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Full length Research Article

Cerebellar and Olfactory Bulb Perturbations Induced by Vanadium Neurotoxicity in the African Giant Rat (*Cricetomys gambianus*, Waterhouse)

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Summary: The African giant rat, AGR (*Cricetomys gambianus*) is a unique rodent known for its keen sense of smell which has enabled its use in the diagnosis of tuberculosis and demining activities in war torn countries. This keen sense of smell and the ability to navigate tight spaces are skills modulated by the olfactory bulb and cerebellum. While the brain is generally susceptible to environmental pollutants such as heavy metals, vanadium has predilection for these two brain regions. This work was thus designed to investigate the probable neurotoxic effect of vanadium on the neuronal cytoarchitecture of the cerebellum and olfactory bulb in this rodent. To achieve this, twelve adults male AGRs were divided into two groups (vanadium and control groups) and were given intraperitoneal injections of 3mg/kg body weight sodium metavanadate and normal saline respectively for 14 days. After which they were sacrificed, and brains harvested for histological investigations using Nissl and Golgi staining techniques. Results from our experiment revealed Purkinje cell degeneration and pyknosis as revealed by a lower intact-pyknotic cell (I-P) ratio, higher pyknotic Purkinje cell density and poor dendritic arborizations in the molecular layer of the cerebellum in the vanadium treated group. In the olfactory bulb, neuronal loss in the glomerular layer was observed as shrunken glomeruli. These neuronal changes have been linked to deficits in motor function and disruption of odor transduction in the olfactory bulb. This work has further demonstrated the neurotoxic effects of vanadium on the cerebellum and olfactory bulb of the AGR and the likely threat it may pose to the translational potentials of this rodent. We therefore propose the use of this rodent as a suitable model for better understanding vanadium induced olfactory and cerebellar dysfunctions.

Keywords*: cerebellum, olfaction, olfactory dysfunction, glomeruli, vanadium, neurotoxicity*

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INTRODUCTION

The African giant rat (*Cricetomys gambianus*), also known as the giant pouched rat, HeroRAT, and Sniff Rat, is a common mammal belonging to the order Rodentia (Cooper 2008; Adekanmbi and Olude, 2021). They are indigenous to sub–Saharan Africa, distributed widely from regions of the West African savannah zones through the Guineo-Congolian forest to the savannahs of east and southern Africa (Musser and Carleton, 2005; Cooper 2008; Olayemi *et al*., 2022). This rodent is unique due to its sheer body size and some noteworthy attributes which include the large cheek pouches, high olfactory acumen and dexterous use of its limbs (Ibe *et al*., 2014; Mustapha *et al*., 2015). These qualities have aroused interest into the scientific and translational applications of these rats. One of such applications is the deployment of their keen sense of smell in successfully sniffing out tuberculosis-infected samples and detecting landmines during demining activities in war ravaged countries particularly in Africa. (Poling *et al*., 2015; Adekanmbi and Olude, 2021; Makoye, 2022). Recent experiments and trainings have seen the potential of these unique rodents in search and rescue operations to save human victims of disasters since they can adequately navigate narrow spaces owing to the adroit use of their limbs (Euronews and Reuters, 2022). The AGR's acute sense of smell and limb dexterity are modulated by the olfactory bulb and cerebellum respectively (Tufo *et al*., 2022; Thau *et al*., 2022).

Generally, the brain has been shown to be susceptible to various perturbations including environmental toxicants. The olfactory system, being the most rostral, most vulnerable, and most exposed part of the brain, is especially liable to neural injury because its primary sensory neurons are in direct contact with the external environment (Czarnecki *et al*., 2012; Rey *et al*., 2018; Calvo-Ochoa and Byrd-James 2019). Quite notably, environmental pollutants such as heavy metal neurotoxicity have been reported in literature (Garcia *et al*., 2005; Azubike *et al*., 2012; Li *et al*., 2021: Mustapha *et al*., 2022; Usende *et al*., 2022).

