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The Effect of *Imperata Cylindrica* (L.) P. Beauv on Learning and memory performance in a single and prolonged exposure treatments of *Drosophila Melanogaster transgenic* flies overexpressing the paralytic gene

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ABSTRACT

Imperata cylindrica root extract is neuropreotective and is used by Luo people in Kenya and Uganda to manage epileptic fits (personal communication); the herb controls seizures in parabss flies by reducing Na^+ and K^+ channel activity. Brain anatomical changes, muscular activity as well as changes in learning and memory function that could arise in these flies from use of the herbas an anti-epileptic molecule during acute and chronic control of seizures were the objectives of our study. Learning and memory response was studied by an Aversive Phototaxic Suppression and brain histomorphological changes were analysed on brain tissues using Haematoxylin and Eosin plus Klüversstaining techniques Our results have further confirmed the promise of Imperata cylindrica as an anti-epileptic drug and have shown that not only treats the convulsions but ameliorates associated electrophysiological and cognitive malfunctions. The extract also ameliorated the abnormal brain histomorphology after prolonged treatment. There was only relief of symptoms in acute exposure treatments unlike in chronic exposure treatments where the extract and sodium valproate cured the brain cellular biological defects.

Keywords: Imperata cylindrical, learning, memory performance, drosophilia, paralytic gene

INTRODUCTION

Epilepsy is a neurological disorder related to seizures that have a habit of recurence without an obvious trigger or cause. The seizures come as a result of simultaneous, excessive firing of nerve signals within the brain. Thus the syndrome is very difficult to treat yet endangering patients and/or indirectly impacting relatives as well [1]. The traditional plant Imperata cylindrica (I. cylindrica) commonly called the 'spear grass' is used by the Luo people to treat epileptic convulsions (personal communication). In a study to determine the anti-epileptic effect of this plant on bang-sensitive paralytic (parabss) Drosophila melanogaster (D. melanogaster) flies conducted by [2], the 'herb' was found to control epileptic seizures in these flies through reduction in the activity of Na+ and K+ channels. The therapeutic and toxic doses of I. cylindrica were also elucidated as were some of the behavioural effects of the extract in parabss flies [2].

MATERIALS AND METHODS

Study Design

The study design had several groups of D. melanogasterbss transgenic flies overexpressing the paralytic gene; these were tested in line with each specific objective. Each objective had three repeated independent experiments and each experiment contained three replicas. A maximum of 6 flies were used in each replica; this was done to ensure quality and reproducibility in results.

Study Location

The study was conducted in the Institute of Biomedical Research (IBR) of KIU- WC; Uganda in Ishaka Municipality, Bushenyi District 350 Km from Kampala, Uganda. Histological techniques were performed at the Central diagnostic Laboratory at the College of Veterinary Medicine Animal Resources and Bio-security, in the Joint National Animal Disease Center (J-NADIC), Makerere University, Kampala, Uganda.

Study Population

The study used laboratory-crossed Drosophila melanogaster flies. These were adults (5-23-days old) D. melanogasterbang-sensitive (bss) transgenic flies overexpressing the paralytic gene (GMR-GAL4>UAS-para). Experiments were performed on male flies only to ensure homogeneity of results. Two wild-type fly strains (GMR-GAL4 and UAS-para) were used in the study as control fly strains; these same strains were crossed in the laboratory to obtain the bss transgenic flies overexpressing the paralytic gene (GMR-GAL4>UAS-para) in accordance with a method by [3].

Sampling Techniques

Each objective included single (acute) exposure and prolonged (chronic) exposure experiments. For any test that was performed there were three replicas (6 flies in each replica) which were randomly selected from a given treatment group; each test was repeated 3 times with different groups (three independent tests). All these aspects ensured quality and reproducibility in the results.

Method of Arriving at the Sample Size

To be able to perform a robust statistical analysis, large sample sizes are used [4].Working with D. melanogaster allows to obtain fairly large sample sizes without incurring big expenses [5; 6]. The usual number of Drosophila flies used in each replica in experiments of exposure to drugs or medicinal herbs is 5-10 and the number of replicas per experiment being 3-5 [7].

Inclusion Criteria

The study involved use of male adult bss transgenic flies overexpressing the paralytic gene (GMR-GAL4>UAS-para) flies whose ages ranged from 5-23-days old. Male flies were recruited because they are always homozygous for the para gene responsible for seizures in Drosophila; female flies on the other hand can either be homozygous (infected) or heterozygous (carriers) for the paralytic (para) gene. Moreover, seizure characteristics in male and female bss flies overexpressing the para gene are expressed differently; female bss flies overexpressing the para gene are more severely affected than their male counterparts because female flies have two alleles yet males have only one allele for the para gene [1].

