# Impact of oral supplementation of Glutamate and GABA on memory performance and neurochemical profile in hippocampus of rats

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**Abstract**: Glutamate (GLU) and gamma-amino butyric acid (GABA) are essential amino acids (AA) for brain function serving as excitatory and inhibitory neurotransmitter respectively. Their tablets are available in market for improving gut function and muscle performance. Despite of having a major role during memory formation and processing, effects of these tablets on brain functioning like learning and memory have not been investigated. Therefore, present study is aimed to investigate the effects of orally supplemented GLU and GABA on learning and memory performance and further to monitor related effects of these orally supplemented GLU and GABA on brain levels of these AA. Three groups of rats were supplemented orally with drinking water (control group) or suspension of tablets of GABA and Glutamate, respectively for four weeks. Cognitive performance was determined using behavioral tests (Novel object recognition test, Morris water maze, Passive avoidance test) measuring recognition, spatial reference and aversive memory. Levels of GLU, GABA and acetylcholine (ACh) were estimated in rat hippocampus. Results showed that chronic oral administration of GLU and GABA tablets has a significant impact on brain function and can alter GLU and GABA content in rat hippocampus. Compared to GABA, GLU supplementation specifically enhances memory performance via increasing ACh. Thus, GLU can be suggested as a useful supplement for improving learning and memory performance and neurochemical status of brain and in future could be effective in the treatment of neurological disorders affecting learning and memory performance.

**Keywords**: Learning, Memory, Glutamate, GABA

## INTRODUCTION

Amino acids (AA), the building blocks of proteins, are required for adequate central nervous system (CNS) functioning (Lieberman, 2000) either as a precursor of neurotransmitters and neuromodulators (like glutamine, tryptophan, tyrosine) or some itself serve as a neurotransmitter like glutamate (GLU) and GABA which are the major excitatory and inhibitory neurotransmitters (Hou *et al.,* 2015). GLU and GABA are found to be involved in variety of functions and any disturbance in their content and balance leads to multiple neurological disorders (Bak *et al.,* 2006). Evidence suggested that for regulation of CNS neurotransmission peripheral concentration of particular AA is thought to be an important factor (Lieberman, 2000). Reports have shown the involvement of GLU and GABA during learning and memory functioning (Tapiero *et al.,* 2002; Thanapreedawat *et al.,* 2013) but very few studies have examined the impact of oral supplementation of these AA on learning and memory performance in either animals or humans because it has long been thought that systemic or oral administration of these AA cannot affect their availability in CNS as GLU and GABA cannot cross the blood brain barrier (BBB) (Hertz *et al.,* 2003). But later researchers found that this concept was wrong as dietary supplements of AA improve the functioning of body and brain (Asrani *et al.,* 2013; Stamoula *et al.,* 2015). In addition, some studies reported that their crossing was possible up to certain quantity and can enter the brain when given in quantities higher than this (Sklenovský, 1967; Boonstra *et al.,* 2015). Although supplements of GLU and GABA are available in market but for other purposes, like GABA supplements are used to treat anxiety, pain, to promote lean muscle growth and to stabilize blood pressure (Aanesen *et al.,* 1995) while GLU is pharmacologically useful to maintain blood glucose level, building skeletal muscle, protects the integrity of gastrointestinal (GI) tract, essential for proper immune functioning other than this it also used as a buffering agent, surfactant, chelating agent (Dharmananda, 2000; Dutta *et al.,* 2013). But effect of their supplementation on process of learning and memory is less likely addressed. So, present study was designed to address this question and aimed to elucidate the relationship between oral AA (GLU and GABA) supplementation and brain functioning in animals. For this we have monitored the effects of chronic oral supplementation of GLU and GABA tablets on cognitive performances and associated alterations in hippocampal GLU and GABA levels to determine whether oral AA supplementation could affect levels of GABA and GLU in hippocampus.

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## MATERIALS AND METHODS

### *Animals*

Twenty-four locally bred Albino-Wistar rats purchased from Dow University of Health Sciences, OJHA campus, Karachi, Pakistan, were used in the study. The animal housing conditions and handling protocols were the same as mentioned previously (Haider *et al.,* 2016; Tabassum and Haider, 2016). All animal experiments were approved by the institutional ethics and animal care committee and performed in strict accordance with National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised1985). All treatment and behavioral monitoring were done in a balanced design to avoid order and time effect.

### *Drugs*

The commercially available Glutamate (Solgar, USA) and GABA (Swanson, USA) tablets purchased from Kousar Medicos were used during the experiments. All chemicals were of analytical grade. The reagents were prepared fresh before starting the experiment. Drug solutions for administration were made fresh each day. Tablets were dissolved in distilled water. Controls received equal volume of distilled water. All drugs were administered via oral route at their respective doses for 1 month in estimated volume of 0.2ml/150g body weight to each animal. The dose selection was based on the prescription given on the tablet. The study was carried out for a period of 5 weeks.

