The Journal of Anatomical Sciences
Email: journalofanatomicalsciences@gmail.com

J. Anat Sci 15(2)

Submitted
Accepted August 23rd, 2024
Published September 30th, 2024

Neurobehavioural and Histomorphologic Study of Mice Hippocampus Following Exposure to Rohypnol

Onyilo PO, Enaohwo TM, Omonigho EM,

Department of Human Anatomy, Faculty of Basic Medical Sciences, Delta State University, Abraka

Corresponding Author: Onyilo PO.

Email: peaceewoma@gmail.com, Tel: +2347030880085

ABSTRACT

Rohypnol (Flunitrazepam), a highly lipophilic benzodiazepine derivative, is commonly used in treating seizures and sleep disorders. This study aimed to evaluate the histomorphological changes in the hippocampus and the associated neurobehavioral effects on spatial memory in mice following exposure to Rohypnol. Forty adult mice, bred in the Animal House of the College of Health Sciences, Delta State University, Abraka, were randomly divided into four groups (n=10 per group). Group A served as the control, while Groups B, C, and D received Rohypnol at doses of 0.5 mg/kg, 1 mg/kg, and 1.5 mg/kg body weight, respectively. Spatial memory was assessed using the Y-maze and Barnes maze tests. Data were analysed with descriptive statistics, and results were expressed as mean ± standard error of the mean. Statistical significance was determined using ANOVA, with $p \leq 0.05$ considered significant. Histological analysis revealed sparsely distributed glial cells within the neuropil, with congested capillaries and vacuolation of glial cells in the treatment groups compared to the control. Behavioural assessments indicated an increase in escape latency and head dips across all treated groups during the final exposure, alongside a significant decrease in alternation patterns in the Barnes maze, indicating impaired spatial memory. These findings suggest that Rohypnol may induce degenerative changes in the hippocampus, potentially impairing its function.

Keywords: behaviour, hippocampus, histology, Rohypnol,

INTRODUCTION

Rohypnol (flunitrazepam) is a benzodiazepine that acts on the gamma-aminobutyric acid (GABA) system in the brain to produce hypnotic, sedative, and anxiolytic effects. Although it is highly effective in the management of severe insomnia, its potential for abuse has been linked to drug-related crime[1, 2]. It is highly lipid soluble and could easily cross the blood-brain barrier acting as a central nervous system depressant. It increases inhibitory neurotransmission by enhancing the effect of the neurotransmitter GABA at the GABA_A receptor. More so, both short and long-term use of benzodiazepines has been linked to cognitive impairments, especially in memory and learning[3]. A related study opines that long-term benzodiazepine use can cause tolerance, dependence, and withdrawal symptoms, which negatively impact cognition and behaviour[4]. Chronic benzodiazepine use has been shown in humans to impair memory[5].

The hippocampus which plays a crucial role in a broad spectrum of cognitive functions and behaviours, including higher-order perception, language and spatial navigation, could be highly susceptible to the effects of benzodiazepines[6]. The neurobehavioral and histological effects of benzodiazepines in rodents have been the subject of numerous studies.

File and Lister (1984) discovered that long-term use of the diazepam, a known benzodiazepine resulted in decreased exploratory behaviour in the open field test (OFT) and impaired performance in the Morris water maze (MWM).

Additionally, histological analyses revealed structural changes and neuronal loss in the hippocampus, indicating that benzodiazepines might be neurotoxic[7]. A study by Owolabi *et al.* demonstrated that while co-administration of Rohypnol and alcohol did not cause extensive structural disruption of the hippocampus, certain regions exhibited morphological aberrations, leading to mild heterogeneity[8]. In a study by Okwuonu *et al.* on the histological changes in the cerebellum of Rohypnol-treated Wistar rats, significant alterations were observed within the cerebellar layers. The granular layer and white matter exhibited severe disruptions, vacuolations, and vascular congestion. Moreso, there was a notable reduction in the number

of Purkinje cells within the Purkinje layer[9]. Udodi and Ezejindu conducted a study evaluating the neurotoxic effects of Rohypnol on the cerebral cortex of adult Wistar rats, revealing a significant increase in relative body weight and organ weight in the test groups. Histological examination of the cerebral cortex in the treated animals showed severe lymphocytic infiltration, focal necrosis, intracerebral haemorrhage, vacuolation, and varying degrees of damage to the tissue layers and neuronal cells[10]. Given the widespread use of Rohypnol and its potential for abuse, it is crucial to understand its long-term impact on brain function and behaviour. While the effects of other benzodiazepines have been investigated, the specific neurobehavioral and histological changes associated with chronic Rohypnol exposure remain underexplored. This study aims to address this gap by examining the impact of prolonged Rohypnol administration on neurobehavioral performance and hippocampal morphology in mice.

