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INFLUENCE OF LEVETIRACETAM ON AGGRAVATION OF MEMORY IMPAIRMENT BY VALPROATE: A STUDY ON PILOCARPINE MOUSE MODEL OF TEMPORAL LOBE EPILEPSY

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Keywords:

Anti-epileptic drug, Levetiracetam, Pilocarpine, Temporal lobe epilepsy

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ABSTRACT: Association of memory impairment (MI) with epilepsy, majorly in temporal lobe epilepsy (TLE) is considerably a tricky condition where existing treatment options involving the administration of anti-epileptic drugs (AED's) tend to worsen the stated incidence. This study evaluates the aggravating effect of valproate (VAL) at normal and reduced doses on MI associated with TLE and additionally assesses the protective effect of levetiracetam (LEV) on this undesirable link. Albino mice of either sex (n = 36) were employed for the study period of 64 days where seizures were induced by intraperitoneal administration of pilocarpine followed by evaluation of anti-epileptic activity by technique of Racine scores. Assessment of errors, a factor for degree of MI was executed by employment of radial arm maze. Finally brain biochemical measures of Acetyl cholinesterase and glutamate along with cresyl violet staining and estimation of total neuronal number of the hippocampus were performed. Both at normal and reduced doses of VAL exhibited augmentation of MI on temporal lobe epileptic mice. Although reduction in VAL dose brought about a decline in the degree of MI, the same diminished the anticonvulsant potency which isn't recommended. However the combinatorial approach with LEV not only brought about the correction in impaired memory but also replaced the lost potency. These findings were reflected in brain studies. This research could thus establish a launch in seizure therapy involving the combination of a memory enhancer with an AED to limit the adverse effect of MI associated with temporal lobe epilepsy.

INTRODUCTION: A memory block / impairment is often associated with epilepsy out of which temporal lobe epilepsy (TLE) has significantly major contribution towards the mentioned problem [1, 2].

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Epileptic therapy involving anti-epileptic drugs (AED's) have been reported to worsen this unfavourable condition thereby resulting in an already present poor health related quality life (HRQL) of the individual associated [3, 4].

The employment of pilocarpine mouse model of status epilepticus was to mimic the human form of TLE where systemic administration of the same could induce robust limbic seizures [5, 6]. Valproate (VAL), which is well established as an AED widely finds application in status epilepticus and

also used in the treatment of both generalised and partial seizures in adults and children [7, 8]. Reports of VAL to pose additional neuro-psychological complaints that are of particular concern in treatment of epilepsy are the reason for taking this AED as the drug of choice [9]. As a variety of racetams (drugs sharing a pyrrolidone ring) have existed as memory enhancers, the selection of levetiracetam (LEV) was based on the fact that it possess an additional benefit to function as an adjunct anti-epileptic in the management of partial seizures [10-12].

Reports showing the use of LEV to correct deficits in learning and memory in an Alzheimer's mouse model and its favourable pharmacokinetic parameters including faster absorption, negligible binding to plasma proteins, lack of enzyme induction, absence of interactions with other drugs, and restricted metabolism outside the liver were considered as the rationale for its selection into this study [13, 14]. Based on the above findings of literature, this study aims to evaluate the extent of aggravation of memory impairment (MI) induced by VAL at normal and reduced doses when administered alone and in combination with LEV on temporal lobe epileptic mice.

MATERIALS AND METHODS:

Animals: Albino mice (n = 36) of either sex of weighing 30-35g were procured from the Central Animal House Facility of JSS Medical College, Mysore (CPCSEA 261/PO/ReBi/2000/CPCSEA, Date: 16/10/2015 – 15/10/2018). They were housed in polypropylene cages with free access to food and water, at an ambient temperature 260 °C, humidity 50-60%, 12:12 light / dark cycle. All efforts were made to minimize animal suffering, and chronic animal protocols were designed to reduce the number of animals used. Experiment protocols were performed in accordance with the approval of Institutional Animal Ethics Committee (IAEC) of JSSCP Mysore; bearing the proposal number 162/2014.

