HIPPOCAMPAL CELLULAR RESPONSES TO *Datura metel* ALKALOID (DATUMETINE) AND N-METHYL-D-ASPARTATE RECEPTOR INTERACTION IN C57BL/6 MICE

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CERTIFICATION

This is to certify that this thesis by ISHOLA, Azeez Olakunle (07/46KA044) has been read and approved as meeting the requirements of the Department of Anatomy, University of Ilorin, Ilorin, Nigeria for the award of Doctor of Philosophy (PhD.) degree in Anatomy.

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DEDICATION

This research work is dedicated to my late father who does not witness the end of this voyage with me, my mother, wife, children and all my tutors in life.

DECLARATION

I, Azeez Olakunle ISHOLA, a Ph.D. student in the Department of Anatomy, University of Ilorin, Ilorin, hereby declare that this thesis entitled "Hippocampal Response to *Datura metel* Alkaloid (Datumetine) and N-Methyl-D-Aspartate Receptors Interaction In C57BL/6 Mice" submitted by me is based on my actual and original work. Any materials obtained from other sources or work done by any other persons or institutions have been duly acknowledged. Also, the research work has been approved by the University of Ilorin Ethical Review Committee.

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Abbreviations

aCSF – artificial cerebrospinal fluid

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor

ANOVA – analysis of variance

APS – ammonium persulfate

BDNF – brain-derived neurotrophic factor

BSA – bovine serum albumin

CA – CornuAmmonis

CREB – cyclic AMP response element-binding protein

DG – Dentate Gyrus

DMSO – Dimethyl sulfoxide

EM – Electron microscopy

GCL – granular cell layer

HRP – horseradish peroxidase

IACUC – Institute of Animal Care and Use Committee

IBI – interburst interval

ISI – inter-spike interval

ISIH – interspike interval histogram

ITI – inter-trial interval

LTD – long term depression

LTP – long term potentiation

ML – Molecular layer

NMDAR – N-methyl-D-aspartate Receptor

NOR – Novel Object Recognition Test

NPS – New Psychoactive Substances

OCT – optimal cutting temperature

OFSS – offline spike sorting software

PAMs – positive allosteric modulators

PBS – phosphate-buffered solution

PBST – phosphate-buffered solution + tween 20

PCA – principal component analysis

PDB – Protein Data Bank

PFC – Prefrontal Cortex

PP1 – protein phosphatase1

PSD – postsynaptic density

PVDF – polyvinylidene difluoride

RIPA – radioimmunoprecipitation assay buffer

SV – synaptic vesicles

TBS – Tris-buffered saline

TBST – Tris-buffered saline + triton-X

TEMED - tetramethylethylenediamine

Tyr – tyrosine

UERC – University of Ilorin Ethical Review Committee

UNODC – United Nations Office of Drug and Crime

VTA – Ventral Tegmental Area

ABSTRACT

Datura metel is a plant that is used as a form of herbal treatment in Nigeria. However, its recreational abuse is increasing among Nigerian youths due to its availability. Which is worrisome because of some untoward side effects like memory loss and hallucinations in humans. This study was designed to investigatethe effects of datumetine (alkaloid) in *Datura* plant on hippocampal N-methyl-D-aspartate receptors (NMDAR) functions. The objectives of the study were to determinethe effects of datumetine on: (i) memory; (ii) some NMDAR signalling molecules (iii) hippocampal cellular morphology; (iv) hippocampal synapse; and (v) hippocampal electrical activity.

Thirty adult male C57/BL6 mice were assigned into three groups of 10 mice each. The mice were administered dimethyl sulfoxide (DMSO/Control), 0.25mg/Kg body weight of datumetine and 1mg/Kg body weight of datumetine intraperitoneally for 14 days. Novel object recognition (NOR) and Y-maze tests were conducted on the animals on days 10 and 13 of administration to assess the level of memory. At the end of treatment, mice were euthanized in isofluorane chamber, perfused transcardially with 1X phosphate buffered solution followed by 4% paraformaldehyde (for immunofluorescence samples). Western blotting was used to assess hippocampal levels of glutamate ionotropic receptor subunit-1 (GluN1), calcium-camodulin kinase-alpha 2-subunit (CamKIIα), phosphorylated calcium-camodulin kinase-alpha-2 subunit at threonine-286 (pCamKIIα-T286), cyclic adenosine-mono-phosphate response element-binding protein (CREB) and brain-derived neurotrophic factor(BDNF) while the distribution of major neuronal subtypes, astrocytes, and microglia were expressed using immunofluorescence antibodies. Expansion and electron microscopy techniques were used to assess neural connections and synapse morphology respectively, while *in vivo* electrophysiology wasperformed for hippocampal electrical activity. Quantitative data were compared using analysis of variance(ANOVA) and/or unpaired t- test at significant level of $p<0.05$.

The findings of the study were that:

i. datumetine binds with NMDAR at its binding sites
ii. memory index from NOR (44.31±5.86%, 45.71±7.91%) and Y-maze (53.05±1.91%, 30.53±4.47%) were significantly reduced in datumetine exposedanimals than control(86.69±8.44%, 66.86±3.53%) ($F(2,12)=7.514$, $F(2,8)=15.96$ $p = 0.0077$, 0.0160)
iii. datumetine significantly increased hippocampal expression of GluN1 (0.0666±0.0088, 0.0987±0.0227) and CamKIIα (0.4276±0.0016, 0.2679±0.0076) than control (0.0406±0.0068, 0.1609±0.0051) ($p = 0.0578$, 0001) while pCamKIIαT286 (0.4062±0.0051, 0.3552±0.0171), CREB (0.2447±0.0258) and BDNF (1.1680±0.0195, 0.8741±0.0287) were significantly reduced in datumetine treated animals than control (0.4326±0.0144, 0.4615±0.0050, 1.2680±0.0337) ($p = 0.00760$, 0022, 0.0001),
iv. increased hippocampal expression of astrocytes, microglia, glutamatergic, GABAergic, cholinergic, and dopaminergic neurons are in datumetine exposed mice.However, there was a

reductionin expression of serotonergic neurons of datumetine exposed mice (791.6±98.2, 953.7±22.3) compared to control (1368.0±64.5) ($F(2,27)=22.49$, $p=0.0016$),

v. datumetine exposure depleted neurofilament expression, arrangement and altered synaptic morphology; and
vi. prolonged duration of interspike interval (2.607±0.772 s), interburst interval (16.08±2.86 s) and burst duration (0.0599±0.0093 s) were observed in datumetine treated mice than control (0.533±0.077 s, 9.265±0.86 s, 0.0464±0.0028 s) ($p = 0.0007, 0.0059, 0.0880$).

The study concluded that datumetine increased hippocampal NMDAR activity, resulting in excitotoxicity, impairment of selected neural diffuse systems thereby leading to memory loss. The study recommended that the use of *Datura metel* plant be discouraged among the populace

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Drug abuse is a global challenge in the present time.According to the United Nations Office of Drug and Crime (UNODC), about two hundred and seventy-five million (275,000,000) people aged fifteen to sixty-four (15 – 64) globally use one form of drug or the other (UNODC, 2018). Although recreational use of cannabis and other psychoactive substances trend is believed to be stable in developed countries, there is still steady rise in people exposed to/using the drug which is attributed to a growing population (UNODC, 2018). In Africa, UNODC estimated that more than fifty per cent (50%) of the global drug abusers reside in the continent (Ndinda, 2018). However, this value was believed not to represent the true situation of drug used in Africa due to paucity of data from member countries and noncompliance of some member states to standardize research and reporting together with some civil unrest in some parts (UNODC, 2018).

Cannabis, heroin,and amphetamines have been the global concern before now, New Psychoactive Substances (NPS) are seen recently flooding the environment with its major health risk and challenges to its users. These new substances are either gotten from plants that grow widely which are often referred to as ‘herbal high’ or pharmaceutical drugs being used without prescription (UNODC, 2017; 2018). These NPS are being marketed as ‘legal high’ due to no legislation in most countries that categorizes the use of such substances as illegal (UNODC, 2017; 2018).

Africa alone saw the emergence of three (3) NPS (khat- *Catha edulis*, *Datura spp.* and phenethylamines) in the year 2016, out of which *Khat and Datura metel* are plants that grow widely in the continent (UNODC, 2017). Use of datura is a new trend among the adolescent especially secondary school students (Stella *et al.,* 2010; Arefi*et al.,* 2016) which may be due to its easy availability as the plant usually grow close to dumpsite and roadside.

In Nigeria, *Daturametel* recreational use accounts for 0.08% of illicit drug use (Moses, 2010). The recreational use of the plant is connected to its hallucinogenic property by causing delirium (Soni*et al.,* 2012). The plant has been reported to contain tropane alkaloid which acts by blocking acetylcholine through muscarinic receptors (Yussuf, 1991; Donatus and Ephraim, 2009; Katzung*et al.,* 2009). Due to its anticholinergic property, the plant is very toxic and at high dose can result inthe death of its users (Prasad and Gowda, 2005). From the available literature, the use of datura is ancient, asthe plant was claimed to be used in the treatment of asthma, relieving of respiratory distress, pupillary dilation in glaucoma, and as antibiotics for wound infections (Hilal *et al.*, 2014).It has been proposed to be useful in cancer treatment (Devi *et al.*, 2011), and employed by cults during their initiation ceremony. Users of the plant usually don't remember any event(s) during its intoxication, this may account for the use of the plant has mind-altering and date rape drug (Devi *et al.,* 2011; Arefi*et al.,* 2016).Also, it is reported that in Italy,some robbers use this plant to dispossess victims of their belongings as theywon't remember anything afterwards (Kanchan and Atreya, 2016).

D. metel abusers are usually adults with a history of alcohol use (Rahmatullah*et al.,* 2010), although there is an increasing trend among adolescence population (Stella *et al.,* 2010; Vearrier and Greenberg, 2010). It is usually used to increase the level of intoxication in alcoholic drinks,

in this case, users add the leaves or dried seeds of the plants to their alcoholic drinks for some time to increase their intoxication level.

Symptoms of datura intoxication are referred as toxidrome; which are: “blind as a bat; mad as a hatter, red as a beet, hot as a hare, dry as a bone, the bowel and bladder lose their tone; and the heart runs alone” (Soni*et al.,* 2012; Adegoke and Alo, 2013) which reflectthe loss of cholinergic activities and neural synchronization. Long consumption has been reported to lead to the onset of paranoid schizophrenia (Khanra*et al.,* 2015).

Datura metel belongs to the *Solanaceae*family which usually grows at the dumpsite, although it is believed to originate from Asia continent but is found in all part of Nigeria (Malamiand Alhassan, 2014), and contains a different alkaloid (PrassadandGowda, 2005). The plant has been found to contain more than thirty alkaloids and other compounds (Maheshwari*et al.,* 2013). Out of which are atropine, hyoscyamine and scopolamine which are anticholinergics (Yussuf, 1991; Donatus and Ephraim, 2009) exerting their action both centrally and peripherally (Katzung*et al.,* 2009). Other compounds isolated from the plant has been reported to have a sedative effect (Malamiand Alhassan, 2014; Babalola *et al.,* 2015), antimicrobial effects (Dabur *et al.,* 2004; 2005; 2007; Bajwa*et al.,* 2008; Khan and Nasreen, 2010; Akharaiyi, 2011), and hypoglycaemic effects (Murthy *et al.,* 2004). Due to its central and peripheral anticholinergic properties, usage of this plant is dangerous as it can cause hallucination, delirium and in some cases death when the excess dose is consumed (Prasad and Gowda, 2005). Most research works focused on the tropane alkaloids of the plant elucidating its hallucinogenic properties and anticholinergic properties and this has been attributed to atropine, scopolamine, and hyoscine (Devi *et al.*, 2011; Hilal *et al.*, 2014; Malami and Alhassan, 2014). Malami and co-workerin 2014 demonstrated that

seed extract of the plant has sedative and hypnotic effect in mice, and this was attributed to the effects of the flavonoids present in the extract.

Datura intoxication is usually achieved by soaking the leaves in water or alcohol, smoking the dried seed, or chewing the stem. Although not only tropane alkaloids constitute major compounds found in datura extract (Nuhu and Gani, 2002; Devi *et al.,* 2011; Kiruthika and Sornaraj, 2011; Monira and Munan, 2012; Maheshwari *et al.,* 2013; Arefi*et al.,* 2016), there is a dearth of information on the biological activity of other classes of compounds found in datura extract. This is necessary to ascertain the level of health risk and the problems it poses to its users.

The brain is the organ that coordinates the physiological process of the body such as circulation, respiration, body coordination etc. (Zhao *et al.,* 2008). It is also involved in the control of human behaviour and memory storage (Xu *et al.*, 2016; Svoboda *et al.*, 2017). Alterations in the normal functioning of the brain have resulted ina myriad of disorders affecting behaviour and memory of individuals (Glass *et al.*, 2010). These disorders range from neurodegenerative diseases like Parkinson's, Alzheimer's diseases, addiction and cognitive impairment (Glass *et al.,* 2010; Soussan and Kjellgren, 2016).

