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Youth and Environmental Enrichment Generate Serum Exosomes Containing miR-219 that Promote CNS Myelination

Aya D. Pusic^{1,2} and Richard P. Kraig^{1,2}

¹Department of Neurology, The University of Chicago, Chicago, Illinois

²Committee on Neurobiology, The University of Chicago, Chicago, Illinois

Abstract

Although commonly considered a disease of white matter, gray matter demyelination is increasingly recognized as an important component of multiple sclerosis (MS) pathogenesis, particularly in the secondary progressive disease phase. Extent of damage to gray matter is strongly correlated to decline in memory and cognitive dysfunction in MS patients. Aging likewise occurs with cognitive decline from myelin loss, and age-associated failure to remyelinate significantly contributes to MS progression. However, recent evidence demonstrates that parabiotic exposure of aged animals to a youthful systemic *milieu* can promote oligodendrocyte precursor cell (OPC) differentiation and improve remyelination. In the current study, we focus on this potential for stimulating remyelination, and show it involves serum exosomes that increase OPCs and their differentiation into mature myelin-producing cells—both under control conditions and after acute demyelination. Environmental enrichment (EE) of aging animals produced exosomes that mimicked this promyelinating effect. Additionally, stimulating OPC differentiation via exosomes derived from environmentally enriched animals is unlikely to deplete progenitors, as EE itself promotes proliferation of neural stem cells. We found that both young and EE serum-derived exosomes were enriched in miR-219, which is necessary and sufficient for production of myelinating oligodendrocytes by reducing the expression of inhibitory regulators of differentiation. Accordingly, protein transcript levels of these miR-219 target mRNAs decreased following exosome application to slice cultures. Finally, nasal administration of exosomes to aging rats also enhanced myelination. Thus, peripheral circulating cells in young or environmentally enriched animals produce exosomes that may be a useful therapy for remyelination.

Keywords

neuroimmune; hippocampal slice culture; remyelination; multiple sclerosis; aging

Introduction

Multiple sclerosis (MS), the leading cause of neurological disability in young adults, is an inflammatory disease involving oligodendrocyte loss, demyelination, and failure to remyelinate. Initially, patients follow a relapsing–remitting disease course, characterized by periods of partial recovery associated with incomplete remyelination (Kuhlmann et al., 2008; Keough and Yong, 2012). However, with advancing age this ability to remyelinate declines and many patients transition into a secondary-progressive disease course.

Remyelination is a spontaneously occurring repair process mediated by recruitment of oligodendrocyte precursor cells (OPCs) to lesions, and their subsequent differentiation into myelinating oligodendrocytes (Kuhlmann et al., 2008). Age-associated impairment of oligodendrocyte differentiation significantly contributes to remyelination failure, as OPCs are present in MS lesions, but do not mature in advanced patients (Sim et al., 2002; Kuhlmann et al., 2008).

Gray matter demyelination has been implicated as a significant cause of the cognitive dysfunction and memory deficits found in 40%–65% of MS patients (Amato et al., 2006), and may be indicative of disease prognosis. Extensive lesions of hippocampal gray matter have been observed in postmortem MS brains, and are characteristic of progressive MS (Geurts et al., 2007).

Here, we used hippocampal slice cultures to model changes in gray matter myelin. Slice cultures retain the three-dimensional organization of the hippocampus, preserve neuron–glia interactions, and exhibit abundant myelination by 21 days *in vitro* (Lin et al., 2008). Mature slice cultures also contain quiescent astrocytes and microglia (Kunkler and Kraig, 1997), and support neural progenitor proliferation and differentiation (Raineteau et al., 2004; Sypecka et al., 2009). Finally, cultures are long-lived, allowing for assessment of repair following demyelination, and are thus an excellent model for the investigation of gray matter myelin (Lin et al., 2008).

Despite increased interest in remyelination therapy, current approaches to treating MS are largely directed at reducing demyelination via immune suppression, and often come with an array of harmful immune sequelae (MSTCG, 2008). No existing treatment is able to prevent the inevitable decline in remyelination or to regenerate already damaged myelin, making this an important therapeutic target. We suggest that use of exosomes to stimulate remyelination would be beneficial to patients undergoing immunomodulatory therapy for MS.

Recent evidence from Robin Franklin's group shows that parabiotic exposure of aged animals to the youthful systemic *milieu* improves recovery from lyssolecithin-induced demyelination (Ruckh et al., 2012). We show that this effect likely involves production of peripheral exosomes that impact OPC differentiation. Exosomes are small, 30–120 nm diameter membrane microvesicles of endocytic origin that are secreted by many cell types (Schorey et al., 2009). They have the potential for directional homing to specific target cells, dependent on the physical properties of their membranes (Liang et al., 2007). Their effect can be local, regional, or systemic. Exosomes do not contain a random sampling of their parent cell's cytoplasm, but are instead enriched with specific mRNA, miRNA, and protein

(Bobrie et al., 2011). This cargo is protected from degradation by proteases and RNases while the vesicle is in the interstitial space, and retains bioactivity once taken up by a recipient cell. In this way, exosomes facilitate the transfer of interactive signaling and enzymatic activities.

We found that exosomes derived from serum of young animals increased OPCs and their differentiation, myelin production, and also improved remyelination following lyssolecithin-induced demyelination in brain slice culture. In addition, serum exosomes from rats exposed to environmental enrichment (EE; volitionally increased intellectual, social, and physical activity) showed similar effects. EE enhances memory, increases production of myelin at all ages, and lessens injury from neurodegenerative disorders including demyelinating disease (Fields, 2008). Importantly, aging animals exposed to EE also produced promyelinating exosomes. Middle-aged (12 months) rats were chosen to represent the “aging” condition, since impaired remyelination is already evident at this time point (Gilson and Blakemore, 1993). Therefore, EE can restore the ability to produce promyelinating exosomes in aging animals. In both youth and EE, we found that this feature of peripheral exosomes involved miR-219, which is required for production of myelinating oligodendrocytes via regulation of multiple mRNA targets in their differentiation pathway (Dugas et al., 2010; Zhao et al., 2010). Furthermore, young exosomes enhanced central nervous system (CNS) myelination *in vivo* in aging rats. Taken together, our results show that the promyelinating effects of youthful systemic *milieu* on aging brain involve peripheral exosome-mediated delivery of miRNAs, and that EE can mimic the nutritive impact of youth on CNS myelination. This work has appeared in preliminary form (Pusic and Kraig, 2012).

Materials and Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were approved by the University of Chicago Animal Care and Use Committee.