Drugs, Chemicals and Materials: VALN-normal 50mg/kg and VALR-reduced 30mg/kg body weight, p.o) and LEV (25mg/kg body weight, i.p) required for the study were procured from Shivari Pharmaceuticals, Mysuru - 570 021, Karnataka, India. The doses were selected with slight variation based on their effectiveness as an anti-epileptic and their toxic profile from available literature [15, 16]. Pilocarpine HCl (300mg/kg body weight, i.p) from Sigma Aldrich was used to produce repetitive limbic seizures. Lab made Radial Arm Maze (RAM) was employed in determining memory impairment activity and Sony Handycam was utilized to record the arm preference and errors. Acetyl thiocholine iodide, Cage hydrate powder of glutamate and Whatman number one chromato-graphy paper from Sigma Aldrich, butanol, acetic acid, ninhydrin, cupric sulphate of analytical grade were used, provided by JSS College of Pharmacy, Mysuru, Karnataka, India. Brain studies involving histopathological staining and total neuronal number was performed by Medall Clumax Diagnostic, Mysuru, Karnataka.

TABLE 1: TREATMENT SCHEDULE

Group	n	Treatment	Evaluation
Normal	6	0.5% Sodium CMC (Vehicle) was administered orally for 64 days	Memory impairment activity was done on days 8, 15, 22, 29, 36, 43, 50, 57 and 64 for a total period of 64 days
Control	6	Vehicle p.o every day + convulsions induced by pilocarpine i.p every 7th day for a period of 64 days. After observation of all racine stages, seizure were terminated by administration of Diazepam 5mg/kg i.p	Convulsive scores (Anti-epileptic activity) on every 7th day followed by Error scores of RAM (memory impairment activity) on days 8, 15, 22, 29, 36, 43, 50, 57 and 64 for a total period of 64 days
VALN	6	VALN p.o every day + convulsions induced by pilocarpine i.p every 7th day for a period of 64 days.	----Same as above----
VALR	6	VALR p.o every day + convulsions induced by pilocarpine i.p every 7th day for a period of 64 days.	---- Same as above ----
VALN+LEV	6	VALN p.o and LEV i.p every day 2hr and 1hr prior to convulsions induced by pilocarpine i.p every 7th day for a period of 64 days.	---- Same as above ----
VALR+LEV	6	VALR p.o and LEV i.p every day 2hr and 1hr prior to convulsions induced by pilocarpine i.p every 7th day for a period of 64 days.	---- Same as above ----

CMC- Carboxymethyl cellulose, VALN - valproate normal dose - 50mg/kg body weight, VALR - valproate reduced dose - 30mg/kg body weight, LEV- Levetiracetam 25mg/kg body weight. Note: For groups VALN + LEV and VALR + LEV, pilocarpine was administered on every 7th day for a period of 14 days prior to the start of treatment. This brought about disruption in the learning curve which aided the reversal or diminishing the effect of LEV on MI.

This study was designed is such a way that pilocarpine administration was followed by anti-epileptic activity determination by Racine scores (on the 7th day). The memory impairment activity was evaluated using radial arm maze on the 8th day. These evaluations were conducted for the entire study period of 64 days.

Pilocarpine Model of Status Epilepticus: In this model, systemic injection of the pilocarpine induces status epilepticus, likely through activation of M1 muscarinic receptors, followed by a seizure-free latent period, and eventually the appearance of recurrent seizures that continue for the rest of the animal's life. By monitoring the behavioural severity of each seizure, scores on a standard 0 - 5 Racine scale were given [17]. The animals were treated as per the schedule (**Table 1**) for 64 days (this period of study was developed as an acute administration of VAL could not provoke worsening in conditions of MI when observed by hippocampal staining although behavioural alterations were present).

Anti-convulsant potential of VALN and VALR in absence / presence of LEV was assessed every 7th day for a period of 64 days. Convulsions were induced by intraperitoneal administration of pilocarpine 300mg/kg on every 6th day. A seizure in control animals were controlled by the administration of Diazepam 5mg/kg i.p. LEV as well as VAL was given orally two and one hour respectively, before induction of convulsions. Convulsive scores were finally given (based on Racine scale for seizures) as follows: 0- No response, 1- Hypoactivity, 2- Monoclonic jerks of the head and head bobbing, 3- Bilateral activity of the whole body, 4- Rearing of limbs, 5- Generalized tonic clonic activity and loss of posture [18].

