Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
http://dx.doi.org/10.21622/AMPDR.2025.05.1.1202

June 2025
Volume 5
Issue 1

Modeling Schizophrenia Endophenotypes In Drosophila Melanogaster: Effects Of Ketamine On Anxiety, Aggression, Locomotion And Inflammatory Responses

Abdulrahman Adesola Bello[1] and Royhaan Olamide Folarin[1]

[1]Group for Biopsychiatry Research and Innovative Neuroscience (G-BRaIN), Department of Anatomy, Olabisi Onabanjo, University, Sagamu, Nigeria

Emails: adesholabaylow@gmail.com, royhaan.folarin@oouagoiwoye.edu.ng

Received on, 22 January 2025 - Accepted on, 06 April 2025 - Published on, 12 May 2025

ABSTRACT:

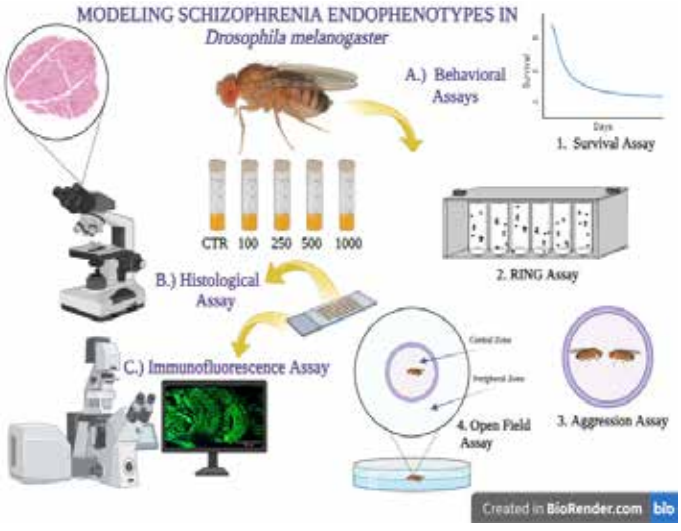
Schizophrenia presents a significant challenge in mental health, characterized by a profound distortion of reality, often accompanied by hallucinations, delusions, cognitive deficits, and neuroinflammatory processes. Ketamine has been widely used as a pharmacological agent to model schizophrenia symptoms in both human and animal studies. However, the potential of ketamine to induce schizophrenia-like phenotypes in Drosophila melanogaster remains under investigated. This study, therefore, investigated the effects of ketamine on anxiety, aggression, locomotor activities, and inflammatory response in Drosophila melanogaster as a preliminary step toward developing a pharmacological model of schizophrenia in this organism.

Virgin male and female Oregon-R flies were collected after eclosion and exposed to four different concentrations of ketamine (100, 250, 500, 1000 µg/mL) for 1 week under standard laboratory conditions (22-25°C, 50-60% humidity). Experimental groups consisted of 10 vials, each containing 10 flies. Anxiety, aggression, and locomotory functions were assessed behaviorally through the open field, aggression, and rapid iterative negative geotaxis (RING) assays. Pro-inflammatory and astrogliotic responses were measured immunohistochemically using Tumor necrosis factor-alpha (TNF-α) and Glial fibrillary acidic protein (GFAP) antibodies. The general neuronal architecture was evaluated using the H&E histological staining techniques.

The results showed a dose-dependent induction of aggressive behavior. Motor function assays demonstrated that ketamine impaired these functions in a dose-dependent manner. Survival assays indicated that higher doses of ketamine reduced survival rates. Immunohistochemical analysis revealed a dose-dependent increase in TNF-α and GFAP mean fluorescence intensity across the treatment groups, indicating upregulation of TNF-α and GFAP expressions. This suggests a robust pro-inflammatory and astrogliotic response to ketamine administration, aligning with the emerging neuroinflammatory endophenotype theory of schizophrenia aetiology and its experimental modeling. Histological analysis displayed significant dose-dependent histopathological changes, including increased cell loss and vacuolization at higher ketamine concentrations.

In conclusion, the findings suggest that ketamine has potential as a pharmacological model of schizophrenia in Drosophila. Overall, these results contribute to the understanding of how ketamine influences key behavioral and neurobiological parameters, offering insights into their potential roles in inducing schizophrenia-like phenotypes like altered behavior and histopathology.