Animal Groups and Environmental Enrichment

Wistar rats for EE and preparation of slice cultures were obtained from Charles River Laboratory. Young male Wistar rats were 2–3 months old. Aging rats were 12 months old (Gilson and Blakemore, 1993). EE of rats was accomplished with an in-house specially designed two-layered Marlau-style enrichment cage (Obiang et al., 2011; Fares et al., 2013) 58-cm wide, 88-cm long, and 65 cm in height. The top layer contained a maze that was changed three times a week to provide a novel stimulus. Rats had to climb to the upper layer, progress through the maze, and descend ramps to access food. One-way doors led from the feeding area to a large socialization and exercise area, from which they once again had access to the upper layer. In the socialization area, rats had free access to water, a running wheel for exercise, and a red plastic resting area. Twelve rats were group-housed in this enrichment cage for 35 days before harvest of young-EE-exosomes (at 4 months old) or 56 days for aging-EE-exosomes (at 12 months old). Age-matched non-enriched (NE) rats were single-housed in standard cages.

Culture Preparation and Maintenance

Untimed pregnant Wistar rats (Charles River) were single-housed with Enviro-dri® paper bedding (Shepherd) and Nestlets (Ancare). For culturing, litters were culled to 10 pups at birth. About 9–10-day-old male and female rat pups were used to make 350- μ m thick hippocampal slice cultures that were maintained in horse serum-based media (Kunkler and Kraig, 1997) until 18 days *in vitro* then changed to serum-free media (Pusic et al., 2011; Mitchell et al., 2011). This movement to serum-free media did not activate microglia (Grinberg et al., 2011), and allowed us to accurately assess the impact of our exosome treatments without potential contamination by exosomes contained in horse serum.

Serum-free media composition, per 100 mL was: neurobasal medium (97 mL; Invitrogen); Gem21 NeuroPlex supplement (2.0 mL; Gemini Bioproducts); GlutaMax (1 mM; Invitrogen); gentamicin (1 μ g/mL; Invitrogen); D-glucose (45%; 680 μ L; Sigma); ascorbic acid (0.5 mM; Sigma); Fungizone (1 mg/mL; Invitrogen); NaCl (41 mM; Sigma); Mg₂Cl₂ (0.8 mM; Sigma); and CaCl₂ (1.6 mM; Sigma). Neurobasal constituents are detailed by Brewer et al. (1993) and included an array of amino acids and vitamins as well as traditional salts. The Gem21 supplement was used as previously described (Chen et al., 2008). This supplement provided insulin, T3, and reduced glutathione as well as the amino acids required to maintain cellular glutathione levels. The media also contained ascorbate, tocopherols, and the antioxidant enzymes catalase and superoxide dismutase (Gemini Bio-Products Material Data Safety Sheet; www.gembio.com).

Primary OPCs cultured either in the absence of mitogens (Barres et al., 1993) or in the presence of T3 (triiodo-L-thyronine; Barres et al., 1994) rapidly differentiated. Although our serum-free media contained both proliferation-promoting insulin and differentiation-inducing T3, we observed robust differentiation of OPCs into myelinating cells. Similarly, the serum-free media contained soluble antioxidants and antioxidant enzymes to ensure adequate and prolonged oxidant-antioxidant homeostasis (Grinberg et al., 2013) which otherwise might inhibit myelination from oxidant stress (Lin et al., 2008).

At 21 days *in vitro*, slice culture vitality was assessed via Sytox, a fluorescent marker of cell death (Hulse et al., 2008). By enriching the care of pregnant rats with additional nesting material, we improved slice culture vitality at 21 days *in vitro* from approximately 80%–85% ($n = 104$ litters) to 95%–100% ($n = 156$ litters). Experiments were performed with 21–35 days *in vitro* slice cultures, when myelination parallels that seen *in vivo* in adolescent rats. The similarity of hippocampal slice cultures to their *in vivo* counterpart extends to neuroimmune signaling, including cytokine expression (Kunkler et al., 2004), microglial behavior (Hailer et al., 1996; Ransohoff and Perry, 2009; Pusic et al., 2011; Grinberg et al., 2011), the presence of T cells (Pusic and Kraig, 2010), and the presence of oligodendrocytes at different developmental stages (Haber et al., 2009).

Lysolecithin Exposure

For lysolecithin-induced demyelination, mature (21–35 days *in vitro*) hippocampal slice cultures were incubated in serum-free media containing lysolecithin (0.5 mg/mL) for 17 hr, rinsed three times in Neurobasal, and returned to standard incubation conditions with or

without exosome treatment. This protocol follows that previously described (Birgbauer et al., 2004), which models MS using lysolecithin in cerebellar slice cultures. While cerebellar slice cultures provide wide expanses of white matter myelin, they contain little gray matter myelin, have limited synaptic circuitry, and reduced longevity when compared with hippocampal slice cultures.

Exosome Isolation and Verification

Exosomes from animal serum were isolated using Systems Bioscience's ExoQuick solutions. Briefly, 500 μ L of serum was incubated with 120 μ L ExoQuick for 30 min at 4°C. Next, exosomes were precipitated by centrifugation at 1,500 $\times$ *g* for 30 min, and resuspended in 100 μ L sterile phosphate buffered saline. Initial dose-response experiments for our exosome treatments suggested a wide effective-dose range. For this study, 20 μ L of resuspended exosomes were considered one "dose." Although exosome recovery varied based on serum sample, on average, a 20 μ L dose was equivalent to 40 μ g of protein. Recovery of exosomes was confirmed via Western blot analysis of two well-characterized exosomal protein markers, namely CD63 and Alix (Schorey et al., 2009; AbD Serotec). The exosome pellet was resuspended in 200 μ L cold lysis buffer for protein extraction. Immunoblot for CD63 and Alix was performed as described below. For electron microscopy (EM), exosomes were diluted 1:50 in 1% phosphotungstic acid and visualized under 300 kV using an FEI Tencai F30 transmission electron microscope with a Gatan CCD camera and Digital Micrograph software (University of Chicago Electron Microscopy Facility). Other methods of isolating exosomes were also tested. Sequential ultracentrifugation (Gallo et al., 2012) and Total Exosome Isolation solution (Invitrogen) yielded exosomes of comparable purity, as assessed by EM and their ability to significantly ($P < 0.001$) increase myelin basic protein (MBP) when applied to slice cultures [with specific values of: control: 1.00 ± 0.19 ; ultracentrifuge: 2.48 ± 0.07 ; Life Technologies: 2.78 ± 0.22 ($n=6$ /group)]. Thus, we believe protein contamination is insignificant and does not play a role in the observed effects. However, since exosome yield was significantly reduced when isolated via sequential ultracentrifugation, we utilized commercial isolation reagents (defined above).

