ORIGINAL ARTICLE

Official Journal of the Neuroscience Society of Nigeria (NSN)

https://doi.org/10.47081/njn2015.7.1/004
ISSN 1116-4182

Gestational Nicotine Exposure Alters Oligodendrocyte Morphology and Axonal Myelination in the Lateral Prefrontal Cortex of Young Wistar Rats

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Received: …….. July 2015
Accepted: …….. October 2015

ABSTRACT

Prenatal exposure of the foetus to chemical insults has implications on the overall growth and development of the baby before and after birth. This study examined the effects of prenatal exposure to nicotine on the morphology of oligodendrocytes which are responsible for the development of myelin in the central nervous system, and are critical in neurodevelopment. Twenty adult female Wistar rats were used for the study. Their oestrous cycle was determined by vaginal smearing, and subsequently exposed to male rats for mating. The female Wistar rats were thereafter grouped into two and each group was further subdivided into a saline-treated control and a nicotine-treated subgroup. Each treatment lasted 5 consecutive days between days 9-13 (Group A) and days 16-20 (Group B) of pregnancy. The pups were allowed to grow and, at postnatal day 15, were euthanized and perfusion-fixed transcardially. The lateral prefrontal cortex was processed for histochemical, immunohistochemical and electron microscopic studies. Findings indicated marked decrease in oligodendrocyte count, reduced expression of myelin basic protein (MBP), and impaired myelin formation. These changes can affect the normal growth and development of the brain with severe implications on cognitive and motor functions after birth.

Keywords: Gestational nicotine, myelin, myelin basic protein, neurodevelopment, lateral prefrontal cortex, oligodendrocytes

INTRODUCTION

Oligodendrocytes are a type of glial cells found in the central nervous system (CNS), and are the most populous of the glial cells (Harris et al. 2013). Their cell bodies reside primarily in the white matter and their processes extend to surround the axons, thereby forming the lipid-rich myelin sheath around the axons of the CNS (Cao et al. 2013). Oligodendrocytes originate from neuroectodermal cells within the subventricular zone (Jackman et al. 2009) as oligodendrocyte precursor cells (OPCs), which eventually differentiate into mature oligodendrocytes (Barca-Mayo and Lu 2012). Mature oligodendrocytes provide critical insulation to facilitate axonal conduction, and are involved in regulating the development and periodicity of nodes of Ranvier, which contain ion channels critical for action potential propagation along the axon (Edgar and Sibille 2012).

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The development of the myelin sheath around CNS axons is essential for normal brain function, and is a cornerstone of human neurodevelopment (Deoni et al. 2011). Myelination in rodents begins at the 3rd week of intrauterine life, few days to the end of gestation, while the greater part of development takes place during the postnatal life (Rice and Barone 2000). Damage to myelin presents as derangement in sensory, motor and cognitive functions (Jarjour et al. 2012), signifying its importance in motor and cognitive functions of the brain. However, not much has been said about the effects of nicotine on brain development and functions as it relates to myelination.

Myelin is a low-capacitance, high-resistance membrane that envelops and electrically insulates the axon, allowing for the rapid and efficient propagation of nerve impulses (Saher and Simons 2010). Rapid saltatory nerve conduction is dependent on its unique composition, highly enriched in glycosphingolipids and cholesterol (Saher et al. 2011). Tri-directional communication between the neuron, the myelinating cell, and the environment is facilitated by myelin. It provides trophic support to the axon and maintains its long-term integrity (Jackman et al. 2009). Myelin has recently been shown to be involved in metabolic phosphorylation (Morelli et al. 2011).

Myelin-related clinical conditions could be in form of hypomyelination, delayed myelination, or demyelination, all leading to some forms of motor and cognitive problems in the CNS. In the first two cases, it is usually a problem with the process or processes leading to myelination, while in demyelination there is loss or removal of myelin from already myelinated axons (Kotter et al. 2011). Conditions that could damage oligodendrocytes or OPCs would result in hypomyelination or delayed myelination, with possibility of significant delay in motor and mental developmental milestones that result in long-term disability (Duncan et al. 2011; Costello et al. 2009).