### *Experimental protocol*

The animals (n=18) (age, 2months, weight, 150-200 g) were randomly divided into three experimental groups each containing 6 rats (n=6) which are as follows:

- *Group 1 (Control):* Received distilled water daily.
- *Group 2 (GABA group):* Received aqueous suspension of GABA tablets via oral route at the dose of 154mg/kg body weight daily for 5 weeks.
- *Group 3 (Glutamate (GLU) group):* Received aqueous suspension of glutamate tablets via oral route at the dose of 103mg/kg body weight daily for 5 weeks.

At the end of 4 weeks' treatment schedules, the behavioral studies were carried out as mentioned in the fig. 1. Behavioral tests performed include Novel Object Recognition test (NORT) to determine recognition ability, Morris Water Maze Test (MWM) and Passive Avoidance Test (PAT) for monitoring the spatial reference and aversive learning and memory performance. After assessing the behavioral activities, rats were then decapitated, and their brains were removed from the skull within 30 s after decapitation. The membrane covering the brain was removed with the help of a fine forceps. The brain taken out using spatula was dipped in ice-cold saline and hippocampus was dissected out as described previously (Haider *et al.,* 2016).

### *Behavioral protocols/test protocols*

*Novel Object Recognition Task (NORT)*

NORT was established by Ennaceur and Delacour in 1988 and was used to assess the cognitive ability and recognition memory in rodents. It assesses the ability of rat to distinguish a novel (new) object in a familiar environment (Ennaceur and Delacour, 1988). The apparatus used and the procedure performed in this experiment was same as described by Haider *et al.,* (2016). The exploration time for the novel and familiar object was measured. Exploration of an object was defined as the rat's ability to sniff, lick, chew or by moving vibrissae while directing its nose towards the object at a distance of <2cm. The preference index (PI) between the two objects was calculated as described by Manchanda and Kaur (2017). It is the ratio of exploration time for new object and total exploration time for both familiar and novel objects.

### *Morris water maze test (MWM)*

MWM is a behavioral test that is used frequently to determine spatial memory in rodents (Morris, 1981). This test requires an animal to use spatial learning and memory to locate a hidden platform just below the surface of a circular pool of water and to remember its location as in the previous trial. The maze used for rats and procedure employed was similar as described by Haider *et al.,* (2016). Learning and memory during a probe trial, performed after a delay of 1 hr and 24 hr since the last acquisition trial, was measured by recording the time spent in the 'target quadrant (QT)' that previously contained the platform and the time spent in other three quadrants (Q1, Q2 and Q3). The retention measure computed was the accuracy ratio (AR) as described by Montarolo *et al.,* (2013). It is calculated as follows:

$$\text{Accuracy Ratio (AR)} = \frac{\text{time spent in the target quadrant (QT) X 3}}{\text{the time spent in the other three quadrants (Q1 + Q2 + Q3)}}$$

An AR=1 corresponds to chance level, indicating no preference for QT relative to the other quadrants. An AR=2 corresponds to 24 s spent in QT, while an AR=3 corresponds to 30 s in QT.

### *Passive avoidance test (PAT)*

PAT, a fear-aggravated test, assesses the aversive memory in rodents where an animal learns to suppress movement in order to avoid an aversive stimulus, thus it is used to assess memory function based on the association formed between an aversive stimulus such as a mild foot shock and a specific environmental context. In this test, rat learns to avoid an environment in which an aversive stimulus (foot-shock) was previously delivered. The paradigm used and the protocol of the task performed was the same as presented previously (Haider *et al.,* 2016). The step-through latency (STL) that indicates the time elapsed before the rat entered the dark compartment was recorded in both training and test sessions to test the retention of the passive avoidance learning.