Hippocampus is the key brain region responsible for learning, memory storage, consolidation and retrieval (Siegel and Sapru, 2011). It is located beneath the temporal cortex on each side (Hayman *et al.,* 1998). It is part of the limbic system that helps control behaviour (Siegel and Sapru, 2011). It receives inputs from different brain areas like prefrontal cortex (PFC), cerebellum, ventral tegmental area (VTA), and projects its fibres to other brain regions as well mainly PFC (Varga*et al.,* 2009; Yi *et al.,* 2015; Takeuchi *et al.,* 2016). Structurally, it is mainly

divided into dentate gyrus (DG), cornuammonis (CA) region, the CA region is subdivided into CA1, CA2 and CA3 (Hayman *et al.,* 1998).

The hippocampus functions mainly using excitatory neurotransmitter glutamate (Tonegawa*et al.*, 2003; Gao *et al.,* 2010). This neurotransmitter acts with two (2) types of receptors viz; N-methyl-D-aspartate Receptor (NMDAR); and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR). NMDAR is ionotropic and allows movement of ions when activated whereas AMPAR is metabotropicand does not allow any movement of ions but causes changes in expression of cellular proteins when activated (Parsons et al., 1998; Schoepp et al., 1999). Out of the two receptors, NMDARhas been greatly implicated in the function of the hippocampus as it relates to memory and behaviour (Tonegawa*et al.,* 2003; Gao *et al.,* 2010).

NMDAR plays an essential role in brain functions ranging from excitatory neurotransmission to learning and memory (Zito and Scheuss, 2009). It functions mainly in the coupling of electrical to biochemical signalling through calcium influx in the neuron (Cull-Candy and Leszkiewicz, 2004). It also acts as an electrical conductance alone for the signal transfer (Dingledine *et al.,* 1999). While NMDAR has also been implicated in brain plasticity with long-term potentiation (LTP), in learning and memory (Dan and Poo, 2006).

1.2 Statement of Problem

One in seven persons of age 15-64 years had used an illicit drug other than tobacco and alcohol, the prevalence of drug use had increased to 14.8% in the year 2018 (UNODC, 2018). With the increase in the socioeconomic status of the country, illicit drug users are tending towards the use of a natural product to achieve intoxication. *D. metel* is one of the plants being used which has very low toxic dose. Although the use of the plant is just gaining ground, it is spreading like

wildfire and several deaths have been recorded. Few that survived the use of the plants don't remember what transpired during the intoxication. Hence, the need for research work to identify the compound(s) that is responsible for short amnesia seen in datura users during intoxication.

1.3 Justification of the Study

Use of datura plants for intoxication is on the increase among adolescents in Nigeria (Moses, 2010), several deaths have been recorded and alluded to datura overdose (Devi *et al.*, 2011; Arefi *et al.*, 2016). Some compounds in datura extract which does not modulate the brain cholinergic system has been identified (Maheshwari *et al.*, 2013; Malamiand Alhassan, 2014; Babalola *et al.*, 2015). Datura users suffer short amnesia during intoxication (Stella *et al.*, 2010). This shows that other compounds present in the plant may directly or indirectly affect the function of the hippocampus which is responsible for memory formation. This alteration(s) may be in part due to NMDAR, which is a major excitatory neurotransmitter receptor responsible for hippocampal function (Gao *et al.*, 2010). Hence, need to research other compounds in the plant and how they affect NMDAR activity in the brain.

1.4 Aim of The Study

The study is designed to assess the hippocampal effect of datumetine-NMDAR interaction

1.5 Specific Objectives of the Study

The specific objectives of the study are to:

- identify the compound of datura that binds well with NMDA receptors
- determine the effect of datumetine on short-term working memory

- check the action of datumetine on NMDAR synapto-nuclear signalling molecules
- ascertain the effect of datumetine on differential hippocampal cells morphology
- evaluate the effect of datumetine on hippocampal synapses
- assess the effect of datumetine on the electrical activity of the hippocampus

1.6 Research Questions

The research question is to determine whether *D. metel* have the modulatory properties on the Central Nervous System NMDAR and the cellular effects in the hippocampus.

1.7 Research Hypothesis

H_0: *Daturametel* plant does not possess compound(s) that modulate NMDAR activity leading to memory decline

H_1: *Daturametel* plant possesses compound(s) that modulate NMDAR activity leading to memory decline

1.8 Scope of Study

This study will use simulation software to identify a compound that can interact favourably with NMDAR. Furthermore, the electrical effect of the compound interaction with NMDAR will be checked using in vivo electrophysiological recording. The memory effect of the compound will be evaluated after two weeks of exposure using novel object recognition (NOR) and Y-maze test. Structural changes to the hippocampus after two weeks exposure of the compound will be checked using immunofluorescence and electron microscopy method whilethe action of NMDAR signalling after compound exposure will be ascertained by western blotting technique.

1.9 Significance of Study

This study will show more of the neurotoxic effect of *Datura metel* plant on the brain and how it affects memory formation. The study will be able to identify the compound in part, responsible for memory loss seen in Datura intoxication and the mechanism involved. This will add to the knowledge of memory processing and formation mechanisms and lead to better treatment of its abusers.

1.10 Expected Outcome of The Study

The expected outcome of the study is that;

i. which of the compound in the plant can interact with NMDAR?
ii. what molecular pathway will be implicated in the modulation of NMDA receptor by the compound?
iii. what structural alterations will the compound exhibit on the hippocampus?
iv. which neuronal subtypes will be greatly affected by the compound in the hippocampus?

1.11 Limitations of the Study

This study is limited to the use of software (autodock vina) to screen for compounds identified in *Datura metel* plant as reported from Maheshwari *et al.*, (2013). In vivo hippocampal recording to detect electrical activity of the brain (hippocampus) after intraperitoneal compound exposure. The use of the memory test (NOR and Y-maze) to check for memory. Use of

immunofluorescence, expansion microscopy and electron microscopy techniques to check for structural changes due to compound exposure.

CHAPTER TWO

LITERATURE REVIEW

2.1 Use and Misuse of *Datura metel* Plant

Datura plants belong to the *Solanaceae* family (Jamdhade*et al.*, 2010; Ganesh *et al.*, 2015; Al-Snafi, 2017). It is locally referred to as ‘gegemu – Yoruba, zakami – Hausa, and myaramuo – Igbo. The plant is usually 0.5-1.5 m tall with a usually dark violet stem (Jamdhade*et al.*, 2010). The leaf is usually ovate or broadly ovate asymmetrical, with erect flowers, white corolla, which can also be yellowish or pale purple funnel form, sometimes doubled or tripled (Jamdhade*et al.*, 2010). The fruits are rounded with spiny bark.It usually grows in the bush and dumpsites.

Figure 2.1: Picture of Datura Plant (Ishola, 2020)

The plant use is well documented since the ancient time from treating illness to religious and occultic practices (Lindley, 1985; Lewis and Elvin-Lewis, 1977; Agharkar, 1991; Agra *et al.*, 2007; Wang *et al.,* 2008; Murch *et al.,* 2009). The plant has been documented in the treatment of asthma, bronchitis, wound healing, sedative, antiseptic etc (Ahgarkar, 1991; Bhattacharjee and Kumar, 1998; Parrotta, 2001; Murthy *et al.,* 2004; Agra *et al.,* 2007). In all, anticholinergic symptoms have been associated with the use of the plant. In recent times, the narcotic use of the plant is becoming popular especially among adolescents (Moses, 2010).

Accidental or intentional ingestion of the plant has been reported due to its wide distribution. Symptoms of ingestion are usually delirium, drowsiness, agitation, hallucination, ataxia, myoclonus jerking, convulsion, coma, dilated pupils, dry mouth, dry skin, hyperthermia, sinus tachycardia, cardiac conduction abnormalities, dysrhythmia, urinary retention, paralytic illnesses (Phua*et al.,* 2008) and in some cases death (Prasad and Gowda, 2005). Extracts from the plant have also been documented to be used as a date rape drug, with the victims experiencing temporary memory loss during intoxication (Kanchan and Atreya, 2016).

The plant is regarded as anticholinergic due to the presence of tropane alkaloids (Yussuf, 1991; Donatus and Ephraim, 2009). Atropine, scopolamine, and hyoscyamine (hyoscine) are found in all parts of the plants (Yussuf, 1991; Afsharypuour*et al.,* 1995; EFSA, 2008; Donatus and Ephraim, 2009; Jamdhade*et al.,* 2010; Ratan *et al.,* 2011; Maheshwari *et al.,* 2013). These alkaloids are shown to vary in different parts of the plants and seasons (Afsharypuour*et al.,* 1995). Other compounds apart from alkaloids have also been identified and isolated from the

plant (Khaleque*et al.,* 1974; Manickam *et al.,* 1993; Pan *et al.*, 2007; Yang *et al.,* 2007; Kuang*et al.,* 2008; Kiruthika and Sonaraj, 2011; Yang *et al.,* 2014a,b; Han *et al.,* 2015).

In recent times, reports have shown the medicinal properties of the plant which have not been yet attributed to compounds present in the plant (Murthy *et al.,* 2004; Al-Snafi, 2017). Extracts from the plant were shown to have antimicrobial (Rajesh and Sharma, 2002; Dhiman *et al.,* 2012; Salma *et al.,* 2015), insecticidal (Yamazaki and Tagaya, 1980; Alacron*et al.,* 1984; Singh and Singh, 2008), anti-inflammatory, analgesic, antipyretic (Abena*et al.,* 2003; Nivedhita*et al.,* 2010), antispasmodic (Prabhakar and Kumar, 1994), antioxidant (Deepa *et al.,* 2014; Roy *et al.,* 2016), cytotoxic (Pan *et al.,* 2007; Bellila*et al.,* 2011; Xue*et al.,* 2015; Roy *et al.,* 2016) wound healing (Anitha and Suseela, 2010), reproductive (Al-Mailay, 2008; Pandiarajan*et al.,* 2012), antidiabetic (Murthy *et al.,* 2004) and neurologic effects (Abena*et al.,* 2004; Babalola *et al.,* 2013; Deepa *et al.,* 2014; Babalola *et al.,* 2015; Etibor*et al.,* 2015).

The antibacterial effect of the plant was found to be the function of 5', 7' dimethyl 6' – hydroxy 3', phenyl 3 a – amine b – yne sitosterol which was isolated from the plant leaves (Okwu and Igara, 2009) while 2beta –(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1'-methylethyl pentanoate also from the leaves possess antifungal abilities (Dabur *et al.,* 2004). Atropine was also reported to have antiviral properties against the enveloped virus by blocking the glycosylation of viral proteins (Yamazaki and Tagaya, 1980; Alacron*et al.,* 1984).

Daturafolisides A and B, baimantuoluosideB and 12-deoxywithasstramonolide isolated from datura leaves possess anti-inflammatory properties (Yang *et al.,* 2014b) dmetelin A, D and 7α,27-dihydroxy-1-oxo-with a-2,5,24-trienolide (also from datura leaves) was shown to inhibit lipopolysaccharide (LPS) induced nitric oxide (NO) production in cell lines (Yang *et al.,* 2014a).

12α-hydroxydaturametelin (a withanolide from datura) exhibits cytotoxic effects against cancer cell lines of murine and man origin (Bellila*et al.,* 2011). Other withanolides gotten from datura plant have also been shown to possess cytotoxic effects against cancer cell lines with lesser potency (Xue*et al.,* 2015).

Extract from the plant has been assessed on the brain using rodents and lower vertebrates. It is reported to increase motor activity, reduced duration of barbituric sleeping, antagonized catalepsy with antidepressant effect at a low dose (Abena*et al.*, 2004). It has an anaesthetic effect in dogs with poor analgesia (Babalola *et al.,* 2013), alters neuronal integrity and induced neural loss with axonal injuries in the medial prefrontal cortex (mPFC) of rats exposed to an ethanolic extract of Datura (Etibor*et al.,* 2015).

The neurotoxic effects of datura plant have been attributed to its anticholinergic effects which may be due to atropine, scopolamine or hyoscine (Etibor*et al.,* 2015). The use of datura is now been associated with memory loss (Kanchan and Atreya, 2016). Since there are more than hundred compounds that have been isolated from the plants (Maheshwari *et al.,* 2013), there may be other compounds present in the plants that can cross the brain barrier to modulate and affect the function of the brain.