Myelin basic protein (MBP) is the second most abundant protein in the myelin sheath and is found only at the cytoplasmic interface (Banquy et al. 2012), where it acts as an intermembrane adhesion protein (Min et al. 2009). MBP expression begins during terminal differentiation of oligodendrocytes (Campagnoni et al. 1988). MBP is useful in assessing CNS myelination, either by direct quantification or by isolation and quantification of myelin (Wang et al. 2009). This study examined the effects of prenatal nicotine exposure on the morphology of oligodendrocytes in the developing prefrontal cortex and its implication on axonal myelination and expression of myelin basic protein.

MATERIALS AND METHODS

Laboratory Animals

Twenty adult female Wistar rats (mean weight: 162.1 ±4.89 g) were used for the study. Their oestrous cycle was determined by vaginal smearing method (Marcondes et al. 2002), and subsequently exposed to male rats for mating during the pro-oestrous phase. They were housed in the Animal House of the College of Health Sciences, University of Ilorin, and kept under standard condition, and were fed on pelletized growers feed *ad libitum*.

Treatment of Animals

There were two experimental groups (A and B) and each was subdivided into two subgroups as saline-treated (0.1ml of normal saline: control) and nicotine-treated (0.1 ml of 13.76 mg/kg). Treatment was for 5 consecutive days, such that Group A was treated from days 9-13 of gestation [2nd gestational week (GW)], while Group B was treated from days 16-20 of gestation [3rd GW].

Animal Sacrifice and Tissue Perfusion Fixation

The pregnant rats were allowed to litter, and the pups were monitored up till postnatal day 15 (P15) when the pups from each subgroup were anaesthetised with intramuscular ketamine injection. Perfusion fixation was thereafter carried out transcardially with saline, and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer or modified Karnovsky's fixative. The prefrontal cortex was excised from the brain and put in appropriate fixative for subsequent tissue processing.

Preparation of Histochemical Sections

Automatic tissue processor (Leica TP1020, Leica Biosystems ®, Germany) was used, and the tissues were embedded in paraffin wax using Tissue Embedding System (Leica EG 1160, Leica Biosystems®, Germany). Tissue blocks were sectioned using a rotary microtome (Leitz Wetzlar® 1512, Germany) at a thickness of 5 µm. The tissues were stained using cresyl fast violet staining technique (Bancroft and Gamble 2008).

Counting of Oligodendroglial cells

The ImageJ –win32 (NIH, USA) software was used. Cell counting was carried out using a Counter Window of 1024 x 768 pixels dimensions. For each slide preparation, five different Counter Windows were captured at different fields of the sections, and analysed, with care taken to ensure consistency in sampling, and to reflect a fair representation of the cortical layers. The average of the five Windows was determined and reported as number of cells per Counter Window. Data were analysed using student's t-test.

Immunohistochemical Techniques (Abcam 2013)
Paraformaldehyde-fixed mounted sections were used. The tissues were deparaffinised in xylene, rehydrated in descending concentration of alcohol and washed in phosphate buffer solution. Heat-mediated antigen retrieval in citrate buffer was carried out, and 5% normal goat serum was used as blocking solution. Primary and secondary antibodies used were anti-myelin basic protein antibody and goat anti-rabbit Alexa Fluor® 468 respectively. The sections were counter-stained with DAPI, stabilised with glycerol as the mounting medium, and viewed under the fluorescence microscope (Olympus).