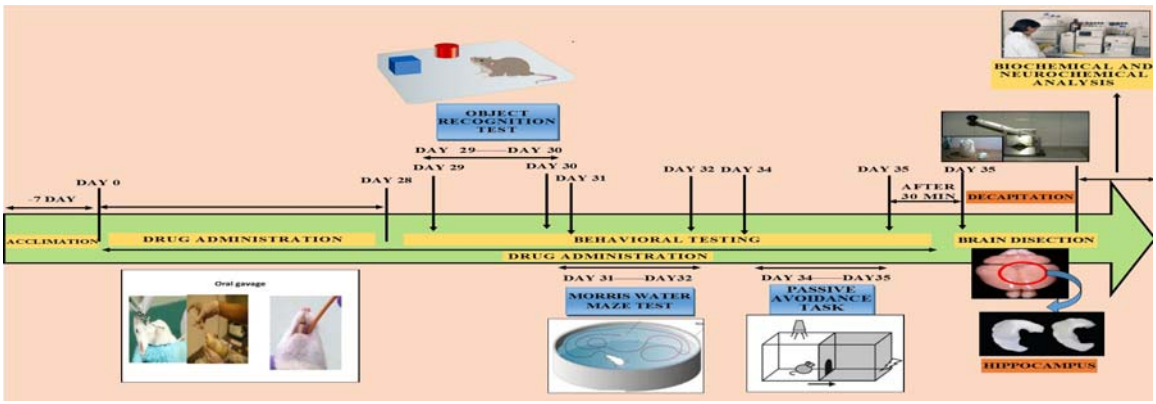



**Fig. 1**: Schematic representation of treatment schedule and Experimental design

The retention scores were obtained for each animal by calculating the inflexion ratio (IF) as described by Ramanathan *et al.,* (2003). The formula for the IF was as follows;

$$\text{Inflexion ratio (IF)} = \frac{\text{Step through latency in test session } (L_1) - \text{Initial step through latency } (L_0)}{\text{Initial step through latency } (L_0)}$$

***Neurochemical analysis***

Hippocampus samples (20□%) were extracted with 5 volume of PCA ($HClO_4$; 70 %) using a simple one-step sample preparation method after that samples were centrifuged at 10,000 rpm for 5 min and supernatant was collected for the assay of GLU and GABA content.

***Estimation of Hippocampus Glutamate (GLU) content***

GLU content in hippocampus is estimated using commercially available Cell Biolabs' Glutamate Assay Kit (STA-674) which is based on a quantitative fluorometric assay. The assay uses GLU-specific enzymes (i.e. Glutamate oxidase (0.08 U/mL) that converts glutamate to α-ketoglutarate and produces $NH_3$ as well as $H_2O_2$, and glutamate-pyruvate transaminase (0.5 U/mL) with L-alanine (200 µM) that regenerates GLU providing significant amplification of $H_2O_2$ production that reacts with sample (50 µL) to generate $H_2O_2$. In the presence of horseradish peroxidase (HRP) (0.2 U/mL), the Fluorometric Probe (100 µM) reacts with $H_2O_2$ to produce highly fluorescent Resorufin which is incubated for 30-45 minutes at 37ºC in dark. The Resorufin product can be easily read by a fluorescence microplate reader with an excitation of 530-560 nm and an emission of 590 nm. Fluorescence values are proportional to the GLU levels within the samples. The concentration of GLU within samples is determined by comparing the sample RFU with a standard curve obtained by using L-Glutamate Standard (20 mM solution).

***Estimation of Hippocampus GABA content***

Concentration of GABA in hippocampus is estimated using commercially available GABA ELISA kit (Bioassay Technology Laboratory, China). During the assay GABA (sample, 40 µl) is added to the wells pre-coated with GABA monoclonal antibody. Then a biotin-conjugated anti-GABA antibody (10 µl) is added that binds to GABA. After that Streptavidin-HRP (50 µl) is added and binds to the biotin-conjugated anti-GABA antibody. The plate was then incubated for 60 minutes at 37ºC. After incubation, a washing step was done to washed away unbound Streptavidin-HRP. Substrate solutions were added after washing step and plate was incubated again for 10 minutes at 37ºC in the dark for developing color proportional to the GABA content. The acidic stop solution was then added to terminate the reaction and absorbance is measured at 450nm using a microplate reader. Concentration of GABA within samples is determined by comparing the sample O.D with a standard curve obtained by using GABA Standard (640 µg/dl) solution.

***Estimation of Hippocampus Acetylcholine (ACh)***

ACh content in hippocampus samples was estimated by method of Hestrin (1949). The hippocampal tissue (0.08g) was boiled in pre-heated tubes in a boiling water bath for 10 min to inactivate the enzyme so that bound ACh was released. The boiled tissue was then homogenized in distilled water (0.8ml) using an electrical homogenizer and then 1ml of TCA (1.84M) was added into the homogenate. The 0.4 ml of homogenate was then mixed with an equal volume of alkaline hydroxylamine hydrochloride (14%) solution and incubated at room temperature for 10 min. After incubation, dilute HCl (0.4ml, 50%) was added into the homogenate mixture and centrifuged at 3500 rpm for 10 min at 4°C. The supernatant was collected and mixed with 0.2 ml of acidic ferric chloride solution (0.37M) resulting in reaction of ACh with ferric chloride to develop brown color that read immediately at 540 nm against the reagent blank. The tissue ACh content was determined using molar extinction coefficient (1005) and is represented as nmol/g of tissue.