2.2 Ligand Docking

Docking is the application of computer-based models to predict the best-fit orientation of a compound (ligand) that binds to a receptor of interest (Kitchen *et al.,* 2004; Gupta *et al.,* 2018). It began in the 1960s, with the advancements in physics, chemistry, informational technology and biochemistry computers are now powerful tools and techniques for drug screening, protein interactions and nanomaterials behaviour (Kavlock*et al.,* 2007; Chen, 2015). It is an important

scientific advancement for understanding chemical compounds, with three top computational scientists winning the 2013 Nobel Prize in chemistry because of this advancement (Chen, 2015). It is widely used to predict the preferred orientation of a designed molecule to a receptor when bound to each other to form a stable complex (Meng *et al.,* 2011). It is widely promoted by the government, academia and industries in stages of the drug development process (Gschwend*et al.,* 1996). Thousands of molecules can be evaluated for potential efficacy and safety at a small cost in a very short time (Schneider and Bohm, 2002).

2.2.1 Types of Docking

Rigid docking fixes protein and ligand so that bond angles or lengths are not changeable (Matsuzaki*et al.,* 2016). It is extremely fast but lacks practical use due to the neglect of conformational changes or freedom of ligands and protein or receptors (Gupta *et al.,* 2018). This is not widely used because biological systems are not rigid. Conformational changes do occur in protein or receptors when activated in a biological system (Rosenfeld *et al.,* 1995). Other variations also cause structures of protein or ligands to change in biological systems e.g. water molecules, pH and ion potential (Forli *et al.,* 2016).

Flexible docking allows conformational degrees of freedom of ligands (Chen, 2015; Gupta *et al.,* 2018). It is widely used and requires more time and computational throughput (Totrov and Abagyan, 2008). It tends to eliminate the problem of fixing by the rigid docking and allows for the mimic of biological systems.

2.2.2 Mechanism of Docking (Docking Algorithms)

The foundation for programs that are used to understand and predict chemical processes was laid by Martin Karplus, Michael Levitt and AriehWarshel in 1970 (Fersht, 2013). Docking predicts the ideal orientation of ligand in the active site of the receptor when bound to each other to form a stable complex (Gupta *et al.,* 2018). Enzyme, proteins or receptors act by forming a complex with their substrates or ligands.

Docking algorithms predict several orientations (poses) for the ligand inside the binding site (Kontoyianni*et al.,* 2004). Algorithms to generate poses are combined with scoring functions that consider the tightness of the protein-ligand interaction. Different algorithms and scoring functions have been described with GOLD, FLEX, DOCK and AUTODOCK constituting the most commonly used (Chen, 2015; Gupta *et al.,* 2018).

Docking simulation works on the principle of thermodynamics and energy change in a reaction (Gupta *et al.,* 2018). Gibbs free energy is a thermodynamic potential that measures the ability of a reaction to occur at a constant temperature and pressure (Weiland and Molinoff, 1981). Protein-ligand binding occurs when the change in Gibbs free energy of the system is negative, at constant temperature and pressure (Gupta, *et al.,* 2018). The extent of binding of a ligand to protein or receptor is determined by the magnitude of negative Gibbs free energy (-ΔG) which represents the stability of any reaction (Jenkins, 2008).

$$\Delta G = \Delta H - T\Delta S$$

The formula above represents the relationship of Gibbs free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) respectively while T represents the temperature of the reaction system in Kelvin. The lower binding energy of ligand-protein complex indicates the high stability of the ligand-protein complex at that pose (Du *et al.,* 2016).

2.2.3 Challenges of Ligand Docking

Numerous challenges have been associated with the use of docking studies. It has been shown that each algorithm has its pitfalls and shortcomings (Sousa *et al.,* 2006). Different algorithms are unable to give the same information with the same level of reliability (Gupta *et al.,* 2018). Docking results only show the binding affinity of ligands to receptors or proteins but do not give the physiological effect of the binding (Chen, 2015). Docking results also give the ideal biological model which is needed to be validated by bioassays (Prinz *et al.*, 2011).

2.3 Hippocampus

Hippocampus is a phylogenetical cortical structure that evolved from the dorsomedial aspects of the cerebral hemispheres (Vida, 2010). Made up of two interlocked folds of a cortical mantel, hippocampus proper and dentate gyrus (Cajal, 1968; Lorente de Nó, 1934). Structurally, curved part of the hippocampus resembles rams' horn, hence its Latin name *cornuammonis*(CA). The cranial part of the hippocampus is closed to the midline in the dorsal hemisphere beneath the *corpus callosum,* and its caudal part (also called temporal part) extends ventrolaterally into the temporal lobes (Vida, 2010; Knierim, 2015).

In coronal sections, the hippocampus proper (CA areas) and dentate gyrus (DG) form two interlocked "C" shape (figure 2.2). The CA regions contain mainly pyramidal cells and can be subdivided into CA1, CA2, and CA3 areas (Lorente de Nó, 1934; van Strien*et al.,* 2009). These regions were further subdivided into three zones along the transverse axis: "a" (closer to the

subiculum), “b” and “c” (closer to the hilus) (Lorente de Nó, 1934; van Strien*et al.*, 2009). DG contains a single population of granule cells (GC). The region between the CA regions and DG is the **hilus** which contains mostly mossy cells (van Strien*et al.*, 2009; Vida, 2010). Hilus is different from other regions of the hippocampus in that it shows no clear lamination and comprises of a similar ratio of interneurons to principal cells (Vida, 2010). Several controversies have been going on as to whether it belongs to the CA region as CA4 (Lorente de Nó, 1934) or to DG as a polymorphic layer (Blackstad, 1956; Amaral, 1978).

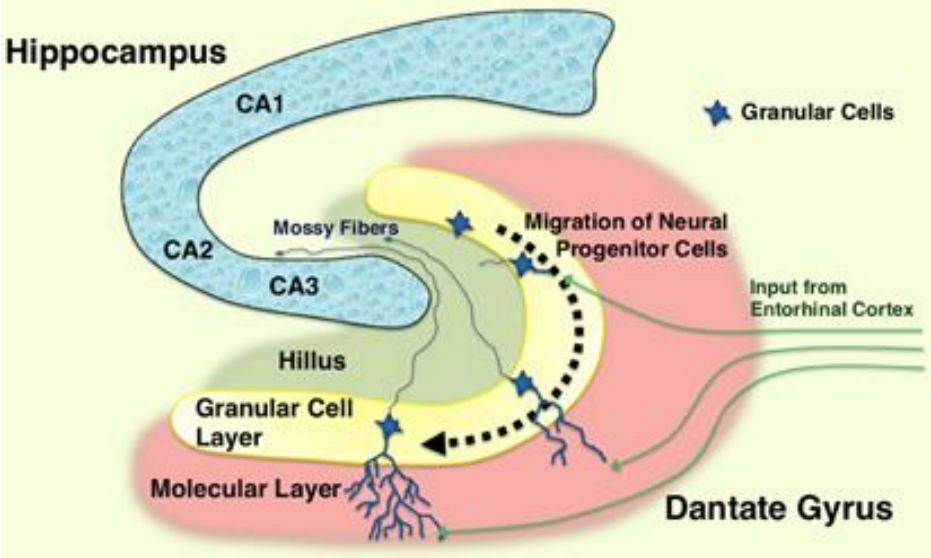


Figure 2.2: Schematic diagram of hippocampus showing its part (Vida, 2010).

The hippocampus is strictly laminated (Förster*et al.*, 2006). The principal cells are tightly arranged with their soma forming well-defined layers called **stratum pyramidale**(str.)in the CA regions and granule cell layer (GCL) in the DG (van Strien*et al.*, 2009; Vida 2010). Due to the multiple curvatures of the hippocampus, the orientation of principal cells depends on their position along the craniocaudal axis (septotemporal) and the transverse axis.It means that vertical positions are about the main axis of the principal neurons (Vida, 2010).

Neuropil in the CA regions are divided into three major layers from basal to apical direction; stratum oriens (beneath the cell body layer), stratum radiatum (above the cell body layer), and stratum lacunosum-moleculare. Stratum oriens and radiatum serve as an afferent path for commissural and association fibres from ipsi- and contralateral CA3 region, while stratum lacunosum-moleculare serve as the afferent path for perforant axons from the entorhinal cortex (van Strien*et al.,* 2009; Vida, 2010; Knierim, 2015). CA3 has an additional narrow layer called stratum lucidum (immediately above cell body layer) which corresponds to the projection of mossy fibres from DG (Vida, 2010). Neuropil above the GC in the DG is referred to as molecular layer which receives the commissural and association fibres from mossy cells (terminate proximally to inner third) and perforant axons (terminate the middle and outer third) (van Strien*et al.,* 2009; Vida, 2010; Knierim, 2015).

2.3.1 Hippocampal Connections

Cross-section of the hippocampus along its long axis shows the classical connectivity referred to as the '**trisynaptic loop'** (Knierim, 2015). In this loop, entorhinal cortex (EC), gives the major cortical input via the perforant pathway to the DG (synapse 1), DG projects to CA3 through mossy fibre (synapse 2), CA3 projects to CA1 through the Schaffer collateral (synapse 3) and CA1 send axons back to EC completing the loop (figure 2.3) (Witter and Amaral, 2004; Witter, 2010; Knierim, 2015). It is also noted that axons from CA3 also form synapses with other CA3 neurons (Knierim, 2015). This classical unidirectional loop was believed to be the main circuitry of hippocampal formations although recent studies have shown otherwise (Witter, 2010).

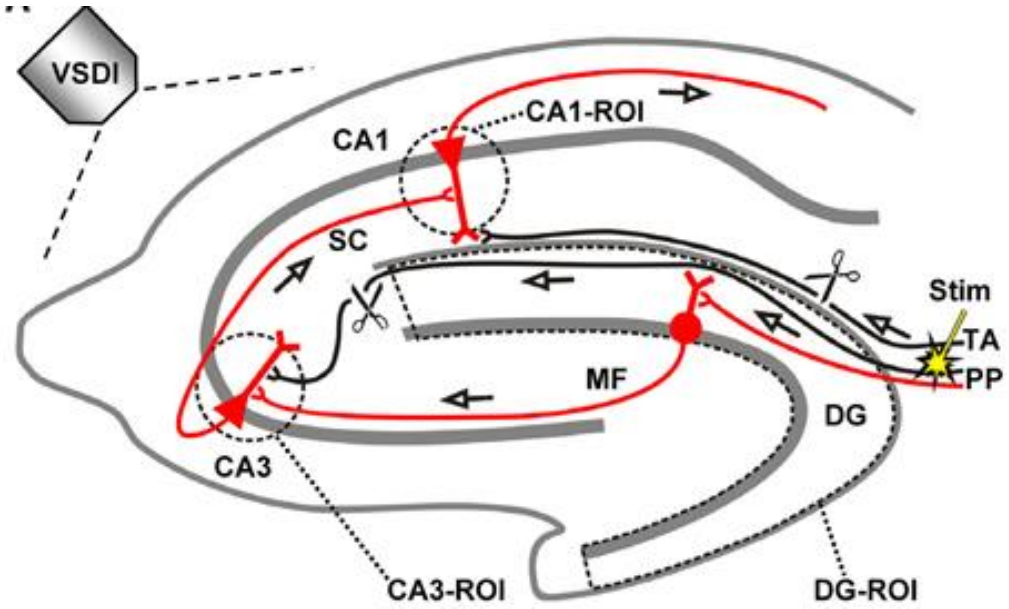


Figure 2.3: Diagram showing the classical trisynaptic loop of the hippocampus (Witter, 2010).

Recent studies have shown that connections within the hippocampus are not unilateral (van Strien*et al.*, 2009). Pyramidal cells in CA3 project back to the hilus and inner molecular layer of DG at all levels (Swanson *et al.*, 1978; Laurberg, 1979; Buckmaster *et al.*, 1993; Li *et al.*, 1994; Wittner*et al.*, 2006; 2007). Another back-projection from CA1 to CA3 has been shown to exist which consist of mainly inhibitory neurons in stratum radiatum and oriens of CA1 (Laurberg, 1979; Swanson *et al.*, 1981; Amaral *et al.*, 1991; Cenquizca and Swanson, 2007). Subiculum also sends back projection to CA1, this contains axons coming from stratum pyramidale and ends in all layers of CA1 (Finch *et al.*, 1983; Kohler, 1985).

The classical loop shows that major input to the hippocampus is through EC. EC is made of two regions namely medial and lateral EC (MEC and LEC respectively) (van Strien*et al.*, 2009; Knierim, 2015). Both regions receive input from prefrontal and olfactory cortices, send reciprocal projections to each other as well and sending axons to the hippocampus (Witter, 2010, Knierim, 2015). MEC and LEC axons end on the same cell in DG and CA3 (Sporns and Tononi,

2007). Whereas axons to CA1 are divergence (Sporns and Tononi, 2007; van Strien*et al.,* 2009). The input of EC has arranged in such that layer II projects to DG and CA3 while layer III projects to CA1 and subiculum and the deep layers receives feedback from the hippocampus (Knierim, 2015). This feedback loop allows for direct control of hippocampal output affect its input.

