

## Dr Francis A. Olaolorun

### Report on ISN/CAEN category 1A August 2016 research grant

**Home institution:** Neuroscience Unit, Department of Veterinary Anatomy, University of Ibadan, Ibadan, Nigeria.

**Host institution:** Section of Anatomy and Histology, Department of Neuroscience, Biomedicine and Movement Sciences, School of Medicine, University of Verona, Italy.

**Host supervisor:** Prof. Marina Bentivoglio

**Project title:** “Effects on the brain of low dose vanadium administration during postnatal development in mice”

**Duration of visit:** January 30 - April 18, 2017.

**Introduction:** I arrived at the University of Verona on the 30<sup>th</sup> of January, 2017. Under the supervision of Drs Anna Andrioli and Idris Azeez, I was shown the laboratories, equipment and office spaces. I was also introduced to staff and graduate students of the Department. I was provided all the necessary assistance in having a smooth and productive stay in Verona. All reagents and antibodies required for the project were generously provided by my host, Prof. Bentivoglio. She also provided me with free accommodation at a guest room of the Biological Institutes of the Medical School throughout my stay.

#### **Experiments:**

##### *Animals and treatment*

The animal experiment was carried out at the University of Ibadan (ethical approval number UI-ACUREC/App/01/2017/001). A total of six nursing mice were used in this study: two nursing mice and their pups (n= 12 pups per group) were randomly assigned to each of three test groups. Only 5 pups per group were, however, sacrificed.

**Group 1 (control):** the nursing mice were given daily intraperitoneal (i.p.) injection of sterile water (vehicle) for the duration of lactation (postnatal day, PND, 1-21). After weaning, the pups received i.p. injection of sterile water every other day till sacrifice on PND 30. The pups were weighed daily using an electronic weighing balance (KERN EW, Germany) throughout the duration of the experiment.

**Groups 2, 3 (vanadium treatment):** the nursing mice were given daily i.p. injection of sodium metavanadate 0.15 mg/kg (Group 2), and 3.0 mg/kg (Group 3; this latter treatment corresponds to a relatively high dose) during the lactation period. The rationale was to expose the neonatal mice to vanadium via the dam’s milk. After weaning on PND 21, the pups received the same dosage of vanadium every other day till sacrifice on PND 30. Body weight was monitored daily as stated for the control group.

**BrdU administration:** to assay for proliferating cells, 5-bromo 2’- deoxyuridine (BrdU; Sigma), dissolved in 0.9% NaCl, was administered i.p at 50 µg/g of body weight on PND22 and 28 to the pups in the three groups.

At the time of sacrifice (P30), the pups were deeply anesthetized (ketamine 100 mg/kg and xylazine 10 mg/kg, i.p) and perfused transcardially with saline followed by 4% paraformaldehyde (Sigma Aldrich, Steinheim, Germany) in 0.1 M phosphate buffer, pH 7.4. The brains were carefully removed from the skull and postfixed for 2 h at room temperature in the same fixative solution. The brains were subsequently stored in 0.1% sodium azide in 0.01M phosphate-buffered saline pH 7.4 (PBS) and kept at 4°C till they were transported to the lab in Verona.

### ***Histology and immunohistochemistry***

The brains were soaked in 30% sucrose in PBS for cryoprotection and kept in the cold room till the brain samples sank to the bottom of the tube. Serial coronal sections were then cut through the brain at a 30 µm thickness with a freezing microtome, and collected in 12 adjacent series in well plates. Cresyl violet stain was first used for cytoarchitectural study. Unfortunately, an artefact was thus revealed (large holes in the tissue), with the high dose vanadium group being the worst affected. We initially thought that the tissue damage (the holes) was a result of freezing artefact and thus I had to try rapid freezing with isopentane in liquid nitrogen and also used other equipment, cryostat and vibratome, to cut the sections; all yielded variable results but the high dose vanadium group was beyond redemption. I was told that probably the artefact was due to problems with fixation. The viable sections were then processed as summarised below and I could pursue the study on the low dose vanadium *versus* control groups.

**Immunohistochemistry for astrocytes, microglia, myelin and proliferating cells:** Series of adjacent sections were chosen at random and processed for immunohistochemistry following the protocol used by Azeez *et al* (2016). The following primary antibodies were used: rat anti-CD11b (Serotec, Oxford, United Kingdom; diluted 1:500) for microglial cells, rabbit anti-gliial fibrillary acidic protein (GFAP; 1: 500; Dako, Denmark), to visualize astrocytes; rabbit anti-myelin basic protein (MBP; 1:300; Abcam) for myelin; mouse monoclonal anti-BrdU (1:400; Dako, Denmark), which recognizes proliferating cells.