Keywords: *Ketamine, Drosophila melanogaster, Neuroinflammation, TNF-α, Schizophrenia, GFAP.*



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1. Introduction

Schizophrenia is a severe mental disorder affecting approximately 1% of the global population, significantly impacting individuals, families, and healthcare systems. It is characterized by positive symptoms (hallucinations, delusions), negative symptoms (social withdrawal, avolition), and cognitive impairments, leading to substantial functional disability and reduced quality of life (1). Current treatments often fail to address the full spectrum of symptoms, especially cognitive and negative symptoms, highlighting the need for novel therapeutic strategies (2).

Ketamine, an NMDA receptor antagonist initially developed as an anesthetic, has gained attention for its psychotomimetic effects and rapid antidepressant properties (3). Its use as a schizophrenia model is due to its ability to induce psychotic-like symptoms and cognitive impairments in healthy individuals, closely resembling those seen in schizophrenia patients (4). Acute ketamine administration produces positive symptoms (perceptual alterations, thought disorder), negative symptoms (blunted affect, social withdrawal), and cognitive deficits in attention, working memory, and executive function. These effects are linked to ketamine's disruption of glutamatergic neurotransmission, aligning with the glutamate hypothesis of schizophrenia (5).

Neuroinflammation has recently emerged as a critical factor in schizophrenia pathophysiology, providing new insights into its etiology and treatment (6). It involves an immune response within the central nervous system, marked by the activation of microglia and astrocytes and the release of pro-inflammatory cytokines (7). Studies have shown elevated inflammatory markers in the blood and cerebrospinal fluid of schizophrenia patients (8), and neuroimaging reveals increased microglial activation in several brain regions (9). GFAP, a key marker for astrocytes, provides insight into neuroinflammatory responses and potential neuropathological states (10). TNF is a critical mediator of neuroinflammation, often exacerbating neurodegeneration, synaptic dysfunction, and cognitive impairments (11).

Drosophila melanogaster is a valuable model organism for studying complex neurological disorders. Despite its simplicity, it shares many molecular and cellular mechanisms with humans, making it a powerful tool for neuroscience research (12). Its advantages include a short lifespan, rapid generation time, and advanced genetic tools. Drosophila also exhibits complex behaviors, such as learning, memory, circadian rhythms, and social interactions, which can be used to model neuropsychiatric disorders and screen for therapeutic compounds (13)autism spectrum disorders and Down syndrome, among others. They are characterized by limitations in adaptive and social behaviors, as well as intellectual disability (ID. While ketamine's effects are well-studied in mammalian models, its impact on Drosophila's cognitive function and neuroinflammatory responses remains underexplored, limiting the fly's utility in schizophrenia research. This study thus investigated the effects of ketamine on anxiety, aggression, and locomotory functions through the open field, aggression chamber, and rapid iterative negative geotaxis (RING) assays, lifespan, neuroinflammatory responses, and neuroarchitecture in Drosophila melanogaster. These findings contribute to our understanding of ketamine's effects on Drosophila and support its use as a model for schizophrenia research.

2. METHODS

Acquisition and Breeding of Flies

Wild-type *Drosophila melanogaster* (Oregon-R strains) were obtained from the Group for Biopsychiatry Research and Innovative Neuroscience at the Department of Anatomy, Olabisi Onabanjo University, Ogun State. The culture and feeding medium, primarily corn-meal-based, was hygienically prepared according to the protocols adopted by (14). The flies were maintained under standard laboratory conditions (22-25°C, 50-60% humidity) with natural day/night cycles in the animal holding facility of the Department of Anatomy at Olabisi Onabanjo University, Sagamu, Nigeria. Ketamine HCl injection (50 mg/ml) was sourced from Jawa® Group in Lagos, Nigeria.

Experimental Design and Dosing

The experimental design for the research included the following groups: a control group, which was maintained on standard fly food ad libitum, and a ketamine-administered group, which received varying concentrations of ketamine hydrochloride (100, 250, 500, and 1000 µg/mL) mixed into the fly food and allowed to feed ad libitum. The dosage of ketamine used in this study was informed by Koksal and Gürbüzel's (2020)(15) study, which provided a foundational understanding of ketamine's effects on *Drosophila*, identifying dosage ranges that elicit significant biological responses

without causing immediate toxicity.

Each vial contained 5 mL of the prepared feed dispensed into standard vials, with the appropriate volumes of ketamine added shortly after cooking the feed, prior to dispensing into the treatment vials. Each experimental group was replicated in ten vials, with each vial containing ten flies. All treatments were administered continuously for one week, and the flies were monitored daily.

