Full Length Research Article

COGNITIVE AND IMMUNOHISTOCHEMICAL STUDY ON THE EFFECT OF NEOSTIGMINE ON KETAMINE INDUCED WISTAR RATS

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ABSTRACT

Background and Objective: Cognitive deficit is one of the symptoms of schizophrenia due to altered acetyl cholinesterase levels and thus treated by cholinesterase inhibitors like neostigmine. We studied the cognitive and immunohistochemical effect of Neostigmine on Ketamine induced cognitive deficit in rats.

Methods: Twenty (20) wistar rats were divided into four groups (n=5). Positive control (group A) received 0.1ml saline; the cognitive deficit groups received 25mg/kg Ketamine for 7days and then divided into B, C, and D groups. The group B and A were sacrificed in day 8, while C and D groups were treated with 0.5mg/kg Neostigmine and negative control treated with 0.1ml saline for 21days. T-maze cognitive test was performed on 8th and 21st days. Thereafter they were anesthetized with 50mg/kg thiopental sodium, and aortic perfusion was performed with 4% paraformaldye. The amygdala was stained for GFAP by immune peroxidase method.

Result: The result showed that the T-maze cognitive deficit induced by Ketamine was reversed by Neostigmine as the rat showed increased number of time entries and time spent in the rewarded arm. GFAP expression increased in Ketamine group was decreased by Neostigmine treatment.

Interpretation and Conclusion: Neostigmine ameliorates the cognitive and decrease GFAP expression induced by Ketamine on the amygdala.

INTRODUCTION

The menace of mental illnesses have continued to stare the face of most communities in Nigeria and Africa at large. These illnesses have overwhelmed the physician as the illness becomes progressively less responsive to the existing conventional therapies. Typically mental illness are referred to as psychosis, common examples are schizophrenia or substances induced psychosis (Betram *et al.*, 2009). Schizophrenia is a complex psychiatric disorder with multiple causes and constitutes a larger part of the global burden of the disease. The cause of schizophrenia is not clearly known but, substance abuse and hereditary factor have been implicated (Scott *et al.*, 2012). The neurobiology facet: neuroanatomical, neurobehavioral and Neurochemistry are major areas explored

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in providing an understanding of the mechanism and pharmacotherapy of this disease (Scott *et al.*, 2012). Ketamine is a dissociative anesthetic agent capable of inducing schizophrenic like cognitive symptoms if given in sub-anesthetic doses in rodents (Aroni *et al.*, 2009). It is also an N-Methyl-D-Aspartate Receptor (NMDAR) antagonist whose hypo function is in sighted in the glutamate hypothesis of schizophrenia (Brown el al., 2012; Scott *et al.*, 2012).

Hence Ketamine cognitive psychosis deficit and that of schizophrenia have similar pharmacotherapeutics responsiveness (Betram *et al.*, 2009). Neostigmine is an acetyl cholinesterase inhibitor that reverses the cognitive defect induced by ketamine. It is also a cholinergic drug that uses acetylcholine neurotransmitter to enhance memory and improve cognition (Alderdiee *et al.*, 1979; Lawrence *et al.*, 1979). This fact was documented in previous pharmacological and biochemical studies on the activity of neostigmine and ketamine on Acetyl cholinesterase enzyme and acetylcholine

neurotransmitter on cognition (Eckermas *et al.*, 1979). But no available literature has shown the anatomical and behavioral changes that may accompany these biochemical and pharmacological changes. Thus, it is worthwhile to investigate the possible changes in brain structures following administration of ketamine and neostigmine particularly on the Amygdala.

MATERIALS AND METHODS

Experimental Animal

Twenty (20) adult Wister rats of both sexes, average weight of 180g were purchased from the animal house of the Department of Physiology, College of Medicine. University of Nigeria Enugu Campus and housed at the Animal facility of Department of Anatomy Abakaliki. The animals were housed in netted iron cages in group of five, fed with grower's mash and given water *ad libitum.* The rats were maintained under laboratory conditions (temperature 24±2 ^{o}C, with relative humidity 60-70%, and a 12- hour light-dark cycle). The animals were acclimatized for two weeks before the experiment; all behavioral experiments were carried out under dim light.

