

Effect of glycine: Studying memory and behavioral changes in mice

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Abstract: Glycine is an important chemical mediator of nervous system that plays a vital role in memory and other neurological functions. Therefore, the effect of glycine on these traits must be studied to understand biological mechanisms of intricate neurological system. We investigated the effect of different doses of glycine on memory and behavior using 30 albino mice models (treated and control). After two weeks of glycine dosing, we performed light and dark activity and novel-object recognition (NOR) tests to assess the cognitive traits. Brain and blood samples were taken and kept at -70°C using ultra-low temperature freezer. Neurochemical estimation of blood glycine level was estimated by high-performance liquid chromatography with electrochemical detectors (HPLC-ECD). Concentration of glycine (100, 300 and 500 mg/kg) is significantly observed ($p < 0.01$) and it changes due to physiological variations in N-methyl-D-aspartate (NMDA) an important neurotransmitter for memory. We observed significant increase in serotonin metabolites including 5-hydroxy tryptophan (5-HT, $p < 0.05$) and 5-hydroxy indole acetic acid (5-HIAA, $p < 0.001$) levels. Similarly, effects were found in case of dopamine (DA, $p < 0.05$) and its metabolites: 3, 4-Dihydroxyphenylacetic acid (DOPAC, $p < 0.001$) and homovanillic acid (HVA, $p < 0.001$). Histopathological investigation of brain tissues showed cellular clumps at cortical junctions at higher doses of glycine as compared to control. These findings revealed that dose dependent concentration of glycine can be useful for memory loss and behavior deficits.

Keywords: Glycine, memory, behavioural effects, histopathology.

INTRODUCTION

Glycine is important neurotransmitter found in CNS, spinal cord and retina and a NMDA receptor co-agonist, and extra-cellular levels of glycine are controlled in the forebrain by the glycine type-1 transporters (GlyT-1) (Castner *et al.*, 2014). It is associated with several neurological functions including memory and behaviour (Castner *et al.*, 2014; Palmer *et al.*, 2008; Betz and Becke, 1988). A typical diet contains glycine that helps to regulate the physiological function, however, the human body can make this vital amino acid from other chemicals that are used for treating schizophrenia, and memory loses (<https://clinicaltrials.gov/ct2/show/NCT00575848>). In central nervous system, glycine has inhibitory effect (Boissiere *et al.*, 1998) and related to memory and behaviour regulated by the signalling of NMDA-receptors (Palmer *et al.*, 2008; Maragos *et al.*, 1987). It has been observed that glycine acts as co-agonist at the NMDA glutamate-receptors (Palmer *et al.*, 2008; Kemp *et al.*, 1988) related to memory and learning (Wu *et al.*, 1991; Borowsky *et al.*, 1993). To regulate and control memory and behavioral effects, NMDAR activation requires postsynaptic de-polarisation and the binding of two agonists including glycine and glutamate. It facilitates NMDA mediated transmission that promotes the brain

functions (Blanke and VanDongen, 2009). Therefore, it has been studied that blockade of NMDA-receptors are associated with memory losses and behavioural problems (Gandolfi *et al.*, 2001). Similarly, serotonergic mechanisms are also associated with memory and other psychiatric disorders (Brown and Praag, 1991). It has been analysed that 5-Hydroxytryptamine (5-HT), one of the serotonin metabolite, have enhanced the performance of rodents in many cognitive tests (Barnes and Cooper, 1992). 5-Hydroxyindolacetic acid (5-HIAA), the degradative product of 5-HT is also associated with memory enhancement (Carli *et al.*, 1992). The role of dopamine (DA) has been studied in psychiatric disorders, memory and learning but nothing is known about precise biological mechanisms (Butterfield and Pocernich, 2003; Kolb, 1984). However, it has been seen in test animals that dopaminergic lesions cause memory and behavioural problems. The positive changes in memory and mental behaviour has been observed by 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) neurotransmitters (Larkin, 2001, Rabat, 2007). Here, we investigated the effect of various concentrations of glycine on memory and behaviour using mice-models. Treatment with glycine at 300mg/kg significantly improved cognitive deficits under this test paradigm. These findings suggest that dose dependent use of glycine could be useful for the cognitive deficits observed in schizophrenic patients or other memory loses. The study

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also shows that enhancing NMDA receptor function is an effective way for treating the cognitive deficits associated with these problems.