Table: Experimental Design

Groups	Regimen	No flies/ vial
Control	Standard fly food	10
KT1	100 µg/mL Ketamine in fly food	10
KT2	250 µg/mL Ketamine in fly food	10
KT3	500 µg/mL Ketamine in fly food	10
KT4	1000 µg/mL Ketamine in fly food	10

Behavioural Assays

1. Survival Assay

Newly eclosed flies were transferred into culture vials containing different concentrations of ketamine mixed into the diet and vials without ketamine for the control with ten flies per vial. The number of dead flies was recorded at regular intervals of three days (16)leading to reduced physical performance and increased risk of disease. Individual aging is manifest at the population level as an increase in age-dependent mortality, which is often measured in the laboratory by observing lifespan in large cohorts of age-matched individuals. Experiments that seek to quantify the extent to which genetic or environmental manipulations impact lifespan in simple model organisms have been remarkably successful for understanding the aspects of aging that are conserved across taxa and for inspiring new strategies for extending lifespan and preventing age-associated disease in mammals. The vinegar fly, Drosophila melanogaster, is an attractive model organism for studying the mechanisms of aging due to its relatively short lifespan, convenient husbandry, and facile genetics. However, demographic measures of aging, including age-specific survival and mortality, are extraordinarily susceptible to even minor variations in experimental design and environment, and the maintenance of strict laboratory practices for the duration of aging experiments is required. These considerations, together with the need to practice careful control of genetic background, are essential for generating robust measurements. Indeed, there are many notable controversies surrounding inference from longevity experiments in yeast, worms, flies and mice that have been traced to environmental or genetic artifacts(1-4. The study examined ketamine's effects on the survival of *Drosophila melanogaster*, with daily monitoring of treatment groups consisting of 50 flies per group, separated by sex.

2. Rapid Iterative Negative Geotaxis (RING) Assay

Ten flies from each treatment vial were introduced into a climbing apparatus consisting of vertical columns made from high-density polyethylene (15 cm in length and 2 cm in diameter) on the final day of administration. After being anesthetized with mild ice, the flies were allowed to recover for one hour. A white background with horizontal lines spaced 1 cm apart was positioned behind the vials. The flies were gently tapped to the bottom of the column by lightly banging it on the assay platform. The number of flies that reached the 8 cm mark within ten seconds was recorded. Each assay was conducted five times for the same group, with a one-minute rest period between trials. The score for each trial was calculated as the average of five measurements (17).

Figure 1: RING Assay Apparatus

Schematic representation of the Rapid Iterative Negative Geotaxis (RING) assay used to assess locomotor function in Drosophila. The setup consists of vertical columns (15 cm in length, 2 cm in diameter) with a white background marked with horizontal lines at 1 cm intervals. Flies are tapped to the bottom, and their climbing ability is measured by recording the number of flies reaching the 8 cm mark within 10 seconds.

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3. Open Field Assay set up

The open field assay provides valuable insights into anxiety-like behaviors and exploratory drive across treatment groups. The open field arena was set up by placing a 10 cm diameter Petri dish on a circular arena drawn on paper, divided into a central zone (50% of the total area) and a peripheral zone (50% of the total area), with a grid overlay for distance measurement and further divided into four quadrants. A video camera was positioned directly above the arena for full visibility.

Flies were acclimatized to the testing room conditions for at least one hour before the assay. A single fly was gently aspirated into the center of the arena without anesthesia to avoid altering its behavior. Video recording began immediately, allowing the fly to explore the arena for ten minutes. After each trial, the fly was removed, and the arena was cleaned with 70% ethanol to eliminate olfactory cues. This process was repeated for all experimental groups, alternating between control and treatment groups to minimize time-of-day effects. The recorded videos were analyzed for parameters including time spent in the central and peripheral zones and frequency of zone crossings (18).

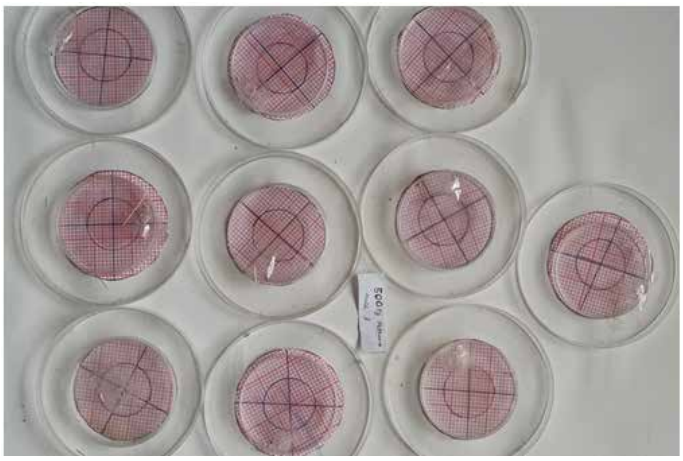

Figure 2: Open Field Assay Setup

Diagram of the open field assay setup used to analyze anxiety-like behavior and exploratory activity in Drosophila. The arena consists of a 10 cm diameter Petri dish placed on a circular zone drawn on paper, divided into a central and peripheral zone. A video camera positioned above records movement patterns, including time spent in each zone and frequency of zone crossings.