Postrhinal and perirhinal cortices also send direct inputs to the hippocampus and the EC (Naber*et al.,* 1997; Burwell and Amaral, 1998a,b). Hippocampus also receives subcortical inputs from the medial septum, locus coeruleus, raphe nucleus, nucleus reuniens and amygdala and sends output through CA1 via fornix to nucleus accumbens, amygdala and prefrontal cortex (Knierim, 2015).

2.3.2 Glutamatergic Neurotransmission in Hippocampus

Stimulation of direct input to the hippocampus (perforant pathway) evokes monosynaptic responses in the dentate granule cells (Tóth, 2010). The principal neurotransmitter of the perforant pathway is glutamate (Lambert and Jones, 1989). Due to the topographical arrangement of the pathway, each exhibit different responses when stimulated (McNaughton, 1980; Tóth, 2010). Medial perforant stimulation shows significant depression while lateral shows less change (McNaughton, 1980; Rush *et al.,* 2002). Differences observed in the excitation pattern of the medial and lateral perforant pathway may be due to silent synapses present on the lateral path (Min *et al.,* 1998). Studies showing the depressive ability of carbachol on the medial perforant pathway indicates that acetylcholine receptors are involved in the medial transmission (Kahle and Cotman, 1989).

Stimulation of perforant input to CA1 also evokes small glutamatergic responses (Tóth, 2010). This input is said to have little effect on the postsynaptic cells (Colbert and Levy, 1992; Empson

and Heinemann, 1995) but may have a feed-forward inhibition capable of regulating Schaffer collateral activity (Empson and Heinemann, 1995; Remondes and Schuman, 2002; Jarsky*et al.,* 2005).

NMDA receptors significantly contribute to excitatory postsynaptic current (EPSP) evoked by a perforant pathway on dentate granule cells (Lambert and Jones, 1989; 1990; Tóth, 2010). Manipulation of NR1 subunit on the granule cells leads to impaired context discrimination in the incremental fear-conditioning paradigm (Tóth, 2010). This gives rise to the hypothesis that NMDA receptors on granule cells are responsible for discrimination between similar contexts (McHugh *et al.,* 2007). Also, selective blocking of NR2-containing NMDA receptors on the medial perforant-granule cell led to learning deficit (Valenzuela-Harrington *et al.,* 2007).

Metabotropic glutamate receptors (mGluRs) also play vital roles in synaptic transmission of the hippocampus (Tóth, 2010). They are mostly located at the presynaptic terminals. mGluR2 is usually located at the medial end and mGluR8 at the lateral end (Shigemoto*et al.,* 1997). It has been shown that mGluRs regulate glutamate release at the perforant pathway while they serve as autoreceptors at the medial perforant pathway (Macek*et al.,* 1996). mGluRs activation reduced synaptic transmission at the medial perforant pathway (Kilbride *et al.,* 2001) but not at the lateral perforant. CA1 perforant input also displays the presence of mGluRs (Tóth, 2010). mGluR7a and mGluR4 are found in active zones while mGluR2 is on the preterminal zones (Shigemoto*et al.,* 1997). This differential arrangement is also seen in perforant input to CA1 interneurons (Price *et al.,* 2005).

Dentate granule cells send their axons to the hilus and CA3 through mossy fibres. Mossy fibresform three distinct types of presynaptic terminals; mossy fibre expansions (Amaral and

Dent 1981), filopodial extensions of mossy boutons and small en passant terminals (Acsády*et al.*, 1998). Large mossy terminals are excitatory and innervate mossy cells at hilus and CA3 pyramidal cells while filopodia extensions terminate on GABA cells in both hilus and CA3 (Acsády*et al.*, 1998).

Mossy fibres input to CA3 pyramidal cells has a linear I-V relationship indicating Ca^{2+}-impermeable receptors at the synapse (Jonas *et al.*, 1993; Koh *et al.*, 1995). Pharmacological studies showed that mossy fibres input to CA3 is transmitted through GluR2-containing Ca^{2+}-impermeable AMPA receptors (Tóth*et al.*, 2000). Although Ca^{2+}-permeable AMPA receptors have been found in these synapses early in postnatal development (Ho *et al.*, 2007). Mossy fibres also synapse on GABA interneurons through either Ca^{2+}-permeable/impermeable AMPA receptors (Tóth and McBain, 1998; Tóth*et al.*, 2000).

NMDA receptors are identified at mossy fibres synapses but show less staining intensity than Schaffer collaterals (Petralia*et al.*, 1994; Watanabe *et al.*, 1998; Takumi *et al.*, 1999). Studies showed that mossy fibres input on pyramidal cells are partly due to NMDA receptors with slower kinetics than AMPA components (Jonas *et al.*, 1993; Sprustone*t al.*, 1995). Activation of postsynaptic NMDA receptors at mossy fibres terminal influenced short term plasticity of kainite-mediated transmission but not AMPA components (Rebola*et al.*, 2007). NMDA receptors distribution at mossy-interneuron synapse follows the trend of Ca^{2+}-permeable and impermeable trend. Ca^{2+}-permeable AMPA receptors occur together with NR2B containing NMDA receptors, while Ca^{2+}-impermeable AMPA receptors are associated with NR2B-lacking NMDA receptors (Lei and McBain, 2002).

Kainate receptors are located on both pre- and postsynaptic site at mossy fibres (Tóth, 2010). Postsynaptic kainate receptors are selectively present at mossy inputs on CA3 pyramidal cells and absent at commissural/association inputs (Tóth, 2010). It is shown that the receptors have slow kinetics than AMPA (Cossart*et al.,* 2002). Presynaptic kainate receptors contribute to the robust frequency and facilitation of mossy fibres (Lauri *et al.,* 2001; Contractor *et al.,* 2001; 2003; Schmitz *et al.,* 2001; 2003; Pinheiro *et al.,* 2007). Presynaptic kainate receptors are sensitive to philanthotoxin; hence it is believed to be Ca^{2+}-permeable (Lauri *et al.,* 2001).

Presynaptic mossy fibres possess two types of mGluRs (Kamiya*et al.,* 1996; Tóth and McBain, 2000; Tóth, 2010). mGluR7 is present on interneuron terminals and depress excitatory transmission (Scanziani*et al.,* 1997; Tóth*et al.,* 2000). mGluR7 has low affinity for glutamate and its activation depresses glutamate responses (O'Connor *et al.,* 1999). mGluR7 antagonist usually doesn't affect baseline transmission but prevented high frequency induced long-term depression (LTD) while the agonist induces chemical LTD (Pelkey *et al.,* 2005). It is now evident that mGluR7 goes through activity-dependent internalization and surface expression (Pelkey *et al.,* 2005; 2007). Postsynaptic mGluRs evoke EPSP independent of G-protein function (Heuss*et al.,* 1999). Their activation also leads to intracellular Ca^{2+} release (Yeckel*et al.,* 1999).

Fibres from CA3 to CA1 through Schaffer collaterals innervate both the stratum radiatum and oriens. Excitatory inputs terminate exclusively on dendritic spines (Megias*et al.,* 2001). Single CA3 pyramidal cell can form 30,000 – 60,000 synapses while single CA1 pyramidal cell receives about 30,000 excitatory synapses (Li *et al.,* 1994; Tóth, 2010).

The excitatory activity of Schaffer collaterals is in part due to AMPA receptors (Kullmann, 1994; Liao *et al.,* 1995; Isaac *et al.,* 2007). Synapses lacking functional AMPA receptors (silent

synapses) do not conduct at resting membrane potentials (Tóth, 2010). Schaffer collaterals AMPA receptors are developmentally regulated; all Schaffer collaterals are silent at early postnatal days and 50% becomes active by the third week (Durand *et al.,* 1996).

NMDA receptors are widely present at excitatory synapses in the CA1 region with little variability (Petralia*et al.,* 1994; Racca*et al.,* 2000). Contrary to AMPA the number of NMDA receptors and kinetics does not change due to activities and location concerning the soma (Andrasfalvy and Magee, 2001).

Kainate receptor activation in CA1 leads to depression of glutamate transmission (Chittajalu*et al.,* 1996; Kamiya and Ozawa, 1998; Vignes*et al.,* 1998; Frerking*et al.,* 2001). It does via a decrease in quantal content with no activity on postsynaptic modulation (Frerking*et al.,* 2001; Tóth, 2010). Kainate receptors also modulate a network of inhibitory neurons by increasing their activation leading to inhibitory input onto pyramidal cells (Tóth, 2010). This is achieved by GluR5 subunit-containing receptors (Cossart*et al.,* 1998). GABA transmission is also affected by presynaptic kainate receptors by altering inhibitory connections between interneurons and serves as enhancing interneurons interaction (Cossart*et al.,* 2001).

mGluRs affect CA1 differently; they excite pyramidal cells (Gereau and Conn, 1995) and interneurons (McBain *et al.,* 1994), decrease excitatory and inhibitory transmission (Bortolotto*et al.,* 1999; Topolnik*et al.,* 2006). Excitation of pyramidal cells and interneurons is by Group I mGluRs located on the postsynapticsomatodendritic membrane (McBain *et al.,* 1994; Gereau and Conn, 1995). Group I and III mGluRssuppressed glutamate release at Schaffer collaterals (Gereau and Conn, 1995). Both are localized presynaptically but regulate glutamate transmission differentially (Tóth, 2010). Group II mGluRs are expressed on granule cells only (Tanabe *et al.,*

1992; 1993).Theireffect on glutamate transmission was found to be mediated by mGluR3 on glialcells (Winder *et al.,* 1996). Group III mGluRs are present on GABAergic presynaptic terminals (Shigemoto*et al.,* 1997). Activation of these receptors decreases inhibitory transmission in GABAergic cells (Tóth, 2010).

2.3.3 Hippocampal Functions

Numerous data has pointed to the fact of hippocampus being majorly responsible for learning and memory (Siegel and Sapru, 2011). Earliest report from animals shows that hippocampal activity (theta rhythm) increases in animal approaching a goal and during different phases of conditioning (Cornwell *et al.,* 2008). Some reports have also pointed out learning and memory deficit in hippocampal lesioned animals (Ramos, 2000).

Discovery of place cells (a group of cells that show activity in positional changes of animals) has led to the discovery that hippocampus plays a significant role in spatial navigation (Eichenbaum*et al.,* 1999). This phenomenon was shown to be correct in a study conducted on taxi drivers in the United Kingdom. The study reveals that hippocampal volume was more in the taxi drivers compared to average citizen of same age group (Maguire *et al.,* 2006). Studies on animals using different paradigms like Morris water maze, Y-maze or radial arm maze showed that animals with hippocampal lesioned do not perform well on this task (Ramos, 2000; Mumby*et al.,* 2002; Clark *et al.,* 2005).

Although most research work on the hippocampus is to understand the working of memory processing, the hippocampus has been implicated in other brain functions. Due to its connections to other brain regions, it is reported that hippocampus indirectly regulates aggression and rage (Siegel and Sapru, 2011). A study conducted on cat shows that stimulation of a temporal part of

hippocampus elicits predatory attack whereas septal part of hippocampus suppresses this behaviour (Leroy *et al.,* 2018). This connection led to the proposal that the septal area may be a relay nucleus of the hippocampus to the hypothalamus (Siegel and Sapru, 2011).

Hippocampal connection to the hypothalamus is also implicated in endocrine regulation (Lathe, 2001; Siegel and Sapru, 2011; Kanoski and Grill,2015). It is shown that estradiol-concentrating neurons are densely located in the ventral region of the hippocampus (Weiland *et al.,* 1997), corticosterone is also present in the hippocampus and has inhibitory roles (Sapolsky *et al.,* 1984; Izumi *et al.,* 2015). Stimulation of the hippocampus inhibits ovulation in normal rats (Carrillo, 1981; Scharfman*et al.,* 2007) and hippocampal lesion (part of fornix) disrupt the diurnal rhythm of adrenocorticotropic hormone (ACTH) (Jankord and Herman, 2008).

2.4 N-Methyl-D-Aspartate Receptors (NMDAR)

N-methyl-D-aspartate (NMDA) receptor is a calcium channel which is both voltage and ligand-gated (Blanke and VanDongen, 2009). The voltage-dependent gating of the NMDA receptor is due to Mg^{2+} block of the receptor pore (Paoletti, 2011). It allows for calcium influx by in part depolarization of its resident membrane which drives out the Mg^{2+} out of the pore and signal transmission (Song *et al.,* 2017). Co-occurrence of glutamate and depolarization of membrane is necessary for the opening of the channel and allow for calcium influx into the postsynaptic cell (Bollmann *et al.,* 1998). These abilities of NMDAR to act based on both glutamate and signal transmission avail it to act as a coincidence detector (Cooke and Bliss, 2006). Calcium influx mediated by NMDA receptor-mediated diverse intracellular signalling processes in the neurons from long-term potentiation (LTP) to synaptic plasticity (Lu *et al.,* 2001; Lau *et al.,* 2009; Lűscher and Malenka, 2012; Park *et al.,* 2014).