Electron Microscopic Technique
The brain tissues for electron microscopic technique were fixed in Modified Karnovsky's fixative: 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). With the aid of a dissecting microscope (Motic SMZ-1 68), about 1x1 mm of prefrontal cortical tissue was excised at the external lateral part of the left hemisphere, and fixed in modified Karnovsky Fixative at 4 °C; and thereafter post fixed in 2% aqueous osmium tetraoxide in the same buffer for 2 hr. The specimens were dehydrated in series of graded alcohols, infiltrated and embedded in araldite 6005 resin or spur resin. Ultra-thin (50–70 nm) sections were made with a glass knife of ultra-microtome (Leica Ultra cut UCT-GA-D/E-1/00), mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead citrate. They were viewed under transmission electron microscope (Hitachi, H-7500, Japan) at required magnification.

RESULTS

Oligodendrocyte Count
Oligodendroglial count was reduced in the lateral prefrontal cortex of animals exposed to nicotine in the 2nd and 3rd GW (Figure 1). This reduction was more marked in those exposed in the 2nd GW, though this difference was not statistically significant ($p > 0.05$).

Histochemical Observation of the Lateral Prefrontal Cortex (LPFC)
The histochemical section of the LPFC at postnatal day 15 revealed mild disruption in the microarchitecture in rats exposed to nicotine prenatally (Figure 2). The population of oligodendrocytes reduced in nicotine-exposed groups, while the cortical layer was slightly wider in animals exposed in the 3rd GW compared with those exposed in the 2nd GW. Nissl staining was less positive in the nicotine-treated groups (Figure 2).
Immunohistochemical Study of Myelin Basic Protein (MBP)
Immunohistochemical study showed reduction in the expression of MBP in myelinated axons in the LPFC of rats exposed to nicotine in the 3rd GW compared with those exposed in the 2nd GW prior to myelination (Figure 3).

Electron Microscopy
The ultrastructure of oligodendrocytes in the LPFC of rats exposed to nicotine in the 2nd and 3rd weeks of gestation revealed nuclei with degenerative changes (Figure 4). Although the nuclear membranes were intact, the perinuclear cisternae were not visible. Nuclear pores were present only in small segments of the entire envelope, and they appeared blocked in a significant section of the nuclear membrane. Nuclei in the 3rd GW group were smaller, and contained more dense bodies and densely packed chromatin within the nucleoplasm. Axons of neurons were either poorly myelinated or lacked myelin sheath, with irregular outline of the axons and associated discoloration (Figure 5).

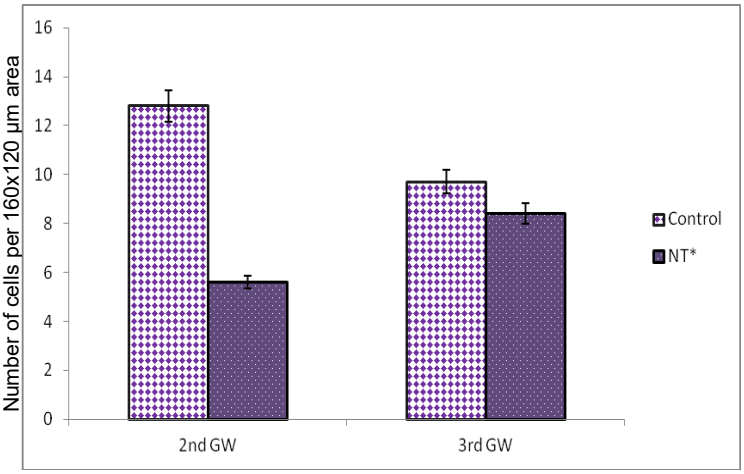

Figure 1: Average oligodendrocyte count. GW: gestational week; NT: nicotine-treated; *$p > 0.05$.

DISCUSSION

The development of oligodendrocytes commences shortly after neuronal cells begin to form in rat's second week of gestation, while myelin begins to form in the third week, with the substantial part of myelination occurring in the postnatal period (Rice and Barone 2000). Exposure of the developing brain to the

the effects of nicotine can adversely impact the role of oligodendrocytes in CNS myelination.