MATERIALS AND METHODS

Ethical Consideration

Ethical clearance for this study was obtained from the Research and Ethics Committee of the Department of Human Anatomy and Cell Biology, Faculty of Basic Medical Sciences, Delta State University with Ethical Number: DELSU/CHS/ANA/2021/23. Ethical guidelines concerning the use of experimental animals were followed strictly.

Experimental Design

Forty adult mice weighing approximately 20-30g were obtained from the animal house of the College of Health Sciences, Delta State University, Abraka, and used for this study. The mice were randomly assigned into four (4) groups, comprising ten (10) animals in each group. Group A was the control group, while groups B-D were the experimental groups. The LD50 of 1200 mg/kg was determined according to a previous study by Airhomwanbor *et al.*[3.] The treatment groups were given daily oral doses of 0.5 mg/kg, 1 mg/kg, and 1.5 mg/kg per body weight of Rohypnol respectively for 28 days. The weights of the rats were also taken weekly throughout the experiment.

Behavioural Study Models

The Barnes maze is a dry-land-based behavioural test created by Carol Barnes to study the basic function of the Barnes maze and to analyse the mouse's ability to learn and remember the location of a target zone using a configuration of distal visual cues placed around the testing area. The mice were simply placed in the open space of the Barnes maze, and the time it took each mouse to find the escape route was recorded.

The Y-maze test was deployed to assess spatial memory, spontaneous alternation and willingness of the treated mice to explore new environments. A Y-shaped maze with three white, opaque plastic arms at an angle of 120° apart was used. The procedures for the behavioural tests were carried out following standard protocol as adopted by Owolabi *et al.*[8]

Animal Sacrifice and Sample Collection

At the end of the treatment period, the mice were sacrificed via cervical dislocation, and the hippocampus was collected. The hippocampal tissues were harvested and fixed immediately in formal saline. Thereafter, the tissues were processed according to standard histological procedures as recommended by Drury and Wallington [11].

Statistical Analysis

Data were analyzed using descriptive statistics and the results were expressed as the mean, standard error of the mean. Differences in mean value were assessed using analysis of variance (ANOVA) and ($p \leq 0.05$) was considered statistically significant.

RESULTS

Effects of Flunitrazepam on Spatial Memory of Adult Mice

Comparison of mean percentage correct alternation in adult mice treated with Rohypnol showed a statistically significant decrease in the percentage of correct alternation among the treated groups compared to the control group ($p < 0.05$). Furthermore, multiple comparisons using the least square difference test indicated statistically significant differences between the control group and each of the Rohypnol-treated groups (Figure 1). These findings suggest that Rohypnol administration at graded doses results in a dose-dependent reduction in correct alternation within the maze model.