### ***Data analysis***

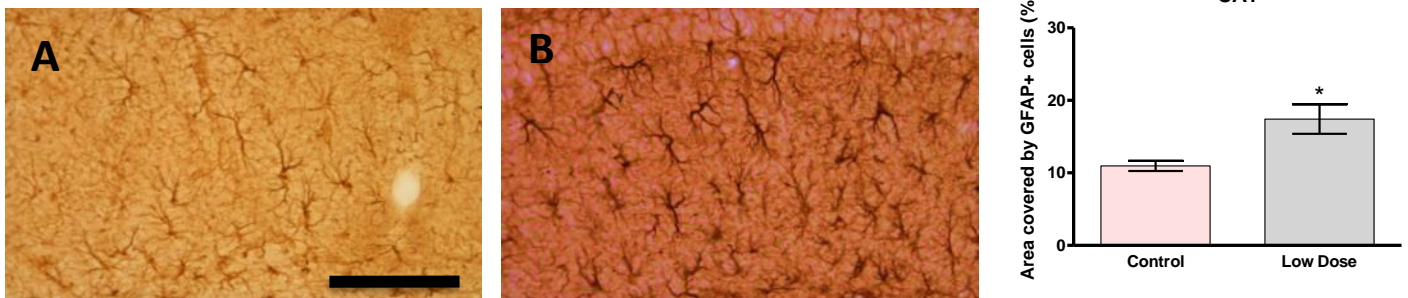
Brain sections processed for immunoperoxidase were viewed under bright-field Olympus BX51 microscope. Quantitative analysis of GFAP immunoreactivity was pursued in the hippocampal CA1 field and dentate gyrus of control and vanadium-treated mice (N=5 animals per group). Briefly, images were acquired with a QUICAM Fast 1394 camera connected to the Olympus BX51 microscope (20X objective). Background intensity was calculated and used as threshold signal; GFAP immunopositivity (cell body and processes) was then measured in each microscopic field with Image Pro Plus 7.0 software and automatically converted by the software into estimated surface area covered. The resulting average total area covered by immunostained cells was expressed as percentage of the total area of the microscopic field.

**Stereology:** BrdU-positive cells were counted bilaterally in the subgranular zone of the dentate gyrus in three sections containing the anterior, middle and posterior portions of the hippocampus, respectively. A semi-automatic stereology system (Stereoinvestigator for Neurolucida, MicroBrightfield) was used to estimate the number of immunopositive cells in each section (40X objective). Average counts were computed from these and statistically analysed using the Student *t* test on GraphPad Prism® 4 software for Windows.

## Results and overall outcome of the award:

The following are the major findings

1. Low dose vanadium caused a mild reactive astrocytosis characterised by increase of intensity of astrocyte immunoreactivity and slight, though significant, hypertrophy (**Fig. 1**), which was observed especially in the hippocampus and olfactory bulb. Astrocyte processes extended over non-overlapping territories (which indicates a mild degree of activation).
2. Low dose vanadium had no significant effect on cell proliferation (here studied for the first time after vanadium treatment), and on myelination, and did not result in microglial activation (**Figs. 2-4**). The latter two parameters were previously found to be affected in a paradigm of longer and high dose vanadium treatment (Azeez *et al.*, 2016).



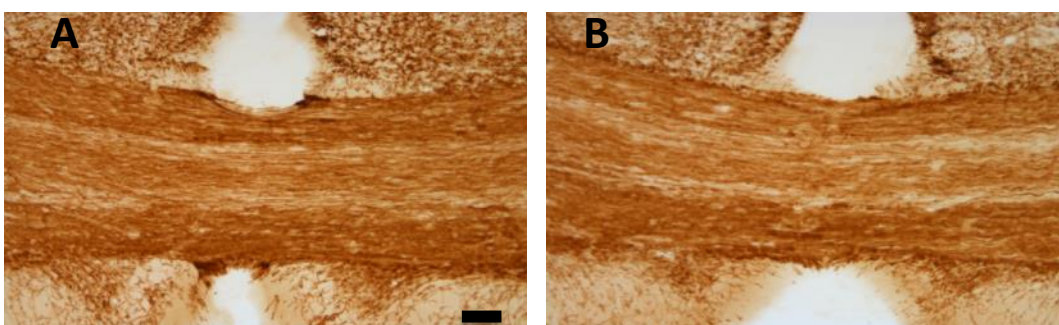
**Fig 1.** GFAP immunostaining in the CA1 region of the hippocampus in a control animal (A) and after low dose vanadium treatment (B). Note in B the slight hypertrophy and intense immunostaining of astrocytes. Scale bar= 100  $\mu$ m

Percentage of area covered by GFAP+ cell bodies and processes. Columns represent mean  $\pm$  SEM. \*  $P < 0.05$ , Student *t* test.