Exosome Tracking

To determine cellular targets, nutritive exosomes were labeled with DiI (3,3'-dioctadecylindocarbocyanine; Invitrogen), a red fluorescent lipophilic dye. DiI is highly photostable when incorporated into membranes, and does not disrupt membrane properties (Baron-Van Evercooren et al., 1996). Young serum exosomes were isolated as before, resuspended in sterile PBS and incubated with 1 μ M DiI for 10 min. DiI-exosomes were then re-isolated, washed, resuspended in sterile PBS, and applied to slice cultures as described above.

Exosome Transfection with miR-219 Inhibitor

Exosomes were isolated from young rat serum as described above and transfected with a fluorescein labeled miRCURY LNATM micro-RNA inhibitor specific to miR-219 (has-miR-219-5p; Exiqon). Transfection was performed using Lipofectamine RNAiMax reagent (Invitrogen) following the manufacturer's protocol. Following transfection, exosomes were

re-isolated and resuspended in phosphate buffered saline. Transfection efficiency was assessed via fluorescence imaging. Roughly 40 µg of these miR-219 inhibitor-transfected exosomes were applied to slice cultures, and collected 3 days later for determination of MBP levels. Untreated control cultures and untransfected young exosome treated cultures were included for comparison.

Nasal Administration

Nasal administration of exosomes to aging rats was performed using a #906 nose cone (Kopf) to deliver isoflurane (Baxter) anesthesia (2%–3% in oxygen) within a fume hood, using a heat lamp and thermoregulator to maintain temperature at 37°C. About 50 µL of exosomes (~100 µg protein) in phosphate buffered saline were administered at a rate of 5 µL every 2 min, alternating nostrils over 20 min (Liu et al., 2001). Brains were harvested 3 days later. Sham exosomes were exposed to ultraviolet (UV) light before administration.

microRNA Expression Assays

ExoRNAs were extracted and converted to cDNA using the SeraMir kit from System Biosciences (SBI). Briefly, exosomes were harvested via ExoQuick and RNA extraction was performed according to manufacturers' protocol, using a phenol-free lysis buffer and small RNA-binding rapid spin columns. A polyadenylation step prepared exoRNAs for subsequent synthesis of qPCR-ready cDNAs. Seventeen microRNA species known to be involved in oligodendrocyte regulation were chosen and primers were taken from SBI's Rat Genome-wide microRNA qPCR Array Panel (based on Sanger miRBase Version 14). qPCR was run on a BioRad iCycler, using SYBR Green chemistry for detection.

For validation of miRNAs of interest from our initial screen, TaqMan Array Rodent MicroRNA Cards (cards A and B v.3.0; Applied Biosystems) were run. In contrast to SBI qPCR arrays, these cards utilize stem-loop RT primers specific to individual miRNAs to synthesize cDNA and detect amplification products via specific TaqMan probes. Total RNA was extracted from exosome pellets (isolated as above) using the mirVana miRNA Isolation kit (Applied Biosystems). cDNA was synthesized from total RNA using Megaplex RT primers and TaqMan miRNA RT kits, followed by preamplification with Megaplex PreAmp Primers (all Applied Biosystems). Samples were loaded to TaqMan microfluidic cards and run on an Applied BioSystems 7900HT thermocycler (University of Chicago Genomics Core Facility).

All analyses included two technical replicates per biological sample, and were performed via the comparative Ct method (Livak and Schmittgen, 2001), relative to reference controls U6 snRNA, RNU43 snoRNA, and U1 snRNA. Greater than two-fold change was considered significantly increased/decreased.

Immunohistochemistry

Slice cultures were fixed in 10% buffered formalin phosphate for 24 hr then stained using immunostaining procedures as previously described (Mitchell et al., 2011). For nasal administration studies, animals were allowed to recover for 3 days and then re-anesthetized with progressive exposure to 100% carbon dioxide followed by decapitation and brain

extraction. Harvested brains were frozen in isopentane at -30°C that was then lowered to -75°C prior to embedding in Optimal Cutting Temperature Compound (Fisher). Sections ($14\text{ }\mu\text{m}$) were cut in a cryostat and directly mounted to slides before fixation in 10% buffered formalin phosphate. Primary antibodies for O4 and O1 immunofluorescence were acquired from Stem Cell Technologies and eBioscience, respectively, and both were used in the ratio 1:100. Ki-67 was obtained from Novus Biologicals (1:500), NG2 from Invitrogen (1:200), and Musashi from Cell Signaling Technology (MSi1/MSi2; 1:50). Oligodendrocyte-specific CNPase antibody for exosome uptake experiments was obtained from Millipore and used in the ratio 1:1,000. Appropriate Alexa Fluor secondary antibodies were obtained from Invitrogen (1:1,000). Immunohistochemistry for MBP distribution and NeuN cytoarchitecture was performed using the peroxidase–antiperoxidase method as previously described (Hulse et al., 2008; Lin et al., 2008). MBP and NeuN antibodies were obtained from Zymed and Santa Cruz Biotechnology, respectively, and both were used in the ratio 1:1,000 followed by horseradish peroxidase (HRP)-conjugated secondary antibody (1:200; Invitrogen) and visualization with 3,3'-diaminobenzidine (Sigma). Representative fluorescent images were acquired using a Leica TCS SP5 II AOBS laser scanning confocal microscope (University of Chicago Integrated Microscopy Core Facility). Imaging section thickness was 772 nm to reduce potential cell body overlap. Images were acquired at 63 $\times$ gain ($1,024 \times 1,024$ pixels @ 12-bit).

O4 and O1 immunofluorescence staining was quantified (integrated optical intensity) using a self-calibrating sensitive CCD digital imaging system consisting of: a QuantEM-512SC camera (Photometrics), an electronic shutter (Lambra SC Smart Shutter; Sutter Instruments), standard 100 W Hg light on a DMIRE2 inverted microscope (Leica) at 10 $\times$ gain, and using stabilized electrical power (#Pro 1500; APC) running on MetaMorph software (v.7.5.4.0; Molecular Devices).

Western Blotting

Samples were collected and processed according to standard protocols. The CA3 hippocampal region was dissected out of slices with a diamond knife (Fine Science Tools) before harvest and lysis. Expression of MBP or miR-219 targets (NeuroD1, PDGFR α , and ELOVL7) was evaluated using standard SDS-PAGE and immunoblotting procedures. Briefly, equal amounts of total protein from hippocampal slice culture homogenates were loaded to SDS-PAGE gels for separation and transferred to nitrocellulose membranes. Primary antibodies used were: NeuroD1 (Sigma; 1:200), PDGFR α (Santa Cruz; 1: 200), and ELOVL7 (Sigma; 1:500), followed by the corresponding HRP-conjugated secondary antibody (Sigma; 1:1,000). Blots were visualized by standard chemiluminescence and densitometric quantification performed with Quantity One[®] 1-D Analysis Software. All proteins were normalized to β -actin (Sigma; 1:1,000).