Memory Impairment Activity of VAL by RAM: The animals were divided into different groups as shown in **Table 1** and memory impairment activity was evaluated every week for a period of 64 days. Each animal was trained prior to the start of experiment on a daily basis for a period of seven days in the maze to collect the food pellets. The maze was kept well illuminated. A modification of rewards by food pellets were replaced by application of butter in the inner areas of the escape box. This was done in order to help the animal in finding the escape box with the aid of olfactory senses. Once trained, the process of application of butter was excluded. During the test, mice were fed once a day and their body weights maintained at 85% of their free feeding weight to motivate the animal to run the maze. The session was terminated after 120 seconds and the animal had to obtain the find the escape box with a minimum number of errors [19].

Estimation of Brain Acetyl Cholinesterase (Ache) Levels: The reagents used were as per **Table 1**. Mice, after 64 days were euthanized using carbon dioxide method and brains were removed quickly and placed in ice-cold saline. Frontal cortex was quickly dissected out on a petridish chilled on crushed ice. The tissues were weighed and homogenized in 0.1M phosphate buffer (pH 8). 0.4ml aliquot of the homogenate was added to a cuvette containing 2.6ml phosphate buffer (0.1M, pH 8) and 100µl of DTNB. The contents of the cuvette were mixed thoroughly by bubbling air and absorbance was measured at 412nm in a spectrophotometer. When absorbance reaches a stable value, it was recorded as the basal reading. 20µl of substrate *i.e.* acetyl thiocholine was added and change in absorbance was recorded. Change in the absorbance per minute is thus determined [20, 21].

$$\text{The enzyme levels} = \Delta A * V_t / €b * V_s * X$$

Where: ΔA - Change in absorbance, V_t - Total volume (3.1), € - 13610*104, b - Path length (1Cm), V_s - Sample volume (0.4ml), X - mg protein of brain tissue.

Estimation of Brain Glutamate Levels:

Preparation of Reagents: Solvent: Butanol: acetic acid: water (12:3:5), To 60 ml of butanol, 15ml of acetic acid and 25ml distilled water were added.

0.25% Ninhydrin: 200mg of Ninhydrin was dissolved in 99ml of acetone. To this solution 1ml of pyridine was added. 0.005% $CuSO_4$ solution: 5mg of cupric sulphate was dissolved in 10ml 75% alcohol.

Standards: 2µM glutamate: 2.942 mg of glutamate was dissolved in 10ml of distilled water. After the 64 day treatment schedule, different brain regions were dissected and were homogenized in 80% double distilled ethanol (for every 100mg of the brain tissue, 2ml of 80% alcohol was used). Homogenates were transferred to polypropylene tubes and centrifuged at 1200rpm for 10 min. One ml of the supernatant was then transferred into small test tubes and evaporated to dryness at 70 °C in an oven. The residue was reconstituted in 100ml distilled water and 10ml was used for spotting on Whatman No.1 Chromatography paper. Standard solutions of glutamate at a concentration of 2mM were also spotted using an Eppendorf micropipette; the spots were dried with a hair drier. The chromatograms were then stitched at the sides and placed in a chromatography chamber containing butanol: acetic acid: water (65: 15: 25, v/v) as solvent. When the solvent front reached the top of the papers, the papers were removed and dried.

A second run was performed similarly, after which the papers were dried, sprayed with ninhydrin (0.25% in acetone with 1% pyridine) and placed in an oven at 100 °C for 4 min. The portions which carry glutamate corresponding with the standard were cut and eluted with 0.005% $CuSO_4$ in 75% ethanol. Their absorbance was read against a blank in a LKB- 4050 spectrophotometer at 515nm and the levels were expressed as µmoles/gram wet weight tissue [22].

Calculations:
Glutamate levels=Unknown OD*Standard (3mg) *100/Standard OD*Volume spotted (10µL)* X;

Where, A = Amino acid content in µmoles/gram wet weight tissue, 1000 = Conversion factor for gram wet weight tissue, X = weight of the tissue in gram.

