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Immunohistochemical Assessment of Thalamic Region in Rat Brain Following Bonny Light Crude Oil Administration

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Abstract

Environmental and occupational exposure to heavy metal-containing products, such as crude oil, poses serious global health concerns. The bioaccumulation of crude oil constituents in humans has been reported to be toxic to the brain, however, much is still unknown about the neurotoxic mechanism of crude oil. The thalamus is a key sensory neuro relay station to various cortical regions involved in numerous cognitive and sensorimotor functions. In the present study, we investigated thalamic glial and neuronal response to Bonny Light crude oil (BLCO) exposure in rats by immunohistochemical evaluations of selected neuro markers, including GFAP, Iba1, Nrf2, parvalbumin, and NeuN. Adult Wistar rats (n = 6) were orally administered either distilled water (control), 1 or 2 ml/kg BLCO for 21 days. Following administration, we performed immunohistochemistry protocols for the markers mentioned above. BLCO induced a marked decrease in Iba1 expression levels with a contrasting increase in GFAP levels reflective of the involvement of glial activity. In contrast, BLCO exposure did not alter Nrf2 levels suggestive of the lack of influence on oxidative stress regulation by the Nrf2 pathway. Furthermore, there was overexpression of thalamic parvalbumin, which could indicate an aberrant increase in inhibitory activity by parvalbumin-expressing GABAergic interneurons, thus possibly disrupting the brain's excitatory-inhibitory balance. Additionally, there was a significant reduction in the number of NeuN-positive cells but no change in immunoreactivity levels, signifying a reduction in thalamic neuronal density in BLCO-exposed rats. Overall, our results suggest that BLCO exposure could alter glial and neuronal functions in the thalamic region of the brain.

Keywords: Crude oil, thalamus, neurotoxicity, GFAP, Iba1, Nrf2, parvalbumin, NeuN.

Introduction

Crude oil and its derivatives are key sources of energy for humans; however, their constituents negatively impact the structure and function of the nervous system [1]. Bonny light crude oil (BLCO) is a special blend of crude oil majorly produced in southern Nigeria. The massive production of BLCO coupled with poor infrastructure results in widespread BLCO pollution [2]. Aside from accidental BLCO exposure, locals use BLCO for "medicinal purposes" such as topical application for burns, oral ingestion in the treatment of gastrointestinal diseases, and management of infertility [3]. However, given the individual constituents of BLCO, it is pragmatic to consider BLCO exposure as a great neurotoxic risk. BLCO contains several hydrocarbons and heavy metals such as vanadium, zinc, lead, iron, copper, and nickel, amongst others [4-6], all of which have been repeatedly reported to trigger neurotoxicity [7-10].

The thalamus is made up of various nuclei, each of which serves a specific function in relaying sensory and motor signals. All sensory inputs, except olfaction, are relayed through the thalamic neurocircuits, which communicate with diverse cortical regions [11, 12]. These intricate neural connections highlight the importance of the thalamus in cognitive processes such as learning and memory [13], decision-making [14], consciousness [15] and motor executions [16] amongst others. Consequently, impaired

thalamic anatomy and associated function by neurotoxicants can potentially alter the proper execution of any of the behaviours mentioned above. The thalamus is organized into distinct nuclei, each with specific neural circuitry and function [12]. These nuclei consist of cellular populations, including parvalbumin-expressing cells, which use gamma-aminobutyric acid (GABA) as a transmitter and provide major inhibition for the thalamocortical neurons. Excitatory-inhibitory imbalance as a result of dyshomeostasis of parvalbumin levels has been linked to neurotoxicity and neurological disorders [17]. Similarly, glia-mediated inflammation involving astrocytes and microglia is a known neurotoxic response to toxicants such as heavy metals and viral infections, amongst others [18]. Biological markers, GFAP and Iba1, are used to demonstrate glial cell activation in neurotoxicological studies [19]. On the other hand, the Nrf2 protein regulates the expression of different genes whose products are involved in antioxidant responses and the detoxification of toxic species produced during oxidative stress [20, 21]. NeuN is a marker of mature neurons and has been used to directly evaluate neuronal loss [22, 23]. All the aforementioned markers: GFAP, Iba1, Nrf2, parvalbumin and NeuN are utilized to evaluate the impact of certain neurotoxicants in the brain. Therefore, in this study, we performed immunohistochemistry demonstrations and quantification of GFAP, Iba1, Nrf2, parvalbumin and NeuN to assess the impacts of BLCO on the thalamus.