Electron Microscopy

Myelin thickness was measured from slice cultures sectioned perpendicular to Schaffer collateral and CA1 output axons. Images were acquired using the University of Chicago Electron Microscopy Core Facility, and g ratios (axon diameter/fiber diameter) quantified

using ImagePro software (v.4.5.0.19, Media Cybernetics) (Guy et al., 1991; Chomiak and Hu, 2009).

Statistical Methods

Data were analyzed using SigmaStat software (v.3.5; Systat). All data were subject to normality testing (P -value to reject: 0.05), equal variance testing (P -value to reject: 0.05), power ($1-\beta$: >0.8), and expressed as mean $\pm$ S.E.M. Mean control data was scaled to 1.00 with all subsequent parameters scaled proportionally to better allow inter-experiment comparisons.

Results

Myelin Distribution in Hippocampal Slice Cultures

Hippocampal slice cultures maintain a structurally and physiologically preserved tri-synaptic loop consisting of dentate gyrus-CA3–CA1 as illustrated via NeuN staining (Fig. 1A). Although predominantly a gray matter structure, the hippocampus contains abundant myelinated fibers. MBP staining of mature (21 days *in vitro*) hippocampal slice cultures demonstrates robust myelination (Fig. 1B). EM image confirms the presence of structurally normal compact myelin sheaths in hippocampal slice cultures (Fig. 1C). Thus, this *ex vivo* system closely parallels its *in vivo* counterpart, while allowing for direct treatment of brain tissue in a controlled environment (Pusic et al., 2011). Since experiential learning increases myelination (Fields et al., 2008), use of hippocampal slice cultures that display both the ability to myelinate and a trisynaptic loop that exhibits long-term potentiation (Gahwiler et al., 1997) is well suited for studying how EE-based exosomes affect myelination.

Young and Environmentally Enriched Exosomes Promote OPC Maturation

Exosomes were isolated from serum and their recovery confirmed via EM, which showed a uniform distribution of vesicles approximately 50 nm in diameter. For further confirmation, Western blot analysis of two well-characterized exosomal protein markers, CD63 and Alix (Schorey et al., 2009) was performed (Fig. 2A). To verify that serum exosomes are non-injurious to slice cultures, exosomes were applied and slices stained with a fluorescent marker of cell death at 3, 5, 7 and 12 days post-treatment. No significant injury was observed (Fig. 2B). Representative images illustrate O4 and O1 immunostaining (Fig. 2C, D, respectively). Quantification of staining intensity revealed that exosomes derived from the serum of young, young-EE, and aging-EE animals significantly increased O4 (Fig. 2C) and O1 (Fig. 2D) immunopositive cells, suggesting that more OPCs were maturing into myelinating oligodendrocytes. Exposure of exosomes to UV light to destroy nucleic acids abrogated this increase, suggesting involvement of RNA species (Fig. 2C, D). For simplicity, exosomes found to increase differentiation of OPCs (i.e., exosomes derived from the serum of young, young-EE, and aging-EE animals) will be collectively referred to as “nutritive” exosomes.

Young and Environmentally Enriched Exosomes Increase OPC Proliferation

Since increases in O1 and O4 positive cells were found, we next aimed to determine if nutritive exosomes impact proliferation of precursor cells. Slice cultures were treated with

exosomes, and fixed 1 day later for immunostaining. Staining with Musashi (Msi1/Msi2), a marker of proliferating multipotent neural precursors (Knerlich-Lukoschus et al., 2010; Fig. 3A) revealed a significantly increased percent of neural stem cells (Musashi⁺ cells per total DAPI⁺ cells) in nutritive exosome-treated cultures versus control and non-nutritive exosome-treated cultures (Fig. 3B). Additionally, we stained for NG2, an early OPC-specific marker (Baumann and Pham-Dinh, 2010), and Ki-67, a marker of proliferation (Fig. 3C). Cell counts revealed that the percent of NG2 and Ki-67 double positive cells (NG2⁺ Ki-67⁺ cells per total NG2⁺ cells) was significantly increased in nutritive exosome-treated cultures compared with control and non-nutritive exosome-treated cultures (Fig. 3D).

Young and Environmentally Enriched Exosomes Increase Myelination

MBP levels were measured via Western blot after application of exosomes derived from young, young-EE, and aging-EE versus their non-stimulated counterparts [i.e., aging, young- NE, and aging-NE exosomes, respectively]. Three days after application to naïve slice cultures, nutritive exosomes significantly increased baseline MBP, whereas exosomes derived from aging and NE animal groups had no effect (Fig. 4A). As with O1 and O4 staining, UV exposure of exosomes prior to application prevented the increase in MBP (Fig. 4A).

To determine if increased MBP expression was reflected in production of structurally normal and thicker compact myelin sheaths, EM images of exosome-treated slice cultures were taken and *g* ratios (axon diameter/fiber diameter) calculated (Guy et al., 1991; Chomiak and Hu, 2009). Young serum-derived exosomes were chosen as representative of the nutritive exosome condition, since they most robustly increased MBP. EM imaging revealed intact and tightly laminated myelin sheaths that were thicker in young exosome-treated samples compared with UV-exposed exosome-treated and control samples (Fig. 4B). The *g* ratios were significantly lower (i.e., thicker myelin) in exosome-treated samples (Fig. 4C), whereas axon diameter did not significantly differ among treatment groups (Fig. 4D). Thus, nutritive exosomes both increased MBP expression and improved *g* ratios, demonstrating that they functionally improved myelination.

Young and Environmentally Enriched Exosomes Improve Recovery From Demyelinating Injury

Lysolecithin exposure induced a stereotypic course of demyelination, with the peak of demyelination seen 3 days post-exposure, the first signs of remyelination seen at 5 days post-exposure, and full recovery seen 12 days after exposure (Fig. 5A). Nutritive exosomes were administered immediately after incubation with lysolecithin, when slice cultures were transferred to fresh media for recovery. Five days later, at the onset of remyelination, nutritive exosome treatment significantly increased MBP expression and O4⁺ cell content compared with sham non-treated lysolecithin-demyelinated slices (Fig. 5B). Demyelinating injury itself triggers proliferation of oligodendrocyte lineage cells (Bruck et al., 2003; Aharoni et al., 2008), as evidenced by the significant increase in O4⁺/O1⁺ cells in cultures exposed to lysolecithin alone. Nutritive exosomes did not have an additive impact on O1⁺ cell content.