Histopathology: Mixing Cresyl violet for 300ml staining wells Cresyl violet Stock solution: 0.2 g cresyl violet-acetate was mixed with a stir bar in 150ml distilled water for 20 minutes. Buffer solution pH 3.5: 282ml of 0.1 M acetic acid (6ml of concentrated acetic acid per 1000ml distilled water) was added to 18ml of 0.1 M sodium acetate (13.6g in 1000ml distilled water). Finally 30ml of cresyl violet stock solution was added to 300ml of buffer and mixed for 30 minutes.

Staining Procedure for Frozen Sections: The well containing stain was placed in an oven or incubator for at least an hour at 60 °C prior to staining. Sectioned tissues were mounted on slides which were loaded on to holders 20 minutes before staining in order to stabilize to room temperature. The holder was then placed into the wells containing the following solutions for the time indicated.

1. Xylene (5 minutes)
2. 95% Alcohol (3 minutes)
3. 70% Alcohol (3 minutes)
4. Deionized distilled water for 3 minutes
5. Cresyl Violet acetate (8 minutes) at 60 °C (oven)
6. Distilled water (3 minutes)
7. 70% Alcohol (3 minutes)
8. 95% Alcohol (1-2 minutes)
9. 100% Alcohol (up to 10 dips to remove any streaks; 1 dip if no streaks were found). Care was taken not to remove all of the stain.
10. Xylene (5 minutes)

Placed in next Xylene well and lid was kept closed. The slides were allowed to stay in the well until cover slipped (Up to 24 hours) using a Xylene based mounting media and top grade cover slips. For fewest air bubbles and best long-term slide storage the slides were placed for a combined total of at least 30 minutes in steps 10 and 11 [23, 24].

Total Neuronal Number: The total neuronal number of sub regions of CA1 and CA3 regions of the hippocampus were estimated by using the method of optical fractionator described by West *et al.*,[25]

Data and Statistical Analysis: The values are expressed as Mean ± SEM, n = 6, analysed by One-way and Two-way ANOVA followed by Tukey's Post Hoc test where $p \leq 0.05$ was considered significant. Graph pad prism version 6.0 was used for statistical analysis.

RESULTS:

Anti-epileptic Activity:

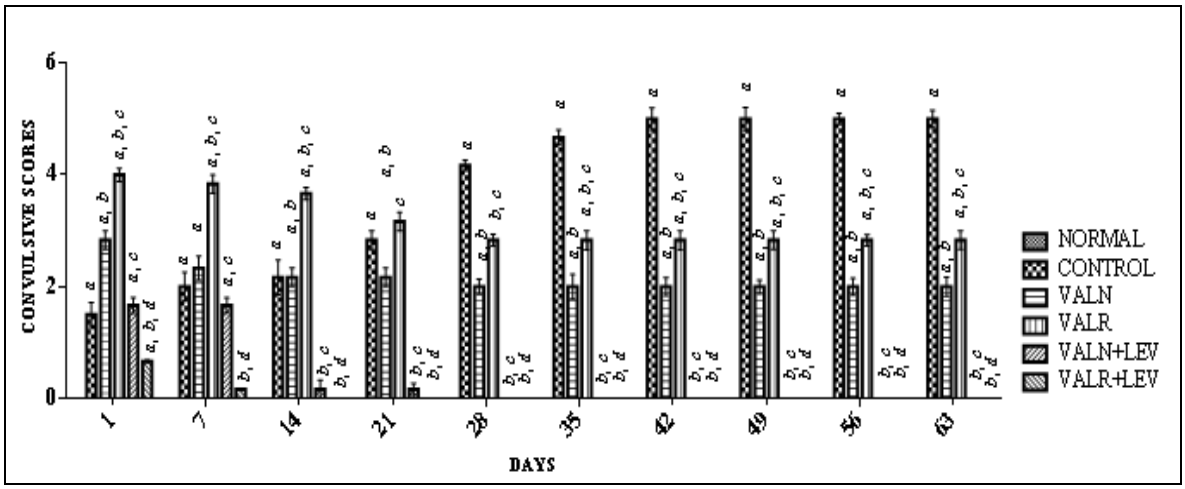

FIG. 1: ANTI-EPILEPTIC ACTIVITY OF VALN AND VALR ALONE AND IN COMBINATION WITH LEV

Values are Mean ± SEM, n = 6, P < 0.05 analysed by Two-way ANOVA followed by Tukey Post Hoc test. a- Significant when compared to normal animals, b- Significant when compared to control animals, c- Significant when compared to VALN animals, d- Significant when compared to VALR animals.