2.0 Methods

2.1 Animal models and treatments

Twenty-four adult male albino strain Wistar rats (150-200 g) were used for the study. All experimental protocols were based on the National Research Council Guide for the Care and Use of Laboratory Animals [24] and approved by the local Institutional Research Committee (FUTA/ETH/23/97). Animals were kept under a 12-hour light-dark cycle with free access to regular food and water. Rats (n = 6) were randomly assigned to receive oral administration of either distilled water (as controls), 1 ml/kg or 2 ml/kg of BLCO every 24 hours for 21 days. Doses were selected based on previous studes [6, 25].

BLCO was obtained from the Warri Refining and Petrochemical Company, Ekpan, Delta State, Nigeria. Gas chromatography–mass spectrometry (GC–MS) on Agilent 7890A/5975C revealed the presence of harmful substances, including several hydrocarbons, benzenedicarboxylic acids, naphthalenes, and carbonic acids.

At day 22, rats were euthanized via isoflurane inhalation; brains were rapidly excised and fixed in 10% neutral buffered formalin for subsequent immunohistochemical evaluations.

2.2 Immunohistochemistry

Fixed brains were embedded in paraffin wax, and 5 µm thin mid-coronal sections were cut on a microtome. To expose the thalamic regions, mid-coronal sections were obtained from Bregma -3.00 to 3.60 mm [26]. Sections were deparaffinised before being heated in a steamer for approximately 30 minutes in a citrate-based antigen unmasking solution, pH 6.0 (Vector®, Burlingame, CA, USA; #H3300) and cooled on the bench at room temperature for thirty minutes. The sections underwent endogenous peroxidase block for ten minutes in 0.3% hydrogen peroxide dissolved in phosphate-buffered saline (pH 7.4, Fisher BioreagentsTM #173844). Sections were then incubated in primary antibodies at room temperature for two hours and thirty minutes in primary antibodies diluted in UltraCruz® Blocking Reagent (Santa Cruz, USA; #SC-516214). The antibodies include GFAP (ThermoFisher, USA; #16825-1-AP) at 1:7500, IBA1 (Cell Signaling, USA; #17198) at 1:1250, Nrf2 (ThermoFisher, USA; #PA1-38312) at 1:100, Parvalbumin (Novus Biologicals, USA; NB120-11427) at 1:1000, NeuN (ThermoFisher, USA; #26975-1-AP) at 1:1500. After washing in phosphate buffer saline twice for five minutes, sections were incubated in ImmPRESS™ (Peroxidase) Polymer Anti-Rabbit IgG Reagent, made in horse (Vector® #MP-7401). DAB Peroxidase (HRP) Substrate Kit (Vector® #SK-4100) was used to develop the brown colour, and sections were counter-stained in haematoxylin [27, 28].

2.3 Digital Image Analysis

Immunostained slides were digitized with the Pannoramic 250 Flash II slide scanner (3D Histech, Budapest, Hungary). The accompanying digital microscopy application, CaseViewer software, was used to capture random non-overlapping 7-12 photomicrographic fields of the thalamic areas at x40 magnification. Non-overlapping images were selected by avoiding the repetition of the same thalamic areas in multiple photomicrographs. Digital images were imported into the NIH-sponsored ImageJ software for analysis using the ImmunoRatio plugin and the cell counter tool. The ImmunoRatio plugin gives a ratio of brown DAB (positive immunoreactivity) and the haematoxylin counterstain. The cell counter tool keeps track of the number of manually selected cell types [27, 29]. Average scores of photomicrographs analysed were used for data analysis.

2.4 Statistics

Data were analysed using One-way ANOVA followed by Tukey's multiple comparison tests with GraphPad Prism Version 8 (GraphPad Inc, San Diego, US) statistical software. Statistical significance was set to $P < 0.05$.

3.0 Results

3.1 BLCO increased the number of GFAP-positive cells and GFAP immunoreactivity levels in the thalamus.

Immunohistochemical analysis with one-way ANOVA demonstrated significant changes in the number of GFAP-positive cells [F (2, 9) = 6.800, p = 0.0159] and the immunoreactivity of GFAP protein [F (2, 9) = 9.891, p = 0.0053] in the thalamus following BLCO exposure in rats. Post-hoc analysis with the Tukey's test showed that there was a significant increase ($p < 0.05$) in the number of GFAP-expressing cells in the thalamus of rats exposed to 1 ml/kg of BLCO but not 2 ml/kg of BLCO (Figure 1b). Similarly, GFAP immunoreactivity was significantly increased ($p < 0.01$) in the 1 ml/kg group compared to the control and the 2 ml/kg group. Also, no significant difference is observed between the control and 2 ml/kg group (Figure 1c).