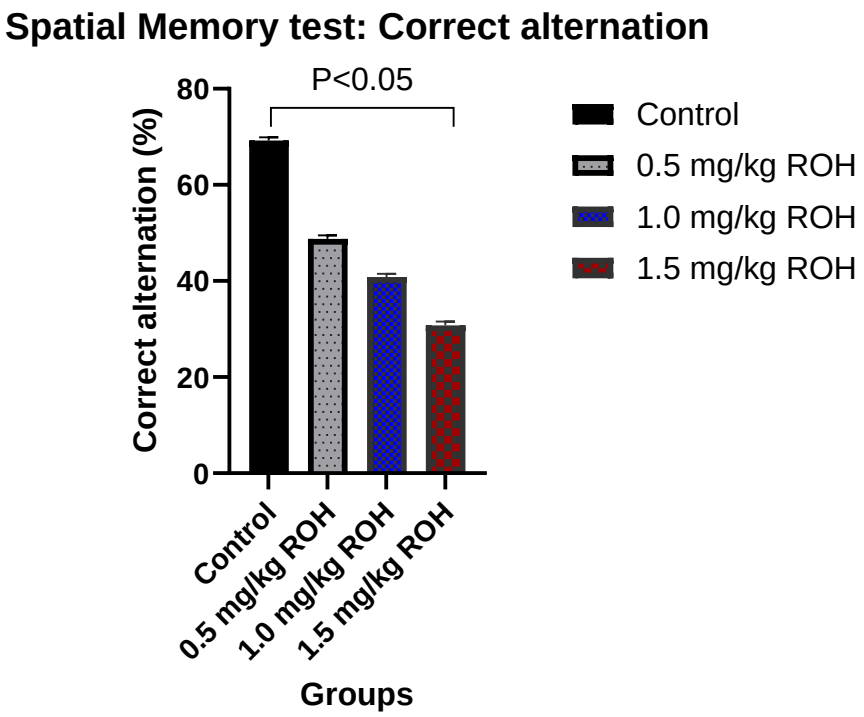

Figure 1: Bar graph showing mean percentage correct alternation pattern (using y-maze) of adult mice exposed to Rohypnol at graded doses of 0.5 mg, 1.0 mg, and 1.5 mg/kg. There was a significant decrease ($p<0.05$) in the percentage of correct alternation of the treated mice compared to the control within the Y-maze.

Comparison of initial and final escape latency in adult mice treated with Rohypnol showed that there was a statistically significant increase ($p < 0.05$) in both initial and final escape latencies in the Rohypnol-treated groups compared to the control group. In the control group, a significant reduction in escape latency was observed during the final exposure compared to the initial exposure. Conversely, in all treated groups, the final escape latency increased compared to the initial exposure. Multiple group comparisons using the least square difference test further confirmed statistically significant differences ($P < 0.05$) in mean escape latencies between each treated group (low, medium, and high doses) and the control group. Additionally, the comparisons between the treated groups were highly significant, indicating that the observed changes were dose-dependent (Figure 2).

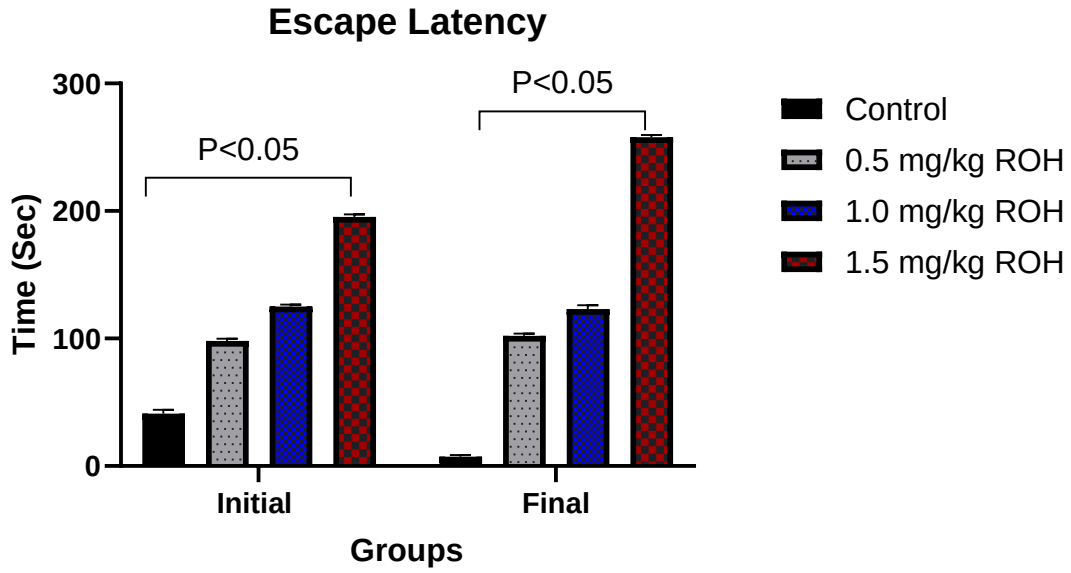

Figure 2: Bar graph showing initial and final escape latency (using the Barnes maze) of adult mice treated with Rohypnol. There was a significant increase ($P<0.05$) in the initial and final escape latency of the treated mice as compared to the control. In the control group, a significant decline in escape latency was observed in the final exposure when compared to the initial exposure; alternatively, there was an increase in latency rate in all treated groups at the final exposure when compared to the initial. Comparison between treated groups was highly significant ($P<0.05$) indicating the changes observed were dose-dependent.