4. Aggression Assay

The aggression assay was conducted to quantify aggressive behaviors in male and female *Drosophila melanogaster* after various treatments. The assay used an arena chamber made from a 96-well plate, a video camera, and a timer. Pairs of male and female flies were gently placed into each cell of the arena chamber using an aspirator. The chamber was then positioned under the video camera to ensure unobstructed recording. After loading the flies, they were given 5 minutes to acclimate to reduce handling stress and encourage natural behavior. Once the acclimation period ended, a 10-minute recording session began to capture the flies' behavior. Aggressive behaviors were observed and scored based on fighting frequency and latency to fighting (19).

Figure 3: Aggression Assay Chamber

Illustration of the aggression assay setup used to assess aggressive interactions in Drosophila. The assay utilizes a 96-well plate as an arena chamber, with pairs of flies placed in individual wells. A video camera records interactions, scoring fighting frequency and latency to aggression.

Histology and Immunofluorescence Analysis

Following administration, the Drosophila were anesthetized on ice and transferred to a dissection dish containing ice-cold phosphate-buffered saline (PBS, pH 7.4). Under a stereomicroscope, the heads were carefully separated from the bodies using fine forceps. Following the protocol of Karmakar & Mishra, 2020, the heads were then immediately placed into a microcentrifuge tube containing 4% paraformaldehyde (PFA) in PBS for fixation, which was carried out for 20 minutes at room temperature with gentle agitation. Following fixation, the samples were washed three times with PBS to remove excess fixative. After anesthetizing the flies on ice, their heads were carefully removed and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 hours at room temperature. Following fixation, the samples were washed in PBS and dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, 100%) for 5 minutes each. The dehydrated heads were cleared in xylene

and embedded in paraffin. Sections were cut at a thickness of 5 µm using a rotary microtome and mounted on gelatin-coated slides.

To assess neuroinflammation and neuronal integrity, two primary antibodies were used: rabbit monoclonal anti-TNF-alpha (1:200, medchem express) to detect neuroinflammatory responses and anti-GFAP (1:500, Sigma-Aldrich) to assess glial reactivity. After fixation and washing, the samples were permeabilized with 0.1% Triton X-100 in PBS for 20 minutes at room temperature, followed by blocking with 5% normal goat serum in PBS for 1 hour to reduce non-specific binding.

Primary antibodies diluted in 0.1% Triton X-100 in PBS were applied to the samples and incubated overnight at 4°C. The next day, samples were washed three times with PBS to remove unbound primary antibodies. Fluorophore-conjugated secondary antibodies (anti-rabbit donkey CF647, 1:500, Sigma-Aldrich) were then applied to the samples for 2 hours at room temperature in the dark, followed by washing three times with PBS. DAPI (1:500 dilution in 0.1% Triton X-100 in PBS) was applied for 10 minutes to counterstain nuclei. After a final PBS wash, the samples were mounted on glass slides using Vectashield anti-fade mounting media and cover-slipped. Image acquisition was conducted using a Zeiss LSM-700 confocal microscope with standardized settings to ensure consistency. The images were processed using ImageJ/Fiji software, where they were converted to 8-bit grayscale for intensity analysis.

Hematoxylin and Eosin (H&E) staining was performed following standard protocols (20). The sections were deparaffinized in xylene, rehydrated through a descending ethanol series, and stained with Harris hematoxylin for 5 minutes. After rinsing in running tap water, the slides were counterstained with eosin for 2 minutes. The sections were then dehydrated through an ascending ethanol series, cleared in xylene, and mounted with DPX mounting medium under coverslips. Images of the H&E-stained sections were captured using an Olympus BX53 bright-field microscope equipped with a digital camera, utilizing 20x and 40x objectives for detailed visualization.

3. Statistical Analysis

Image analysis was performed using ImageJ software (FIJI) to measure fluorescence intensity.