Heavy metals are naturally occurring elements that are found throughout the earth's crust. Though some have biological functions in trace amounts (Tchounwou *et al*., 2011; Jyothi 2020), bioaccumulation due to prolonged exposure to heavy metals can result in disruption of many biological system including the CNS where it has been shown to be neurotoxic and increase risk of neurodegenerative diseases (Naseri *et al*., 2021; Li *et al*., 2021).

Large quantities of vanadium compounds are released into the environment mainly through the burning of fossil fuels and gas flaring activities as in seen the Niger Delta region of Nigeria, with vanadium reported as the most abundant heavy metal in petroleum samples (Igado *et al*., 2012; Mustapha *et al*., 2018; Olaolorun *et al*., 2021). Vanadium accumulates in soil, groundwater, and plants, and is consumed by animals and humans (Pyrzynska and Wierzbicki, 2004; Chen *et al*., 2021). Vanadium exposure to humans has been shown to cause motor deficits (Li *et al*., 2013; Ngwa *et al*., 2014). Putatively, vanadium exerts its neurotoxic effects by causing oxidative damages to neural tissues in several regions within the brain (Ścibior *et al*., 2019; Rojas-Lemus *et al*., 2020; Xiong *et al*., 2020). Although, Mustapha *et al*., (2023) reported the effect of vanadium on the hippocampal-neuronal circuitry of the AGR, the neurotoxic effect of this metal on the olfactory bulb and cerebellum is yet to be documented. This work is thus designed to investigate the possible neurotoxic effect of vanadium on the neuronal cytoarchitecture of the cerebellum and olfactory bulb.

MATERIALS AND METHODS

Ethical consideration: All experimental procedures followed the criteria established by the College of Veterinary Medicine Research Ethics Committee (CREC) of the Federal University of Agriculture Abeokuta. Ethical Approval Reference Number: FUNNAB/COLVET/ CREC/007/18.

Animals: Twelve adult male AGRs were purchased from local hunters who had captured these rodents from the wild in Southwest, Nigeria. Physical examination was conducted on all animals to ensure they are devoid of any physical deformities that may interfere with the study. They were transferred to the Animal House of the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Ibadan, and housed in locally made cages (60cm long x 32cm wide x 21cm high) with a metal cap to mimic the nocturnal environment of the rodents and to provide them with a dark sleeping compartment. Feeding troughs containing nuts and rat pellets (Ladoke® type) and water troughs were fixed to the wall of this chamber. Screen wires were fixed at the other end of the cage to allow for light and proper ventilation.

Experimental procedure: Rats were stabilized for 48 hours before commencement of the experiment. Animals were randomly assigned into two groups of six animals each: vanadium (treatment) and control (sterile water). AGR in vanadium group were dosed with sodium metavanadate daily (Sigma-Aldrich, St. Louis, MO, USA; pH7.7; 3mg/kg body weight; intra peritoneally, IP) according to Garcia *et al*., (2004) and Olude *et al*., (2018) while control group were given sterile water, IP. All animals were dosed daily for a period of 14 days after which they were sacrificed. The rats were anaesthetized by intraperitoneal injections of 100mg/kg Ketamine (Ketanir®, Gujarat, India) and 10mg/kg Xylazine (Xylazine 20 Inj® KEPRO, Holland) after which they were perfused with 4% paraformaldehyde transcardia. Their brains were harvested with the aid of a pair of bone nippers as described by Olude *et al*., (2018). The olfactory bulbs and cerebellum were carefully dissected from the rest of the brain and later post fixed in 4% paraformaldehyde for 48 hours and subsequently processed histologically for Nissl (Cresyl Violet) and Golgi stains. Nissl stain was used for illustrating the neuronal somata and cytoarchitecture of the olfactory bulb and cerebellar cortex while the Rapid Golgi Technique was used to elucidate the Purkinje neurons with their axonal and dendritic projections in the cerebellum.