TABLE 2: ANTI-EPILEPTIC ACTIVITY OF NORMAL AND REDUCED DOSES OF VAL BOTH ALONE AND IN COMBINATION WITH LEV (PERCENTAGE PROTECTION)

Day	VALN	VALR	VALN+LEV	VALR+LEV
	% protection compared to control			
63	85.0	40.0	100.0	100.0

VALN - Valproate normal dose, VALR - Valproate reduced dose, LEV – Levetiracetam.

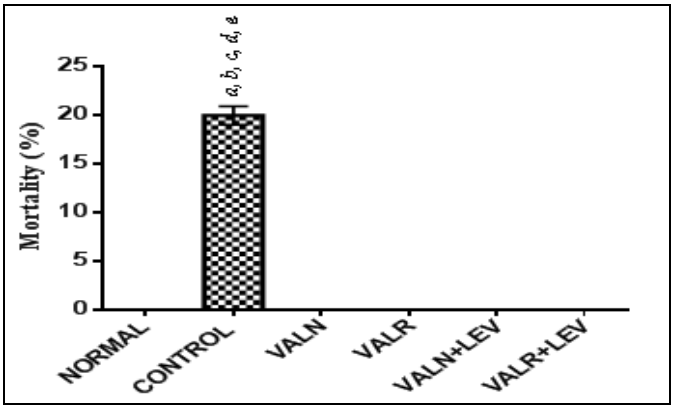

FIG. 2: PERCENTAGE MORTALITY

Values are Mean ± SEM, n = 6, p ≤ 0.05 analysed by One-way ANOVA followed by Tukey's Post Hoc test. a- Significant when compared to normal, b- Significant when compared to VALN, c- Significant when compared to VALR, d- Significant when compared to VALN+LEV and e- Significant when compared to VALR+LEV.

The convulsive scores by pilocarpine induced status epilepticus could be styled as, lesser the convulsive score, better the anti-epileptic property. Illustration of **Fig. 1** and **Table 1** denote convulsive scores based on Racine scale for seizures.

A significant decline in convulsive score was observed both in VALN (2.00 ± 0.14; p ≤ 0.05) and VALR (3.00 ± 0.27; p ≤ 0.05) than the control group having a convulsive score of 5.00 ±0 .17. Combining LEV for both groups of VAL (*i.e.* VALN+LEV and VALR+LEV), further reduced the convulsive scores significantly to 0.00 ± 0.00; p ≤ 0.05 and 0.00 ± 0.00; p ≤ 0.05. **Fig. 2** displays the percentage mortality of mice for the study period of 64 days where the outcomes of control or pilocarpine treated group were found to be 20.00 ± 0.94; p ≤ 0.05.

Memory Impairment Activity: The error scores obtained from RAM are depicted in **Fig. 3**, where the control group increased the same after a study period of 64 days by 21.20 ± 0.39; p ≤ 0.05 than the normal group (0.00 ± 0.00). A relatable increase in errors were identified in VALN group by 34.17 ± 0.54; p ≤ 0.05 which was found to be more than that of control. Out of these, noticeable measurement of errors was revealed in the reduced dose of VAL *i.e.* in VALR (27.54 ± 0.81; p ≤ 0.05) which was found to be less than that of VALN. When looking at the combination groups linking LEV, there was an extreme drop in error scores of groups VALN+LEV (1.83 ± 0.33; p ≤ 0.05 compared to control and VALN) and VALR+LEV (1.00 ± 0.17; p ≤ 0.05 compared to VALR).