Statistical analyses were conducted using Microsoft Excel. For each quantitative measure, the mean, standard deviation, and standard error of the mean were calculated.

4. RESULTS AND DISCUSSION

Survival Assay

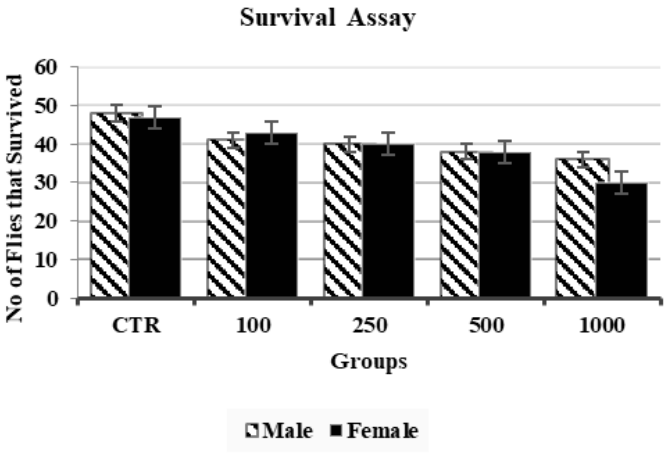

Figure 4: Graph of Survival Assay

Results indicated a clear dose-dependent decrease in survival $p \approx 0.018$ (< 0.05) for males and $p \approx 0.028$ (< 0.05) for females, with the control group exhibiting high survival rates (48 males, 47 females), while the highest ketamine dose (1000 µg/mL) led to significant reductions (36 males, 37 females surviving). These findings are consistent with previous studies, such as Zou et al. (2009) (21), which reported dose dependent neurotoxicity in rat models.

In vivo, reduced survival rates imply significant physiological stress and potential neurotoxicity at higher ketamine doses. Interestingly, our results showed similar survival trends between males and females across all treatment groups, suggesting that ketamine's effects on Drosophila survival may not be strongly sex-dependent. This observation contrasts with some mammalian studies, such as (Franceschelli et al., 2015) (22), who reported sex-specific behavioral responses to ketamine in mice.

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Open Field Assay

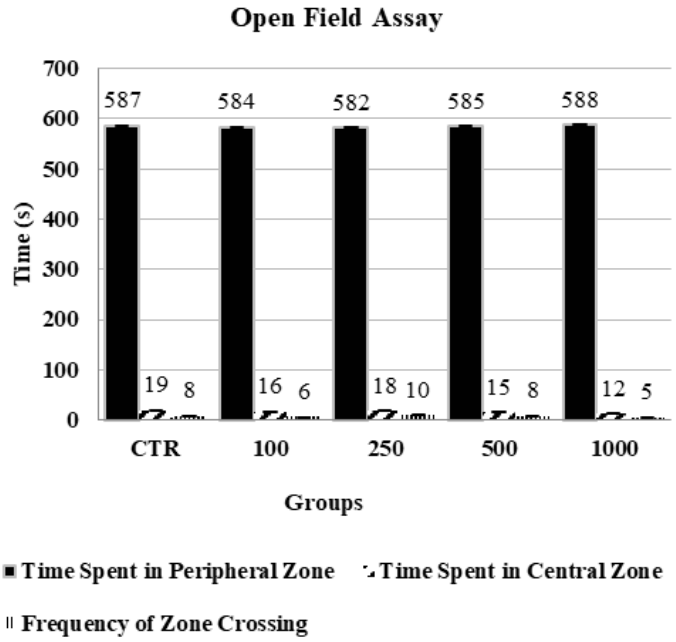

Figure 5: Graph of Open field Assay

The control group established baseline exploratory behavior, spending 19 seconds in the central zone and 587 seconds in the peripheral zone, with an average of 8 zone crossings.

Ketamine treatment showed no statistically significant differences despite appearing notable. For time spent in the central zone, p-value ≈ 0.089; for Time in the Peripheral Zone:

p ≈ 0.94 while For Zone Crossings: p ≈ 0.132 (> 0.05).

Although not significant, the trend suggests a possible dose-dependent decrease with higher doses leading to increased thigmotaxis (preference for the peripheral zone), which is often interpreted as increased anxiety-like behavior in rodent models Simon et al., (1994) (23). This suggests that while ketamine may influence anxiety-like behaviors, its effects in Drosophila may be more subtle than those observed in mammalian models.