Drugs and Chemicals

Neostigmine was used as standard reference cholinesterase inhibitor. Ketamine hydrochloride injection (Rotex Medica. Trittau. Germany) was used to induce cognitive deficit in the rats. The drugs were procured from registered pharmacy in Enugu and the drug doses were selected based on data from literature and drug information leaflets. Routine histological reagents were purchased from chemical stores, GFAP was procured from Novacastra Leica Germany.

Ethical Considerations

The experimental procedures and techniques used in the study were in accordance with accepted principles for laboratory animal use and care and all the protocols used were approved by the ethics and research committee of the Faculty of Basic Medical sciences, Ebonyi State University Abakaliki.

Ketamine Induction

Twenty (20) rats were divided into Group A and Group B. Group A (control, n=5) received 0.1ml of 0.9% saline while group B (n=15) received 25mg.kg.-1 ketamine hydrochloride (i. p), for 7days. On the 8 day, the ketamine group A (n=15) where divided into three (3) groups as B1, B2 and B3. Groups A and B1 were sacrificed on the 8th day. Group B2 received 0.5mg/kg of neostigmine while Group B3 which is the negative placebo was given 0.1ml of 0.9% saline for 21 days respectively.

On the 8th and 21st day the rats in all the groups were subjected to behavioral study. On the 22nd day the rats were anaesthetized with thiopental sodium and aortic perfusion-fixation with 4% paraformaldehyde was performed on the rats. The brain was dissected out and further fixed in 10% paraformaldehyde overnight for immunohistochemical studies.

Elevated T-Maze Test behavioral study

The T Maze is a simple maze used in animal cognition experiments to study how rodents function with memory and spatial learning through applying various stimuli. The elevated T-maze apparatus was made of wood, shaped like the letter T with two turns- left and right arm and a base.

The rat was placed at the base (starting point) of the maze and reward was placed in the left arm of the maze, the rat was allowed to make the choice of which arm to enter. The study was repeated after removal of the reward (food) .The choice of the left arm was considered a right choice (memory) for two trials.

Immunohistochemical Method for GFAP

The Avidin - Biotin immunoperoxidase method was used for the work. The antibody dilution factor used was 1:100 dilutions for the antibody markers. The paraffin processed tissue were sectioned at 2 microns on the rotary microtome and placed on the hot plate at 70 degree for at least 1hour.Sections were brought down to water by passing them on 2 changes of xylene, then 3 changes of descending grades of alcohol and finally to water.

Antigen retrieval was performed on the sections by heating them on a citric acid solution of pH 6.0 using the microwave at power 100 for 15minutes.The sections were equilibrated gradually with cool water to displace the hot citric acid for at least 5minutes for the section to cool.Peroxidase blocking was done on the sections by simply covering section with 3% hydrogen peroxide (H_2O_2) for 15minutes.Sections were washed with PBS and protein blocking were performed using avidin for 15minutes.Sections were washed with PBS and endogenous biotins in tissue were blocked using biotin for 15minutes.

After washing with PBS sections were incubated with the respective diluted primary antibody, example GFAP antibody diluted 1:100 for 60mintes.Excess antibodies were washed off with PBS and a secondary antibody (LINK) was applied on section for 15mintes.Sections were washed and the (LABEL) which is the horseradish peroxidase (HRP) were applied on the sections for 15minutes.A working DAB solution is made up by mixing 1 drop (20microns) of the DAB chromogen to 1ml of the DAB substrate.

This working solution is applied on sections after washing off the HRP with PBS for at least 5minutes. The brown section begins to appear at this moment especially for positive target.Excess DAB solution and precipitate are washes off with water.Sections are counterstained with haematoxylin solution for at least 2minutes and blued briefly.Sections are dehydrated in alcohol, cleared in xylene and mounted in DPX.