2.4.1 Structure of NMDAR

NMDA receptor is a multiunit transmembrane protein (Zhou and Sheng, 2013). It consists of tetrameric hetero-oligomeric protein containing two glutamate binding sites (Luo *et al.,* 2011) and other binding sites for zinc, polyamines, and glycine (which act as co-agonist necessary for action) (Sweatt, 2010). It is made up of two subunits GluN1 (NR1) and GluN2 (NR2) encoded by four different genes GluN2A-D) (Zhou and Sheng, 2013). Functional NMDA receptor composed of one or more GluN1 with one or more GluN2-type subunit (Luo *et al.,* 2011; Zhou and Sheng, 2013). GluN2 subunit determines the calcium permeability and magnesium sensitivity of the functional NMDAR (Cull-Candy *et al.,* 2001; Paoletti, 2011).

Most synaptic hippocampal NMDA receptors are either NR2A (i.e. contains two subunits of GluN2A) or NR2B (i.e. contains two subunits of GluN2B) (Luo *et al.,* 2011). However, triheteromeric NMDA receptors containing one GluN1, GluN2A, and one GluN2B have been identified at the synapse (Sheng *et al.,* 1994; Luo *et al.,* 1997; Al-Hallaq*et al.,* 2007; Luo *et al.,* 2011). Although, little information is known about triheteromeric NMDA receptors they have distinct properties from the GluN2A- and GluN2B-NMDA receptors (Neyton and Paoletti, 2006; Rauner and Köhr, 2011).

Each subunit consists of four distinct domains; extracellular N-terminal domain, extracellular ligand-binding domain, a transmembrane domain and intracellular C-terminal domain of varied length (figure 2.4) (Dingledine *et al.,* 1999; Traynelis*et al.,* 2010). The transmembrane domain has three membrane-spanning helices and membrane re-entrant loop which contributes to the

channel pore and the cytoplasmic domain is responsible for protein-protein interactions in the cell (Luo *et al.,* 2011).

GluN2A- and GluN2B-NMDA receptors are the most widely studied since their location and signalling process are distinct (Thomas *et al.,* 2006; Martel *et al.,* 2009). GluN1 is usually phosphorylated by cyclin-dependent kinase 5 and GluN2B by tyrosine kinases (Sweatt, 2010). Distribution of GluN2A- and GluN2B-NMDA receptors are tightly regulated during brain development, GluN2A expression increased switching from GluN2B dominating NMDA receptors to GluN2A-NMDA receptors (Cull-Candy *et al.,* 2001). GluN2A-NMDA receptors are usually at the synapse while GluN2B-NMDA receptors are extra-synaptic in the mature brain (Steigerwald*et al.,* 2000; Groc*et al.,* 2006; Martel *et al.,* 2009). Signalling pathway studies on this type of receptors showed that GluN2A-NMDA receptors promote cellular survival while GluN2B-NMDA receptors promote cell death (Liu *et al.,* 2007; Lai *et al.,* 2011). These preferential functions of GluN2A- and GluN2B-NMDA receptors in cell survival and death are proposed to be due to the different cytoplasmic domain coupling (Salter and Kalia, 2004). GluN2A-NMDA receptors are usually coupled with Homer and ß-Catenin (Al-Hallaq*et al.,* 2007) while GluN2B-NMDA receptors are coupled with Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and synaptic Ras GTPase activating protein (SynGAP) (Leonard *et al.,* 1999; Kim *et al.,* 2005). However, they also share some common cytoplasmic coupling like postsynaptic density protein 95 (PSD-95) (Sheng and Kim, 2011).

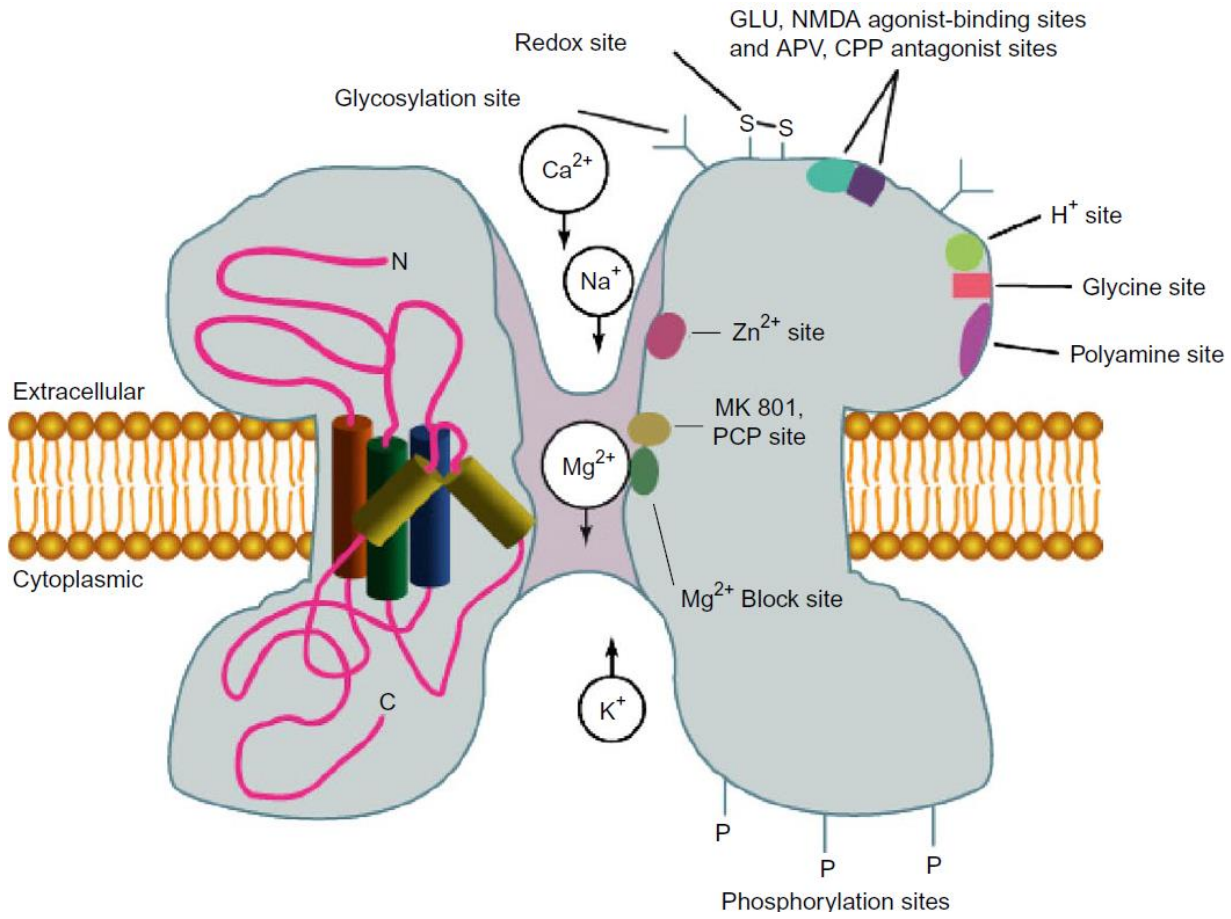


Figure 2.4: Schematic diagram of the structure of the NMDAR showing the binding sites, domains and phosphorylation sites (Hollmann and Heinemann, 1994).

2.4.2 NMDAR Signalling

Activation of NMDA receptors allows for Ca^{2+} influx into the cells. As earlier stated, major types of NMDA receptors in the brain exhibit different functions and signalling pathway due in part to their cytoplasmic coupling (figure 2.5). As stated earlier, GluN2A (NR2A) and GluN2B (NR2B) are extensively studied and has been proposed to be responsible for the death or survival of neurons in the brain (Luo *et al.*, 2011).

Activation of GluN2A-NMDA receptors allows the influx of Ca^{2+} ions which then activates different second messenger molecules. Highly understood of such pathway is the Ca^{2+}/calmodulin-dependent protein (CaM) kinase-cAMP response element-binding protein (CREB) signalling pathway. Calcium influx activates nuclear CaMK IV which increases phosphorylation of the transcription factor CREB on its regulatory residue (Serine 133) (Hardingham *et al.,* 2002; Sasaki *et al.,* 2011). The phosphorylated CREB recruits CREB coactivator CREB binding protein (CBP) to stabilize preinitiation complex and increase CRE promoter activity (Mayr and Montminy, 2001). CREB activation induces expression of pro-survival molecules like brain-derived neurotrophic factor (BDNF) and represses the expression of death molecules (Hardingham *et al.,* 2002). GluN2A-NMDA receptors activation also activate the activity of salt-inducible kinase 2 (SIK2) phosphorylation by CaMK I/IV which will then induce transducer of regulated CREB activity (TORC1) and its nuclear transfer (Sasaki *et al.,* 2011). TORC1 also can activate CREB independent of phosphorylation at Ser133 (Luo *et al.,* 2011). GluN2A-NMDA receptor also regulates the expression of forkhead box protein O (FOXO) transcription factors (Luo *et al.,* 2011). GluN2A-NMDA receptors activation suppresses FOXO activity by triggering nuclear export of FOXO through activation of PI3-Akt pathway (Brunet *et al.,* 1999; Dick and Bading, 2010) and by reducing FOXO1 expression (Al-Mubarak *et al.,* 2009). Suppression of FOXO reduces the expression of death molecules (Papadia*et al.,* 2008; Al-Mubarak *et al.,* 2009). Extracellular signal-regulated kinases 1/2 (ERK1/2) is also activated by Ca^{2+} influx through GluN2A (Chandler *et al.,* 2001; Ivanov *et al.,* 2006) which promotes cell survival. It is now proposed that CREB activation and FOXO repression are responsible for the neuronal survival function of GluN2A-NMDA receptors.

GluN2B-NMDA receptors activation also activates CREB transiently (Luo *et al.,* 2011) and gets dephosphorylated back by protein phosphatase 1 (PP1) (Hardingham *et al.,* 2002). This later leads to inhibition of CREB functions. GluN2B-NMDA receptors also promote nuclear import of FOXO factors and activate FOXO genes (Dick and Bading, 2010) which later leads to cell death. Death-associated protein kinase 1 (DAPK1) is physically bound to GluN2B-NMDA receptors and phosphorylates it at serine 1303 which enhances its conductance (Luo *et al.,* 2011) and initiates brain damage (Tu *et al.,* 2010). ERK signalling is suppressed by GluN2B-NMDA receptors through SynGAP activation (Kim *et al.,* 2005). Neuronal nitric oxide synthase (nNOS) which catalyzes the production of nitric oxide (NO), a neurotoxic substance, is coupled to GluN2B through PSD-95 (Sattler *et al.,* 1999). Ca^{2+} influx through GluN2B can also activate the activity of nNOS leading to cell death (Aarts*et al.,* 2002; Cui *et al.,* 2007; Zhou *et al.,* 2010).

Figure 2.5: Schematic diagram showing different signalling pathway of NMDAR. (A) GluN2A-NMDA down-regulates the expression of pro-death genes by inhibiting forkhead box protein O (FOXO) activity through PI3K-Akt pathway (red arrow) and can also activate CaMKIV and upregulate a battery of pro-survival genes by activation of cAMP response element-binding protein (CREB) (blue arrow). (B) GluN2B-NMDA receptor signal blocks CREB-dependent pro-survival gene expression (red arrow), FOXOs are also targets of extrasynaptic NMDAR activity to activate expression of pro-death genes (blue arrow) (Luo *et al.*, 2011).