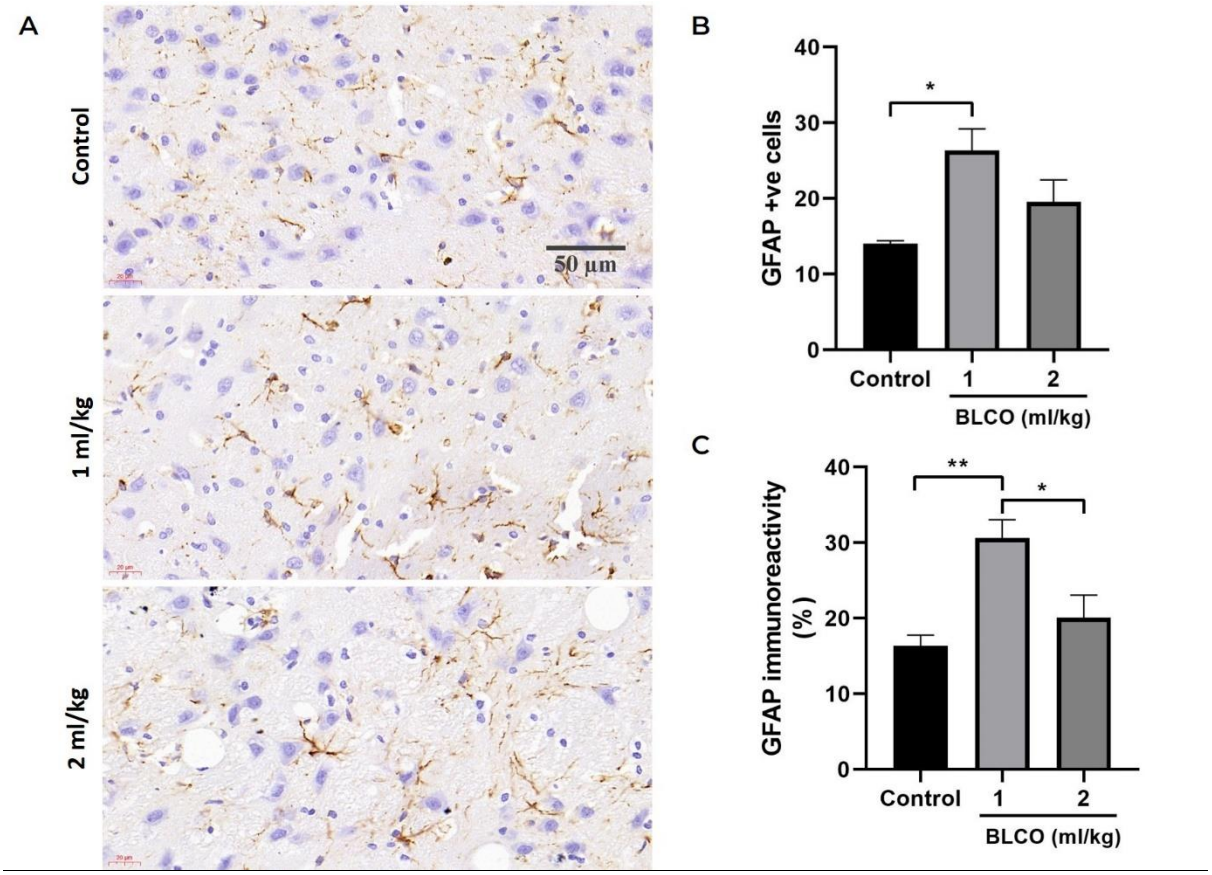

Figure 1: Immunohistochemical demonstration of GFAP in the thalamus of rats exposed to either 1 or 2 ml/kg of BLCO (A). Bar graphs depict the number of cells positive for GFAP (B) and their levels of immunoreactivity (C) in experimental rats. Each column represents mean ± S.E.M. Data were analysed using one-way ANOVA followed by Tukey's post-test. *p < .05, **p < .01.

3.2 Reduction in the number of Iba1-positive cells in the thalamus following BLCO exposure.

Immunohistochemical analysis with one-way ANOVA showed significant changes in the number of Iba1-positive cells in the thalamus [F (2, 9) = 4.272, p = 0.0496] of rats exposed to BLCO. Conversely, the immunoreactivity of thalamic Iba1 protein showed no significant change [F (2, 9) = 2.198, p = 0.1670] following BLCO exposure in rats. Post-hoc analysis with the Tukey's test showed a significant reduction ($p < 0.05$) of Iba1 positive cells only in the thalamus of rats exposed to 2 ml/kg of BLCO compared to control (0 ml/kg) (Figure 2b). The immunoreactivity level of Iba1 protein showed no significant difference in the thalamus of rats exposed to either 1 or 2 ml/kg of BLCO compared to the control (Figure 2c).