Statistical Analysis

All data were presented as mean ± SEM and analyzed using one-way analysis of variance (ANOVA) with drug treatments as a between-subjects factor.
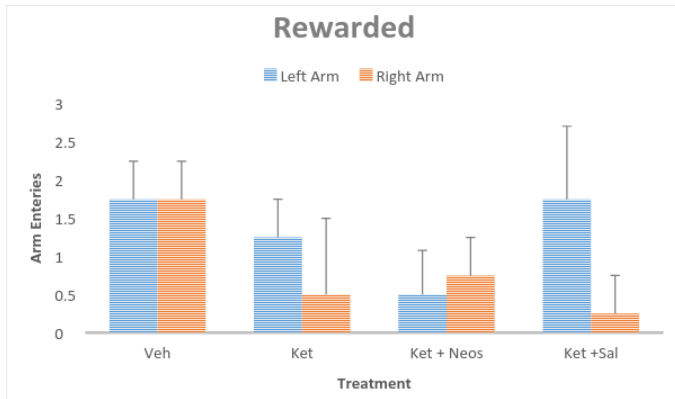
RESULTS

Elevated T-Maze

Figure 1. Graph showing the Effect of Saline, Ketamine and Neostigmine on the Arm Enteries of Elevated T-Maze with left arm rewarded. Each bar represents mean S.E.M. *P* values for group comparisons were obtained by one way ANOVA followed by Student-Newman–Keuls test. †*P* <0.05; ††*P* <0.01 compared to the vehicle-treated group.
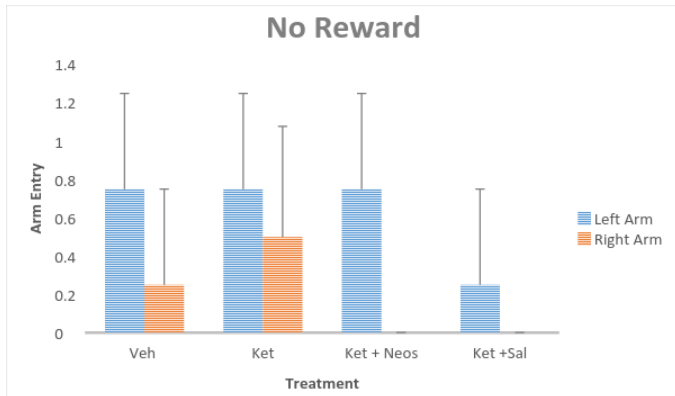

Figure 2. Graph showing the effect of Saline, Ketamine and Neostigmine on the Arm Enteries of the Elevated T-Maze when not rewarded. Each bar represents mean S.E.M. *P* values for group comparisons were obtained by one way ANOVA followed by Student-Newman–Keuls test. †*P* <0.05; ††*P* <0.01 compared to the vehicle-treated group.
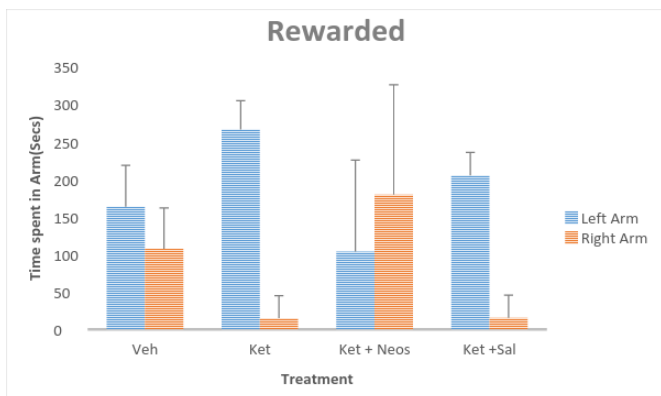