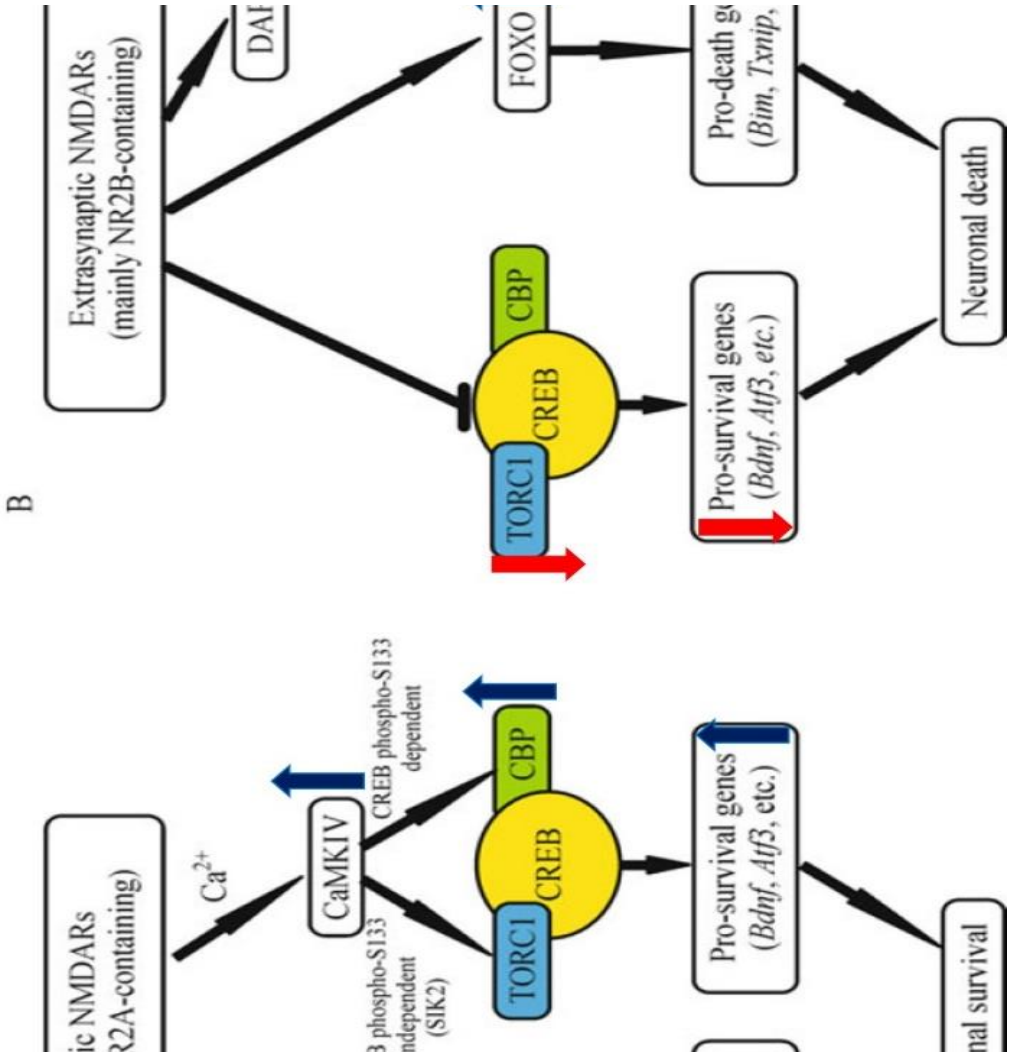


Figure 2.5

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethical Approval

Ethical approval was received from the University of Ilorin Ethical Review Committee (UERC) with approval number UERC/ASN/2018/1277 and protocol identification code UERC/BMS/108.

Together with the approval of Louisiana State University Institute of Animal Care and Use Committee (IACUC) with protocol number 17-4203.

3.2 Ligand Docking

Structure of compounds that have been isolated from Datura plants (from review Maheshwari *et 1al.,* 2013) was simulated against NMDAR. The simulation was done using Autodock Vina (Trott and Olson, 2010) version 4.2.6 (USA). Compounds were simulated using a flexible docking to get compounds that bind to the binding sites of NMDAR. Structure of NMDAR and compounds were received from protein data bank (PDB) and PubChem respectively. The compound with the lowest binding energy (i.e. lowest ΔG), function of stable binding was selected, and the biological activities subsequently evaluated *in vivo*.

3.3 Purchase and Preparation of Datumetine

Datumetine (CAS No.: 67078-20-0) was purchased from ChemFaces Biochemical Company China. The compound was dissolved in dimethyl sulfoxide (DMSO) to achieve 1 mg/mL stock solution and stored at 4^0C. It was then serially diluted based on the dosage of animals for working solution (kept at room temperature) to administer to the animal.

3.4 Animal Care

Long term exposure of datumetine on hippocampal activity was further assessed. Thirty (30) adult C57BL/6 miceprocured from Jackson's Laboratory (Bar Harbor, ME, US) were used for the experiment. They were housed under standard laboratory conditions of 12 hours alternating light and dark cycle. They were kept in standard cages of five animals/cage. Feed and water wereprovided*ad libitum.*

3.5 Animal Grouping and Treatment

The animals were divided into three groups of ten (10) mice each and were administered 0.25 mg/kg (0.25 Datumetine) and 1.0 mg/kg (1.0 Datumetine) body weight of datumetine and 0.1 mL of DMSO (DMSO/Control) respectively. All treatments were done intraperitoneally for fourteen (14) days.

3.6 Behavioural studies

Memory function of the animals was assessed using Y-maze and Novel object recognition (NOR) test. Animals were familiarised with the behavioural room and apparatus three days before the experimental days. The behavioural protocol was captured and analyzed using EthoVision version 13 tracking software (Noldus Information Tech. the Netherlands).

3.6.1 Y-maze

This was done to measure the hippocampal-dependent spatial working memory of the mice. The mice were placed facing the edge where two arms meet and were left to make their own decisions of which arm to follow. Visiting three arms consecutively was termed right decision (right) and visiting one arm twice in three alternations was termed a wrong decision (wrong). The durationof the test was for five (5) minutes. Percentage alternation (index of memory) was calculated for the mice using the expression:

$$\text{Percentage (\%) alternation} = \frac{\text{Number of the right decision}}{\text{Number of total arms entry} - 2} \times 100$$

3.6.2 Novel Object Recognition (NOR) Test

This was to assess the cortical-dependent non-spatial working memory. The mice were exposed in an open chamber to two identical objects to acclimatize with them for five minutes which is termed as trial 1 (T1). The mice were then kept back to their home cage with food and water. One hour later i.e.,intertrial interval (ITI), the mice were placed back in the chamber with one of the objects being replaced by a novel one for five minutes. The time used in rearing the old (old time) and a new object (new time) was recorded. Animals were considered rearing the object when the centre body of the animal was less than 2cm from the object while sitting on the object was not considered. The memory index was calculated using the expression:

$$\text{Memory index (\%)} = \frac{Timespentonnewobject}{Totaltimespentonrearingbotholdandnewobject} \times 100$$

3.7 Animal Sacrifice

The animals were sacrificed 24 hours after the last administration. The animals were deeply anaesthetized in an isoflurane gas chamber. They were then perfused transcardially with 10 mM phosphate-buffered solution (1X PBS pH 7.4). Samples for western blotting and electron microscopy have their brains excised and the hippocampus dissected out on a cold surface. Hippocampal samples for electron microscopy were quickly transferred into electron microscopy (EM) fixative (1.6% paraformaldehyde, 2.5% glutaraldehyde, 0.03% Calcium chloride in cacodylate buffer pH 7.4) and at 4^0C, while blotting samples were placed inside 1X PBS and stored at -20^0C. Samples for immunofluorescence were further perfused with 4% paraformaldehyde after 1X PBS. The brains were then excised and placed in specimen bottle containing the fixatives and stored at 4^0C, these were then post-fixed in 4% paraformaldehyde with 30% sucrose solution at 4^0C till further processing.

3.8 Western Blotting

Samples for western blotting were further processed as follows;

3.8.1 Tissue Pre-treatment

1. Hippocampus samples for western blotting were homogenized in 10 times volume of radioimmunoprecipitation assay (RIPA) buffer (Lot 2912060 EMD Millipore Corp. Billerica USA) to the weight of the specimen, using hand-held homogenizer and kept on ice for 30 minutes and vortexed every 10 minutes.
2. They were then saponified (using PRO200 Bio-Gen series homogenizer Oxford USA) on ice for 5 minutes.
3. They were then centrifuged (using accuspin Micro 17R Fisher Scientific USA) at 12,000 rpm for 15 minutes at 4^0C.
4. The supernatant was collected in another tube.

3.8.2 Total Protein Assay

Total protein concentration was checked using Pierce BCA assay kit (Lot 23225 Thermo scientific Rockford USA) and the protein concentration was evaluated based on the standards using microplate reader(Tecan Spark®Männedorf, Switzerland) at 562 nm.

Proportional dilution was made based on the estimated protein concentration and 10 µg/µL solution was made and half volume of laemmli SDS buffer (Lot Y18C501) was added for loading on the gel well.

3.8.3 Gel Electrophoresis

Gel Electrophoresis was done using 8% gel (for protein >100kDa) and 12% gel (for protein <100kDa). See table 3.1 and 3.2 for details of the gel composition. Samples were loaded in the well starting with the ladder (loading standard) and the samples (5 µL each well). Electrophoresis was done at 140 mA, 300 V for 80 minutes (current was constant) using PS300B 300 V supply (AA Hoefer Holliston, USA).

3.8.4 Transfer of Protein to Membrane

This was done after the completion of electrophoresis. This is done to transfer separated protein on a membrane. The following procedures were followed;

1. The polyvinylidene difluoride (PVDF) membrane was placed in methanol for 1 minute.
2. PVDF membrane was later transferred to transfer buffer (Tris base, glycine, methanol, and distilled water) and rock for 10 minutes.
3. At the end of electrophoresis, the machine was turned off and disconnected.
4. The moulds were released to get the gel.
5. The loading gel was cut off and discarded while the stacking gel was quickly transferred to transfer buffer.
6. Transfer sandwich was made by taking the cassettes and the black part facing down inside transfer buffer.

7. The sandwich was made by placing foam, blotting-pad, gel, membrane, blotting-pad and foam in that order.
8. The cassettes closed and clipped and placed in the transfer machine with all-black part facing back.
9. The transfer machine was filled with running buffer and ran at 100 V, 400 mA (current constant) for 70 minutes.
10. When completed, the membrane was transferred to 10mM Tris-buffered saline (1X TBS) and stored at 4^0C untilstaining.

Table 3.1: Composition of Separating and Stacking Gel for 8% gel

S/N	Components	Volume (Separating gel)	Volume (Stacking gel)
1	Double distilled water	21.2 mL	10.1 mL

2	40% Bis-Acrylamide Mix (29:1)	8 mL	1.6 mL
3	1.5M Tris (pH 8.8)	10 mL	-
4	0.5M Tris (pH 6.8)	-	4 mL
5	10% Sodium dodecyl sulfate (SDS)	400 µL	200 µL
6	10% Ammonium Persulfate (APS)	400 µL	200 µL
7	Tetramethylethylenediamine (TEMED)	40 µL	23 µL

Table 3.2: Composition of Separating and Stacking Gel for 12% gel

S/N	**Components**	**Volume (Separating gel)**	**Volume (Stacking gel)**
1	Double distilled water	17.2 mL	10.1 mL
2	40% Bis-Acrylamide Mix (29:1)	12 mL	1.6 mL
3	1.5M Tris (pH 8.8)	10 mL	-
4	0.5M Tris (pH 6.8)	-	4 mL
5	10% Sodium dodecyl sulfate (SDS)	400 µL	200 µL
6	10% Ammonium Persulfate (APS)	400 µL	200 µL
7	Tetramethylethylenediamine (TEMED)	40 µL	23 µL

3.8.5 Membrane Staining

1. Membranes were washed twice in 1X TBS containing 2% triton-X (1X TBST) for 10 minutes each

2. Membranes were blocked in 3% bovine serum albumin (BSA) (Cat: BP671-10, Fisher Scientific USA) prepared in 1X TBST for 55 minutes at room temperature on a gentle rocker.
3. Membranes were then incubated in primary antibodies diluted in 3% BSA; anti-CREB(9197S 1:1000, 43 kDa), anti-Trk/BDNF (92991S 1:1000, 120-140 kDa), anti-CamKII-α (3357S 1:1000, 50 kDa), anti-pCamKII-αT286 (12716S 1:1000, 60 kDa) and anti-NMDAR (5704S 1:1000, 120 kDa) all from Cell Signaling Tech. Danvas MA, the USA at 4^0C overnight.The next day membranes were rinsed twice in 1X TBST followed by washing twice in 1X TBST for 10 minutes each.
4. Membranes were then incubated in HRP conjugated Chicken anti-rabbit antibody (3:10,000 A15987 Invitrogen Fisher Scientific USA) for 60 minutes at room temperature.
5. Membranes were then washed in 1X TBST twice for 10 minutes and stored in 1X TBS for viewing.

3.8.6 Membrane Imaging

Stained membranes were viewed by developing the substrate using ECL WB substrate (TF 268244 Thermoscientific Rockford USA).

1. 2 mL of solution A and B were mixed
2. Stained membranes were incubated in the solution for 70 seconds.
3. They were then placed inside a plastic film and rolled to sleek.
4. These were then viewed using the Bio-RadChemidoc Touch Imaging machine (V3 Bio-Rad USA) at 2 minutes exposure time.

3.8.7 House-keeping Protein Staining

1. Membranes were then stripped off antibodies and staining molecules using 10 mL of stripping buffer (Restore plus WB stripping buffer 46430 Thermoscientific USA).
2. They were then rinsed 6 times in 1X PBST and washed twice in 1X PBST for 10 minutes each.
3. Later they were washed in 1X PBS and 1X TBST twice for 10 minutes each.
4. They were blocked in 3% BSA for 55 minutes
5. Then incubated inhorseradish peroxidase (HRP) conjugated ß-actin antibody (3:10000, 12262S Cell Signaling Tech. Danvas MA, USA) for 60 minutes at room temperature.
6. The steps after antibody incubation to imaging was repeated to capture the expression of ß-actin.