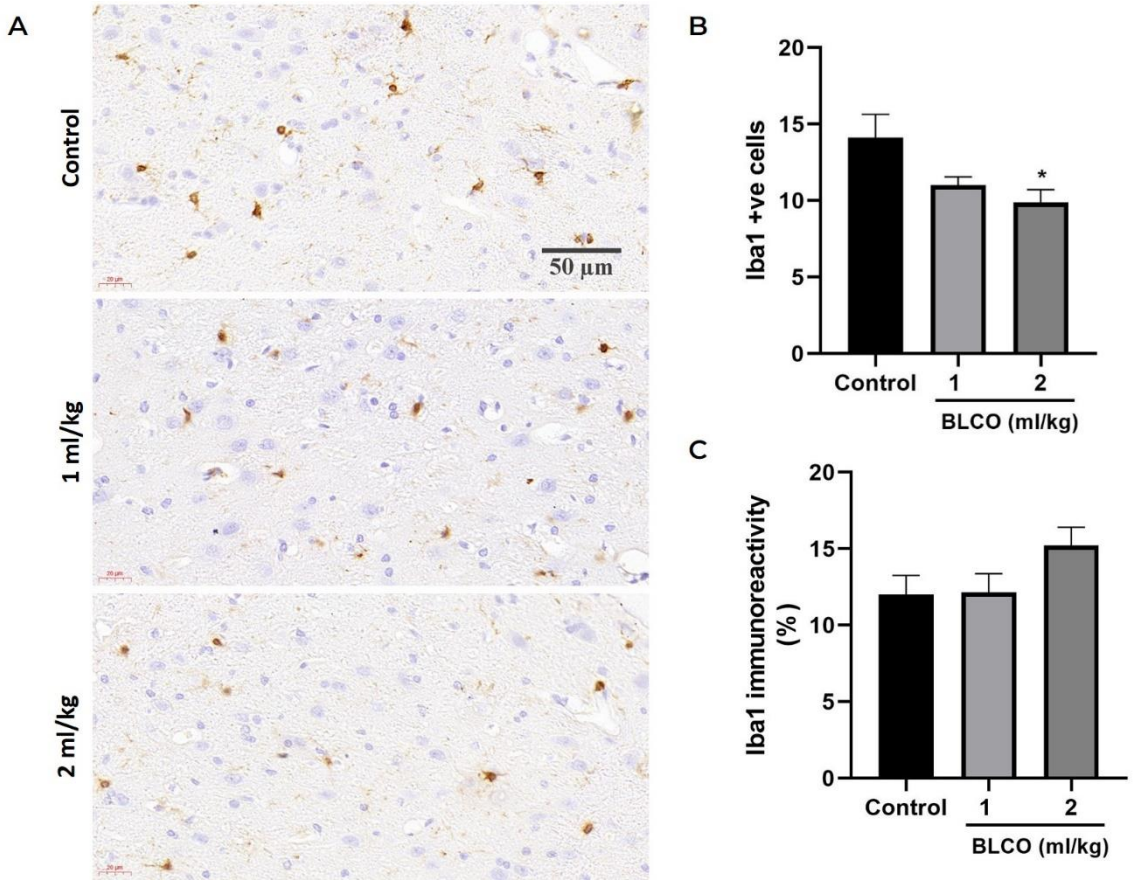

Figure 2: Immunohistochemical demonstration of Iba1 in the thalamus of rats exposed to either 1 or 2 ml/kg of BLCO (A). Bar graphs depict the number of cells positive for Iba1 (B) and their levels of immunoreactivity (C) in experimental rats. Each column represents mean ± S.E.M. Data were analysed using one-way ANOVA followed by Tukey's post-test. *p < .05.

3.3 Elevated parvalbumin levels and increased number of parvalbumin-positive cells following BLCO exposure.

Immunohistochemical analysis with one-way ANOVA demonstrated significant changes in the number of parvalbumin-positive cells [F (2, 9) = 15.00, p = 0.0014] and the immunoreactivity of parvalbumin protein [F (2, 9) = 13.93, p = 0.0018] in the thalamus following BLCO exposure in rats. Post-hoc analysis with Tukey's test indicated that there were more parvalbumin-positive cells in the thalamus of rats exposed to either 1 ml/kg ($p < 0.05$) or 2 ml/kg ($p < 0.01$) of BLCO than in control rats (Figure 3a). Likewise, parvalbumin immunoreactivity was increased in both 1 ml/kg (insignificant; $p > 0.05$) and 2 ml/kg (significant; $p < 0.01$) of BLCO compared to the control. In addition, parvalbumin immunoexpression was increased ($p < 0.05$) in the 2 ml/kg group than in the 1 ml/kg group (Figure 3b).