Comparison of initial and final number of Error Head Dips in adult mice treated with Rohypnol showed there was a statistically significant increase ($p < 0.05$) in the number of error head dips in the Rohypnol-treated groups compared to the control group. This increase was dose-dependent, with higher mean values observed in the higher dosage groups. Further analysis using multiple comparisons via the least square difference test showed statistically significant ($p < 0.05$) differences in the mean number of error head dips between the initial and final exposures within each treatment group compared to the control. However, the comparison between the treatment groups themselves was only significant at the final exposure indicating a dose-dependent response (Figure 3).

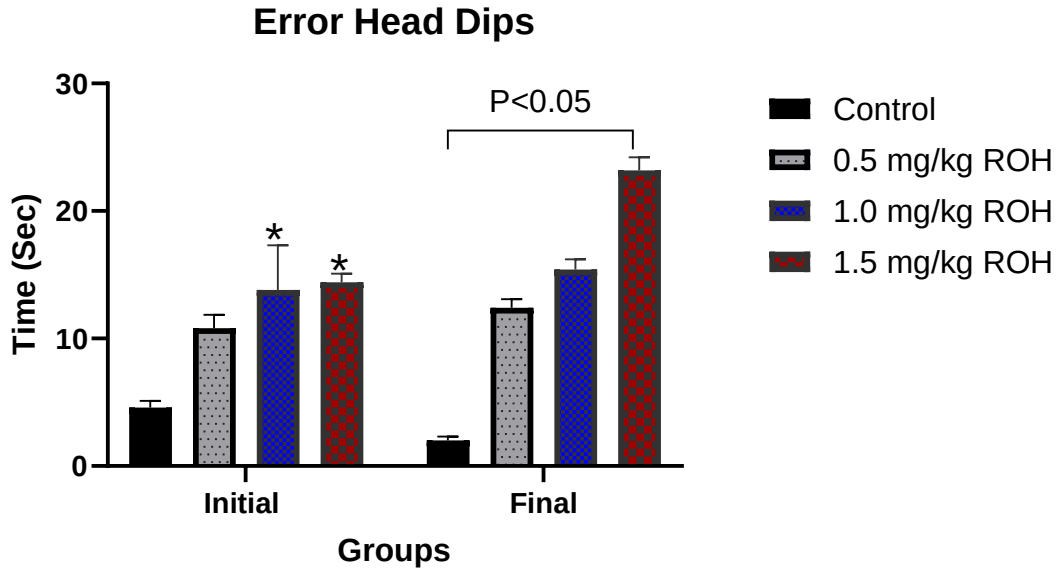

Figure 3: Bar graph showing the initial and final number of error head dips (using the Barnes maze) of adult mice treated with Rohypnol. There was a significant increase ($P<0.05$) in the number of error head dips of the treated mice relative to the control. The initial head dips showed a significant increase ($p<0.05$) in mid and high-dose treatment groups compared to the control; however, a significant increase ($p<0.05$) was observed in the final head dips for all treatment groups compared to the control.

Effects of Flunitrazepam on the Histoarchitecture of the Hippocampus of Adult Mice

The hippocampus of control mice showed features consistent with normal hippocampal microanatomy notably *cornu ammonis* (CA) regions made of giant pyramidal cells and the Dentate Gyrus (DG) containing the granular cells, the Glia cells (arrow) are distributed sparsely within the neuropil (NP), the capillaries (arrow) appeared distinctly without congestion, erosion or any other form of microangiopathy (Fig. 4A).

Mice given 0.5 mg/kg of ROH showed CA regions made of the giant pyramidal cells and the dentate gyrus (DG) containing the granular cells, the glial cells (arrow) are distributed sparsely within the neuropil (NP), the capillaries appeared congested (star) with glia cells vacuolation. Features suggestive of cellular reaction to injury (Fig. 4B).

Mice treated with 1 mg/kg of ROH showed CA regions made of giant pyramidal cells and the DG containing the granular cells, the Glia cells (arrow) are distributed sparsely within the neuropil (NP), the capillaries appeared congested (star), features suggestive of cellular reaction to injury (Fig. 4C).

The hippocampus of mice treated with 1.5 mg/kg of ROH as shown in Fig. 4D showed CA regions made of the giant pyramidal cells and the Dentate Gyrus (DG) containing the granular cells, the Glia cells (arrow) are distributed sparsely within the neuropil (NP), the capillaries appeared congested (star) with glia cells vacuolation. Features suggestive of cellular reaction to injury.