Aggression Assay

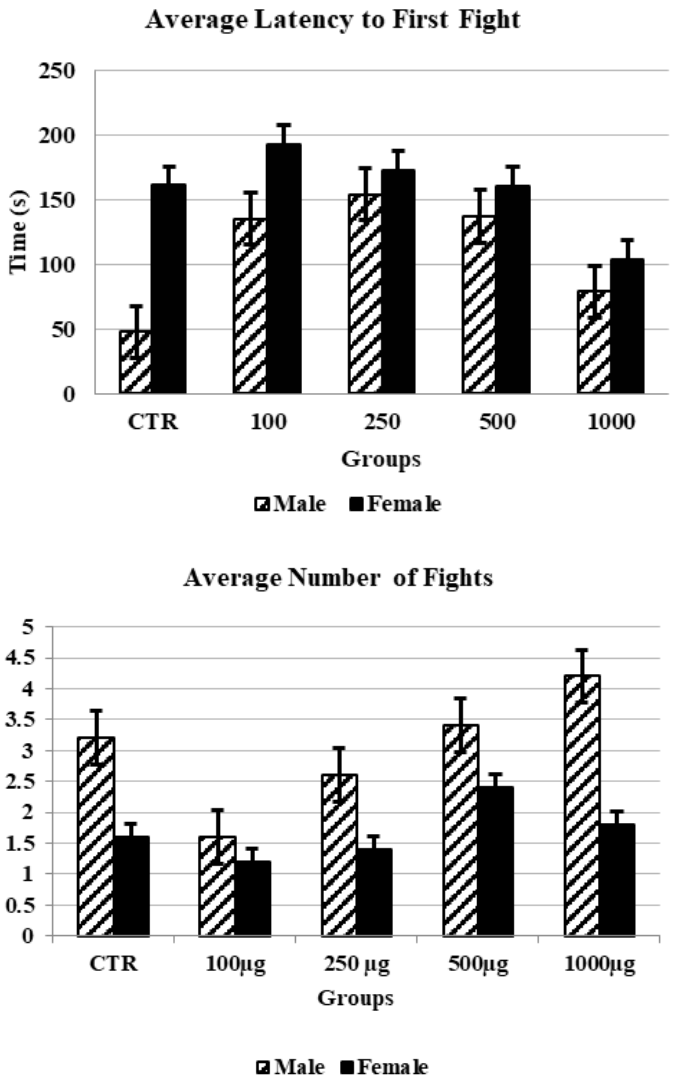

Figure 6: Graph of Aggression Assay

In the control group, males exhibited a 50-second latency and an average of 3.2 fights, while females showed a 160-second latency and an average of 1.6 fights. Ketamine treatment resulted in a significant dose-dependent increase in number of fights, p ≈ 0.031 in male although there was an initial suppression of aggression at lower doses and p ≈ 0.047 in female with moderate reduction at lower doses and peak increase at 500 µg/mL.

Lower doses (100 µL) lead to mild aggression, longer latencies (140 seconds for males, 190 seconds for females), and fewer fights (1.6 and 1.2 fights for males and females, respectively). As doses increased (500 µL, 1000 µL), latencies decreased, and fighting frequency rose, with the highest aggression observed at 1000 µL (65 seconds for males, 102 seconds for females; 4.2 and 1.8 fights). These findings align with studies like Ye et al. (2019) (24)6R, linking ketamine metabolites to altered aggression via its effects on glutamatergic neurotransmission.

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Negative Geotaxis Assay

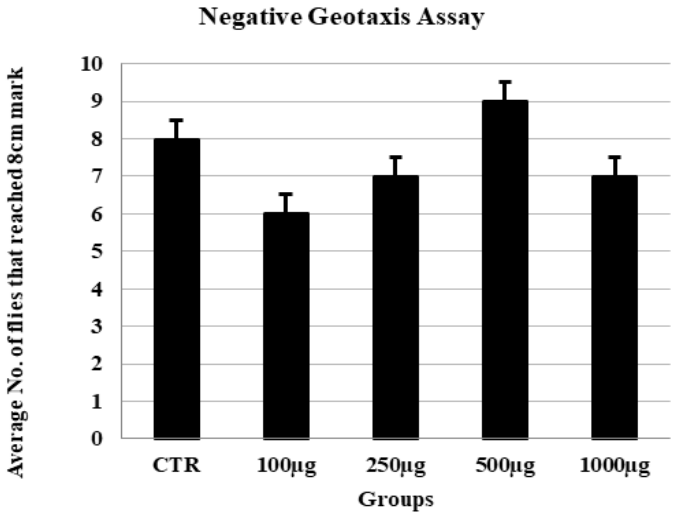

Figure 7: Graph of Negative Geotaxis Assay

The control group established a baseline climbing performance, while ketamine-treated groups showed a dose-dependent increase, most notably in the 500 µL group, where climbing ability was markedly increased. Although the differences between the groups are not statistically significant at p ≈ 0.086