Exclusion Criteria

The study excluded female flies. This was done to ensure that only flies which were homozygous (males) for the para gene were included in the study and those which might have been carriers for the gene (females) were eliminated; this ensured uniformity in the results since a single sex of flies was used. The study excluded flylarvae or flies that were too young or too old since these would interfere with cognitive abilities; larvae as well as flies younger than 5 days or older than 23 days were eliminated in the study.

Collection of *Imperata cylindrica* Samples

The plant materials were harvested from the banks of Rwizi River located in Mbarara Municipality, Mbarara district, Western Uganda. This area is located 295 Km by road from Kampala. A sample of the plant, roots and leaves were placed in a dry moisturized container and transported by road to the department of botany, Mbarara University of Science and Technology for botanical identification by Dr. Olet Eunice (Botanist). The plant was identified as Imperata cylindrical (L.) P. Beauv., Collection number: Fred Kalanzi #001. The plant roots from which the extract was obtained were stored in a fridge at Kampala International University, Western Campus in the Institute of Biomedical Research Laboratory for preservation.

Fly Stock

The control fly stocks used in this study included two wild-typeD. Melanogaster flies (GMR-GAL4 and UAS-para). In UAS-para (Upstream Activating Sequences-para) flies the para gene responsible the bss phenotype is kept silenced and it is

only triggered when crossed with an activator gene kept in GMR-GAL4 (glass multiple reporter-GAL4) system; therefore UAS-para flies are regarded as 'silencer' and GMR-GAL4 are regarded as 'activator' wild-type flies [3]. To obtain transgenic bss fly strains overexpressing the para gene for use in the study, female virgin UAS-paraflies were crossed with male GMR-GAL4 flies in a method similar to that used by [3]. The larvae obtained from the cross were isolated from the adult flies to ensure that progeny is not mixed with the parents. Then these larvae were raised to obtain first generation adult GMR-GAL4>UAS-paraflies. The resultant F1 progeny were sexed to isolate the males from the females; the male flies were kept as the bss transgenic flies overexpressing the para gene. These transgenic flies were kept as the test stock
flies for the study.

Determination of Activity of Imperata cylindrica Extract

The activity of the plant was determined by using the working concentrations that were established by [2], and the change in CT, PT and MRT before and after exposure to different concentrations of the extract was noted. To do this, 50 independent GMR-GAL4>UAS-paraflies were exposed to relatively safe doses of the plant extract as established in Oginga's study. In acute exposure treatments the safe dose range was established by Oginga as being below 1.0g/ml; therefore doses of 0.0, 0.6, 0.8 and 1.0g/ml of the extract were used to determine the effect of single exposure (2hr-exposure) on CT, PT and MRT. In prolonged exposure treatments (12-day exposure) the safe dose range was established by [2] as being below 0.1g/ml; therefore we used doses of 0.0, 0.0125, 0.025 and 0.05 g/ml to determine the effect of prolonged exposure on CT, PT and MRT. All experiments were performed at room temperature (22- 25oC) unless stated otherwise [7].

Data Analysis and Statistical Measures

To test our scientific hypothesis, experimental results were analysed to test whether there was enough evidence to reject the null hypotheses: that the extract causes no brain histomorphological improvement on the transgenic D. melanogaster flies overexpressing the para gene, the extract does not ameliorate muscular activity of giant fibers in DLMs in the flies and that the extract does not improve learning and memory function in the transgenic flies. In general, Microsoft statistical computer package called 'Microsoft Excel Windows 2010' was used to analyze data obtained from the study and took the form of frequency tables, graphs and charts. A statistician was consulted in data analysis. Data on histomorphological changes was recorded in form of photomicrographs taken with a digital camera (Nikon digital sight DS Fi 1) mounted to the light microscope (Nikon Eclipse Ci, 104c type) and connected to a Toshiba computer with NIS-Elements F3.00, SP7; Build 547 software.

ETHICAL CONSIDERATIONS

Proper Handling of Flies

The fly strains and reagents used in the study were acquired by using the stipulated proper channels to get them (ordered from Bloomington Drosophila Stock Center) and ethics were followed to ensure that the flies were handled well and not intentionally injured or harmed. All procedures and techniques used in this study were in accordance with the care and use of laboratory animals from the Uganda National Council of Research and approval was sought from the Postgraduate Ethics and Research Committee (PGERC) and Institutional Review and Ethics Committee (IREC) of KIU-WC. Less invasive techniques were employed to avoid causing pain on the flies; inflicting pain to live flies was minimized by sacrificing the flies under ether anaesthesia. Flies were provided with necessary requirements for their survival such as food, water and favorable environment.