The Nissl Technique: The brain samples were passed through the serial process of washing, dehydration, clearing, and embedding in paraffin. The embedded samples were then cut into 5µm thick mid- and para-sagittal sections. Sections were stained following a standard Nissl protocol as described by Olude *et al*., (2014). Briefly, sections were deparaffinized in xylene for 5 min, followed by a hydration series in graded alcohols for 3 min each. After 3 min in distilled water, sections were stained in 0.1% cresyl violet solution for 10 min at 57°C. Sections were then differentiated in 95% alcohol for 20 min. After rinsing in distilled water, sections followed an ascending series of dehydration in graded alcohols for 3 min each, and finally 5 min in xylene. The sections were then covered with mounting medium and glass coverslip.

The Rapid Golgi Technique: Brain slices of not more than 4mm thickness were cut in horizontal and frontal planes and immersed in osmium-dichromate solution at room temperature for 7 days. The pieces were transferred into 0.75% aqueous silver nitrate and kept for 24 hours in the dark. Then the pieces were placed back into the same osmium-dichromate solution used in the first step in which they were left in for 6 days. This step was followed by the immersion of the pieces into silver nitrate solution for 48 hours in the dark. Then they were transferred into a new osmium-dichromate solution for 5 days. Again, the step with silver nitrate was repeated with increased time solution for 3 days. The pieces were then dehydrated in absolute alcohol for 5 minutes and embedded in soft paraffin matrix by pressing each piece gently into the melted paraffin. 200µm sections were made on a sliding microtome. The sections were transferred into absolute alcohol for 15 minutes, after which the sections were transferred into oil cloves for 15 minutes (Faulogy *et al*., 2008).

Photomicrography and Histology: All stained sections were examined and imaged under the light microscope with a built-in 1.3 megapixel digital camera (OMAX® MD82ES10) and advanced image software (OMAX ToupView Version x64, 4.11.19627). The observed histological features and cytoarchitecture in the cerebellum and olfactory bulbs were appropriately described.

Quantitative Densitometric Analysis of Purkinje Cells: Purkinje cell density (PCD) was estimated in the Purkinje cell layer of AGR cerebellum using a computer-based image analysis software (FIJI: ImageJ 1.53t; Java 1.8.0_172; 64bit NIH, USA). For each AGR, the number of Purkinje cells (intact and pyknotic) were counted from three different cerebellar folia of each section. Pyknotic neurons (with signs of injury) were identified and delineated from intact (healthy) neurons based on criteria described below (Kamal and Kamal, 2013; Ribeiro *et al*., 2017):

- **Cellular oedema:** represented by cells with visibly intact nuclei that showed an increase in cytoplasm/nucleus size ratio compared to adjacent cells
- **Autolysis:** anuclear cells or cells exhibiting abnormal nuclear morphology; and
- **Darkened and small cells:** shrunken cells with increased nuclear compactness

Number of intact and pyknotic neurons were counted over a surface length of 500µm using Nissl-stained sections at ×100 magnification according to methods described by Ribeiro *et al*., (2017). PCD was calculated as the sum of intact and pyknotic neurons in the Purkinje cell layer of the cerebellum over a surface length of 500µm while Intact-Pyknotic (I-P) ratio was also calculated for both control and vanadium groups.

Data Analysis: PCD were reported as mean ± standard error of mean. Inferential statistics was carried out with unpaired student t test. Differences in the means of intact and pyknotic neurons were analyzed using GraphPad Prism Software (version 7.00; GraphPad Software Inc., San Diego, CA, USA). A value of $p < 0.05$ was set to be statistically significant.

RESULTS

Effect of Vanadium Neurotoxicity on the histomorphology of the AGR Cerebellar Cortex: In Nissl stained sections, the cytoarchitecture of the cerebellar cortex was preserved in the control group, showing a distinct monolayer of intact Purkinje cells characterized by large pyriform shaped somata with prominent nucleoli and cytoplasm. These cells were located between an outer relatively hypocellular molecular layer and an inner densely packed granular cell layer (Plate 1). In contrast, the vanadium group showed a loss of cellular integrity of the Purkinje cells (Figure 1). Furthermore, granular cell degeneration was seen in the cerebellar cortex of vanadium-dosed AGR while the cytoarchitecture of these cells were preserved in control groups

Similarly, Golgi-stained sections of the vanadium group demonstrated Purkinje cells with poor dendritic arborizations into the molecular layer of the cerebellar cortex, while the control group had distinct and highly ramified dendritic projections in the molecular layer (Plate 2).