Young and Environmentally Enriched Exosomes Contain miRNAs Involved in Oligodendrocyte Differentiation

Since UV exposure ablated positive effects of exosomes, we screened exosome content for relevant RNA species. Myelination in the CNS is highly regulated by miRNA control of oligodendrocyte differentiation (Dugas et al., 2010; Zhao et al., 2010; Li and Yao, 2012), so we focused on a panel of 17 miRNAs known to be involved (Fig. 6).

Young, young-EE, and aging-EE exosomes influenced myelination to a similar degree. Thus, we looked for miRNA species enriched in exosomes of all three groups relative to aging-NE exosomes, which had no significant effect on myelination. Our initial screen using SBI's Rat Genome-wide microRNA qPCR Array Panel showed that several miRNAs of interest were present/enriched in nutritive exosomes (Fig. 6). We focused on members of the miR-219 family since they were most highly and consistently enriched. TaqMan Array Rodent MicroRNA Cards were used for validation, and confirmed miR-219 enrichment.

To determine if exosomes are taken up by oligodendrocytes, we next labeled young exosomes with DiI and applied them to slice cultures. One hour after application, treated cultures were fixed and stained with CNPase to label oligodendrocytes. DiI labeled exosomes were found colocalized with CNPase positive cells throughout the culture. This indicates that exosomes derived from serum are indeed able to enter oligodendrocytes, and perhaps transfer miRNA contents to produce a functional impact.

Young and Environmentally Enriched Exosomes Deliver Functional miR-219 that Impact Oligodendrocyte Differentiation and Myelination

All three classes of nutritive exosomes contained higher levels of mature transcripts of the miR-219 family than their non-nutritive counterparts. miR-219 is deficient in MS lesions (Junker et al., 2009), is induced during OPC differentiation (Dugas et al., 2010; de Faria et al., 2012), and plays an essential role in OPC maturation and maintenance of compact myelin (Li and Yao, 2012; Shin et al., 2009). This suggests that miR-219 deficiency in MS may contribute to remyelination failure by blocking OPC differentiation (Fig. 7A), and that exosome-mediated delivery of miR-219 may alleviate this block and thus facilitate remyelination (Fig. 7B).

To confirm the functional effect of miR-219 from nutritive exosomes on slice cultures, Western blots for known targets were performed. Specific targets were: NeuroD1, a pro-neurogenic factor that diverts stem cells to a neuronal fate; PDGFR α , a receptor for a mitogenic factor that promotes proliferation and inhibits differentiation; and ELOVL7, a regulator of lipid metabolism and redox homeostasis in mature oligodendrocytes (Shin et al., 2009; Dugas et al., 2010; Zhao et al., 2010). Twenty-four hours after application, exosome-treated naïve cultures contained significantly lower levels of NeuroD1, PDGFR α , and ELOVL7 protein than untreated controls (Fig. 7C–E).

Finally, transfection of young exosomes with an inhibitor of miR-219 ablated the increase in MBP seen after treatment with young exosomes, providing additional support for the delivery of functional of miR-219 by exosomes (Fig. 7F).

Nasal Administration of Young Serum-Derived Exosomes Increase Myelin in Aging Rats

Exosomes taken from the serum of young animals significantly enhanced myelination when nasally administered to aging rats. Three days after nasal administration, brains were harvested and processed for assessment of myelin changes. MBP immunostaining demonstrates typical myelin content of paramedian motor cortex (Fig. 8A). Sections from treated brains were stained with FluoroMyelin as an initial step to measure compact myelin (Dugas et al., 2010). Quantification of FluoroMyelin staining intensity showed a significant increase of myelin in the motor cortex of animals administered young serum exosomes compared with sham treated animals (Fig. 8B). This significant increase was confirmed by Western blot analysis of MBP from the motor cortex area (Fig. 8C), which was used as a representative brain region for demonstration of nutritive effects.

Discussion

The capacity for remyelination in the adult CNS is robust, and extensive repair can be seen in MS patients (Lassmann et al., 1997). However, as MS becomes chronic, aging-related impairment of regenerative processes is a major determinant of remyelination failure and resultant disability (Rist and Franklin, 2008). Decline in repair cannot be explained by exhaustion of progenitor pools, as even repeated demyelination/remyelination does not deplete the OPC pool (Penderis et al., 2003), and abundant OPCs have been observed in the absence of remyelination in advanced MS lesions (Chang et al., 2002). Instead, experimental evidence suggests that age-associated deficits in remyelination result from failure of OPCs to differentiate into myelinating oligodendrocytes (Woodruff et al., 2004; Kuhlmann et al., 2008). Here, we show that serum exosomes derived from young, young-EE and aging-EE animals improve CNS myelination in part via delivery of miR-219.

Since exposure of aging animals to young systemic *milieu* increases oligodendrocyte proliferation (Ruckh et al., 2012), we first applied exosomes derived from youthful serum to hippocampal slice cultures then stained for O4 and O1, O-antigens expressed at specific stages during oligodendrocyte maturation (Sommer and Schachner, 1981; Baumann and Pham-Dinh, 2010). Although some controversy exists as to the timing at which these markers are expressed, they are known to label mid- to late-stages of oligodendrocyte maturation. We used them here as a means to determine OPC maturation toward a myelinating phenotype. Slice culture content of pre-oligodendrocytes ($O4^+/O1^-$) and immature oligodendrocytes ($O4^+/O1^+$) increased following treatment with nutritive exosomes, indicating increased efficiency of OPC maturation. Notably, we observed increased proliferation of neural stem cells ($Musashi^+$), which was reflected in increased early OPCs ($NG2^+/Ki-67^+$). These data suggest that increasing differentiation through exosome treatment is not depleting, and plays a role in enhancing, the endogenous progenitor population.

To determine if the measured increase in OPC proliferation and maturation also led to terminal differentiation of oligodendrocytes, we next evaluated myelination. Indeed, application of nutritive exosomes to slice cultures resulted in increased expression of MBP that was reflected in increased production of structurally normal and thicker myelin (i.e., compact myelin). Although myelin g ratios were significantly improved, average axon

diameter was not significantly different between treatment groups, indicating that a change in the distribution of axon diameters is not responsible for the observed increase in myelination.