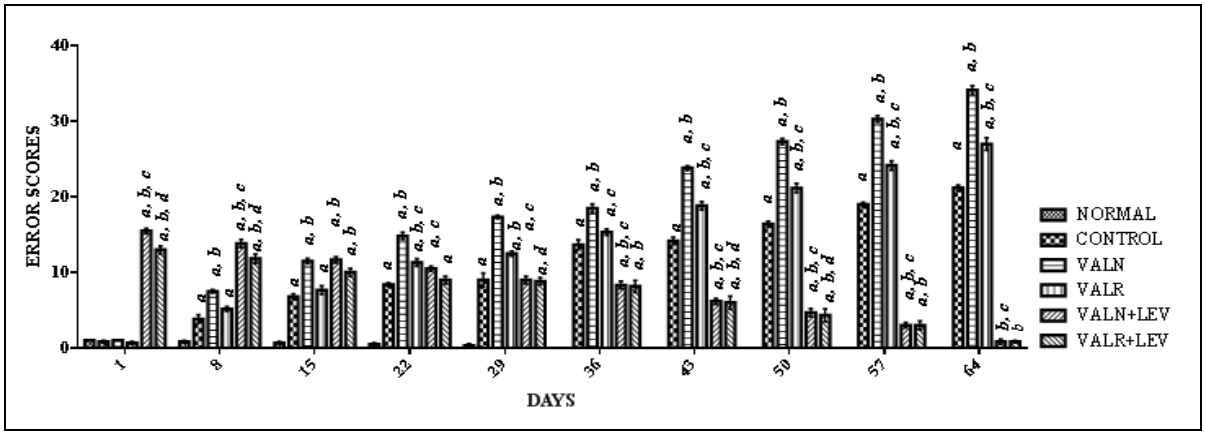

FIG. 3: MEMORY IMPAIRMENT ACTIVITY OF VALN AND VALR ALONE AND IN COMBINATION WITH LEV

Values are Mean ± SEM, n = 6, $p \leq 0.05$ analysed by Two-way ANOVA followed by Tukey's Post Hoc test. a- Significant when compared to normal, b- Significant when compared to control, c- Significant when compared to VALN, d- Significant when compared to VALR and e- Significant when compared to VALN+LEV.

Acetylcholinesterase (AChE) Levels: Fig. 4 is a representation of brain AChE levels, in which the pilocarpine / control group (7.52 ± 0.71; $p \leq 0.05$) significantly increased the enzyme levels than the normal (2.84 ± 0.48). An intensification of the same was found in cases of VALN (15.78 ± 0.86; $p \leq 0.05$) and VALR (9.24 ± 0.50; $p \leq 0.05$) than the control. However, a fall in the above enzyme levels was brought about in groups of VALN+LEV (6.23 ± 0.94; $p \leq 0.05$) and VALR+LEV (4.48 ± 0.87; $p \leq 0.05$) upon comparison with control.

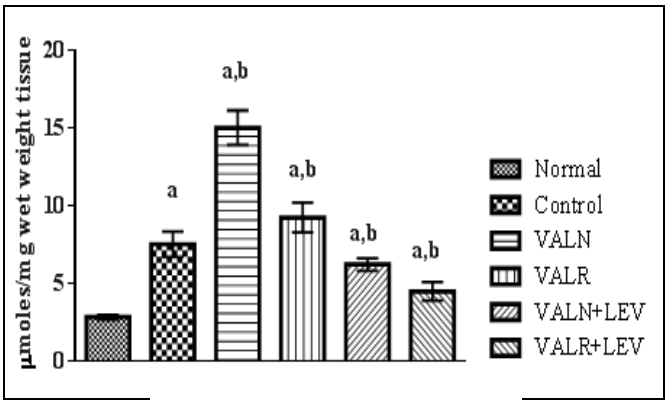

FIG. 4: BRAIN ACHE LEVELS

Values are Mean ± SEM, n = 6, $p \leq 0.05$ analysed by One-way ANOVA followed by Tukey's Post Hoc test. a- Significant when compared to normal and b- Significant when compared to control.

Glutamate Levels: Brain glutamate levels are as shown in **Fig. 5**. This measure was found to increase in the control (8.23 ± 0.40; $p \leq 0.05$) compared to the normal of 2.07 ± 0.31. Both treatment groups of VALN and VALR, witnessed a significant rise in levels of glutamate (15.78 ± 0.91 and 12.78 ± 0.83; $p \leq 0.05$) when compared to that of normal and control groups. In the combination groups of VALN+LEV (7.58 ± 0.79; $p \leq 0.05$) and VALR+LEV (5.51 ± 0.53; $p \leq 0.05$), a decrease in the levels of the amino acid were identified than normal and control groups.