Figure 3. Graph showing the percentage arm entries, and percentage time spent on the arms of the elevated T-Maze, over a five-minute test period in rat when rewarded. Each bar represents mean ± S.E.M. *P* values for group comparisons were obtained by one way ANOVA followed by Student Newman–Keuls test. †*P* <0.05; ††*P* <0.01 compared to the vehicle-treated group.
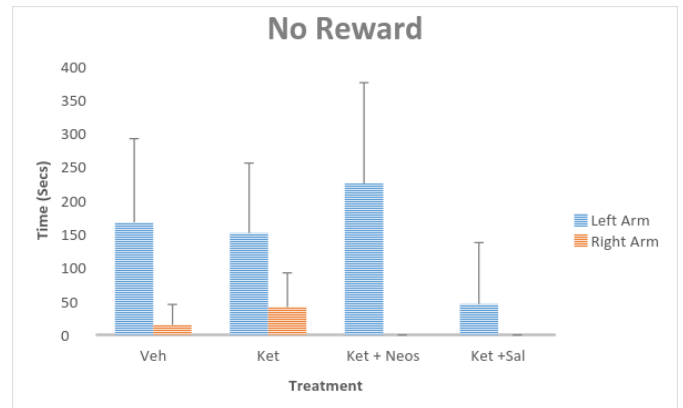

Figure 4. Graph showing the percentage arm entries, and percentage time spent on the arms of the elevated T-Maze, over a five-minute test period in rat when not rewarded. Each bar represents mean ± S.E.M. *P* values for group comparisons were obtained by one way ANOVA followed by Student Newman–Keuls test. †*P* <0.05; ††*P* <0.01 compared to the vehicle-treated group.

Immunohistochemical finding

Plate 1. Section of Amygdala given 0.1ml saline (control).normal expression.GFAP.x200
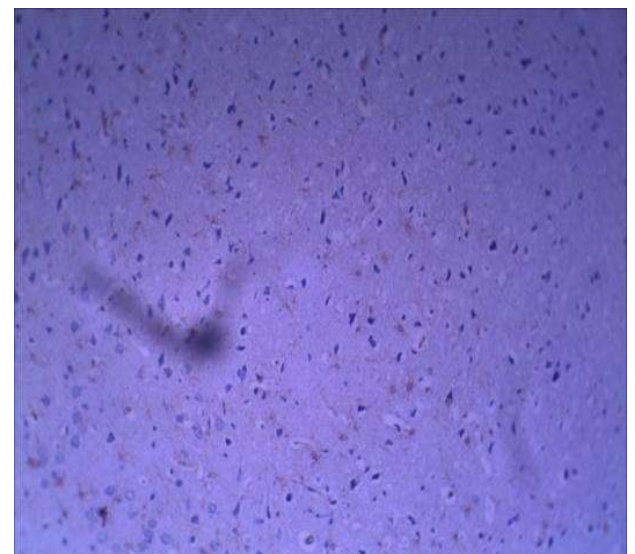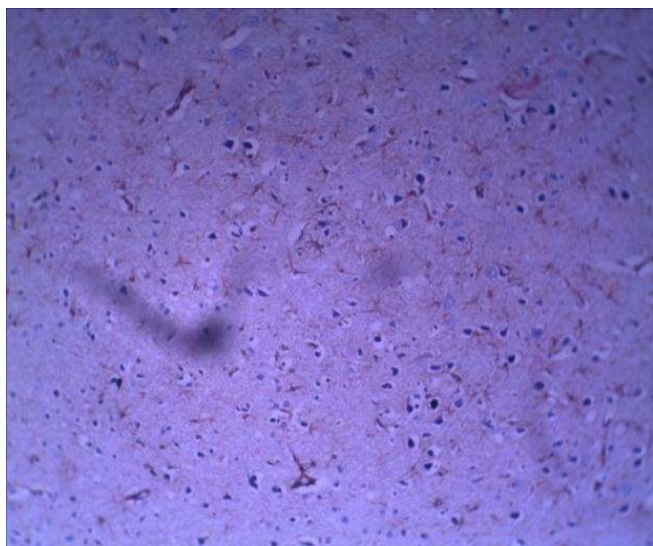

Plate 2. Section of Amygdala induced with 25mg/kg ketamine. Increase expression. GFAP. x200

Plate 3. Section of Amygdala treated with 0.5mg/kg Neostgmine. Decrease expression. GFAP. x200

Plate 4. Section of Amygdala treated with 0.1ml saline (control). Moderate expression. GFAP. x200