RESULTS

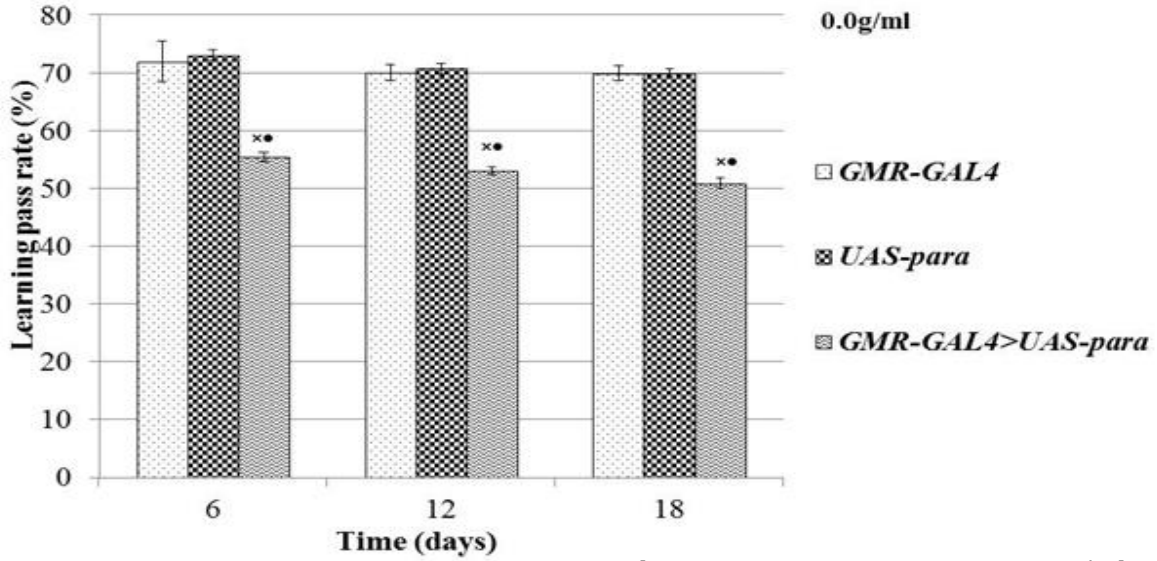

Figure 1: Learning pass rate variations in single exposure treatments at 0.0g/ml of extract. Learning pass rates for Drophila melanogaster flies overexpressing paralytic gene (GMR-GAL4>UAS-para) and those that do not overexpress paralyticgene (GMR-GAL4 and UAS-para) on acute exposure to 0.0g/ml of extract.

This data was from three experiments with three replicas in each experiment. Each replica had 6 flies. Mean learning pass rate in percentage (y-axis) was plotted against time (hours) of exposure to 0.0g/ml of extract. Error bars represent the standard error of mean. ANOVA p-value < 0.05; student's t-test for learning comparisons of GMR-GAL4>UAS-para against that for flies that do not overexpress para at 0.0g/ml; comparisons for GMR-GAL4>UAS-para against GMR-GAL4; × p-value < 0.05; comparisons for GMR-GAL4>UAS-para against UAS-para; ● p-value < 0.05.

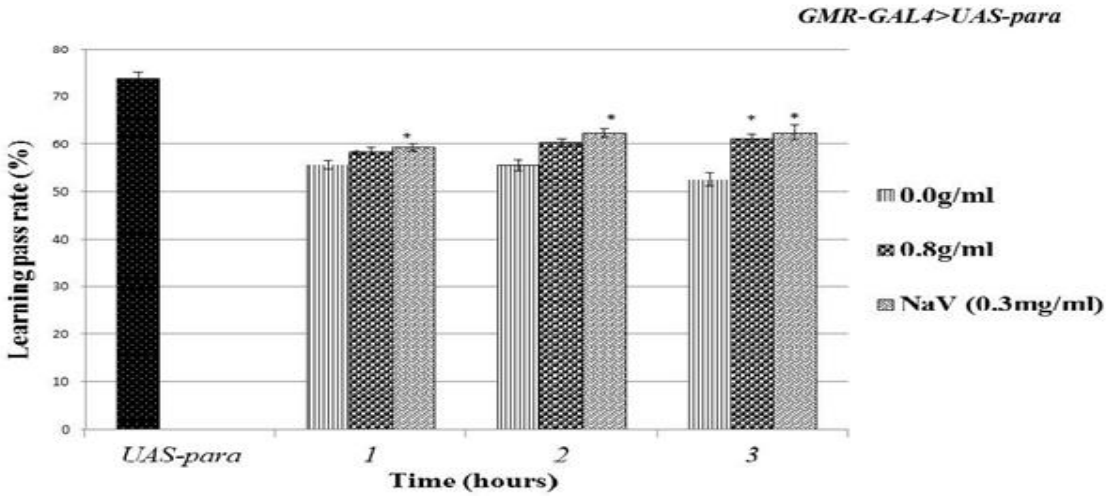

Figure 2: Learning pass rate variations for flies overexpressing paralytic gene in single exposure treatments Learning pass rates for GMR-GAL4>UAS-para Drosophila melanogaster flies on prolonged exposure to extract and standard anti-epileptic drug.