Having established that nutritive exosomes increase baseline myelin levels in healthy, mature hippocampal slice cultures, we next assessed if they could facilitate remyelination following a demyelinating injury. Lysolecithin, a chemical compound that partially hydrolyses phosphatidylcholines to produce acute demyelination, was used as a means to focus on remyelination without the additional immune component seen in rodent autoimmune models of MS (Ruckh et al., 2012). Treatment with nutritive exosomes significantly improved recovery. However, the increase in O4 positive cells and MBP levels despite no corresponding increase in O1 positive cells implies that while early differentiation events were affected (i.e., cell cycle withdrawal and expression of O4) cells had not yet fully matured, and so exosomes must also directly increase compact myelin formation by already present mature oligodendrocytes.

UV exposure negated the impact of nutritive exosomes, suggesting involvement of RNA species. Although UV exposure can also degrade proteins, we focused on RNA species and miRNA species specifically, as myelination in the CNS is highly regulated by miRNA species (Dugas et al., 2010; Zhao et al., 2010; Li and Yao, 2012). However, future studies should also assess the relative contribution of proteins and mRNA species contained within nutritive exosomes. miRNAs are small non-coding RNAs that post-transcriptionally regulate gene expression by binding to the 3' untranslated region of target mRNAs to either inhibit translation or promote degradation (Bartel, 2009). Seventeen miRNAs were chosen based on the published literature on miRNA expression profiles in oligodendrocytes during differentiation and myelination (Shin et al., 2009; Dugas et al., 2010; Li and Yao 2010; Zhao et al., 2010; de Faria et al., 2012). Several of these miRNAs were present/enriched in nutritive exosomes. We further pursued miR-219, as all three classes of nutritive exosomes contained high levels of mature transcripts of miR-219 relative to exosomes derived from NE and aged animals.

miR-219 precursors are transcribed as either miR-219-1 from chromosome 6 or miR-219-2 from chromosome 9. Both precursors produce the same mature miR-219-5p, but generate different 3p miRs: miR-219-1-3p and miR-219-2-3p. We found significant elevation of miR-219-5p and miR-219-1-3p. miR-219 promotes entry of neural stem cells into the OPC lineage and their subsequent differentiation into mature oligodendrocytes by repressing a number of negative regulators of this pathway. Others have confirmed that overexpression of miR-219 is sufficient to induce differentiation *in vitro* (Ebrahimi-Barough et al., 2013) and *in vivo* (Zhao et al., 2010). miR-219 is also deficient in MS lesions (Junker et al., 2009), suggesting that its absence contributes to diminished OPC differentiation and remyelination in later stages of the disease.

To determine if nutritive exosomes deliver functional miR-219 that may be responsible for increased differentiation and myelination, we looked for evidence of miR-219 translational repression and/or mRNA degradation in exosome treated slices. We selected targets involved in two distinct stages of OPC maturation, as well as one involved in maintenance

of myelin by mature cells. NeuroD1 is a transcription factor that is required for generation of neurons, and overexpression of NeuroD1 is sufficient to promote neuronal differentiation from neural stem cells (Boutin et al., 2010). miR-219 mediated downregulation of NeuroD1 would suppress neuronal differentiation and shift the transition of neural stem cells toward the oligodendrocyte lineage (Dugas et al., 2010; Shin et al., 2010). PDGFR α is expressed on OPCs, and is the receptor for PDGF, a mitogen that keeps OPCs in a non-differentiated, proliferative state (Fruttiger et al., 1999). Our third target, ELOVL7, regulates lipid metabolism and is necessary for maintenance of myelin in mature oligodendrocytes. Overexpression of ELOVL7 in oligodendrocytes leads to excessive accumulation of very long chain fatty acids in myelin membranes, which both destabilize the membrane and cause oxidative damage, ultimately leading to demyelination (Shin et al., 2010). Reduced expression of the protein products of all three targets confirmed that nutritive exosomes are able to deliver biologically active miR-219 to affect cells in slice culture, and regulate OPC differentiation and myelination on multiple levels.

Here, it is important to discuss our finding of a significant increase in NG2⁺ cells concurrent with a decrease in PDGFR α protein. In the literature, NG2 and PDGFR α are often used as interchangeable markers for OPCs (Kang et al., 2010). However, some evidence suggests that this colocalization diminishes as cells mature (Nishiyama et al., 1996), and the relative expression of NG2 and PDGFR α during the transition from a proliferation-competent oligodendrocyte precursor to a post-mitotic, differentiating cell is not well characterized. Loss of PDGFR α occurs as OPCs begin to mature, and is necessary for cell cycle withdrawal and subsequent differentiation (Nave, 2010). It is possible that a precursor cell positive for both NG2 and PDGFR α begins to reduce PDGFR α expression before losing NG2 expression. Alternatively, transient NG2 expression by microglia/macrophages has been reported (Baracska et al., 2007) and could also account for the observed increase in NG2⁺ staining. Nevertheless, the significant increase in Musashi⁺ neural stem cells after exosome treatment is consistent with increased NG2 staining, and is evidence that exosomes do not deplete, but instead augment, the endogenous progenitor pool.

As further confirmation of miR-219 function, we inhibited miR-219 in young serum-derived exosomes and found that these altered exosomes no longer increased myelination in slice culture. However, we would like to stress the importance of further studies to determine the complete miRNA profile of nutritive exosomes and their respective contributions to improved brain health. Such studies are underway, but beyond the scope of this article. For example, preliminary work has shown that these exosomes also improve oxidative tolerance (unpublished observations) and may do so by miRNA-mediated alteration of microglial inflammatory phenotype (Ponomarev et al., 2013), which would have important implications for the role of EE in improving outcome of neuroinflammatory diseases.

Finally, we began investigating the translational potential of exosomes by testing their efficacy *in vivo*. Nasal administration of young serum-derived exosomes significantly increased myelin in aged rats. This increase in myelin was evident in motor cortex, suggesting that intranasal delivery provides an effective, non-invasive method for exosome administration (Zhuang et al., 2011). Since limited ability to penetrate the blood-brain barrier presents a major problem in the development of CNS-directed therapeutics, it is

significant that exosome treatment was achieved without need of an additional delivery vehicle. Future studies will be necessary to determine the extent to which myelin is affected by nasal delivery of nutritive exosome, identify the most advantageous route of delivery, and assess the efficacy of nutritive exosomes in whole animal models of MS.