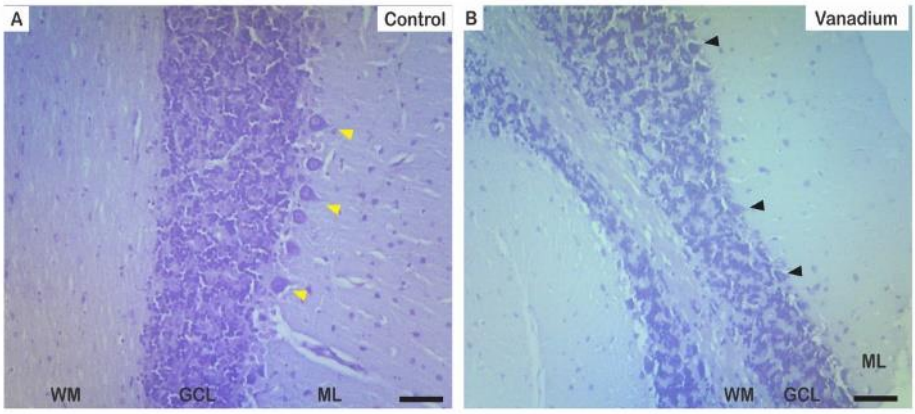

Plate 1:
Nissl-stained sections of AGR cerebellar cortex showing intact Purkinje cells (yellow arrows) in the control group [**A**], while a loss of Purkinje cells with numerous pyknotic cells (black arrows) in the vanadium dosed group. ML: molecular layer; GCL: granular cell layer; WM: white matter. Scale bar: 100µm.

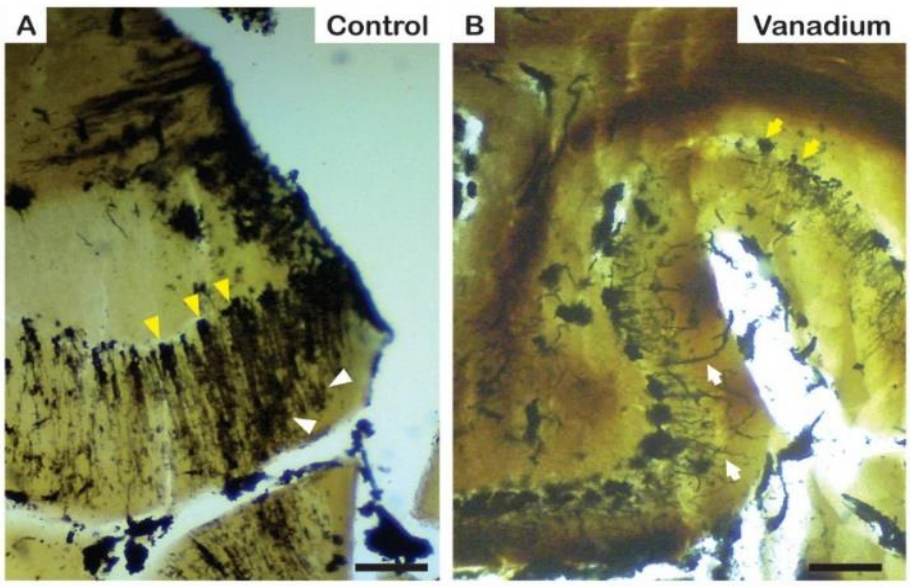

Plate 2:
Golgi-stained sections of the AGR cerebellar cortex. [A]: Control group - intact Purkinje somata (yellow arrowhead) with distinct dendritic projections (white arrowheads) into the molecular layer in the control group; [B]: Vanadium group - fewer Purkinje somata (yellow arrows) with reduced dendritic arborizations (white arrows). Scale bar: 50µm