This data was from three experiments with three replicas in each experiment. Each replica had 6 flies. Mean learning pass rate in percentage (y-axis) was

plotted against time (hours) of exposure to the extract and NaV (standard anti-epileptic drug). Error bars represent the standard error of mean. ANOVA p-value < 0.05; student's t-test for learning pass rate in-comparisons against negative control; * p-value < 0.05; learning pass rate in-comparisons at 0.8g/ml and 0.3mg/ml of sodium valproate; # p-value < 0.05.

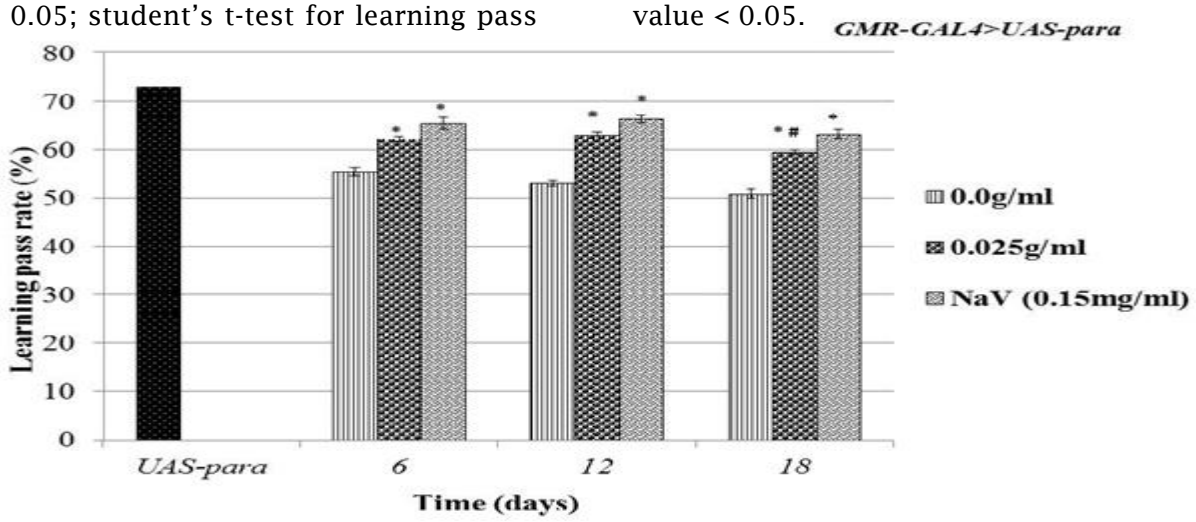

Figure 3: Learning pass rate variations in prolonged exposure treatments at 0.0g/ml of extract. Learning pass rates for Drosophila melanogaster flies overexpressing paralytic gene (GMR-GAL4>UAS-para) and those that do not overexpress paralytic gene (GMR-GAL4 and UAS-para) on prolonged exposure to 0.0g/ml of extract.

This data was from three experiments with three replicas in each experiment. Each replica had 6 flies. Mean learning pass rate in percentage (y-axis) was plotted against time (days) of exposure to 0.0g/ml of extract. Error bars represent the standard error of mean. ANOVA p-value < 0.05; student's t-test for learning pass rate comparisons of GMR-GAL4>UAS-para against that for flies that do not overexpress para at 0.0g/ml; comparisons for GMR-GAL4>UAS-para against GMR-GAL4; × p-value < 0.05; comparisons for GMR-GAL4>UAS-para against UAS-para; • p-value < 0.05.