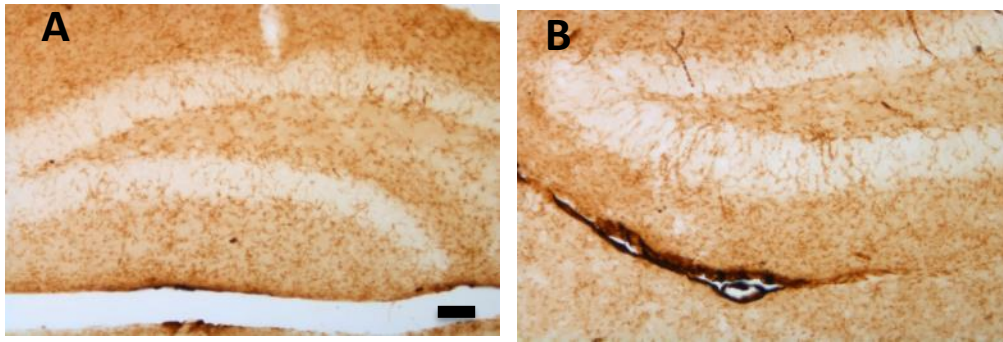


**Fig. 2.** BrDU-positive cells (arrows point to some examples) in the subgranular zone of the dentate gyrus. A: control; B: low dose vanadium. Scale bar= 50  $\mu$ m

Average number of BrDU-positive cells between control ( $36.90 \pm 4.189$  N=5) and low dose vanadium ( $44.53 \pm 6.384$  N=5) groups.  $P=0.2173$  (no significant difference)



**Fig. 3.** Myelin basic protein immunostaining of the midline corpus callosum. A; control, B; low dose vanadium. Scale bar= 50  $\mu$ m



**Fig. 4.** CD11b immunostaining of microglia in the dentate gyrus: A; control; B; low dose vanadium. Note that no microglia activation is evident. Scale bar= 50  $\mu$ m

The results therefore indicated that low dose vanadium exposure during the first postnatal month affects the brain, eliciting signalling to which astrocytes are sensitive. However, this exposure paradigm does not seem to affect postnatal cell proliferation and to exert myelin toxicity and activate microglial cells, as previously documented after high dose vanadium exposure (Azeez *et al.*, 2016). The effect of high vanadium exposure on postnatal cell proliferation remains instead to be assessed, since, as explained above, I could not use for the analyses the tissue of high vanadium dose group during my stay in Verona.

### **Benefits of the award**

One of the silver linings of the challenge I had with my samples; aside from teaching me the value of perseverance in research, is that during the process of salvaging the situation, I gained proficiency in the use of the cryostat, vibratome, as well as the freezing sliding microtome. During my stay in Verona, I had hands-on experience in routine histological staining (cresyl violet), and immunohistochemistry on free-floating sections. I also gained valuable experience in quantitative image analysis at the microscope: stereological cell counting using Stereoinvestigator (NeuroLucida) and image analysis using Image Pro-Plus 7.0.

On my departure from Verona, Prof. Bentivoglio made a donation of 100 mg BrDU to help in a repeat of the experiment for the study of proliferating cells after high dose vanadium exposure, with a pledge of support to host me if I return to analyse the samples in Verona.

I am currently writing my MSc dissertation with the work done in Verona contributing a considerable proportion of the data. I am grateful for the tutelage of Drs Azeez and Andrioli as well as Professors Marina Bentivoglio and James Olopade.

Finally, I would like to say *grazie mille* (thanks a lot) to ISN-CAEN for the invaluable opportunity of carrying out my MSc bench work at the University of Verona and also experiencing the culture of Verona.

### **Reference cited:**

Azeez IA, Olopade F, Laperchia C, Andrioli A, Scambi I, Onwuka SK, Bentivoglio M, Olopade JO (2016). Regional myelin and axon damage and neuroinflammation in the mouse brain after long-term postnatal vanadium exposure. *J. Neuropathol. Exp. Neurol.* 75, 843-854.



My picture while sectioning with the freezing microtome in the lab at the University of Verona.