## STATISTICAL ANALYSIS

All the data are presented as mean ± S.D. The statistical analysis was performed by using SPSS software version

20.0 for windows program on a computer. The results of behavioral and neurochemical assessment were analyzed by one-way analysis of variance (ANOVA) for the three experimental groups followed by Tukey's *post hoc* test for multiple comparisons. The significance level for all comparisons was set at $p<0.05$. Differences in experimental groups were determined by two-tailed analysis of variance

## RESULTS

The current study aims to investigate the effects of chronic oral supplementation of commercially available GABA and GLU tablets on learning and memory performance along with determining relationship between oral AA (GLU and GABA) supplementation and alterations in hippocampal levels of GLU and GABA which may help in future to use these tablets for cognitive aspects other than the cause for which these are available and secondly to propose their use for future experiments. Effect of these tablets on spatial and non-spatial learning and memory performance and cognitive functions was assessed during this study using memory specific behavioral tasks such as ORT, MWM and PA. Recognition memory of all experimental groups was assessed by ORT in terms of sniffing time for novel and familiar (Old) objects and then by evaluating PI which is presented in fig. 2. It was observed that recognition memory was improved following both GABA and GLU supplementation with varying degree as evident by increased exploration time for new object and enhancement in PI. Data analysis of PI by one-way ANOVA showed significant ($F(2, 17) = 243.356$, $p<0.0001$) differences between groups. Post-hoc comparisons by Tukey's test indicated significant increment in PI in GABA ($0.79\pm0.026$, $p<0.01$) and GLU ($0.93\pm0.012$, $p<0.01$) compared to control group ($0.68\pm0.018$) as shown in fig. 2.

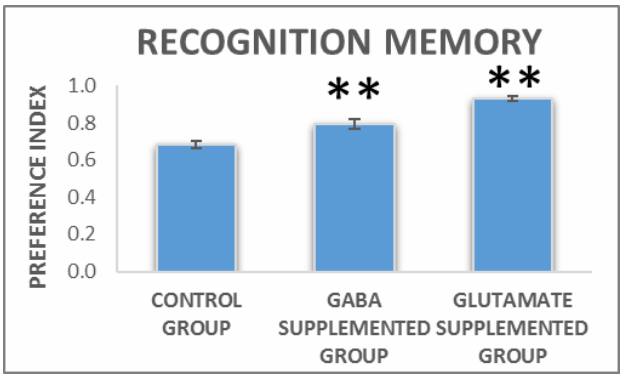
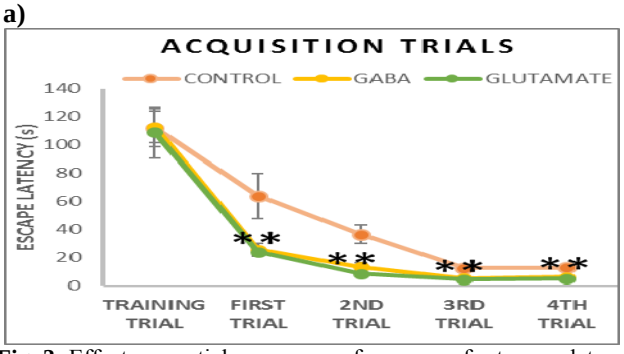



**Fig. 2**: Effect on recognition memory was evaluated by NORT in terms of sniffing time for familiar (old) and novel object during 3 min choice phase then for determining animal's recognition ability preference index was calculated. For each group n=6 and values are presented as mean ± S.D. Significant differences were obtained by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons and expressed as *=p<0.05, **=p<0.01, ***=p<0.001 compared to control group.

Effect on spatial cognitive abilities of animals was determined by using MWM task. Animals from each experimental group were exposed to 4 acquisition training trials in MWM following initial training trial. To find the difference in escape latencies of animals during 4 acquisition trials two-way ANOVA (repeated measures) was performed. Results indicated significant effect of trials ($F(4, 60) =554.469$, $p<0.0001$), group ($F(2, 15) =32.77$, $p<0.0001$) and interaction between trials x groups ($F(4, 60) =8.161$, $p<0.001$). Pair-wise comparisons by Bonferroni test revealed that escape latencies of animals started decreasing significantly ($p<0.001$) over trials (shown in fig. 3a) indicating that memory performance was enhanced in animals over trials. Pair-wise comparisons on escape latencies among groups showed that there was a significant decline ($p<0.001$) in escape latencies in both GLU and GABA group compared to control group. Reference memory and cognitive performance was examined by performing the probe trials (free swimming without platform) in MWM tank. In our study, we performed two probe trials; one was performed 1hr after the last training trial while the other was performed 24hr after the last training trial via monitoring the time spent in the QT and the other quadrants and then by computing AR. Data analysis of AR by one-way ANOVA showed significant differences between groups ($F(2, 17) =20.399$, $p<0.0001$) during 1hr probe trial and ($F(2, 17) =16.744$, $p<0.0001$) during 24hr probe trial as shown in fig. 3. Post-hoc comparisons by Tukey's test revealed significant enhancement in AR in GLU group after 1 hr ($2.028\pm0.325$, $p<0.01$) compared to control group ($1.138\pm0.178$) and after 24-hr ($1.68\pm0.14$, $p<0.01$) compared to control group ($1.123\pm0.07$) while the performance of GABA group remained comparable to control in both 1hr ($1.304\pm0.245$, $p=0.514$) and 24 hr ($1.14\pm0.288$, $p=0.986$) probe trial sessions as shown in fig. 3b.