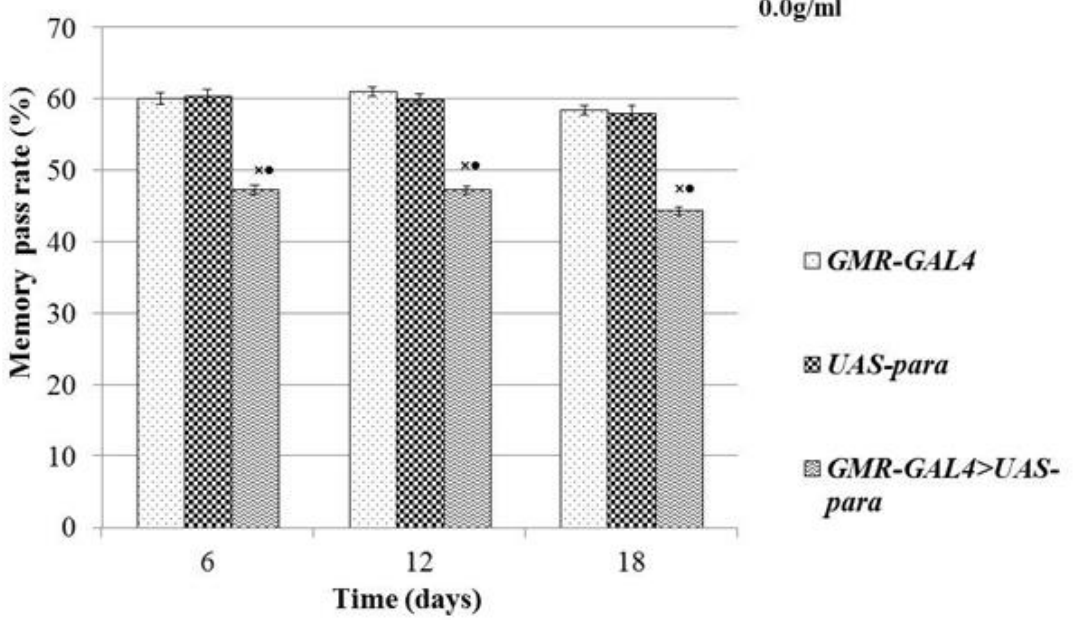

Figure 4: Learning pass rate variations for flies overexpressing paralytic gene in prolonged exposure treatments. Learning pass rates for GMR-GAL4>UAS-para flies on prolonged exposure to extract.
This data was from three experiments with three replicas in each experiment. Each replica had 6 flies. Mean learning pass rate in percentage (y-axis) was plotted against time (days) of exposure to the extract and NaV (standard anti-epileptic drug). Error bars represent the standard error of mean. ANOVA p-value <0.05; student's t-test for learning pass rate in-comparisons against negative control; * p-value < 0.05; learning pass rate in-comparisons at 0.0g/ml and 0.15mg/ml of sodium valproate; # p-value < 0.05.

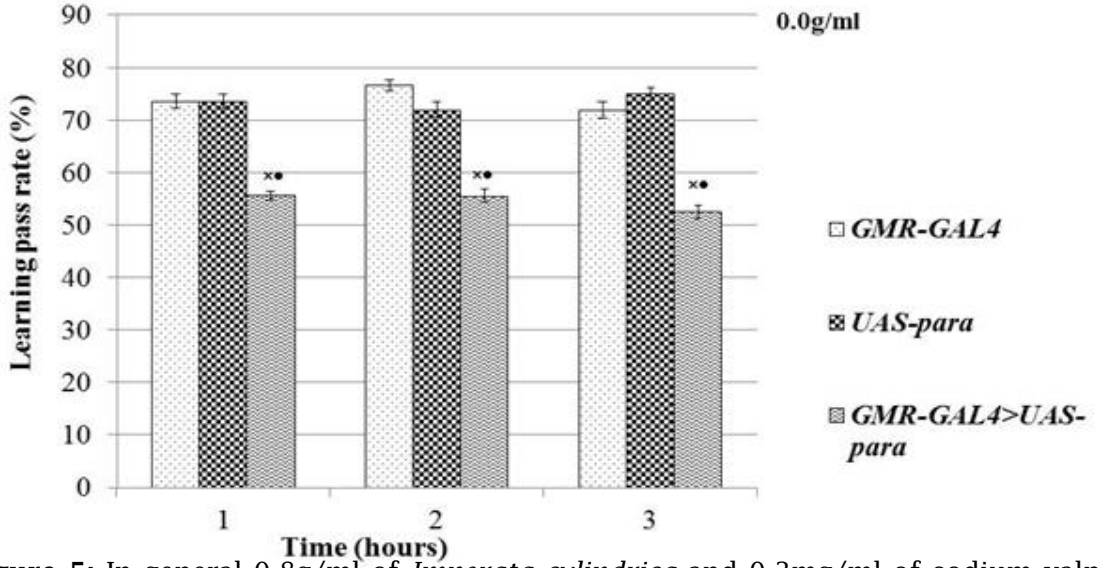

Figure 5: In general 0.8g/ml of *Imperata cylindrica* and 0.3mg/ml of sodium valproate did not affect memory in the flies that do not overexpress paralytic gene (control flies). *Imperata cylindrica* gave 6% memory retrieval and sodium valproate retrieved memory by 8% in the flies overexpressing paralytic gene.