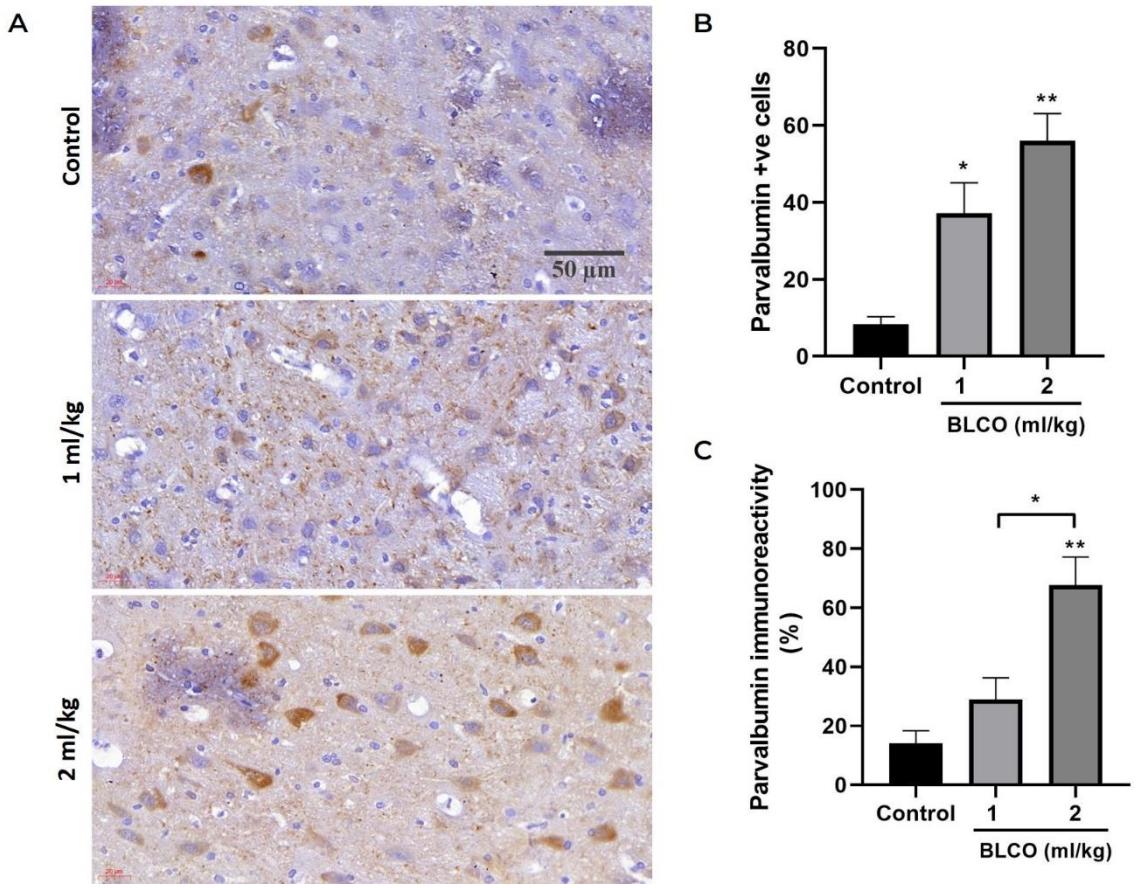

Figure 3: Immunohistochemical demonstration of parvalbumin in the thalamus of rats exposed to either 1, or 2 ml/kg of BLCO (A). Bar graphs depict the number of cells positive for parvalbumin (B) and their levels of immunoreactivity (C) in experimental rats. Each column represents mean ± S.E.M. Data were analysed using one-way ANOVA followed by Tukey's post-test. *p < .05, **p < .01.

3.4 Loss of NeuN-positive cells following BLCO exposure.

Immunohistochemical analysis with one-way ANOVA showed significant changes in the number of NeuN-positive cells in the thalamus [F (2, 9) = 11.65, p = 0.0032] of rats exposed to BLCO. Conversely, the immunoreactivity level of NeuN protein showed no significant change [F (2, 9) = 2.273, p = 0.1589]. Post-hoc analysis with Tukey's test showed that at doses of either 1 ml/kg ($p < 0.05$) or 2 ml/kg ($p < 0.01$), BLCO significantly reduced the number of NeuN-positive cells (Figure 4a). Conversely, there was no significant difference in NeuN immunoreactivity across the three groups: 0 ml/kg, 1 ml/kg, and 2 ml/kg (Figure 4b).