MATERIALS AND METHODS

Study approval and animals handling

This study was permitted by Ethical Review Committee of Institute of Molecular Biology and Biotechnology (Letter No. IMBB/002/2014), Bahauddin Zakariya University, Multan, Pakistan. Thirty locally breed albino male mice (8-10-week-old) with average weight of 30 grams were used for this study in animal facility of the University. They were caged individually at 20-25°C with free access to standard rodent diet and clean and filtered tap water under 12-hours of light/dark cycle. These mice models were randomly assigned to control and test groups (each containing 15 animals) (Kaidanovich-Beilin *et al.*, 2011).

Preparation and administration of glycine doses

Analytical grade glycine was dissolved in clean and filtered tap water (mg/L) (Mirza *et al.*, 2013) and introduced 100, 300 and 500 mg/kg doses once in a day to test mice for consecutive two weeks. A dose was administrated orally using micropipettes. The same concentration of tap-water was administered orally to control group. After two weeks, behavioral tests were performed.

Behavioral test: The light-dark box activity

We performed this test to observe behavioral activity (Bourin and Martine, 2003; Mirza *et al.*, 2013). This test contains two perspex boxes in which one third area was kept dark and remaining illuminated with the external size of 47-24-32cm (*l, b, h*). The distance between two areas was not more than 6cm. Treated mice were positioned in the center of illuminated area and total entries and retention time to this area was recorded. We applied simple t-test for of values of means \pm SD.

Behavioral test: Novel object recognition test

The Novel Object Recognition (NOR) test is used to evaluate recognition memory. This test is based on the spontaneous affinity of mice to spend more time recognizing a novel object than a familiar one (Moscardo, 2012; Mirza *et al.*, 2013) and it reflects the use of learning and recognition memory. We performed the Novel Object Recognition task in an open field area with two dissimilar types of objects. Both objects were similar in height and volume, but vary in shape and appearance. During familiarization, the animals were allowed to identify an empty side. After 24 hours of exposure, the mice were exposed to the acquainted site with two similar objects kept at an equal distance. The next day, the mice were allowed to recognize the open field in the presence of the accustomed object and a novel-object to test long-term

recognition memory. The time taken to recognize each object and the discernment index percentage were calculated.

Mice dissection

After two weeks of glycine administration, mice were dissected as per standard protocol (Ikram *et al.*, 2012; Mirza *et al.*, 2013). Blood was collected from cardiac puncher and centrifuged at 3000 rpm for plasma at 25°C. Brain tissues were removed, washed with ice-chilled saline and preserved for histopathological and neuro-chemical estimation. All samples were stored at -70°C.

Neuro-chemical estimation by HPLC-ECD

We executed neurochemical estimation by high performance liquid chromatography (HPLC) linked with electrochemical detection (ECD) to examine the effect of glycine (Ikram *et al.*, 2012, Mirza *et al.*, 2013) (shimpack-ODS column: 5 μ , diameter: 0.4mm, length: 150mm) using isocratic elution of 0.1M phosphate buffer with 2.9 pH, 14% methanol and 0.023% octyl-sodium sulphate, 0.0035% EDTA with operating potential of 2000-3000 psi. We detected electrochemical-signals (Schimadzu LEC 6A detector) at +0.8V.

Histopathology analysis

The brain tissue samples were washed with 0.9% normal saline solution and preserved in 10% formalin (Lim *et al.*, 2010). The posterior section of brain was treated as: (a) 70% alcohol for 1hour (b) 80% alcohol for 1.5hour (c) 90% alcohol for 1.5hour (d) pure alcohol for 1.5hour (e) xylene for 1.5 hour twice (f) in molten wax at 65°C for 3hour. The processed brain tissues were packed in paraffin, sectioned at 5 μ thickness, kept on frosted glass slides and dry these slides on 70°C for 30 minutes. The brain tissues were stained with hematoxylin and eosin dyes. The sections were treated with absolute ethanol for 5min each, washed with water and stained. After staining, tissues were re-treated with ethanol for 4 minutes and rinsed with xylene for 2 minutes. The digital camera integrated with microscope (Olympus CX21 microscope at 40x10 magnification power) was used to take images.