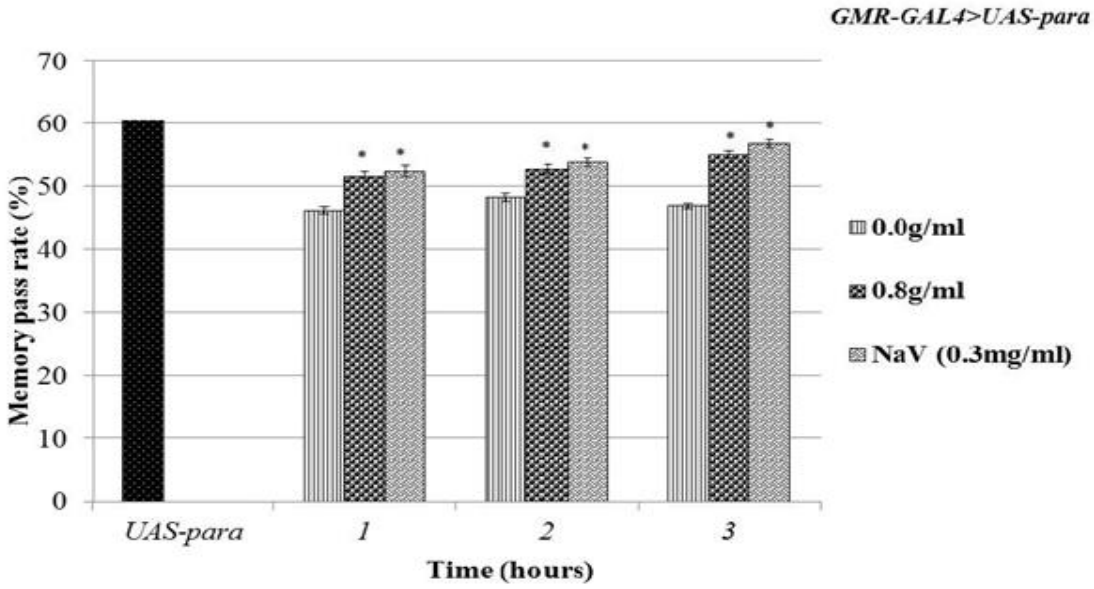

Figure 6: Memory pass rate variations for flies overexpressing paralyticgene in single exposure treatments.

Memory pass rates for GMR-GAL4>UAS-para Drosophila melanogaster flies on single exposure to extract. This data was from three experiments with three replicas in each experiment. Each replica had 6 flies. Mean memory pass rate in percentage (y-axis) was plotted against time (hours) of exposure to the extract and NaV (standard anti-epileptic drug). Error bars represent the standard error of mean. ANOVA p-value < 0.05; student's t-test for memory pass rate in-comparisons against negativecontrol; * p-value < 0.05; memory pass rate in-comparisons at 0.0g/ml and 0.3mg/ml of sodium valproate; # p-value < 0.05.

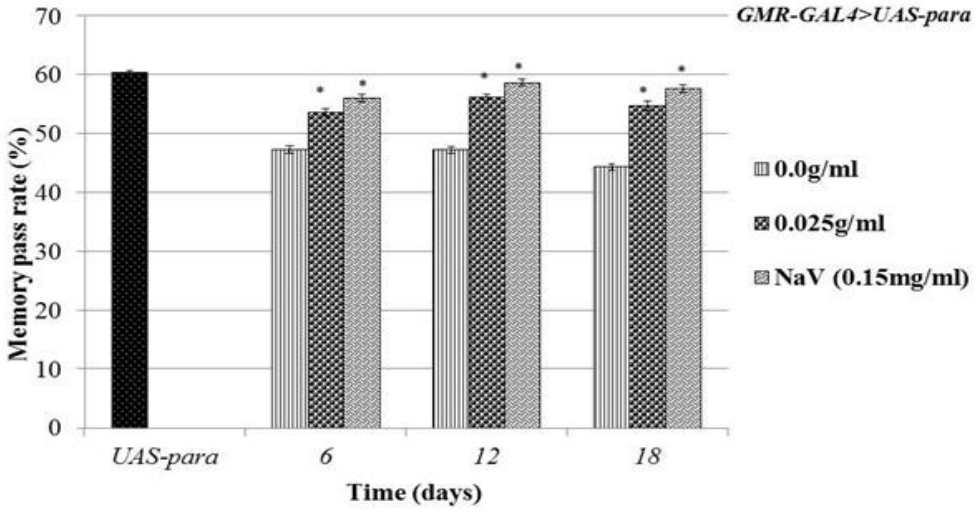

Figure 7: Memory pass rate variations in prolonged exposure treatments at 0.0g/ml of extract.