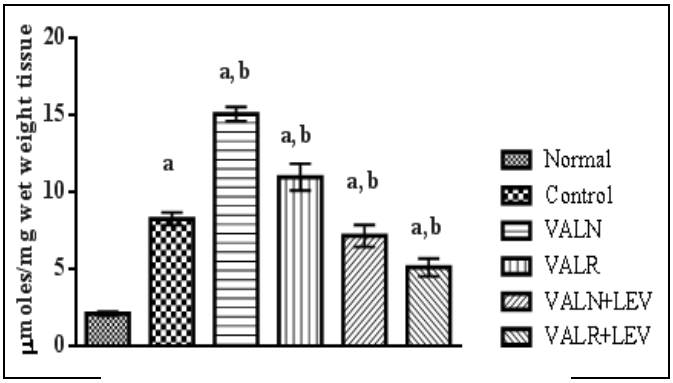

FIG. 5: BRAIN GLUTAMATE LEVELS

Values are Mean ± SEM, n = 6, $p \leq 0.05$ analysed by One-way ANOVA followed by Tukey's Post Hoc test. a- Significant when compared to normal and b- Significant when compared to control.

Histopathology: The damage of neurons process in areas of CA1 and CA3 (sub regions of the hippocampus mainly involved in memory processes) upon cresyl violet staining is explained in **Fig. 6**. Apart from this, a quantitative method called the optical fractionator was employed to estimate the number of neurons in regions of CA1 and CA3 as shown and described in **Fig. 7**. It was found that VALN reduced the total number of neurons by CA1 – 45521 ± 5350 and CA3 – 49763 ± 7701; $p \leq 0.05$ when compared to control (CA1 – 89564 ± 8314 and CA3 -99280 ± 4552; $p \leq 0.05$) and normal (CA1 – 164350 ± 13917 and CA3 – 179460 ± 17205; $p \leq 0.05$) groups.

However the reduction was less in case of VALR (CA1 – 49051 ± 5840 and CA3 – 56504 ± 4722; $p \leq 0.05$). In cases of VALN (CA1 – 143933 ± 13172 and CA3 - 167098 ± 12518.9; $p \leq 0.05$) and VALR (CA1 – 157769 ± 13441 and CA3 – 177337 ± 11332; $p \leq 0.05$) in combination with LEV, the extent of reduction in total neuronal number was very much less when compared to control, VALN and VALR groups.