STATISTICAL ANALYSIS

It was performed by two-way ANOVA. Post-hoc comparison of treated and control groups was performed by Newman-Keuls test following ANOVA using IBM SPSS Statistics 20. Values of $p < 0.05$ were considered as significant.

RESULTS

Analyzing cognitive traits by light and dark activity

We observed memory and behavior of mice in novel conditions, open-area seemed to have aversive characteristics which constrain investigative actions. In

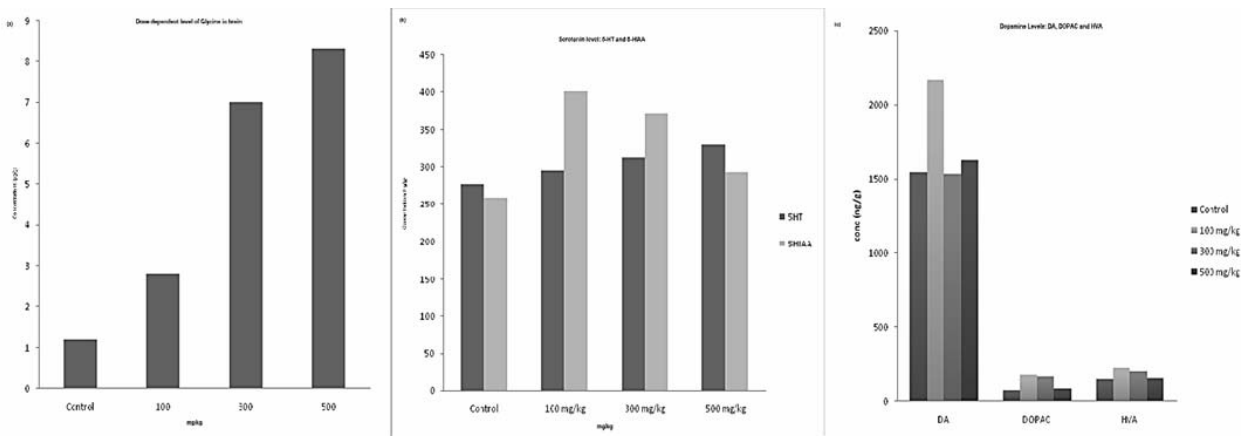


Fig. 1: (a) Effects of dose dependent glycine supplementation on brain (b) glycine effect on brain 5-HT and 5-HIAA at 100, 300 and 500mg/kg doses (c) glycine effect on brain dopamine and its metabolites at 100, 300 and 500mg/kg doses.