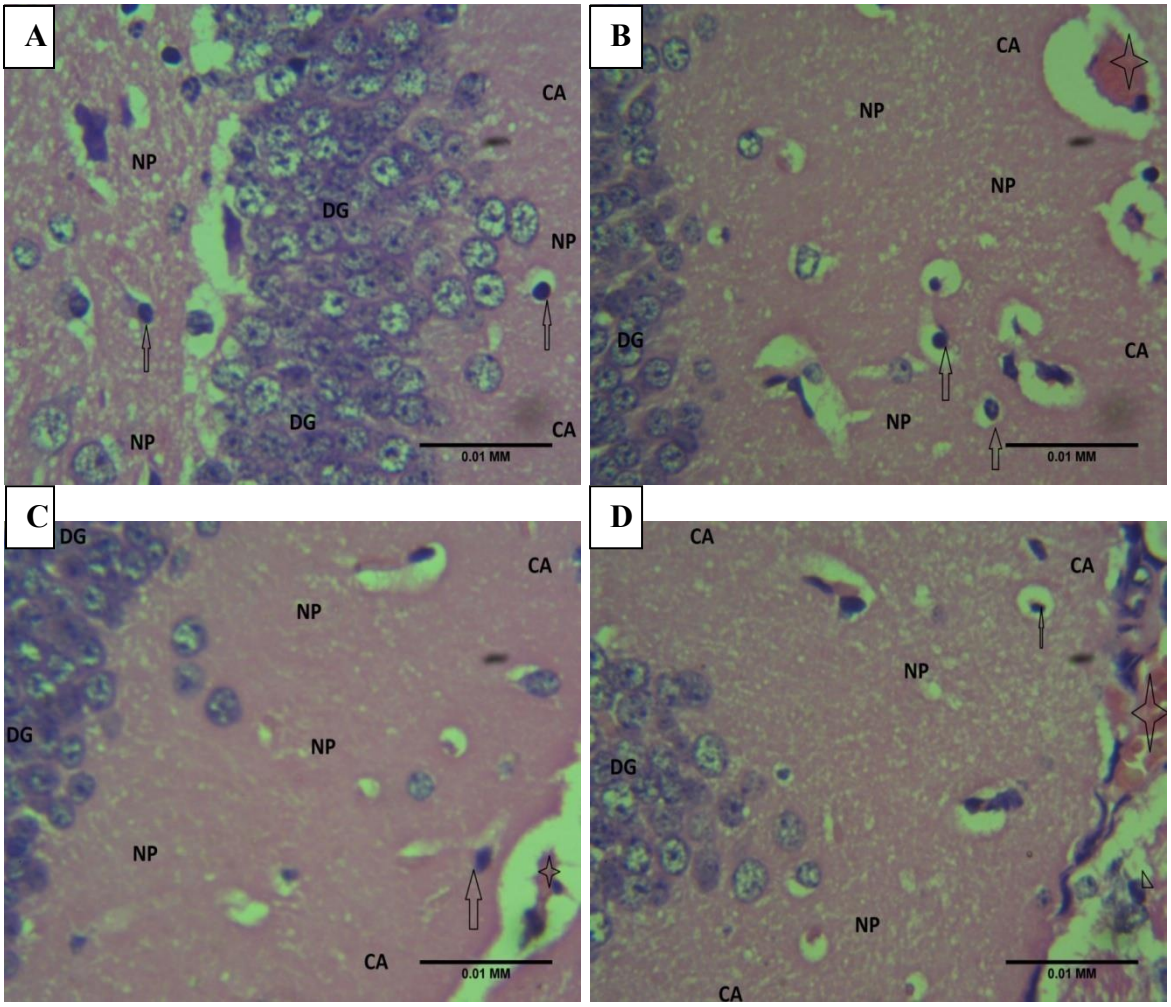

Figure 4: Photomicrograph of mice hippocampus (H&E x400). The control (A) shows normal histoarchitecture of the cornu ammonis (CA) regions made of giant pyramidal cells and the Dentate Gyrus (DG) containing the granular cells, the Glia cells (arrow) sparsely distributed within the neuropil (NP). In the treatment groups (B, C, D) the capillaries appeared congested (star) with glial cell vacuolation which could be suggestive of cellular reaction to injury.