Nicotine negatively affects the number and size of oligodendrocytes, and the ultrastructure revealed irregular nuclear contour, congested perinuclear cisternae probably due to the shrinkage of the cells and closely packed nuclear components. The nuclear pores were occluded in some segments of the membrane, with numerous dense bodies within the nucleoplasm, and heavily clumped heterochromatin, especially in rats exposed in the third gestational week.

Gestational nicotine exposure in this study appeared to adversely affect the differentiation and maturation of oligodendrocytes probably at the level of the oligodendrocytes precursor cells, as reduction in cell count was more marked at the commencement of neurodevelopment in the second week of gestation. This might be responsible for the impairment in the formation of myelin around the axons during the late gestational period, and the poor consistency of the myelin sheath. Deformed myelin sheath would affect the integrity of the axons and their functions.

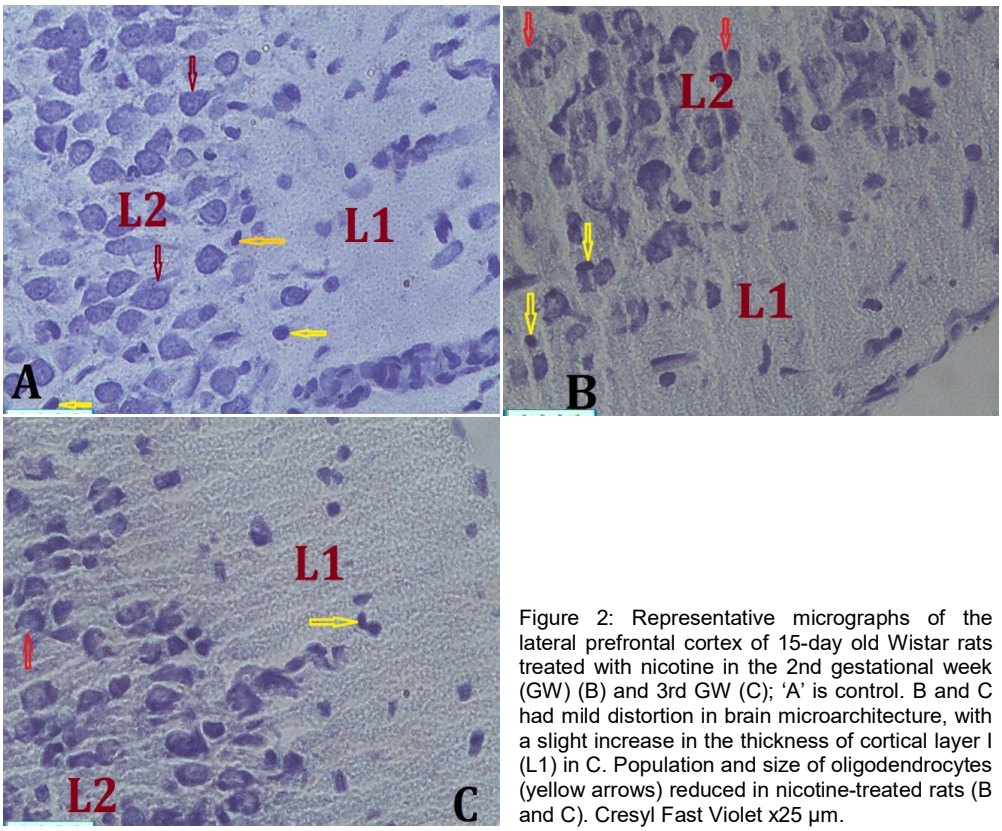

Figure 2: Representative micrographs of the lateral prefrontal cortex of 15-day old Wistar rats treated with nicotine in the 2nd gestational week (GW) (B) and 3rd GW (C); 'A' is control. B and C had mild distortion in brain microarchitecture, with a slight increase in the thickness of cortical layer I (L1) in C. Population and size of oligodendrocytes (yellow arrows) reduced in nicotine-treated rats (B and C). Cresyl Fast Violet x25 µm.