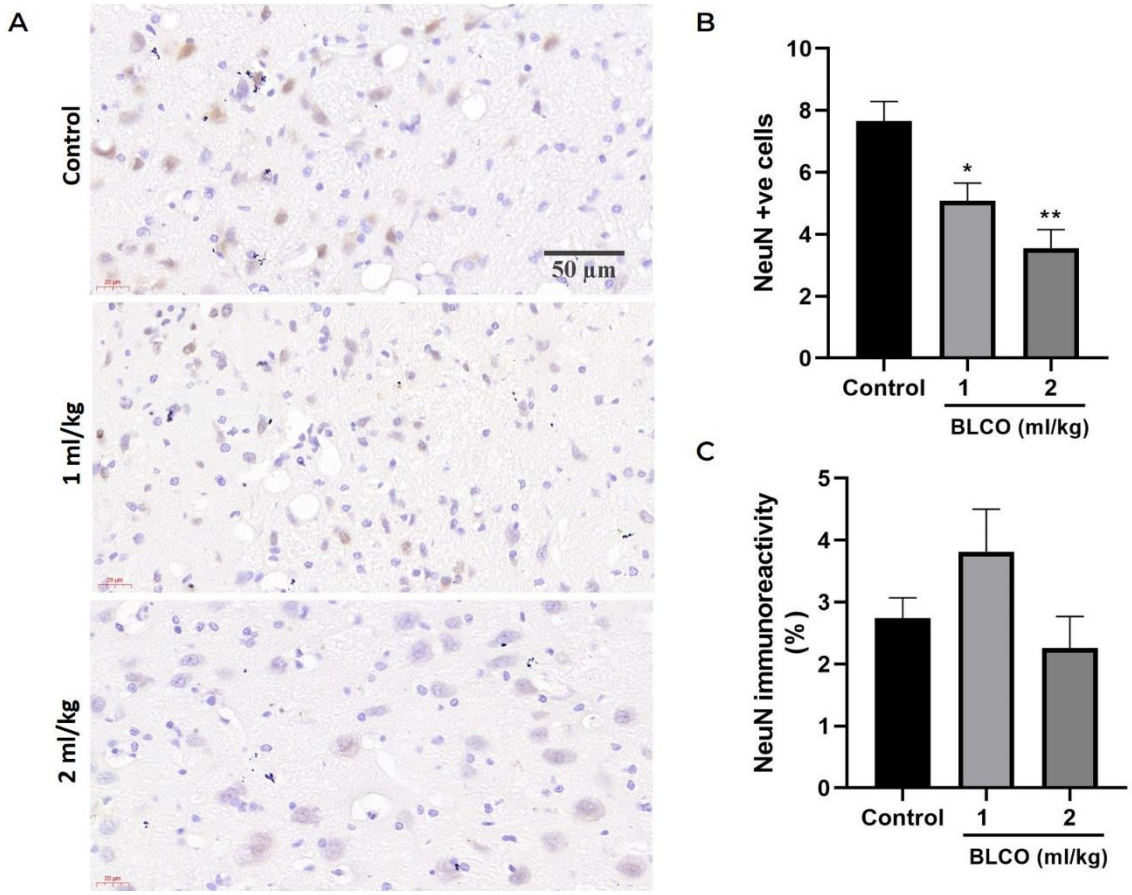

Figure 4: Immunohistochemical demonstration of NeuN in the thalamus of rats exposed to either 1 or 2 ml/kg of BLCO (A). Bar graphs depict the number of cells positive for NeuN (B) and their levels of immunoreactivity (C) in experimental rats. Each column represents mean ± S.E.M. Data were analysed using one-way ANOVA followed by Tukey's post-test. *p < .05, **p < .01.

3.5 Lack of Nrf2 activation in the thalamus following BLCO exposure.

One-way ANOVA showed that exposure of rats to BLCO showed no significant change in the number of Nrf2 positive cells [F (2, 9) = 1.269, p = 0.3269] as well as the immunoreactivity levels of Nrf2 protein [F (2, 9) = 0.4111, p = 0.1589] when compared with control (Figure 5b & 5c).