Memory pass rates for Drosophila melanogaster flies overexpressing paralytic gene (GMR-GAL4>UAS-para) and those that do not overexpress paralytic gene (GMR-GAL4 and UAS-para) on prolonged exposure to 0.0g/ml of extract. This data was from three experiments with three replicas in each experiment. Each replica had 6 flies. Mean memory pass rate in percentage (y-axis) was plotted against time (days) of exposure to 0.0g/ml of extract. Error bars represent the standard error of mean. ANOVA p-value < 0.05; student's t-test for learning pass rate comparisons of GMR-GAL4>UAS-para against that for flies that do not overexpress para at 0.0g/ml; comparisons for GMR-GAL4>UAS-para against GMR-GAL4; × p-value < 0.05; comparisons for GMR-

GAL4>UAS-para against UAS-para; ● p-value < 0.05.

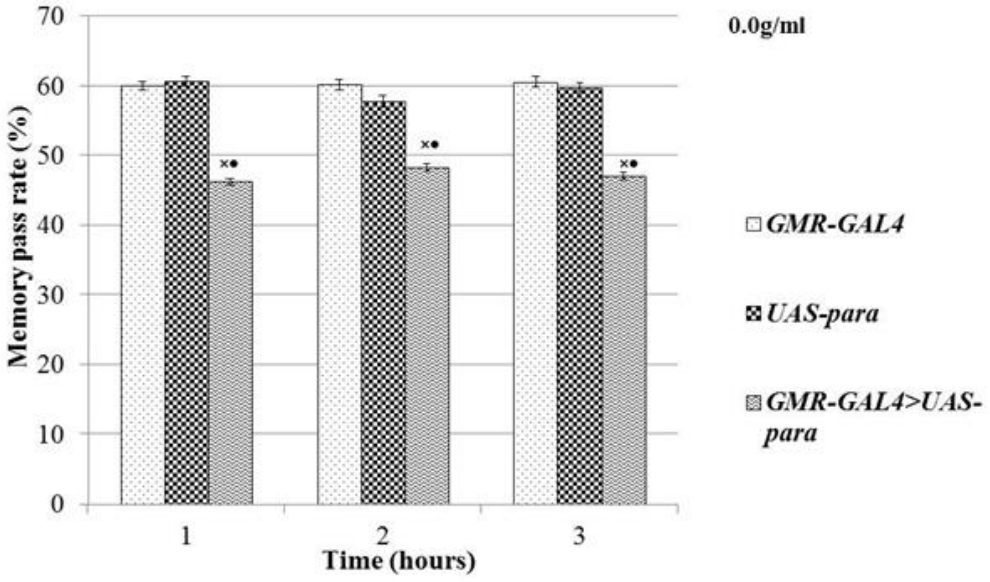

Figure 8: Memory pass rate variations for flies overexpressing paralytic gene in Prolonged Exposure treatments.

Memory pass rates for GMR-GAL4>UAS-para Drosophila melanogaster flies on prolonged exposure to extract. This data was from three experiments with three replicas in each experiment. Each replica had 6 flies. Mean memory pass rate in percentage (y-axis) was plotted against time (days) of exposure to the extract and NaV (standard anti-epileptic drug). Error bars represent the standard error of mean. ANOVA p-value < 0.05; student's t-test for memory pass rate in-comparisons against negative control; * p-value < 0.05; memory pass rate in-comparisons at 0.025g/ml and 0.15mg/ml of sodium valproate; # p-value < 0.05.

DISCUSSION

Our interest was to assess the effect of the therapeutic concentrations of I. cylindrica on the defective learning and memory in our transgenic model for epilepsy following single and prolonged exposure of the transgenic flies to the extract. Overexpression of the para gene led to 19% reduction in performance in the learning test and to13% reduction in memory performance in single exposure treatments. Similarly, overexpression of the para gene led to 18% reduction in performance in the learning test and reduced memory performance by 14% in prolonged exposure treatments. Therefore, GMR-GAL4>UAS-para flies show reduction in learning and memory performance similar to the established parabss model for seizures; therefore these flies reproduce the phenotype of the established model [8]. In a study to elicit how overexpression of the para gene in parabss and in other bang-sensitive mutant flies impacts on learning and memory performance, it was revealed that the gene causes disruption of metabolites and metabolic pathways that affect the Bangsensitive (bss)

D. melanogaster seizure mutants. This increased metabolic disruption was associated with seizure susceptibility in these flies which in turn interferes with learning and memory [9] a finding similar to that seen in our study. This concurs with earlier findings from our study where it was suggested that overexpression of the para gene could have caused higher amounts of neuronal activity that spread first to the motor brain center and subsequently to the learning and memory brain center (the mushroom body) [10]. This could be the cause for interference in learning and memory pathways of the flies since cognitive functioning in bang-sensitive

Paralytic flies are found to be more severely affected during phases of high electrophysiological activity in these flies which leads to reduced general welfare of the flies in concentrating to learn and memorise [11].