Aversive memory of all experimental groups was assessed by PAT in terms of STL to enter the dark compartment during both training and test sessions and the IF was computing which was presented in fig. 4. It was observed that aversive memory was improved following treatment with GLU and GABA as evident by increase in STL and IF. Data analysis of IF evaluated after 1 hr of last training trial by one-way ANOVA revealed significant ($F(2, 17) = 39.026$, $p<0.0001$) differences between groups. Post-hoc comparisons by Tukey's test indicated significant increment in IF of GLU ($17.604\pm3.69$, $p<0.01$) compared to control group ($9.836\pm2.06$) while a significant decline in IF was seen following GABA ($4.82\pm1.08$, $p<0.01$) treatment as shown

a)

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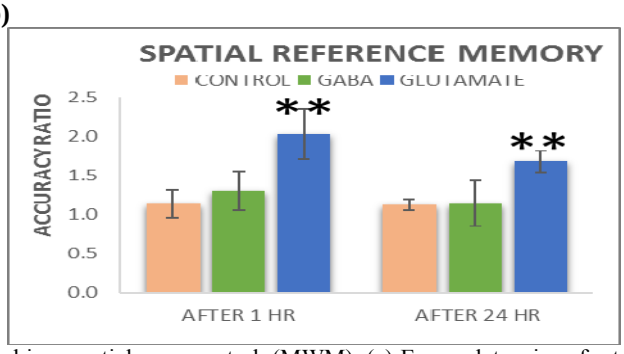


**Fig. 3**: Effect on spatial memory performance of rats was determined in a spatial memory task (MWM). (a) Escape latencies of rats in a spatial memory task during exposure to acquisition training trials which were measured for 120 seconds for each trial. (b) Effect of treatment on spatial memory performance of after exposure to training trials on spatial memory task (MWM) assessed by performing probe trial measured for 120 s in MWM after 1 h (acquisition) and 24 h (retention) of last acquisition trial. Spatial memory performance in acquisition and retention sessions was evaluated by monitoring duration of time spent (sec) in the target quadrant (NW) in absence of platform and presented by computing accuracy ratio. For each group n=6 and values are presented as mean ± S.D. Significant differences were obtained by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons and expressed as *=p<0.05, **=p<0.01 compared to control group. For training trials 2-way ANOVA (repeated measures) was performed followed by Tukey's post hoc test for multiple comparisons and significant differences were presented as *=p<0.05, **=p<0.01 compared to trial 1.

in fig. 4. Data analysis of IF evaluated after 24 hr of last training trial by one-way ANOVA revealed significant ($F(2, 17) = 45.904$, $p<0.0001$) differences between groups. Post-hoc comparisons by Tukey's test indicated significant increment in IF of GLU (17.205±3.297, $p<0.01$) group compared to control group (10.85±0.757) while following GABA (5.71±1.24, $p<0.01$) treatment a significant decline in IF was seen as shown in fig. 4.

These results suggest that associative memory was improved following intake of GLU while impaired following supplementation of GABA tablets.

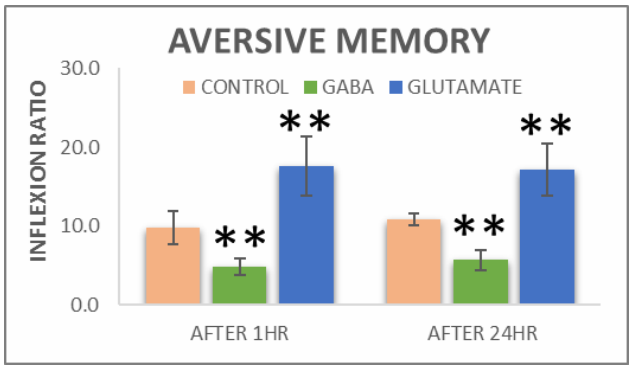


**Fig. 4**: Effect of treatment on aversive memory performance of rats was assessed in PAT for duration of 3 min by recording the step-through latencies (the time taken by rats to enter aversive stimulus associated dark compartment) during initial training trial, during acquisition phase performed after 60 minutes of initial training trial and during retention phase performed after 24hr of initial training trial. The inflexion ratio was computed for each animal for determining retention ability of rats during both acquisition (after 1 hr) and retention (after 24 hr) sessions. For each group n=6 and values are presented as mean ± S.D. Significant differences were obtained by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons and expressed as *=p<0.05, **=p<0.01 compared to control group.