3.8.8 Data Acquisition

Quantification of the blotting volume/density was done using Image Lab version 6.0.1 Standard edition Bio-Rad Laboratories Inc. The USA.

3.9 Immunofluorescence (IF)

Brain samples for immunofluorescence were further processed as stated below;

1. They were embedded in optimal cutting temperature (OCT) compound (4585 Fisher Healthcare Houston USA) in coronal plane on cryomold.
2. They were serially sectioned at 40 µm thick sections on a cryostat (Leica CM 3050 S USA).
3. Sections were then stored in 48 well microplates containing 1X PBS and stored at 4^0C till further processing.

Staining Procedures

1. Sections were washed with 1X PBS containing 0.5% tween 20 (1X PBST) twice for 10 minutes.
2. Sections were then blocked in 5% goat anti-rabbit serum (S-1000 Vector Laboratories USA) prepared in 1X PBST for 60 minutes at room temperature.
3. Sections were then incubated in primary antibodies as stated in table 3.3 (prepared in goat anti-rabbit 5% serum) for either overnight at 4^0C or 120 minutes at room temperature.
4. Sections were washed in 1X PBST twice for 10 minutes and then incubated in Alexa Fluor-488 goat anti-rabbit secondary antibody (2:500, A11034 Invitrogen Fisher Scientific USA); or Alexa Fluor-568 goat anti-rabbit (2:500, A11036 Invitrogen Fisher Scientific USA) for 60 minutes at room temperature in the dark.
5. Sections were washed twice in 1X PBST for 10 minutes and then transferred to 1X PBS.
6. Sections were mounted on gelatin-coated glass slides with antifade mounting medium with DAPI (VECTASHIELD H-1500 Vector laboratories the USA) and coverslippedand stored on slide tray in the dark.
7. Slides were viewed and the image was taken using a Nikkon fluorescent microscope attached with a CCD camera.

Table 3.3: List of primary antibodies used and their dilution

Probe	**Antibody (Cat. Number)**	**Dilution**	**Company**
Neurons	Anti-NeuN* (24307S)	1:100	Cell Signaling Tech. Danvas MA, USA
Astrocytes	Anti-GFAP (3670S)	1:300	

Microglia	Anti-ITGAM/CD11B (49420S)	1:300		
Dopaminergic neurons	Anti-TH (58844S)	1:100		
Glutamatergic neurons	Anti-vGlut2 (71555S)	1:150		
Serotonergic neurons	Anti-TPH-1 (12339S)	1:200		
Synapse	Anti-PSD-95 (3450S)	1:200		
Cholinergic neurons	Anti-CHRNA7 (PA5-37280)	1:300	Invitrogen Fisher Scientific USA	
GABAergic neurons	Anti-GABA (PA5-32241)	1:5000		

*Incubation was done for 120 minutes at room temperature; others were overnight at 4^0C.

3.10 Expansion Microscopy

This was done to check for neural connections. This is done by embedding already stained sections in a gel polymer which is later digested and made to expand uniformly in water. This is

to increase the clarity and magnification of the specimen. Immunofluorescence protocol was followed for the staining of neurofilament using rabbit anti-neurofilament-L (1:200 2837S Cell Signaling Tech. Danvas MA, USA) at 4^0C overnight, followed by incubation in Alexa Fluor-568 goat anti-rabbit secondary antibody for 60 minutes at room temperature.

Additional Expansion Techniques was done as follows;

1. The stainedsections were rinsed in 5% goat serum twice for 15 minutes each.
2. Sections are placed in 1% acryloyl-X solution (prepared in 1X PBS) at 4^0C overnight.
3. Sections were rinsed twice in 1X PBS for 15 minutes each
4. Sections were placed in gelling solution (94% monomer solution, 2% of 0.5% 4-hydroxytempo, 10% tetramethylethylenediamine- TEMED and 10% ammonium persulfate - APS) for 5 and 25 minutes at 4^0C respectively.
5. Each section was then incubated separately in a slide mounting chamber with a new gelling solution for 2 hours at 37^0C.
6. The excess gel was trimmed off and sections were placed in a digestion buffer (for digestion of the gelling polymer) at room temperature overnight.
7. Sections were then rinsed in distilled water for 15 minutes for uniform expansion.
8. Sections were then mounted on a glass slide and coverslipped using DAPI mountant.

3.11 Electron Microscopy

Hippocampal samples for electron microscopy were further processed as follows;

1. Microdissected hippocampal tissue was trimmed into 1mm sections using a sharp razor.

2. Dissected tissues were transferred into a fresh fixative for 2 hours at room temperature.
3. The samples were then washed in 0.1 M cacodylate buffer supplemented with 5% sucrose and fixed in 2% osmium tetroxide for 1 hour at room temperature.
4. The sections were washed in water, then in-block stained with 2% uranyl acetate prepared in 0.2 M sodium acetate buffer (pH 3.5), for 2 hours.
5. Stained sections were dehydrated in ascending grades of alcohol and propylene oxide.
6. The processed sections were embedded in Epon-Araldite mixture and polymerized for 24 hours at 60^0C.
7. Tissue blocks were sectioned using a Leica Ultratome (Leica EM UC7).
8. Thin (80 nm) sections were recovered and stained with lead citrate for 5 minutes.
9. Transmission electron photomicrographs were obtained in a JEOL 1400 TEM microscope, equipped with a GATAN digital camera. All reagents for electron microscopy were from EMS (Hatfield, PA).

3.12 Electrophysiology

The electrical activity of the brain was recorded on anaesthetized animals to evaluate the datumetine effect on NMDAR linked Ca^{2+}current. This is to show the effect of datumetine binding on the electrical activity of hippocampal neuron mediated by NMDAR. This was done on four animals. To reduce the pain of the procedure, the animals were anaesthetized using 100 mg/kg of ketamine + 10 mg/kg xylazine cocktail. When the animals were deeply sedated, they were tested for toe and tail pinch reflex. The procedure was as follows;

1. They were fixed on a stereotaxic frame.

2. A 7 mm by 7 mm craniotomy was done to expose the dura and drops of artificial cerebrospinal fluid (aCSFThomas Recording) was applied to the exposed area to prevent dryness.
3. The dura of the exposed area was carefully excised.
4. A 10 mm long and 50 µm thick shank acute neural probe was used (Neuronexus, Michigan, USA). The probe shank carries four electrodes arranged as a tetrode, with an inter-electrode distance of 25 µm was used.
5. The electrodes were connected to a pre-amplifier head stage (Intantech, California, USA), tethered to a recording controller and amplifier system (Intantech, California, USA) (see figure 3.1).
6. The electrode was gently lowered down into the brain tissue using an ultrafine (µm range) hydraulic micromanipulator (Narishige, Japan) to reach the CA1-DG field at stereotaxic coordinates (AP: 1.94 mm, ML: 1.0 mm, DV: 1.5 mm) relative to the Bregma.
7. Stainless steel ground wires soldered onto the head stage-electrode adapter (Neuronexus; A4 to Omnetics CM32 adapter) were tied to ground screw that was fixed on the occipital bone.
8. The stereotaxic apparatus, micromanipulator, electrode and subject mouse were kept in a Faraday cage and connected to the amplifier ground.

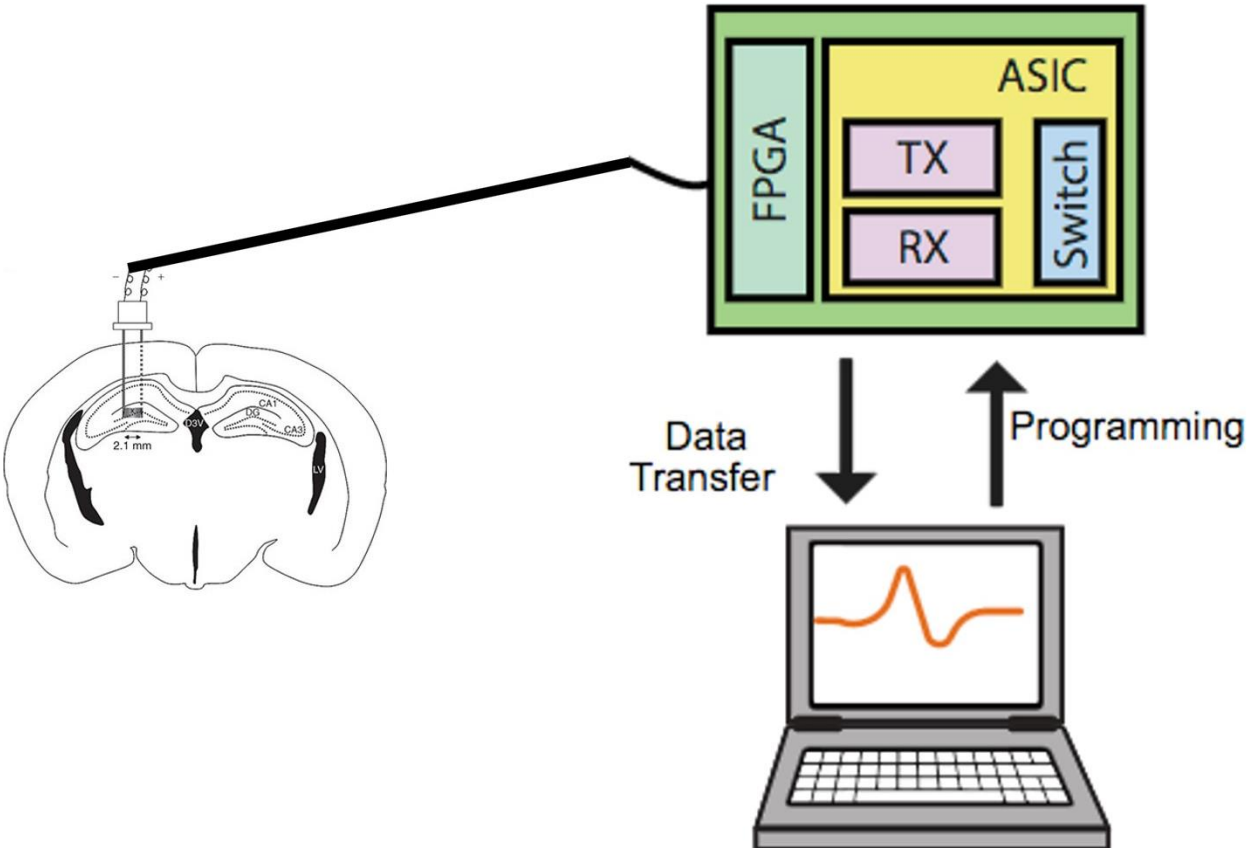


Figure 3.1: Schematic diagram showing the placement and connection of electrode in the animal brain.

At the onset of each recording procedure;

1. The tested impedance of the electrodes was at 1kHz. For all recording sessions in this experiment, impedance measurement for the tetrodes ranged between 0.6 – 3.1 megΩ.
2. Single unit activity was recorded by setting the cut-off frequency like 250 Hz and 7.5 kHz for proper and lower limit respectively, sampled at 30 kHz/s.
3. Neural activity was monitored for 20 minutes to ensure the stability of the animal's vitals before the commencement of recordings.

4. Baseline recording was done for 30 minutes for each animal, after which 0.1 mL of 0.07 µM of datumetine was injected to the animals and another recording was taken for 30 minutes.
5. Each recording was saved differently.

Spike Processing

1. The continuously recorded action potential spike trains were analyzed in an Offline Spike Sorting Software (OFSS) Version 4 (Plexon Inc., Dallas, USA).
2. Neural spikes were extracted from the continuous data through threshold crossing in the OFSS.
3. The extracted spikes were sorted into clusters using a combination of Valley seeking and K-means clustering methods.
4. Spikes were assigned to single unit clusters through a 3-dimensional space principal component analysis (PCA) projection.
6. Sorted neural spike waveforms, clustered units, and upsampled continuous data were exported into the Neuroexplorer software Version 5 (Nex Technologies USA) for further analysis of spike properties.

3.13 Statistical Analysis

Quantitative data werepresented as mean±SEM in barcharts. Electrophysiology data were analysed using unpaired t-test with Welch's correction if variance showed a significant difference. Other data were analysed using one- way analysis of variance (ANOVA) and Tukey post-hoc test was done when ANOVA shows significant *p*-value at 0.05.

CHAPTER FOUR

RESULTS

4.1 Ligand Docking

Simulation of structures of compounds found in *Datura*plants against NMDAR at its orthostatic and allosteric binding site revealed that datumetine (an alkaloid) will form a stable binding complex with the receptor at both sites (figure 4.1). The free energy was found to be -8.3 $kJmol^{-1}$ at the allosteric binding site and -8.6 $kJ\ mol^{-1}$ at the orthostatic binding site.