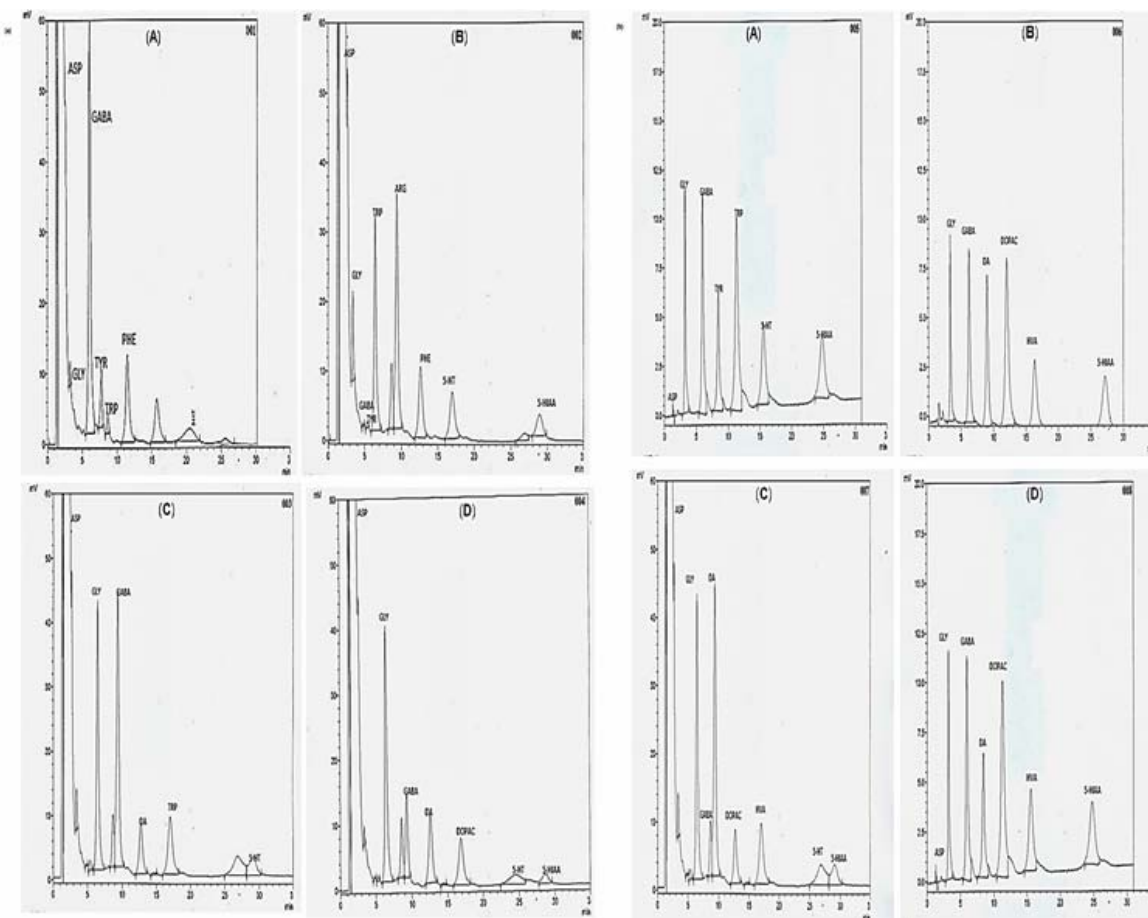


Fig. 2: (a) Neuro-chemical estimation of glycine and other metabolites for A. Control, B. 100mg/kg, C. 300mg/kg, D. 500mg/kg (b) Neuro-chemical estimation of 5-HT, 5-HIAA, DA and its metabolites for A. Control, B. 100mg/kg, C. 300mg/kg, D. 500mg/kg

table 1, the effect of recurrent doses of glycine on light and dark activity was observed after 2- weeks. Differences among individuals were found significant. At all doses of glycine, total movements and retention time of mice in light compartment was significant (p-value: 0.443) as compared to dark area (p-value: 0.826).

Analyzing cognitive traits by novel object recognition test

The statistical t-test showed significant improvement in memory of the mice after giving repeated dose, however the prominent results were recorded at 300mg/kg as compared to water-treated control group. Table 2 showed