Concentration of GLU and GABA in hippocampus of all experimental groups was determined by spectrophotometric and fluorometric assay ELISA kits. Significant alterations in GLU and GABA content in hippocampus was observed following their supplementation. Data analysis by one-way ANOVA revealed significant differences between groups for GLU ($F(2, 17) = 35.103$, $p<0.0001$) and GABA ($F(2, 17) = 20.954$, $p<0.0001$). Post-hoc comparisons by Tukey's test indicated significant increment in GLU levels in GLU supplemented group (25.55±2.07, $p<0.01$) compared to control group (20.92±1.57) while decreased following GABA (16.857±1.71, $p<0.01$) supplementation as shown in fig. 5a. Post-hoc comparisons on hippocampal GABA content by Tukey's test indicated significant enhancement in GABA levels in both GLU supplemented (25.545±3.83, $p<0.01$) and GABA supplemented (19.123±3.403, $p<0.05$) group compared to control group (12.73±3.01) as shown in fig. 5b.

Acetylcholine (ACh) content in hippocampus of all experimental groups was determined by spectrophotometric analysis. It was observed that ACh content was enhanced following treatment with GLU. Data analysis by one-way ANOVA revealed significant ($F(2, 17) = 62.947$, $p<0.0001$) differences between groups. Post-hoc comparisons by Tukey's test indicated significant increment in ACh levels in GLU supplemented group (1582.78±284.36, $p<0.01$) compared to control group (668.62±55.2) while in GABA supplemented (498.42±115.49, $p=0.344$) group no significant change in ACh levels was observed as shown in fig. 6.

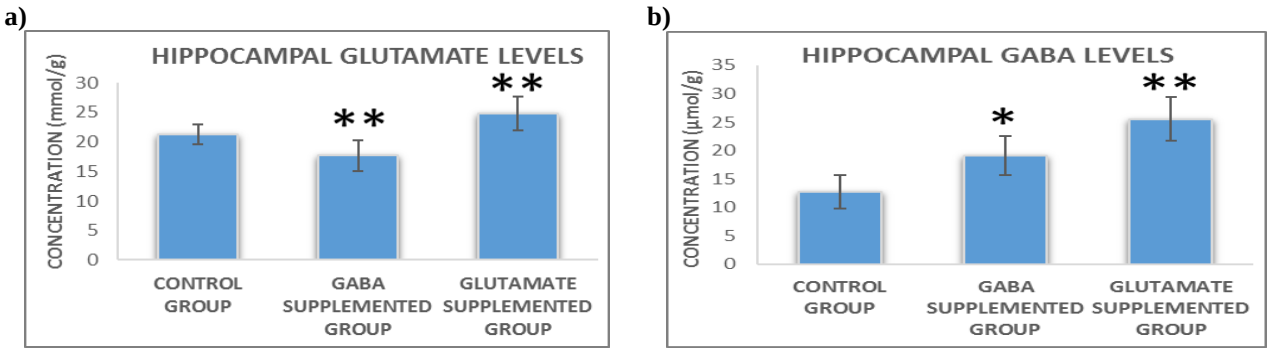


**Fig. 5**: Effect on Glutamate content (a) and GABA levels (b) in hippocampus following GLU and GABA supplementation was determined by fluorometric assay. For each group n=6 and values are presented as mean ± S.D. Significant differences were obtained by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons and expressed as *=p<0.05, **=p<0.01 compared to control group.

## DISCUSSION

The current study showed that chronic dietary supplementation of GLU and GABA altered the learning and memory performance via modulating the neurochemical status in brain. In addition, we found that GLU supplementation was more effective in improving learning and memory performance across all three domains; recognition, spatial reference and aversive memory. Current findings are in agreement with the studies that reported improved functioning of body and brain following dietary AA supplementation (Stamoula *et al.,* 2015, Dutta et al., 2013) while contradict the studies which showed that dietary supplementation of aminergic neurotransmitter substances is ineffective as they cannot cross the BBB (Hertz *et al.,* 2003; Albrecht *et al.,* 2010). Although the dietary supplements of these AA are available in market and are being used for treatment of various disorders but their effect on learning and memory performance has not been addressed. The current study focuses on whether dietary AA supplementation would be effective in modulating brain functioning and neurochemical status or not. For this purpose, dietary supplements of GLU and GABA were given orally to rats for 4 weeks and changes in their behavioral performance and alterations in neurochemical status of brain were monitored. Evidence shows that ability to learn and remember is essential for our survival (McGaugh, 2013) as memory is the process of acquiring, storing and retrieving information (Kandel and Schwartz, 1982) and AA neurotransmitter shown to play an important role in controlling learning and memory processes (Liu *et al.,* 2010). Present findings showed that supplementation of GLU and GABA improved the learning and memory function which was more pronounced following GLU supplementation across all three domains (recognition, spatial and aversive memory). Results showed that following GLU and GABA supplementation recognition memory was improved evident by increment in preference index suggesting that chronic GLU and GABA supplementation is beneficial to enhance recognition capacity and recognition memory. These results are in agreement with previous reports that found enhancement in recognition index and recognition memory following GABA administration (Thanapreedawat *et al.,* 2013). Present results also showed marked improvement in spatial reference memory and cognitive ability of rats in both time frames (1hr and 24hr probe trials) following chronic GLU and GABA supplementation evident by significant decline in escape latency during acquisition trials and significant enhancement in AR and time spent in target quadrant during probe trials. This improvement was more pronounced following GLU supplementation which is consistent with reports that showed contribution of GLU to learning and memory through use-dependent changes in synaptic efficacy (Tapiero *et al.,* 2002). The observed improvement in memory performance in this study was attributed to rise in GLU/GLN content and enhancement in glutamatergic activity which is implicated in memory formation and consolidation (Hertz *et al.,* 2003).