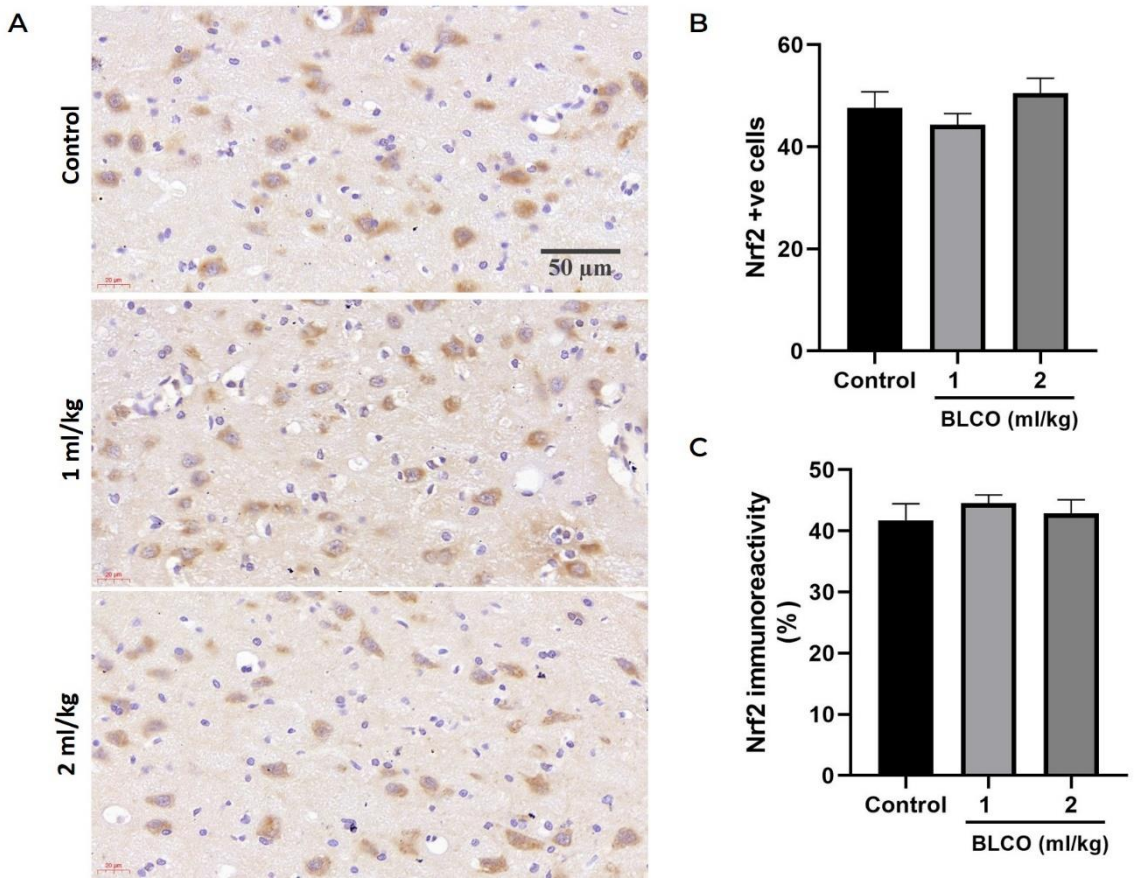

Figure 5: Immunohistochemical demonstration of Nrf2 in the thalamus of rats exposed to either 1, or 2 ml/kg of BLCO (A). Bar graphs depict the number of cells positive for Nrf2 (B) and their levels of immunoreactivity (C) in experimental rats. Each column represents mean ± S.E.M. Data were analysed using one-way ANOVA followed by Tukey's post-test.

Discussion

Organ toxicity following crude oil exposure is well-established [3, 4], particularly in the nervous system [1, 30, 31]. Crude oil contributes massively to Nigeria's revenue. As such, at least for the foreseeable future, there is a certainty for continuous economic exploitation of crude oil as well as its increased environmental exposure [2]. Deliberate and aprioristic usage of BLCO in medicinal practices by locals also contributes to the increased occurrence of BCLO exposure [3]. The thalamus is a critical relay station for all sensory information except olfaction and is for important processes such as learning and memory [13], decision-making [14], and motor execution [16]. Thus, in this study, we evaluated the neurotoxic impact of BLCO, a special blend of crude oil majorly produced in southern Nigeria, in the thalamus.