In summary, exosomes from serum of young and EE animals improve myelination, in part via delivery of miR-219, which promotes OPC differentiation into mature myelin-producing cells. Importantly, EE of aging animals stimulates production of exosomes enriched in miR-219 that are as effective as young exosomes at improving myelination and restoring remyelination. This suggests that peripheral exosomes that are endogenously produced during EE can enter the CNS to deliver miRNAs that functionally impact myelination. It is notable that peripheral immune cells can state-dependently produce miR-219 (Favreau et al., 2011; Montecalvo et al., 2012; Fredman et al., 2012), previously considered a brain-specific miRNA (Liang et al., 2007). Thus, exosome-mediated transfer of miRNAs may represent an additional mechanism for neuro-immune interactions. We propose that the age-related decrease in remyelination associated with immunosenescence involves deficiencies in peripheral production of exosomes containing nutritive microRNAs, including miR-219. Similarly, the well-documented benefits of EE on brain health may involve improved immune function (De la Fuente and Gimenez-Llort, 2012) and related changes in exosome composition.

EE-exosomes are well-suited for development as therapeutics to “jump-start” and enhance the promyelinating effects of EE. They are naturally occurring, deliver endogenous miRNA species that promote myelination by enhancing oligodendrocyte differentiation, cross the blood–brain barrier without need for an additive delivery platform, do not deplete the OPC progenitor population, and can be administered intravenously or nasally (Alvarez-Erviti et al., 2011; El Andaloussi et al., 2012; Zhuang et al., 2011), and can be modified for targeting of specific cell types (Alvarez-Erviti et al., 2011; El Andaloussi et al., 2012). Furthermore, exosomes are non-toxic and do not provoke adverse immune reactions (O’Loughlin et al., 2012), traits that collectively support the possibility of designing a novel exosome-based regenerative therapy to be used in conjunction with immunomodulatory strategies.

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FIGURE 1.

Myelin distribution in hippocampal slice cultures. **(A)** NeuN staining of hippocampal slice culture illustrates neuronal cytoarchitecture consisting of structurally preserved trisynaptic loop (dentate gyrus-CA3-CA1). **(B)** Immunostaining for MBP shows regional distribution of gray matter myelin in 21 days *in vitro* hippocampal slice culture that closely parallels that seen *in vivo*. Scale bar=250 μ m. **(C)** EM confirmation of compact myelin in hippocampal slice culture. Representative image demonstrates presence of structurally normal, tightly laminated myelin sheath. Scale bar=200 nm.

FIGURE 2.

Exosomes derived from serum were non-toxic and increased pre-oligodendrocyte levels. **(A)** Exosome isolation from serum was confirmed by EM and Western blot for surface markers CD63 and Alix. Representative electron micrograph image illustrates a uniform population of vesicles approximately 50 nm diameter in size. Scale bar=20 nm. **(B)** Exosome application to hippocampal slice cultures was non-toxic. Exosomes were resuspended in phosphate buffered saline and 20 μL (~40 μg protein) applied to 21 days *in vitro* slice cultures. Slices were then stained with Sytox, a fluorescent marker of cell death, at 3, 5, 7, and 12 days post-treatment. NeuN immunostaining image (**left**) is shown to illustrate neuronal architecture. Sytox positive image (**center**) shows control with neuronal injury induced by 24 hr exposure to 20 μM *N*-methyl-D-aspartate. Quantification of Sytox intensity ($n=9/\text{group}$) confirmed that exosome application caused no significant injury (**right**). Scale bar=250 μm. **(C)** Representative confocal images of O4 positive cells in control (**left**) and nutritive exosome-treated slice cultures (**right**). Scale bar=25 μm. Low power images were used for quantification of staining intensity from a stereotypic area of interest in CA3. One day after treatment with 20 μL (~40 μg protein) of young, young-EE, and aging-EE exosomes, O4⁺ fluorescence intensity was significantly ($*P = 0.001$; $n=6-19/\text{group}$) increased relative to untreated control slices and slices treated with aging, NE, and aging-NE exosomes. Exposure of exosomes to 254 nm UV light for 1 hr before application to slices eliminated this increase. **(D)** Representative confocal images of O1 positive cells in control (**left**) and nutritive exosome-treated slice cultures (**right**). Scale bar=25 μm. As before, 1 day after exosome treatment, quantification of low power images revealed significantly ($*P = 0.001$; $n=6-19/\text{group}$) increased O1⁺ fluorescence intensity in nutritive exosome-treated

cultures compared with control and nonnutritive exosome-treated cultures. Once again, exosomes exposed to UV light lost their effect, indicating that the responsible factor is an RNA species. Significance was determined by ANOVA plus *post hoc* Holm-Sidak testing.

FIGURE 3.

Young and environmentally enriched exosomes increase OPC proliferation. Naïve slice cultures were treated with exosomes (20 μ L, ~40 μ g protein) and fixed one day later for subsequent staining for neural stem cells and OPCs. **(A)** Representative confocal images of Musashi (green) with DAPI nuclear counterstain (blue) in slice cultures. Scale bar=10 μ m. Arrows indicate Musashi positive neural stem cells (blue-green). **(B)** Quantification of percent Musashi positive cells (Musashi⁺ cells/total DAPI⁺ cells) per field (five images per slice culture, $n=3$ slices/group) revealed significantly ($P < 0.001$) increased neural stem cells in nutritive exosome-treated compared with control and non-nutritive exosome-treated cultures. **(C)** Representative confocal images of NG2 (green) and Ki-67 (red) double stain in slice cultures. Scale bar=10 μ m. Arrowheads indicate NG2 positive progenitor cells. Arrows indicate NG2 positive cells co-labeled with proliferation marker Ki-67. **(D)** Quantification of percent NG2/Ki-67 double positive cells (NG2⁺ Ki-67⁺ cells/total NG2⁺ cells) per field (three images per slice culture, $n=4$ slices/group) revealed significantly ($P < 0.001$) increased proliferation in nutritive exosome-treated cultures compared with control and non-

nutritive exosome-treated cultures. Significance was determined by ANOVA plus *post hoc* Holm-Sidak testing.

FIGURE 4.

Young and environmentally enriched exosomes enhanced myelination. **(A)** Nutritive exosomes enhanced baseline slice culture myelin levels. Slice cultures were treated with 20 μ L exosomes ($\sim$ 40 μ g protein) and harvested three days later for Western blot analysis of MBP content. Young, young-EE, and aging-EE rat serum-derived exosomes ($n=3-18$) all significantly ($*P=0.004$, 0.008 , and 0.003 , respectively) increased MBP content of slice cultures above control, whereas their aging, NE, and aging-NE counterparts did not. Exposure of exosomes to 254 nm UV light for 1 hr before application to slices ablated their effect. **(B)** Representative electron micrograph images show myelin thickness in control cultures (**left**) and 3 days after exposure to young serum-derived (**middle**) or UV-exposed exosomes (**right**). Scale bars=200 nm. **(C)** The g ratio (axon diameter/fiber diameter) calculation ($n=3$ /group, with 20–27 axons measured per group) revealed a significant ($*P < 0.001$) decrease in young exosome-treated versus control and UV-exosome treated samples, indicating improved myelin thickness. **(D)** Axon diameter was not significantly different in young exosome-treated versus control and UV-exosome treated samples ($n=3$ /group, with 20–27 axons measured per group). Significance was determined by ANOVA plus *post hoc* Holm-Sidak testing.