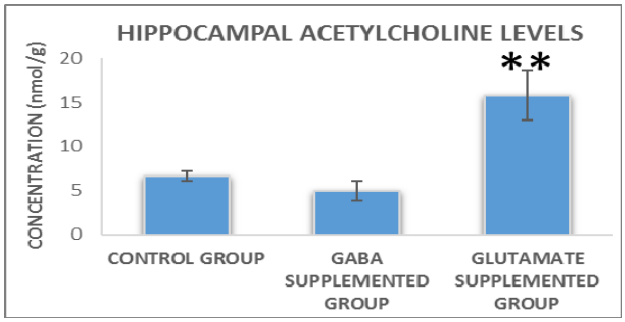


**Fig. 6**: Effect on cholinergic neurotransmission was assessed via determining the levels of ACh content following GLU and GABA supplementation. For each group n=6 and values are presented as mean ± S.D. Significant differences were obtained by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons and expressed as *=p<0.05, **=p<0.01 pared to control group.

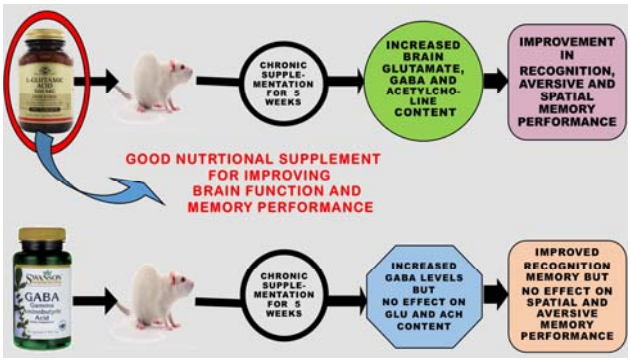


**Fig. 7**: Diagrammatical representation of proposed findings of the study.

Moreover, regarding the impact of GLU and GABA supplementation on aversive memory performance present findings showed a marked rise in learning acquisition and retention as there was a significant enhancement in IF during both acquisition and retention session following GLU supplementation while following GABA supplementation reduction in aversive memory performance was observed. Results of behavioral analysis showed that although recognition, spatial and aversive memory performance was improved following both GLU and GABA supplementation but was more prominent following GLU administration. Further, to understand the underlying mechanism responsible for this improvement, neurochemical alterations was determined via assessing levels of GLU and GABA in the hippocampus which is recognized as an important region implicated in learning and memory (Abu-Taweel *et al.,* 2013). Alterations in aminergic neurotransmission was monitored via assessing the content of GLU and GABA in hippocampus following GLU and GABA supplementation to find out whether oral AA supplementation is responsible to induce neurological alterations in brain. It is evident that in the CNS, GLU serves as a major excitatory neurotransmitter while GABA carrying the inhibitory signals and an adequate balance between fast excitatory and slower inhibitory signals is required for memory formation (Staubli *et al.,* 1994). Present findings showed an enhancement in GLU and GABA levels following chronic GLU supplementation while following GABA supplementation hippocampal GABA content was increased but GLU content was declined. The improvement in learning and memory function observed in present study may be attributed to the enhancement in aminergic neurotransmission possibility of alterations in hippocampal GLU and GABA levels following chronic dietary intake of these AA. The ability of GLU and GABA to cross the BBB is controversial. Previously it was assumed that dietary intake of GLU and GABA was unable to affect the brain functioning as it was assumed that GLU and GABA were not able to cross the BBB (Hertz *et al.,* 2003; Janeczko *et al.,* 2007; Albrecht *et al.,* 2010). But later researchers reported that dietary supplements of these AA improved the functioning of body and brain (Asrani *et al.,* 2013, Stamoula *et al.,* 2015) and they can cross the BBB but in specific amount (Sklenovský, 1967; Boonstra *et al.,* 2015, Mazzoli and Pessione, 2016). The improvement in GLU and GABA content was more pronounced following GLU supplementation as it improved the balance between GABA and GLU neurotransmission in hippocampus as GLU/GABA ratio has been implicated in various neurological disorders. Increases in glutamatergic neurotransmission observed in present study are supported by reports that showed enhancement in memory formation and encoding via facilitation of glutamatergic transmission (Staubli *et al.,* 1994). It is thought to be involved in regulating neurogenesis and neuronal plasticity (Mattson, 2008) and plays important role in enhancing learning and memory as glutamatergic neurons are widely distributed in the hippocampus (Onaolapo *et al.,* 2016), hippocampus is therefore largely dependent on GLU signaling for learning and memory processing (Tamminga *et al.,* 2012). In addition to alterations in aminergic transmission we also observed alterations in cholinergic neurotransmitter content following GLU and GABA supplementation which further supports the results of behavioral and amino acid analysis. Evidence has shown that in cognitive function cholinergic neurons have a vital role (Brosnan and Brosnan, 2013) determined by variations in levels of ACh, the important neurotransmitter that play important role in memory retention and cognitive function regulation (Mufson *et al.,* 2007; Haider *et al.,* 2016). Evidence showed that both ACh and GLU have an important role in memory formation (Aigner, 1995). In the current study, we found a significant rise in ACh content following chronic GLU supplementation but not after GABA supplementation which suggests that brain function was also improved via enhancing cholinergic neurotransmission following GLU supplementation. These results are in agreement with the study that report a facilitation of GLU activity by ACh in cortex and hippocampus during acquisition and recall states (Aigner, 1995) and observed enhancement in memory retention following intrahippocampal infusion of ACh and GLU receptor agonists (Fan *et al.,* 2000).