This dose-dependent effect of ketamine on motor function aligns with findings from Imre et al. (2006) (25) and Chen et al. (2023) (26), who reported dose-dependent effects of ketamine on locomotor activity in rats. A dose-dependent enhanced negative geotaxis in Drosophila has previously been reported using Dizocilpine, an NMDA receptor agonist widely used to model schizophrenia-like-phenotypes (27).

However, the 1000 µL group showed a reduction in motor function, suggesting that high doses of ketamine could impair locomotion.

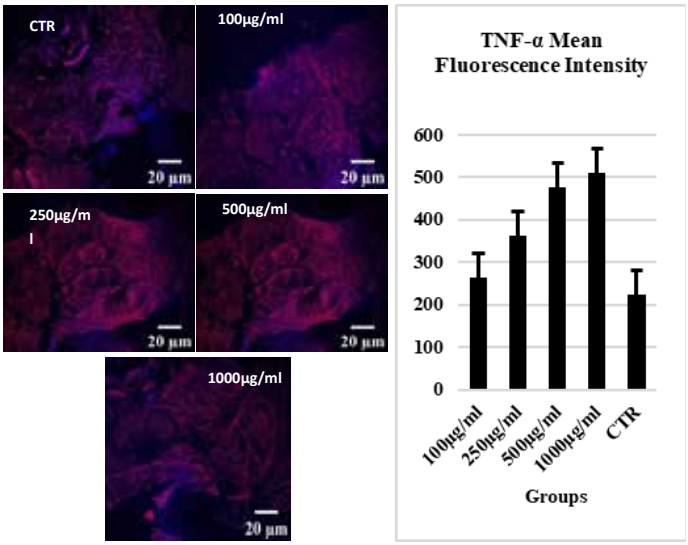

Figure 8: Section of Drosophila brain showing the distribution and localization of TNF-alpha (red).

Immunofluorescence and Histological Analysis

Figure 8: Section of Drosophila brain showing the distribution and localization of TNF-alpha (red). DAPI (Blue) is used as a nuclear stain to visualize the whole brain morphology, and the graph shows a comparison of the mean fluorescence intensity of TNF-alpha across different experimental groups.

TNF-α expression analysis revealed highly significant differences between groups ($p < 0.0092$), characterized by a strong dose-dependent increase. The highest ketamine concentration resulted in more than doubled TNF-α expression compared to control levels, providing clear evidence of an inflammatory response. This pattern aligns with prior studies that link increased doses of pro-inflammatory agents to heightened TNF levels, reinforcing the inflammatory cascade's sensitivity to modulation (28)

The pronounced increase in TNF-α expression indicates robust neuroinflammatory activation at higher ketamine doses. Similar findings were reported by Wang et al. (2015) (29), who observed that chronic ketamine administration led to elevated pro-inflammatory cytokine levels, including TNF-α, in the hippocampus of rodents. Additionally, Li et al. (2017) (30) found that Single administration of ketamine increased the level of TNF-α, whereas multiple and long-term administration decreased it significantly. In Drosophila, elevated TNF levels likely mirror mammalian inflammatory processes, given the conservation of TNF pathways across species (31).

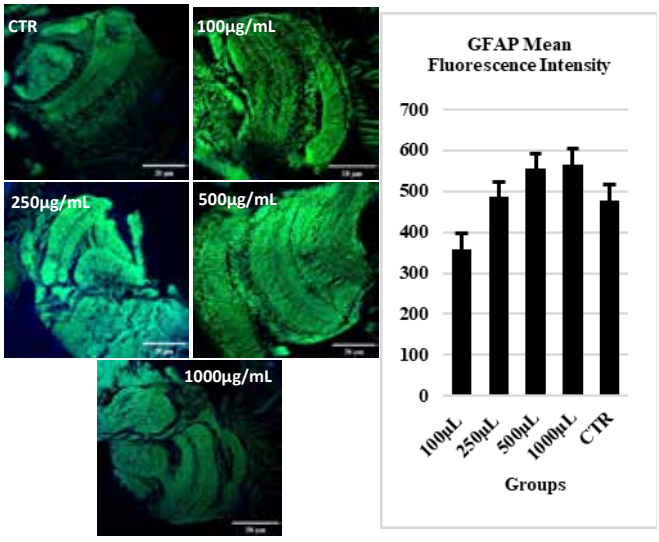

Figure 9: Section of Drosophila brain showing the distribution and localization of GFAP (green).