Inflammation is a key cellular response to biological and chemical toxins. Glia-mediated inflammation is specific to the brain and is the local immune response that deals with a threat to the neuronal microenvironment [32]. In this study, there is a decreased number of recruited microglia after BLCO exposure, which was particularly significant at 2 ml/kg. There is no significant difference in the number and activation levels of microglial cells in 1 ml/kg- compared to 2 ml/kg-exposed thalamus. Typically, the local density of microglia rises in response to toxic invasions [19], which is contrary to the observations in this study. It is plausible that BLCO could have caused microglia toxicity and triggered their death, possibly via apoptosis [33]. Also, this study reported increased astrocytic activity, particularly following 1 ml/kg BLCO exposure. The activation levels, but not the number of astrocytes were significantly reduced in 2 ml/kg- compared to 1 ml/kg-exposed thalamus. This finding of astrocytic activation following BLCO is consistent with other cases of neurotoxicity [34-36]. Another interesting observation of the glia activation pattern in this study is the increased astrocytic activity in the thalamus at low BLCO exposure in comparison to reduced microglia cell number at the same dose. It is not particularly clear why these contrasting responses; however, Ni, Li [37] provided evidence that

microglial cells are more vulnerable to MeHg toxicity than astrocytes, which could account for the observed microglia but not astrocyte loss. Notwithstanding, all these observations are evidence of glia activity following BLCO neurotoxicity in the thalamus and suggest further understanding of differential glia dynamics in cases of crude oil neurotoxicity.

Oxidative response to BLCO neurotoxicity in the thalamus was explored via the quantification of the Nrf2 protein. It is well established that ROS produced during oxidative stress disrupts the sequestration of Nrf2 by Keap1, leading to the nuclear translocation of Nrf2. In the nucleus, Nrf2 binds to the antioxidant response element in the promoters of its target antioxidant genes to increase their production [38]. In this study, there is no significant change in levels of the Nrf2 protein as well as the number of Nrf2-positive cells. It is characteristic for Nrf2 levels to increase to protect cells from oxidant injuries [39-41]. Therefore, it is surprising that we found no significant change in Nrf2-expressing cells and levels. We speculate that BLCO elicitation of oxidative stress is unable to impart activation of the Nrf2 antioxidant system. Evidence from several works supports oxidative stress as a cellular response mechanism to BLCO [3, 31, 42, 43]. Thus, we suggest that the lack of change to Nrf2 in our study might involve the inhibition of keap-1/Nrf2 dissociation, which is key to the nuclear translocation of Nrf2 to activate antioxidant genes [38] Since we failed to find any study that evaluates BLCO neurotoxicity with keap-1 activity, this hypothesis is untested and warrants further investigation.

Parvalbumin (PV) interneurons are GABAergic (PVGs) cells that play an essential role in maintaining a fine-tuned excitation-inhibition balance in the brain [44, 45]. Given the inhibitory role of PVGs, any aberration in their function results in the dysregulation of excitatory-inhibitory balance and subsequently neurologic disorders such as epilepsy and schizophrenia [46-49]. In this study, there was a dose-dependent significant increase in the number of PV-positive cells and their expression levels after BLCO exposure, which indicates increased inhibitory activity in the thalamus. It is unclear why this increase occurred as we anticipated the potential loss of PV cells and subsequent PV expression as seen in cases of neurotoxicity [50, 51]. Nonetheless, we suggest that the over-inhibitory activity in the thalamus may be a compensatory response to increased excitatory influences from other neocortical regions, with which the thalamus forms neural circuits [11]. These neocortical regions are packed with numerous glutamatergic neurons [52] implicated in brain excitotoxicity [53, 54].

Finally, this study explored neuronal loss in the thalamus via the quantification of the NeuN protein. NeuN is a marker for mature neurons and has been used to directly evaluate neuronal loss [22, 23]. Therefore, the reduction in the density of NeuN-expressing cells suggests an actual loss of neuronal density in the thalamus following BLCO exposure, as demonstrated in other neurotoxicity and neurological studies [55-57].

In summary, the study clearly showed evidence of significant glia activity, no evidence for the activation of the Nrf2 antioxidant system, a surprising over-inhibitory mechanism in the thalamus, and a significant reduction in thalamic neuronal density in BLCO-exposed rats. Importantly, the findings reported here pose a few questions about (i) the nature of microglia loss caused by BLCO; (ii) the interaction of BLCO with the Nrf2/keap1 antioxidant system; and (iii) the unexpected over-inhibitory activity of GABAergic interneurons in the thalamus following BLCO exposure. It is worth stating that the present study was limited to the use of immunohistochemical techniques to evaluate expression of the selected neuromarkers. Other methods of evaluating the expressions of these markers including western blotting, chromatin immunoprecipitation, may shed provide further information. Hence, further studies are needed to proffer insights into these outlined queries.

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Conflict of interest

The authors declare no conflict of interest.

Author contribution

Conceptualization – OMI; Study design – OKI and OMI; Animal experiments – JDI, OSA, VOA, HDA; Immunohistochemistry, image acquisition and data analysis – JDI, OKI; Manuscript draft – JDI; Critical revisions and editing – OKI and OMI. All authors approved the final draft.

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