The significant finding of current study is the enhancement in cholinergic and aminergic neurotransmission accompanied with improved memory function which was more pronounced following GLU supplementation compared to GABA supplementation. Although previous research showed a discrepancy regarding the effects of GLU as some have reported its beneficial effects while others have reported the harmful effects. In the earlier research GLU has been shown as a neurotoxin which is responsible for various neurodegenerative diseases via increasing intracellular calcium influx causing excitotoxicity (Mehta *et al.,* 2013). Appearance of brain lesions and neuronal degeneration

have been reported following subcutaneous GLU injection (Tapiero *et al.,* 2002) and it has been speculated that in excessive amount it may cause neuronal damage associated with neurodegenerative disorders like Alzheimer's disease (Sklenovský, 1967). But later studies have reported that GLU plays an important role in neurological functions like learning, memory, long-term potentiation and synaptic plasticity (Platt, 2007; Peng *et al.,* 2011) has proposed safer dietary supplementation of monosodium glutamate that improved growth performance (Rezaei *et al.,* 2013) or may be nootropic at low doses (Onaolapo *et al.,* 2016). However, the role of free L-glutamate in brain functioning was less addressed. So, present study was focused on this aspect. Reports in recent years suggest the promising role of GLU in future treatment of neurological conditions as it helps in transportation of $K^+$ across BBB (Dutta *et al.,* 2013). Furthermore, a recent study, showing the dubious nature of GLU could be related to its extracellular levels which need to be maintained at low levels as its excessive amount is excitotoxic, so regulation of extracellular GLU levels is required to maintain neuronal homeostasis (Stamoula *et al.,* 2015; Dong *et al.,* 2009) thus, suggesting a role of GLU as a neuroprotective and cytoprotective agent (Stamoula *et al.,* 2015). Present findings are in agreement with the above finding as it also highlights the beneficial effects of GLU supplementation (Stamoula *et al.,* 2015).

## CONCLUSION

In conclusion, GLU and GABA supplementation-induced alterations in their hippocampal levels found in current study endorses the previous research that theses AA can cross the BBB to modulate neurochemical status in brain. Current findings therefore suggest a beneficial role of oral AA (GLU and GABA) supplementation on brain functioning and learning and memory formation associated with improved aminergic and cholinergic neurotransmission in the hippocampus (presented in fig. 7). Furthermore, current findings also demonstrated that in comparison to GABA, GLU supplementation may be more effective for improvement in learning and memory function accompanied by marked enhancement in GLU, GABA as well as ACh levels in hippocampus which suggests its potential role as a therapeutic agent for treatment of neurological conditions. However, to draw a better conclusion a detailed study addressing the effect of GLU on redox profile and monoaminergic neurotransmission is required to demonstrate its nootropic potential.

## FUNDING AND DISCLOSURE

The funds for experimental material and animals for the present study were provided by University of Karachi and Higher Education Commission (HEC), Pakistan. Authors also declare that there are no competing financial interests in relation to the work described.

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