somatosensory cortex ($F(6,21)= 49.40$, $p < .0001$) and for the total brain analysis ($F(6,21)= 198.018$, $p < .0001$) (see Figure 2).

Post-hoc analyses were completed using Tukey's HSD test with alpha set to 0.05. Analyses of total brain staining indicated that mice younger than 20 days of age had significantly less myelin compared to older subjects. Mice 20-25 days of age had similar levels but then myelination increased again at 28 and 60 days post-partum (see Figure 2A).

Myelination in the corpus callosum (see Figure 2B) and area S1BF of the cortex (see Figure 2C) was similar at days 5 and 10 but then increased significantly at 15 days. Myelin staining intensity did not increase significantly after day 15. However, in area S1BF intensity plateaued from day 15-28 before rising significantly by day 60.

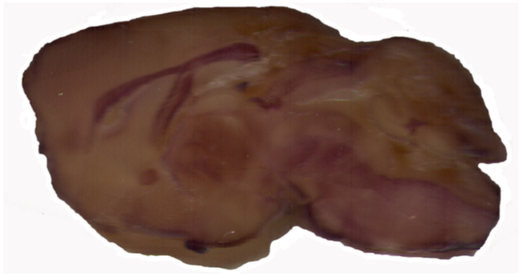

Figure 1. Gold chloride method for myelin (based on Wahlsten, Colbourne, & Pleus, 2003). Tissue is stained in blocks but cannot be sectioned because the stain only penetrates 1-2 mm.

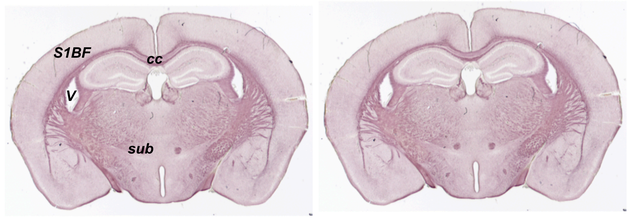

Figure 4. Examples of stain intensity in selected regions for a slide taken at age 28 days. CC: corpus callosum raw score = 173.063 (r= 115-227) with an adjusted score of 81.937; S1BF: primary somatosensory cortex S1 barrel field raw score = 211.952 (r= 122-250); SUB: submedius thalamic nucleus raw score = 151.655 (r= 122-189) . The raw score for the entire slide was 199.423. V= lateral ventricle raw score = 255 or no staining.

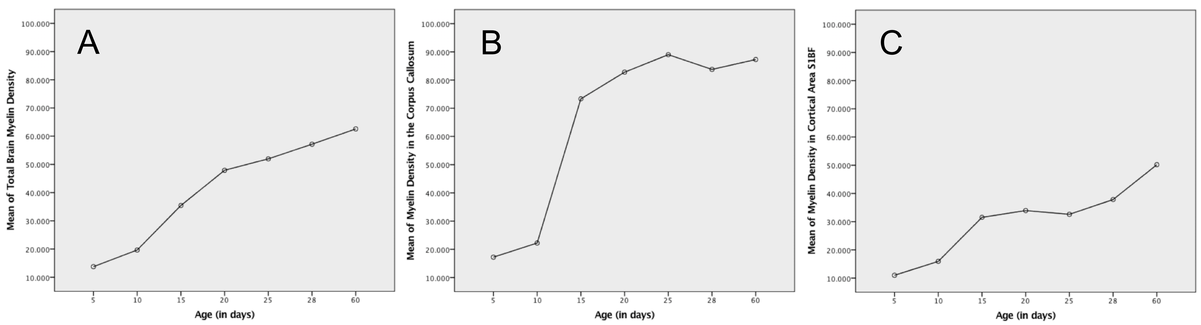

Figure 2. Analysis of staining intensity in the total brain (**A**), corpus callosum (**B**) and cortical area SBF1 (**C**). Scores were obtained by subtracting the raw scores from 255 (no staining) since more intense binding would be represented by a lower number (i.e., 0 = full saturation to 255 for no staining).

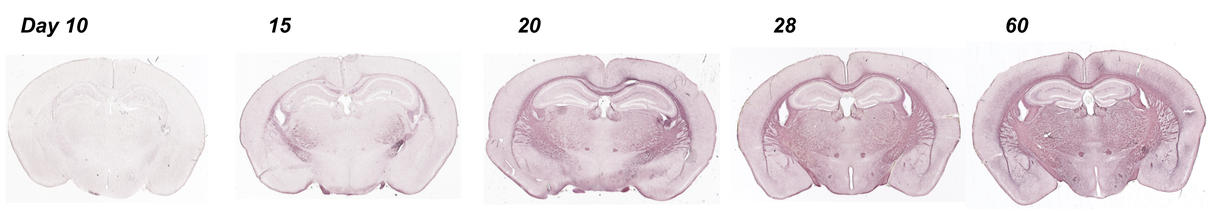

Figure 3. Representative slides by age.

Discussion

Black-Gold II staining increased in intensity at rates that varied by region but that with the exception of the corpus callosum continued to at least 60 days (see Figures 2 and 3). In this study we did not go beyond day 60, as we wanted to control for possible litter effects by including subjects from each litter in each age group tested. Given the pattern of staining it is clearly necessary to continue testing beyond 60 days.

The most active myelination of the axons within the corpus callosum appears to be after day 10 through day 20-25 based on the intensity of staining. The cortex (e.g., area S1BF), however, showed slower but relatively stable increases in growth at all points tested.

In summary, these findings indicate that the Black-Gold II staining method may be appropriately used to produce an atlas of myelination in the developing mouse brain. The data also show that the stain is sensitive enough to detect and quantify regional variations.

Table 1. Summary of Mean Raw Scores for Staining Densities of Selected Regions.

Age	Total Brain	S1BF	Corpus Callosum
5	241.2507	244.018	237.783
10	235.359	239.079	232.786
15	219.572	223.482	181.660
20	207.081	221.071	172.199
25	203.031	222.407	171.230
28	197.840	217.152	166.003
60	192.423	204.814	167.717

Note: 255 = no staining.

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References

Schmued, L., Bowyer, J., Cozart, M., Heard, D., Binienda, Z. & Paule, M. *Brain Res*, 2008,1229: 210-7.

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