MBP was poorly expressed in rats exposed to nicotine after the onset of myelination in the third gestational week. The low content of this protein impairs the normal process of myelin formation and neurodevelopment in general, and its function as an intermembrane adhesion protein (Min et al. 2009) is also lost.

The reduced oligodendrocyte count could be as a result of destruction

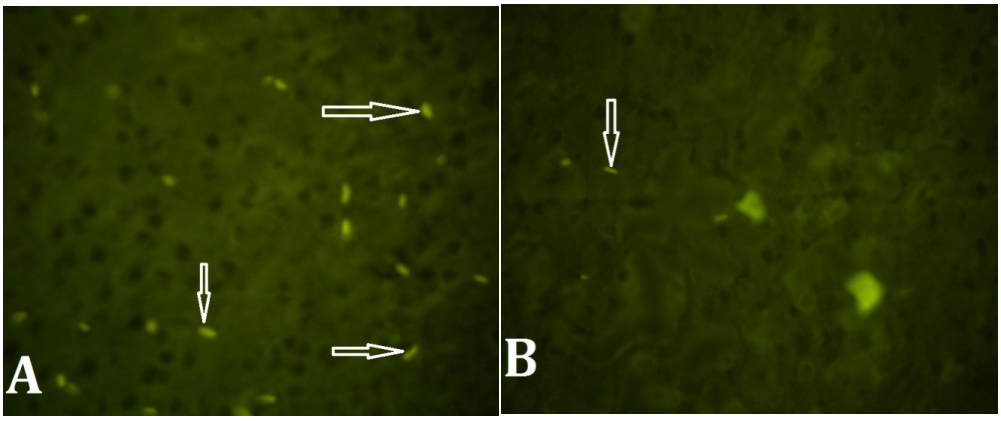

Figure 3: Representative Immunofluorescence micrographs of myelin basic protein (MBP)-positive cells in rats exposed to 13.76 mg/kg/d nicotine during the 2nd GW before myelination (A) and 3rd GW when myelination has commenced (B). The expression of MBP (arrows) was markedly reduced in myelinated axons of B compared to the expression in A.

of already formed oligodendrocytes, or an effect on the oligodendrocyte precursor cells (OPCs) and the processes involved in their eventual differentiation into mature oligodendrocytes. Affectation of OPCs could result in delayed cell differentiation, leading to a low population of oligodendrocytes. As earlier observed, absence of molecules, such as semaphorins, that control OPCs migration results in delay or arrest of oligodendrocytes differentiation, with a consequent reduction in MBP expression (Bernard et al. 2012).

Reduction in the number and size of oligodendrocytes could affect their regulatory role in the development and periodicity of nodes of Ranvier (Edgar and Sibille 2012), with a resultant adverse effect on axonal conduction of impulses. The functions of myelin component of the axons such as the rapid saltatory nerve conduction, the insulating property as well as the metabolic capability, are impaired, with a resultant reduction in the speed and efficiency of propagation of nerve impulses (Saher and Simons 2010). With impaired myelin formation, the integrity of axons is also lost (Jackman et al., 2009).