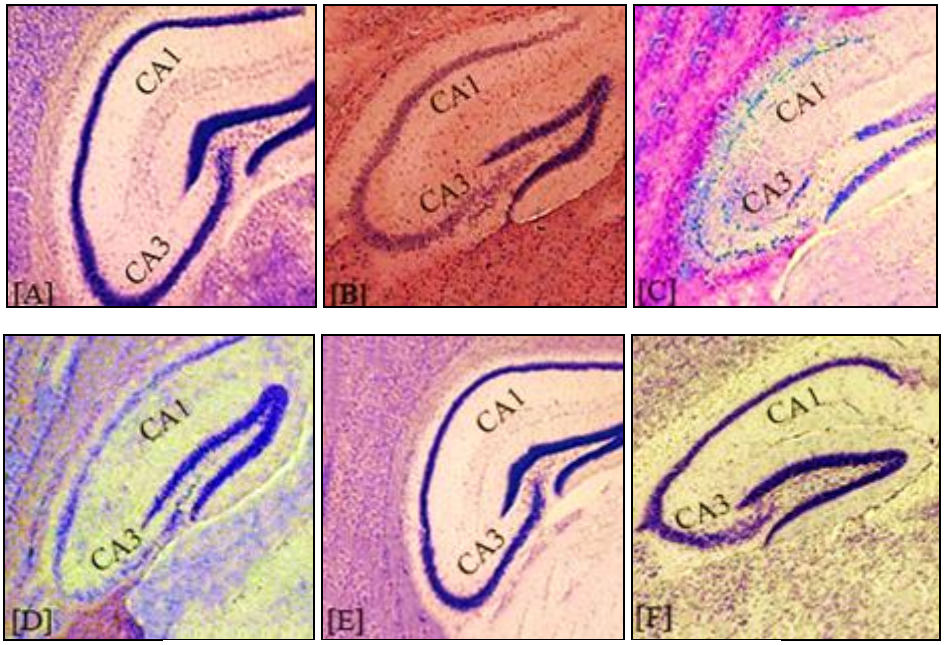

FIG. 6: NISSL STAINING OF THE HIPPOCAMPUS

[A] Normal group showing no disruption in CA1 and CA3 regions. [B] Control group showing significant destruction of regions of CA1 and CA3 regions. [C, D] Worsening of the hippocampal regions when VAL was administered at normal and reduced doses. [E, F] Correction by combination LEV with VALN and VALR exhibiting no destruction in regions of the hippocampus involved in memory. Magnification-10X, Scale bar-0.25 mm.

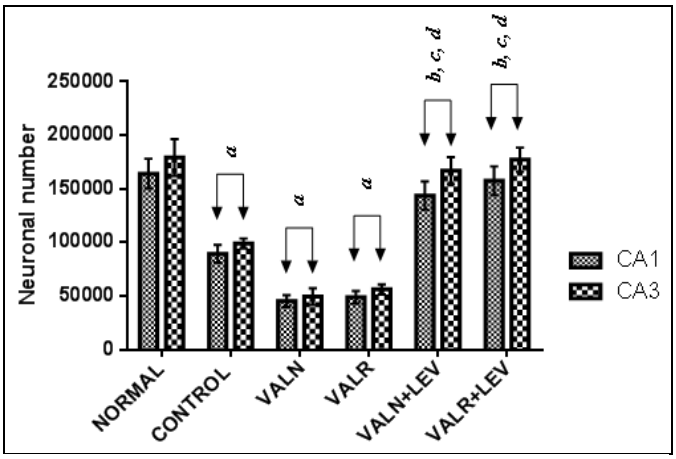

FIG. 7: TOTAL NEURONAL NUMBER OF CA1 AND CA3 REGIONS OF THE HIPPOCAMPUS

Values are Mean ± SEM, n = 6, $p \leq 0.05$ analysed by Two-way ANOVA followed by Tukey's Post Hoc test. a- Significant when compared to normal, b- Significant when compared to control, c- Significant when compared to PHTN and d- Significant when compared to PHTR

DISCUSSION: Since the disease *i.e.* epilepsy itself has memory impairment associated with it, a worsening of the same state is least tolerated [26, 27]. This was seen with a significant increase in control group errors than healthy / normal group. Although RAM was first used in studying of spatial memory in rats, this study was designed to evaluate the memory impairment in mouse [28, 29].

VALN and VALR had an increase in error scores when compared to normal and control groups which hence substantiates the fact that an AED upon treatment could worsen the scenario of an already present MI in TLE [26, 30, 31]. It could be assumed that magnitude of the disruption mechanism of action may not be interrelated [32]. Although this was undesirable, the degrees of errors were found to be less in the case of VALR where the tactic of dose reduction seemed to be a success. However when it came to the evaluation of anti-epileptic property of VAL, there was a reduction in potency of the AED upon dose reduction, which is unfavourable as the treatment of the disease is of primary significance and should not be neglected.

The combinatorial approach using LEV with VAL brought about a decline in error scores. This may be due to the acetyl cholinesterase inhibitory activity of LEV since reports of this memory enhancer to improve memory deficits in Alzheimer's disease could be taken into account as revealed in **Fig. 4** [33]. LEV additionally synergized the anti-epileptic property of VAL as seen in **Table 1** and **Fig. 1** which is reflected in reduction of brain glutamate levels which could be by means of modulating pre-synaptic P/Q-type voltage dependent calcium channel [34].

Variation in these brain biochemical measures were supported by Nissl/Cresyl violet staining of hippocampus and estimation of total neuronal number.

CONCLUSION: These research findings could thus find aid as an implementable directive to achieve reduction in degree of memory impairment in the case of TLE by combining a memory enhancer to the primary drug of choice. However, an add-on and in depth research to reinforce this study is of great importance and must not be disregarded.

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CONFLICT OF INTEREST: The authors declare no conflict of interest for this research.

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