FIGURE 5.

Young and environmentally enriched exosomes improved recovery from demyelinating injury. **(A)** Lysolecithin (0.5 mg/mL) exposure for 17 hr was used as a means to evoke demyelination followed by remyelination in slice cultures. Time course of recovery was determined via staining for MBP. Control slice shows typical MBP immunostaining in a healthy, mature slice culture. Dotted line follows the CA3 pyramidal neuron body layer. Lysolecithin induced demyelination that peaked at 2 days, showed first signs of recovery at 5 days, and progressively returned to normal by 12 days. Sham treatment (17 hr exposure to fresh media without lysolecithin) had no injurious effect on myelin content and/or distribution. Scale bar=250 μ m. **(B)** Immediately after exposure to lysolecithin, cultures were treated with 20 μ L exosomes (~40 μ g protein) derived from young, young-EE, or aging-EE animals. Five days later, at the onset of remyelination, cultures were collected and MBP content analyzed via Western blot. While lysolecithin (Lyso) exposure triggered a significant ($*P < 0.001$; $n=6-16$) reduction of MBP in all groups compared with control, exosome exposure prompted a significant ($\#P < 0.001$) increase in remyelination compared with that seen with lysolecithin alone. Similarly, slice cultures collected 5 days after lysolecithin treatment also revealed significantly ($*P < 0.001$; $n=4-8$ /group) increased presence of O4 positive cells. All exosome treatments significantly enhanced O4 staining ($\#P < 0.001$) above that seen with lysolecithin treatment alone. While O1 staining also significantly increased ($*P < 0.001$; $n=3-4$ /group) 5 days after lysolecithin treatment, no additional increase was seen with exosome treatment, suggesting that exosomes may directly increase remyelination by surviving mature oligodendrocytes. Significance was determined by ANOVA plus *post hoc* Holm-Sidak testing.

FIGURE 6. Young and environmentally enriched exosomes were enriched in miRNAs necessary for oligodendrocyte differentiation. **(A)** To determine the specific miRNA species responsible for nutritive exosome increase of myelin, the miRNA content of young, young-EE, and aging-EE exosomes were compared with that of non-nutritive aged-NE exosomal miRNA utilizing two different methods: SBI's Rat Genome-wide microRNA qPCR Array Panel (**left**), and TaqMan Array Rodent MicroRNA Cards (**right**). Mature species that were significantly (i.e., >2 fold, $n=2-3$) enriched in each class of exosomes are shown in red. miRNAs that were readily detectable but not significantly enriched are shown in white. miRNAs that could not be detected are shown in grey. [†]TaqMan microarray cards contained mmu-miR-219, which likely cross-reacted with rno-miR-219 species due to the high sequence homology. To determine if exosomes are taken up by oligodendrocytes, young exosomes were labeled with DiI and applied them to slice cultures (20 μ L, ~40 μ g protein). **(B)** Representative confocal image illustrates DiI labeled exosomes colocalized with a CNPase⁺ oligodendrocyte. Scale bar = 10 μ m. Although exosomes are typically 30–120 nm, the limit of resolution via confocal imaging is 250 nm. Thus, this image represents an accumulation of DiI labeled exosomes in a CNPase⁺ cell, suggesting that nutritive exosomes may enter oligodendrocytes and transfer their cargo of specifically enriched microRNAs (A).

FIGURE 7.

Young and environmentally enriched exosomes deliver functional miR-219 that impacts oligodendrocyte differentiation and myelination. Schematic illustration of the involvement of miR-219 in oligodendrocyte differentiation. **(A)** In MS OPCs are actively prevented from differentiating into myelin producing cells in part due to the deficiency of miR-219. **(B)** However, upon exposure to nutritive exosomes, neural stem cells preferentially enter the oligodendrocyte lineage due to the inhibition of the proneurogenic factor NeuroD1. miR-219 also suppresses expression of a number of other factors that inhibit OPC differentiation,

such as PDGFR α , a receptor for a mitogenic factor that promotes proliferation and prohibits differentiation. Finally, miR-219 decreases levels of ELOVL7, a regulator of lipid metabolism whose over-activity could lead to demyelination. (C–E) Naïve slice cultures were treated with nutritive exosomes (20 μ L, ~40 μ g protein) and harvested 1 day later for protein extraction. Protein products of aforementioned miR-219 target mRNAs were measured via Western blot. Exosome-treated slices ($n=3-10$ /group) expressed significantly (*) less NeuroD1, PDGFR α and ELOVL7 (P value ranges of 0.04–0.002, 0.03–0.003, and 0.02–0.001, for C, D, and E, respectively) relative to levels in untreated control slices. (F) Young-EE exosomes were transfected with a miR-219 inhibitor to assess the role of miR-219 from nutritive exosomes in increasing myelination. Three days after application, Young-EE exosome treatment significantly (* $P=0.008$) increased MBP content of slice cultures relative to untreated controls. Inhibitor-transfected Young-EE exosomes had no significant effect. Significance determined by Student's t -test (D–E) and plus *post hoc* Holm-Sidak testing (F).

FIGURE 8.

Nasal administration of young serum-derived exosomes increased myelin in aging rats. About 50 μL of exosomes ($\sim 100 \mu\text{g}$ protein) were intranasally delivered to aging rats. Three days later, brains were harvested, frozen, and motor cortex sectioned ($14 \mu\text{m}$) for staining. **(A)** Staining for MBP demonstrates myelin distribution in parasagittal motor cortex (**left**). Representative images illustrate corresponding cytochemical staining with FluoroMyelin to measure levels of compact myelin after nasal administration of UV-exposed sham exosomes (**center**) or young serum-derived exosomes (**right**). Stronger staining intensity at bottom of all images delineates the underlying white matter. Scale bar= $250 \mu\text{m}$. **(B)** Quantification of staining intensity shows significantly ($*P=0.001$; $n=3$ animals/group, with nine images quantified per animal) increased compact myelin in cortex of animals treated with young exosomes. **(C)** Western blot for MBP confirms staining results and shows significantly ($*P=0.01$; $n=3$ animals/group) increased MBP in cortex of animals treated with young exosomes. Significance was determined by Student's *t*-test.