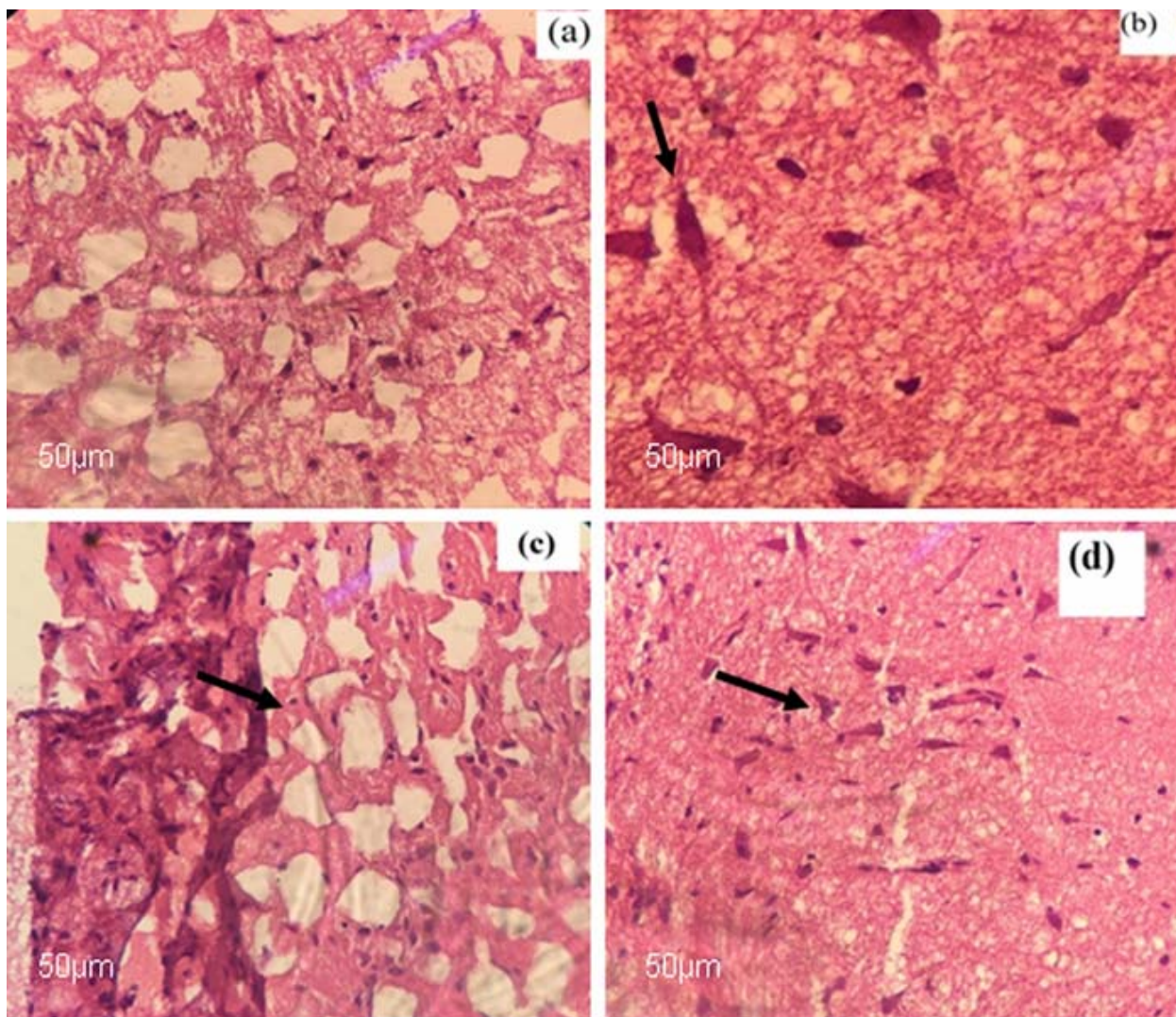


Fig 3: Histopathological studies photomicrographs (100, 200x) of mice after 2 weeks of glycine exposure (a) Control (b) 500mg/kg (c) 300mg/kg (d) 100mg/kg.

the effects of high, moderate and low doses of glycine in mice. Glycine treated mice approached and recognized novel object significantly (p-value: 0.098).

Neuro-chemical estimation by HPLC-ECD

The HPLC connected with electrochemical detection (ECD) system was used to analyze the effect of glycine on brain. Data on brain glycine level was analyzed by simple t-test (D.F.=3, 20; $p < 0.01$). Glycine level was considerably increased $p < 0.01$, however we observed more significant and positive change at 300mg/kg dose (fig. 1a).

Effect of glycine on serotonin and its metabolite

The concentration of glycine showed significant increase of serotonin metabolites including 5-hydroxy tryptophan (5-HT) (D.F.=3, 20; $F=36.785$, $p < 0.05$) and 5-hydroxyindolacetic acid (5-HIAA) (D.F.=3, 21; $F=44$, $p < 0.001$) with prominent spectral level. The increased

level of serotonin and its metabolites has positive influence on memory and behavior (fig. 1b).