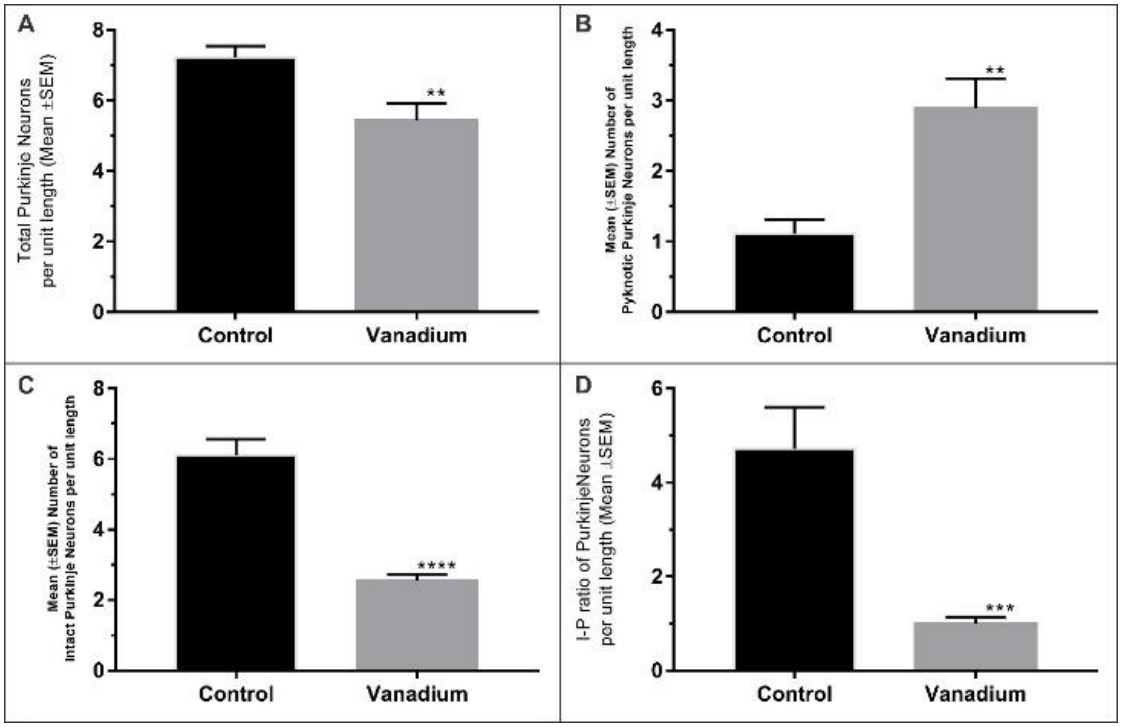

Figure 1:
Quantitative cell counts of: (A) total Purkinje neurons, (B): mean pyknotic Purkinje neurons, (C): mean intact Purkinje neurons, (D): Intact - Pyknotic Purkinje neuron ratio in the AGR cerebellar cortex. Cell count was done over a linear distance of approximately 500µm. Statistical significance was set @ $p < 0.05$.

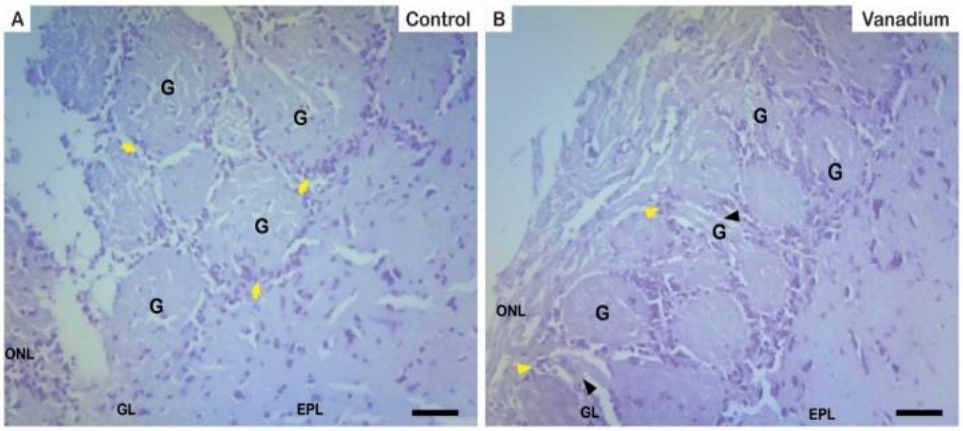

Plate 3:
Nissl-stained sections of the AGR olfactory bulb. Control group **[A]:** normal glomeruli (G) surrounded by periglomerular cells (yellow arrows) in the control group. Vanadium group **[B]** shrunken glomeruli (black arrowheads) surrounded by periglomerular cells (yellow arrowheads). ONL: olfactory nerve layer; GL: glomerular layer; EPL: external plexiform layer. Scale bar: 100µm