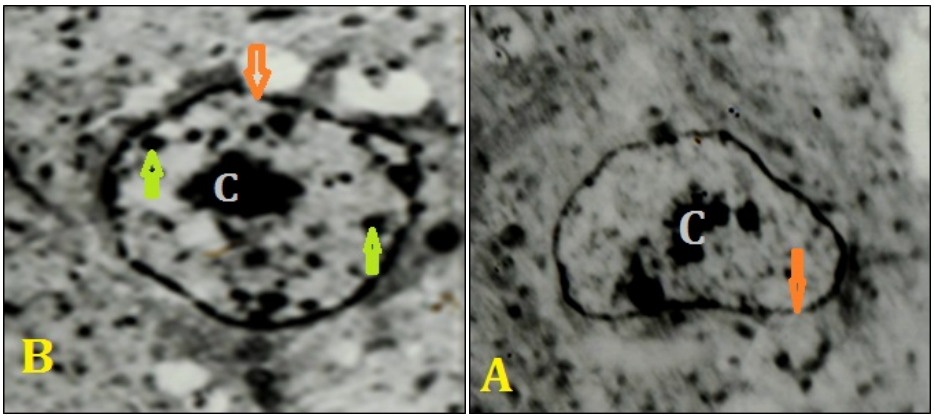

Figure 4: Electron micrographs of the LPFC of 15 days old rats prenatally exposed to nicotine in the 2nd (A) and 3rd GW (B), showing irregular contour of the nuclei of degenerating oligodendrocytes. The perinuclear cisternae in both were not visible, though the nuclear pores were present at few segments of the membrane (brown arrows), while they appeared blocked largely on the right half of the nuclear membrane; the nucleus of B had more dense bodies (green arrows) and densely packed chromatin components (C) within the nucleoplasm compared with A. (A: 3474x; B: 3860x).

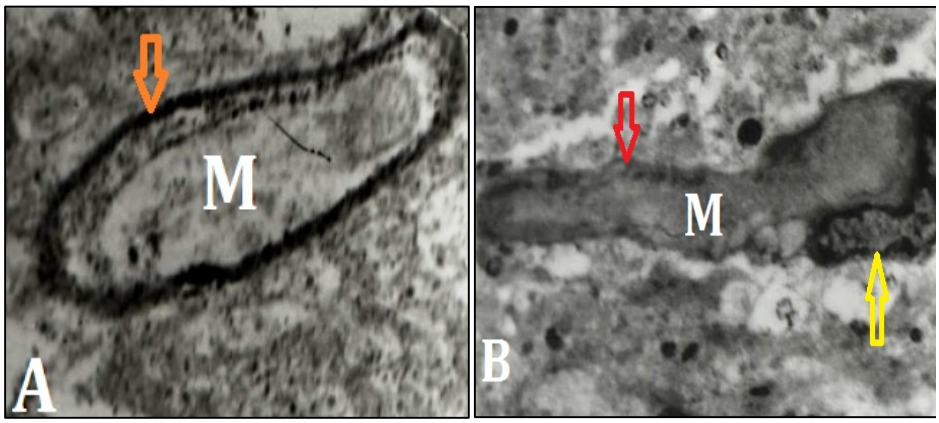

Figure 5: Electron micrographs of the LPFC of 15 day old Wistar rats showing the Control (A) and nicotine-exposed rat in 3rd GW (B). B was poorly myelinated (red arrow) compared with A (brown arrow) whose myelin layer was smoothly wrapped around the myelinated axon (M). The axonal outline in B was poorly defined and irregular. Close to the myelinated axon in B was the nucleus of an oligodendrocyte (yellow arrow) with an irregular contour. A: 2316x; B: 5790x.

Nicotine-induced dysmyelination could mimic any of the clinical conditions associated with hypomyelination, delayed myelination, or failure of development of myelin, with features of motor and cognitive impairments (Costello et al. 2009). This, however, could readily be excluded while taking a clinical history of such individuals.

These findings have serious implications on cognitive and motor functions, and could be responsible for some of the neurologic dysfunctions seen in children prenatally exposed to nicotine, via maternal tobacco use or nicotine replacement therapy.

Conflict of Interest

None declared.

Acknowledgements

The authors wish to acknowledge the support from the CV Raman International Fellowship for African Researchers, and also recognise the assistance of the following people: Dr. Joby Joseph, Centre for Neural and Cognitive Sciences, University of Hyderabad, India; Prof. and Mrs. B. Senthilkumaran, and Dr. Suraj, School of Life Sciences, University of Hyderabad; and Dr. M. Lakshman, SV Veterinary University, Rajendranagar, India.

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