In general therapeutic doses of I. cylindrica and NaV did not affect learning and memory in the flies that do not overexpress the para gene (control flies) in single and prolonged exposure treatments. This suggests that therapeutic doses of I. cylindrica and NaV are not toxic in learning and memory performance in normal flies. This could be suggesting presence of intact homeostatic control pathways that ensure stability of function in the wild-type flies [12]. In single exposure treatments, I. cylindrica improved learning and memory by 5% and 6% respectively and NaV improved the parameters by 6% and 8% respectively in the flies overexpressing the para gene. In prolonged exposure treatments, I. cylindrica improved learning and memory by 9% and NaV improved the parameters by 12% in the flies overexpressing the para gene.I. cylindrical and NaV are seen to be partially rescuing learning and memory defects and have similar potential which could be suggesting related mechanisms of action. I. cylindrica has the ability to reduce the activity of Na+ and K+ channels [2] and NaV is a stardard Na^+ channel blocker [13]. The reduction in activity of Na^+ and K^+ channels by the extract and Na+ channel blockade by NaV both modulate neuronal electrophysiological activity in the brain of the flies by increasing the threshold level of neuronal stimulation making the neurons less easily stimulated. This in turn reduces occurrence of seizures in the brain and convulsions in muscles [11]. The end result is stabilization of the learning and memory pathways in the mushroom bodies and improvement in the general welfare of the flies to be able to coordinate learning and memory activities better [14; 10]. The result is improved 'general well-being' and concentration of the flies to be able to learn better [11].

The ability to inhibit spread of seizures and improvement in the 'general well-being' of the flies proposed in our study could be one mechanism put forward to explain the improvement in life-span; in the frequency of the resting membrane potential of muscles and the occurrence of the mild negative geotaxic effect seen in the climbing seen in the mutant epileptic fly models (parabss) in a previous study by [2]. In general, our findings concur with studies in Drosophila models for seizures that have revealed that anti-seizure drugs have the ability to ameliorate bss behaviour including muscular activity as well as cognitive function in these flies [15; 11]. However, there was only partial rescue of the learning and memory defects in the transgenic flies following treatment with the extract and NaV; the reason for this occurence is beyond the explanation of this study.

Another mechanism put forward to explain how learning and memory might have been improved by the extract was explored in the current study as discussed in detail in 5.2.3 of this dissertation. The extract and NaV were able to improve the abnormal brain neuroanatomical defects (demyelination and brain vacuolation) after treating the flies with the extract and NaV for several days but no improvement in brain cell biology was seen following acute exposure treatments. This is an indicator that both the extract and NaV act to relieve symptoms related to the defective learning and memory in acute exposure treatments but act by curing the defective brain cell biology in chronic exposure treatments. This reversal of the progressive neurodegeneration and demyelination by the extract and standard anti-epileptic drug could be associated with the improvement in histomorphological nature of the mushroom bodies which in turn ensures that the numerous learning and memory pathways located here function better to improve learning and memory function [16]. Studies have shown that I. cylindrica and sodium valproate are neuroprotective in Drosophila models of seizures [17] and sodium valproate is a known mood stabilising drug [18]; therefore it is not surprising to see these two molecules having a positive impact on learning and memory function. Going forward, one possibility that is worth exploration is

whether this improvement in the general well-being or health coupled with amelioration of brain neuroanatomical defects and improvement in learning and memory performance could also be extrapolated to the increase in life-span; negative geotaxis and reduced frequency in resting membrane potential of muscles previously seen with I. cylindrica extract.

CONCLUSION

Imperata cylindrica and sodium valproate ameliorate the basal membrane potential amplitude defects. Despite the fact that both sodium valproate and Imperata cylindrica extract have the ability to improve the basal membrane potential defects in flies overexpressing paralytic gene, sodium valproate was a better modulator than the extract. GMR-GAL4>UAS-para flies show reduced performance in both learning and memory test; Imperata cylindrica and sodium valproate partially ameliorate the learning and memory defects of the flies overexpressing paralytic gene in single exposure treatments. Overexpression of paralytic gene leads to reduction of learning and memory function being aggravated with time; Imperata cylindrica and sodium valproate partially ameliorate the learning and memory defects and the effect increases with time.I. cylindrica extract seems to be relieving symptoms in acute exposure treatments and curative by improving brain cell biology which in turn ameliorates muscular basal membrane potential, learning and memory defects following prolonged exposure treatments.

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