DISCUSSION

T. maze have been used to test learning and memory, preference for stimuli or rewards or spontaneous alternation behaviors (Eric *et al*., 2011). In this study all the groups exhibited spontaneous alternation even with reward placed in the left arm (fig 1). These indicate normal rodent behavior in their natural environment (Eric *et al*., 2011). Upon removal of the reward placed in the left arm, the rats treated with 0.5mg/kg of Neostigmine visited the rewarded arm and spent the whole time in the left rewarded arm ever (fig 2 and 4). Hence this study confirmed that Neostigmine administration acted as treatment to the Ketamine induced psychotic cognitive deficit rats by reversing it effect. The observation also agreed with the pharmacological and biochemical studies earlier reported (Eckernas, 1981; Birchwood *et al*., 2006). Astrocytes played an active role in CNS function by influencing or even directing the neuronal activities. It has been shown that accumulation of glial fibers is a histological

landmarks of astrocytes response to nervous system injury, appropriately named reactive gliosis (Scott *et al*., 2012). Such response is characterized by intense astrocytes proliferation and over expression of Glial Fibrillary Acidic Protein (GFAP). The Glial Fibrillary Acidic Protein (GFAP) is expressed by astrocyte only in the brain. In this present study the control groups given 0.1ml of saline showed normal GFAP expressions (Plate 1). The group induced with 25mg/kg of Ketamine revealed increased expression of astrocyte (Plate 2). Decreased expression was observed in the treatment group treated with 0.5mg/kg of neostigmine (Plate 3) and saline treated group showed moderate expression of astrocyte compared to the control group and Ketamine group (Plate 4). The finding signifies that Ketamine actually induced astrocyte expression, but was resolved following treatment with the Neostigmine. Ketamine induced cognitive deficit was reversed by Neostigmine treatment and resolved further progression of the effect of Ketamine. Likewise the finding showed that withdrawal of Ketamine administration produces a slow resolution of effect as seen in the saline treated group (negative placebo). This finding can translate into improved cognitive, structural, physiological and therapeutic responses if neostigmine is being use alongside antipsychotic treatments.

REFERENCES

Aggleton, JP. and Passingham, RE. 1981. Syndrome produced by lesions of the amygdala in monkeys (Macaca mulatta). *Journal of Comparative and Physiology Psychology*95(6): 961-977.

Alderdice, MT. 1979. Physiology, but not Neostigmine, inhibits acetylcholine release; *Brain Res*178(2-3):596-9.

Aroni, F., Iacovidou, N., Dontas, I. and Pourzitaki, C. 2009. Pharmacological aspects and potential new clinical applications of ketamine: Reevaluation of an old drug. *Journal of Clinical Pharmacology*49 (8): 957–64.

Bertram, G. K., Serzan, B. M. and Anthony, T.T. 2009. Sedative-Hypnotic drugs: *Basic and Clinical pharmacology*.11th International edition, McGraw Hill Companies inc 371-509.

Birchwood, M. and Trower, P. 2006. "The future of cognitive-behavioral therapy for psychosis: not a quasi-neuroleptic". *British Journal of Psychotic*188(2): 108-108.

Brown, EC., Tas, C. and Brüne, M. 2012. "Potential therapeutic avenues to tackle social cognition problems in schizophrenia". *Expert Rev Neurother*12 (1): 71–81.

Eckernas, SA. 1981. Pharmacokinetics of Neostigmine and pyridostigmine in man and its correlation to clinical effects in myasthenia gravis; *Adv Behav Biol*25 (cholinergic mech);879-90.

Eric, W., Wonder, K. M. A. and Abraham, Y. M. 2011. Anxiolytic and Anti - depressant like effects of an ethanolic extract of aerial part of *Hilleria Latifolia* (Lam) H. Walt in mice. *Journal of Natural Pharmaceutical.* 2(2):62-71.

Lawrence, D. and Livingston, A. 1979. The effect of physosstigmine and Neostigmine on Ketamine anesthesia and analgesia; *Br J Pharmacol* 67(3):426.

Stephens, D. N. and Duka, T. 2008. "Cognitive and emotional consequences of binge drinking: Role of amygdala and prefrontal cortex". *Philosophical Transactions of the Royal Society B*363 (1507): 3169–79.

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