Effect of Vanadium Neurotoxicity on the Purkinje cell density in the AGR Cerebellar Cortex: Morphometric analysis revealed a statistically significant decrease in total PCD in the vanadium-dosed group compared to control ($P = 0.0070$; Figure 1a). A higher pyknotic PCD, lower intact PCD and lower I-P ratio was noted for AGR dosed with vanadium compared to the controls which had a lower pyknotic PCD, higher intact PCD and higher I-P ratio (Figure 1b - d). The observed differences in mean were statistically significant with P values given as $P=0.0016$, $P<0.0001$ and $P=0.0007$ respectively.

Effect of Vanadium Neurotoxicity on the histology of the AGR Olfactory Bulb: Disruption in the cellular integrity evidenced by the shrinkage of few glomeruli in the glomerular cell layer was noted for AGR dosed with vanadium. Compared to vanadium, the control group had intact, glomeruli surrounded by periglomerular cells (Plate 3).

DISCUSSION

This study documents vanadium induced neural perturbations in the cerebellum and olfactory bulb of the AGR – a rodent known for its profound olfactory acuity, cognition, and limb dexterity. We highlighted the neurotoxic effect of vanadium on the glomeruli (an important waystation in the pathway for signal transduction of odours) in the olfactory bulb, and the Purkinje neurons of the cerebellar cortex.

Vanadium has been shown to cross the blood-brain barrier and accumulate in the brain where it exerts its deleterious effect by generating reactive oxygen species and

causing lipid peroxidation (Mustapha *et al*., 2014; Azeez *et al*., 2016; Fatola *et al*., 2019). Current findings have established that vanadium has preferential predilection for the olfactory bulb, brain stem and cerebellum (Haider *et al*., 1998, Garcia *et al*., 2005; Ngwa *et al*., 2014; Colín-Barenque *et al*., 2015; Folarin *et al*., 2017; Rojas-Lemus *et al*., 2020).

In the olfactory bulb, for instance, vanadium has been shown to induce olfactory dysfunction typified by decreased olfactory bulb volume and, severe dopaminergic neuronal loss in the glomerular layer of the olfactory bulb (Ngwa *et al*., 2014). From this study, we observed focal lesions of glomeruli shrinkage at the glomerular layer of the OB of AGR dosed with vanadium. This observation may reflect a destruction and/or disruption of the dopaminergic neurotransmission system of the OB, and consequential reduction of the synaptic surface area in this layer. Indeed, dopamine plays a significant role in olfaction (Duchamp-Viret *et al*., 1997, Hsia *et al*., 1999, Koster *et al*., 1999; Ngwa *et al*., 2014) and have been shown to be expressed in high abundance in the glomerular layer of the OB (Halasz *et al*., 1981, Davila *et al*., 2003), with changes in dopamine levels shown to affect olfaction. Dopamine regulates transmission between the olfactory bulb epithelium and the olfactory bulb glomeruli to mediate entry of sensory olfactory information into the brain (Hsia *et al*., 1999). Ngwa *et al*., (2014) posited that the depletion of dopamine in the olfactory bulb may impair odor identification and discrimination by disinhibition of neural transmission in olfactory glomeruli consequently leading to impaired olfactory processing.