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http://dx.doi.org/10.21622/AMPDR.2025.05.1.1202

DAPI (Blue) is used as a nuclear stain to visualize the whole brain morphology, and Graphs show a comparison of the mean fluorescence intensity of GFAP across different experimental groups.

The analysis of GFAP expression revealed significant differences between treatment groups ($p < 0.038$), demonstrating a complex dose-dependent response to ketamine exposure. At the lowest concentration (100 µg/mL), GFAP expression showed an initial decrease compared to control levels, followed by a return to baseline at 250 µg/mL. Higher concentrations (500-1000 µg/mL) induced a progressive increase in GFAP expression, with peak levels observed at 1000 µg/mL, suggesting dose-dependent astrogliosis at higher concentrations.

The biphasic response of GFAP expression is particularly noteworthy. The initial decrease at low doses followed by elevation at higher concentrations suggests that ketamine's effects on astrocyte activation are dose-threshold dependent. This pattern aligns with findings from Keilhoff et al. (2004) (32), who demonstrated that repeated ketamine exposure in rats led to reactive gliosis and increased GFAP immunoreactivity.

This dose-dependent trend is consistent with previous studies showing increased astrocyte activation at higher ketamine doses (33). Lower GFAP expression at 100µL may reflect neuroprotective effects, whereas higher doses likely indicate pro-inflammatory or neurotoxic effects (34).

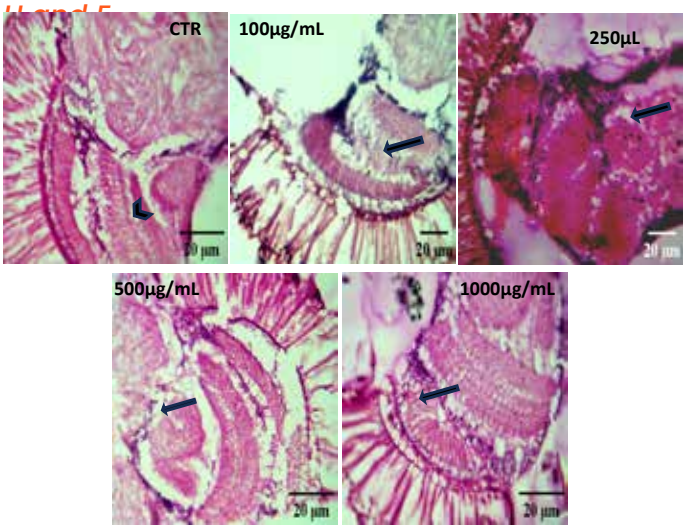

Figure 10: Histological Analysis of Drosophila Brain Sections

Hematoxylin and eosin (H&E)-stained brain sections of Drosophila showing structural changes across treatment groups.

Brains from control flies exhibited normal histological features with intact neuropil (Arrowhead) and no significant vacuolation across all brain regions. 100 µL Ketamine groups displayed mild degenerative changes as scattered vacuoles were observed (arrow). Moderate vacuolation was observed in the 250 µL Ketamine groups with increased disorganization of neuropil fibers (Figure 10). Marked degeneration was evident in the 500 µL Ketamine with prominent vacuolar lesions (arrow) disrupting the neuropil and necrotic cell bodies. Severe neurodegeneration was apparent, with extensive vacuolation and fragmentation in the 1000 µL Ketamine group. These findings are consistent with previous studies on ketamine's neurotoxic effects in both invertebrate and vertebrate models. For instance, Liu et al., (2013) (35) reported that ketamine induced widespread neuroapoptosis in the developing rat brain, with particular vulnerability in certain brain regions.

5. CONCLUSION

In conclusion, our study demonstrates the potential utility of Drosophila as a model organism for investigating complex neuropsychiatric phenomena. The observed effects of ketamine, particularly its ability to induce some behavioral and histopathological changes, suggest potential avenues for its use as a pharmacological model of schizophrenia. However, future research should focus on elucidating the molecular mechanisms underlying these effects and exploring their relevance in mammalian models. The complex effects underscore the need for careful, nuanced approaches in translating these findings to clinical applications.

Acknowledgment
Chan Zuckerberg Initiative and the BioRTC Fellowship

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Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
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