Estimation of glycine on dopamine and its metabolites

We observed the positive level of dopamine (DA) in glycine treated animals as compared to control. fig. 1c showed these significant levels (D.F.=3, 44; 9.030 , $p < 0.05$). The concentration of 3,4-Dihydroxyphenylacetic acid (DOPAC) increased significantly (D.F.=3, 45; $F=18.34$, $p < 0.001$) in mice which is one of the important metabolite of DA. Homovanillic acid (HVA) is another key metabolic component of DOPAC that shows a major role in dopaminergic activity in CNS. A significant increase in brain HVA (D.F.=3, 44; $F=43.32$, $p < 0.001$) was observed. The most significant outcomes were seen at 300mg/kg dose. Glycine supplementation positively affected dopamine (DA) and its metabolites (DOPAC and HVA) improve the memory and mental behavior of the mice. Electrographs of glycine levels and other metabolic

Table 1: Light and dark activity test of glycine dependent groups

Parameters	Control (n=5)	500mg/kg (n=5)	P-value	300mg/kg (n=5)	P-value	100mg/kg (n=5)	P-value
Latency time	10.00 ± 2.16	19.50 ± 2.65	0.003*	8.00±4.97	0.501	6.25±5.44	0.290
Transitions	5.00 ± 2.16	4.50 ± 1.29	0.711	5.25±2.50	0.886	1.00±1.15	0.031*
Light movements	13.00 ± 2.94	16.75 ± 1.71	0.092	12.50±2.08	0.793	14.50±2.08	0.443
Time spend	0.1005±0.0686	0.0984±0.03	0.960	0.0663±0.012	0.399	0.0748±0.040	0.553
Dark movements	11.50 ± 5.07	15.25 ± 4.43	0.316	12.50±3.00	0.751	12.25±4.03	0.826
Time spend in dark	0.1113±0.0511	0.0964±0.03	0.649	0.1101±0.041	0.972	0.1297±0.042	0.602
Rears	16.75 ± 1.71	13.25 ± 2.22	0.052*	11.50±3.11	0.042*	19.75 ± 9.98	0.595

Table 2: Novel Object Recognition of Glycine treated Groups

NOR 1st Trail of Glycine Treated Groups							
Parameters	Control	500mg/kg	P-value	300mg/kg	P-value	100mg/kg	P-value
Line crossing	14.00±3.92	11.25±3.50	0.343	18.75±2.63	0.1	14.0±2.94	1
Rearing	10.50±5.26	5.25±2.63	0.149	15.75±2.22	0.14	5.00±1.83	0.143
Stretching	9.00±4.32	8.75±4.86	0.942	9.50±1.29	0.839	13.75±3.40	0.145
Approach to Object 'A'	11.75±4.65	9.00±4.55	0.436	16.00±2.83	0.193	8.25±4.57	0.332
Time spent at 'A' Object	0.03±0.026	0.03±0.039	0.961	0.03±0.012	0.823	0.006±0.002	0.186
Approach to Object 'B'	13.00±2.16	7.75±4.92	0.123	16.50±2.08	0.052*	10.50±1.29	0.118
Time spent at 'B' Object	0.03±0.017	0.007±0.002	0.109	0.004±0.002	0.034*	0.009±0.003	0.135
NOR 2nd Trail of Glycine Treated Groups							
Parameters	Control	500mg/kg	P-value	300mg/kg	P-value	100mg/kg	P-value
Line crossing	7.50±1.29	14.50±2.38	0.007*	9.50±1.29	0.071	8.75±2.99	0.485
Rearing	2.25±0.957	2.25±1.26	1	4.50±1.29	0.038*	4.00±3.37	0.391
Stretching	3.25±1.71	4.50±1.29	0.296	4.50±2.08	0.396	4.75±2.22	0.333
Approach to 'Novel object'	8.00±1.41	7.00±2.71	0.548	10.25±1.71	0.098	7.25±4.03	0.749
Time spent at 'Novel object'	0.02±0.004	0.03±0.014	0.207	0.024±0.01	0.262	0.015±0.005	0.732
Approach to Object 'B'	4.25±1.71	4.50±1.29	0.825	7.00±1.41	0.054*	4.25±2.06	1
Time spent at 'B' Object	0.002±0.0009	0.003±0.001	0.139	0.02±0.04	0.365	0.006±0.003	0.116

(*): P-values: significant observations

components 5-HT, 5-HIAA, DA, DOPAC and HVA has been shown as compared to control (fig. 2).