Purkinje neurons are the primary processing units and sole output neurons of the cerebellar circuitry (Kemp *et al*., 2016; White *et al*., 2021). They send inhibitory GABA-ergic inputs to the deep cerebellar nuclei in the cerebellum and play pivotal roles in motor coordination, control, and learning (Purves *et al*., 2001; Kano and Watanabe, 2020). These neurons have been shown to be susceptible to both genetic and environmental perturbations that may disrupt their regular functions (Garcia *et al*., 2005; Hegarty *et al*., 2020). Pathologies associated with Purkinje cell loss have been associated with motor deficits and incoordination. Thus, optimal Purkinje cell function is essential to the overall function of the cerebellum (Xia *et al*., 2013; Louis *et al*., 2014; Redondo *et al*., 2015; Folarin *et al*., 2017). From this study, Purkinje cell degeneration and pyknosis substantiated by a higher pyknotic PCD, lower total PCD, and lower I-P ratio in the Purkinje cell layer of the AGR cerebellum dosed with vanadium may suggest a likely deficit in motor coordination and learning – an essential neurobehavioural forte of this rodent. This Purkinje neuronal degeneration was further corroborated with Golgi stain which revealed significant loss of dendritic arborizations of the Purkinje neurons in the molecular layer. Reduction in dendritic arborizations is a structural change that is usually associated with neuronal dysfunction, leading to dysfunctional neural circuits and it is thought to precede neuronal death (Ferrer *et al*., 1984; Beckers and Moons, 2019). Previous works have reported a reduction of Purkinje cell dendritic branching in patients with essential tremor (Louis *et al*., 2014); hereditary ataxias (Shintaku and Kaneda, 2009), chronic alcoholics (Ferrer *et al*., 1984) and in Alzheimer's disease (Mavroudis *et al*., 2013).

Interestingly, olfactory dysfunction induced by the dopamine depletion has been shown to be associated with cerebellar motor deficits, as seen in Parkinson's disease patients, via the nigrostriatal dopaminergic system. More intriguing is that, recently the striatum has been shown to have a di-synaptic communication with the cerebellum via the cerebello-thalamo-cortical circuit (Bostan *et al*., 2010; Bostan and Strick, 2018) while the dentate nucleus of the cerebellum also has a di-synaptic projection to an input stage of the striatum (Bostan *et al*., 2010). Thus, providing an anatomical substrate for substantial two-way integrated functional network that play a pivotal role in a variety of motor and non-motor functions (Milardi *et al*., 2019).

Neuronal death is an important process in many neurodegenerative diseases that is associated with oxidative stress. One of the probable mechanisms by which vanadium might have caused the observed neuropathologies in this study is by ROS-mediated oxidative damages to cellular macromolecules such as lipids, proteins and DNA, leading to a cascade of cellular injuries and metabolic dysfunctions, and ultimately apoptosis and necrosis (Bandyopadhyay *et al*., 1999; Mustapha *et al*., 2014 & 2019; Rojas- Lemus *et al*., 2020; Xiong *et al*., 2021; Folarin *et al*., 2018; Usende *et al*., 2018 & 2022). Vanadium has been shown to increase the upregulation of apoptotic proteins such as p53, induce mitochondrial membrane permeability, mitochondrial membrane potential disruption, cytochrome c release, and activation of proteases such as caspase 9 and 3 (Chakraborty *et al*. 2005; Roos and Kaina, 2006; Caicedo *et al*., 2008; Zhao *et al*., 2010; Colín-Barenque *et al*., 2015; Xiong *et al*., 2021).

Although stereological analysis allows for an unbiased approach to tissue sampling (Choe *et al*., 2016), there remain real concerns on the feasibility for stereological studies of the entire brain region selected (case in point: the cerebellum), as the entire cerebellum or even a hemi-cerebellar study is rare if ever available, as is required for such analyses. Our results should thus be interpreted within the context of this limitation. Also, a detailed neurochemical profiling of these brain regions would provide more credence to these pathologies and will be considered in future studies.

In conclusion, this work has put together some evidences of the vanadium-induced neuropathology in the cerebellum and olfactory bulb of the AGR, characterized by neuronal degeneration, loss of dendritic arborizations, pyknosis, and neuronal cell loss. It has also demonstrated the potential threat environmental pollution may contribute to translational benefits of the rodent. We propose this rodent as a suitable model for a better understanding of olfactory dysfunctions and cerebellar disorders.

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