DISCUSSION

Rohypnol is a benzodiazepine commonly used for the treatment of insomnia; however, it could aid sedation, and muscle relaxation, reduce anxiety, and prevent convulsions. Exposure to this drug has been shown to alter the development of specific areas of the brain, especially the cerebellum and hippocampus in both embryonic and adult mice [1].

The result of this study showed a mild to moderate degree of cellular alterations in the histoarchitecture of the treated mice exposed to oral intake of Rohypnol groups. There was significant neuronal loss and morphological alterations in the hippocampus of Rohypnol-treated mice, particularly in the CA1 and CA3 regions. The reduction may suggest neurodegenerative and neuroinflammatory processes, which may grossly affect cognition and result in behavioural impairments. These findings are consistent with studies by Gilman *et al.* (2007) and Mohler (2012), which reported decreased neurogenesis and synaptic plasticity in the hippocampus following chronic benzodiazepine exposure. Prolonged exposure to benzodiazepines downregulates GABA_A receptors and alters the receptor subunit composition, contributing to tolerance and dependence. More so, it has been associated with decreased neurogenesis, increased apoptosis, glial activation and neuroinflammation in the hippocampus[12, 13].

Stewart (2005) proposed that benzodiazepine-induced cognitive impairments might also involve disruptions in hippocampal long-term potentiation (LTP), a cellular mechanism underlying learning and memory[14]. Also**,** Tewari *et al* reported the deleterious effect Rohypnol treatment on the hippocampus as well as significant depletion of the white core of the cerebellum with congested capillaries [15]. These disruptions were significantly seen mostly in the

capillaries with glial cell vacuolation. Similar studies by Owolabi *et al.* and Adekomi *et al.* reported that the hippocampal profile of treated rats showed marked neuronal necrosis, vacuolation, and neuronal loss as well as gross morphological alterations in the hippocampus [7, 16]. The structural distortion of the cells observed in this study was dose-dependent with moderate to severe capillary congestion suggestive of cellular reaction to injury evident in mid and high-dose treated groups when compared to control. The alterations observed in the glial cells could be attributed to the action of Rohypnol as an inhibitory neurotransmitter that elicits a negative effect on general brain structure and function, decreasing neuronal connectivity and thereby inducing depressive-like behaviours. Rohypnol abuse has been associated with varying degrees of cognitive and motor skills impairment as evidence-based studies affirmed cases of intoxication, lack of coordination, and aggressive behaviour when taken in combination with addictive substances like alcohol and heroin[17]. Empirical evidence from previous studies by

Owolabi *et al.,* Udodi and Ezejindu, and Gerald and Kevin also affirm that Rohypnol caused severe lymphocyte infiltration, vacuolation, necrosis, and intracerebral haemorrhage [8,10, 18].

Neurobehavioral evaluation of spatial memory in the treated mice using Y-maze and Barnes maze models showed symptoms of memory impairment evident in the orientation of the treated rats when introduced into the model compared to the control. Spatial memory is responsible for the recording, recovery, and retrieval of information in the brain required to plan a path to a desired location as well as to recall where an object or an event occurred. For navigation in memory space, maintaining orientation to the environment and the ability to recover after being disoriented is critical[19]. In this study, spatial memory was assessed using correct alternations and escape latency of mice within the maze models. There was a reduction in correct alternation patterns in the treated groups relative to the control. The treated mice's initial and final escape latency at varying doses significantly increased in the treatment groups. Related study also demonstrated significant cognitive deficits in long-term benzodiazepine users, including impairments in memory, and executive function as well as impaired performance in spatial learning[5,7]. This finding is in tandem with the study of Prabhakar *et al.,* who reported an interesting dose-related effect on memory. Although the use of Rohypnol has been found helpful in the management of insomnia, it has been linked to a lack of memory coordination, and distorted psychomotor and cognitive function among addicted users [20]. The deficits observed in this study, characterized by increased latency and distance travelled to find the hidden platform, reinforce the notion that prolonged benzodiazepine use adversely affects hippocampal-dependent cognitive functions.

CONCLUSION

This study demonstrates that chronic exposure to Rohypnol results in significant neurobehavioral impairments and histological changes in the hippocampus of mice. Hence a need for cautious prescription of the medication to prevent untoward effects on brain function and structure.

Conflict of Interest

The authors declare no conflict of interest.

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