Histopathological studies

The histopathology investigation showed no positive change at normal doses of glycine. However, at high doses of glycine (500mg/kg) cellular clumps were observed at cortical junctions. fig. 3 showed the effect of the glycine in different concentrations on brain tissues.

DISCUSSION

Memory loses and mental-behaviors are severe and mounting public health issues caused by the physical and chemical factors. In 2008, the World Health Organization (WHO) predicted that number of individuals with behavioral problems remains to increase and reaches the level of 35.6 million (Takeda *et al.*, 2010; WHO guidelines, 2008). The N-methyl D-aspartate receptor is associated with the long-term potentiation, which is considered as the biological mechanism of learning and memory. This mechanism is mediated through glycine receptor co-agonist site (File *et al.*, 1999). The positive effects of glycine doses were observed in patients with schizophrenia (<https://clinicaltrials.gov/ct2/show/NCT00575848>). The underlying biological and neurological mechanisms of these cognitive deficits are not clear and

molecular studies are required to find the potential reasons. In this regard, glycine is building block to several natural products, involved in many neurological functions and its role has also been studied positively for schizophrenia (Coyle and Tsai, 2004, Castner *et al.*, 2014) and other behavioral problems. To observe the dose dependent effect of glycine, this study shows an improvement in the cerebral and cognitive functions at successive doses of glycine including 100, 300, and 500mg/kg. Such results would be helpful to manage emerging problem of memory loss and mental behavior.

Glycine is a major inhibitory neurotransmitter in CNS and associated with important neurological and memory functions (Greenamyre, 1986, Castner *et al.*, 2014). The neurocognitive and functional effects of glycine have demonstrated positive outcomes in different studies. Goff and Coyle (2001) found a highly significant (P=0.003) improvement by using glutamate (Goff and Coyle, 2001). In another study, Heresco-Levy *et al.* (1999) also found a significant reduction in negative symptoms of memory losses (P<0.0008) (Heresco-Levy *et al.*, 1999). In our study, after glycine doses we observe progress in memory by significant increase of serotonin metabolites including 5-hydroxy tryptophan (5-HT) (D.F.=3, 20; F=36.785, p<0.05) and 5-hydroxyindolacetic acid (5-HIAA) (D.F.= 3, 21; F=44, p<0.001) with prominent spectral level.

Although, this substantial improvement was observed at the 300mg/kg but higher dose does not have positive effect. In other study, oral intake of glycine increased 5-HT and 5-HIAA (Markus *et al.*, 2002). The cognitive functions are affected by reducing the concentrations of serotonin and dopamine (Haider *et al.*, 2006, Porter *et al.*, 2003; Riedel *et al.*, 2003).

Memory improved by increasing the concentrations of 5-HT and 5-HIAA levels of serotonin and metabolites of dopamine (DOPAC, HVA) and it was observed that the effect was dose dependent and better outcome were achieved at 300mg/kg. Therefore, the progress in the synthesis of 5-HT improve the cognitive functions and decrease in glycine level have adverse effect on memory (Markus *et al.*, 2002). NOR-performance is associated with long-term memory, which is used to assess the working-memory (Calkins *et al.*, 2005). We observe positive change in mice by the light and dark and NOR tests after glycine administration (Ennaceur and Delacour, 1988). However, at higher dose the response was negative which showed that 300mg/kg of glycine is more effective as compared to other doses. In histopathological analysis, we observed cellular clumps and some necrosis at higher doses (500mg/kg). These finding suggested that dose dependent effect of glycine can improve the memory and cognitive functions without negative impact on neurological tissues.

CONCLUSION

The improve performance of the mice is due to rise in brain glycine level. The moderate-dose 300mg/kg is more effective and significant than high doses. The results of this study are helpful to find cure of cognitive impairment.

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