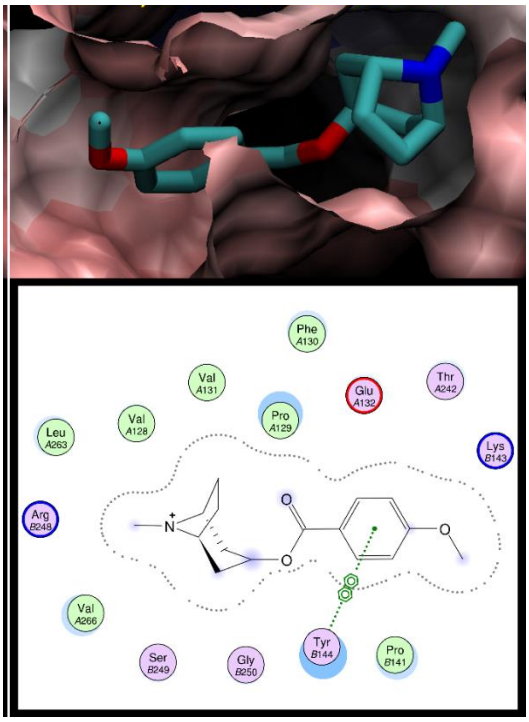


Figure 4.1: Simulation diagram showing the mode of binding of datumetine to NMDAR. Upper panel showing the orientation of datumetine on NMDAR binding site. The lower panel showed the skeleton of datumetine compound binding with NMDAR at tyrosine 144 (Tyr B144).

4.2 Datumetine Induces Seizures in Mice

Animals treated with datumetine exhibits seizures characterized by long contraction of lower limb muscles and tail with an abnormal orientation of the body immediately after treatment. This persists for some minutes and reverses. Animals treated with 1 mg/kg body weight exhibit these signs longer than those treated with 0.25 mg/kg body weight. This symptom later reduces with time as the treatment days progresses and animals show little signs of seizures towards the end of treatment. However, it persists in animals treated with 1mg/kg bodyweight of datumetine.

4.3 Memory Deficit in Datumetine Exposed Animals

In the Y-maze protocol, the memory index (% alternation) showed a declining trend in animals exposed to datumetine compared to control animals (figure 4.2a). This decline was significant in animals treated with 1 mg/kg body (**p<0.01) compared to the control but not in 0.25mg/kg datumetine exposed animals.

Heatmap visualization from the tracking software (figure 4.2b) showing the activities of the animals on the Y-maze. The region showing blue indicate areas of moderate activities while areas showing green to yellow indicate increased activities and red to black colourations indicates regions of maximum activities. From the heatmap, DMSO (control) animals exhibit maximum activities at the centre of the arm and increased activities in all the three arms of the maze. A similar trend was observed in .25 mg Datumetine animals but only exhibit maximum

activities in two arms out of the three of the Y-maze. While 1.0 mg Datumetine animals only showed maximum activities at the centre and one arm of the maze.

Novel object recognition test results showed a similar trend with Y-maze which has both groups of animals exposed to datumetine showed reduced memory index compared to control animals (figure 4.3a). These changes were significant (*p<0.05, **p<0.01) compared to control.

Heatmap visualization from the tracking software (figure 4.3b) showed that control animals explore areas outside of the two exposed objects as indicated with the red colouration. Although, the animals still explore the novel object more than the old object indicated by more blue colour clusters than the area of the old object which is less. The increased locomotor activity was observed in animals exposed to 0.25 mg/kg datumetine, indicated by the presence of blue clusters filling the box and more areas of maximum activities indicated by the yellow to red colour. It is observed that compared to areas around the novel object, 0.25 mg Datumetine animals showed maximum activities in the areas around the old object indicated by the yellow and red colour. Locomotor activities of animals exposed to 1.0 mg/kg datumetine are less than that of 0.25 mg Datumetine but still more than the control. 1.0 mg Datumetine animals showed activity more at the corner of the box than the centre. Like 0.25 mg Datumetine animals, they showed maximum activities in the area around the old object than the novel object.

Figure 4.2: Results of the Y-maze protocol showing (a): % alternation of experimental animals, and (b): Representative heatmap visualization of animal activity during the Y-maze test. (n= 10 per group, $^{**}p<0.01$ one-way ANOVA with Tukey post-hoc test). Animals exposed to datumetine showed reduced % alternation compared to the control.

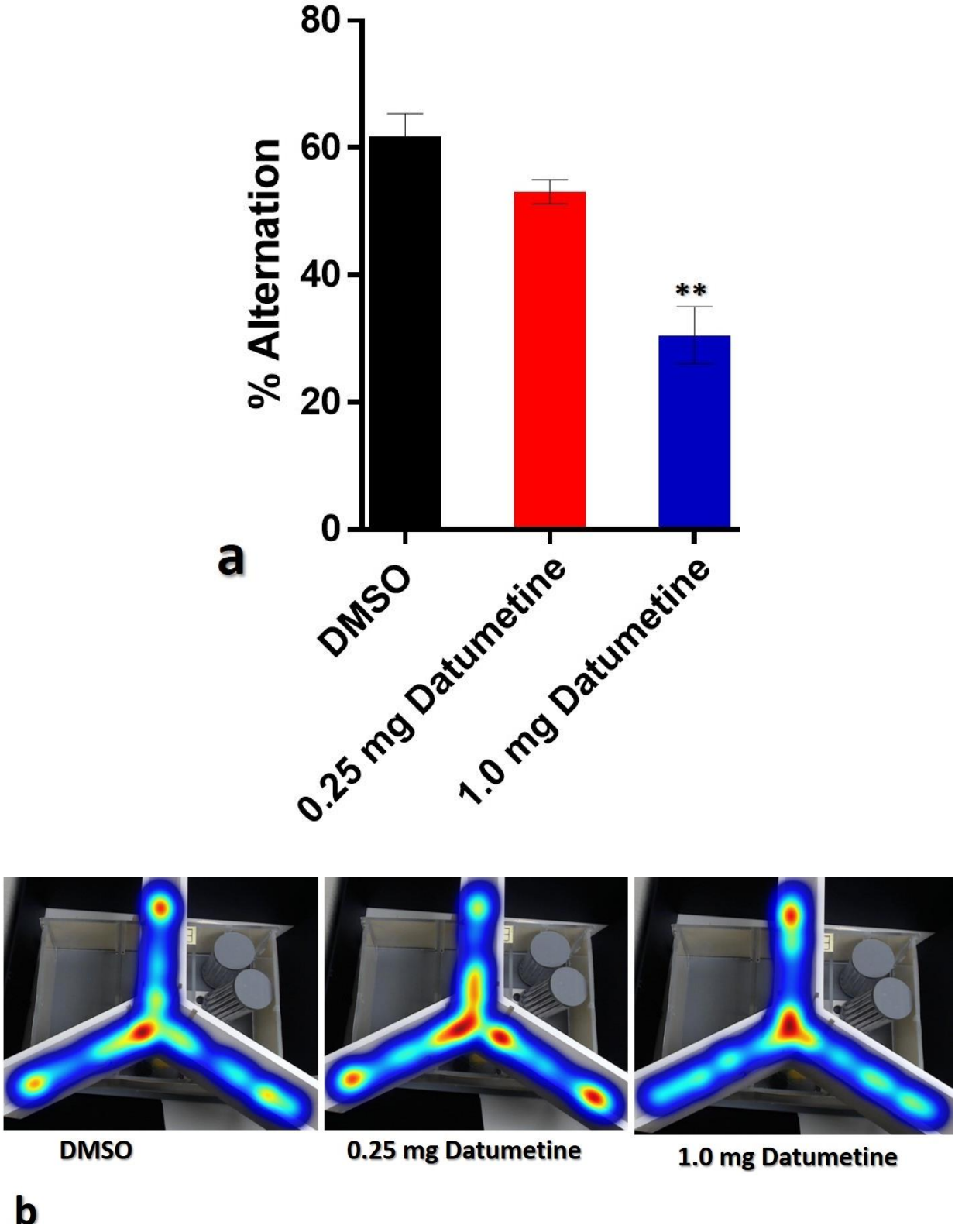

80
60
40
20
0
% Alternation
**
a
DMSO
0.25 mg Datumetine
1.0 mg Datumetine
DMSO
0.25 mg Datumetine
1.0 mg Datumetine
b

Figure 4.2

Figure 4.3: Results of NOR test showing (a): memory index of experimental animals, and (b): representative heatmap visualization of animal activity during NOR test. (n= 10 per group, *p<0.05, **p<0.01 one-way ANOVA with Tukey post-hoc test). Animals exposed to datumetine showed reduced memory index compared to the control.

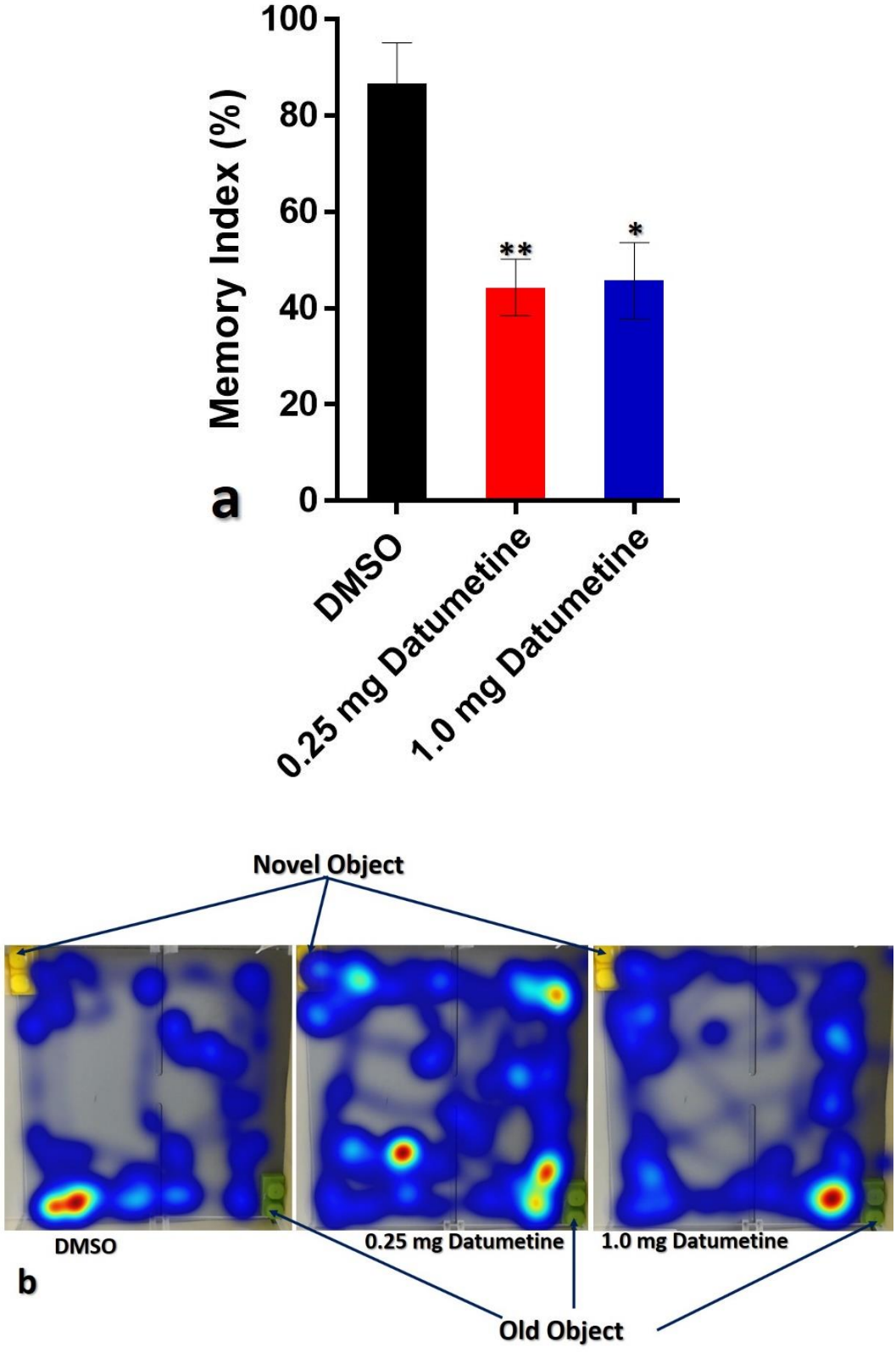

100
80
60
40
20
0
Memory Index (%)
a
**
*
DMSO
0.25 mg Datumetine
1.0 mg Datumetine
Novel Object
DMSO
0.25 mg Datumetine
1.0 mg Datumetine
b
Old Object

Figure 4.3

4.4 Datumetine Increases the Expression of NMDAR with Downregulation of Downstream Signalling Molecules

Expression of NMDAR was assessed using the GluN1 antibody. The expression of GluN1 from the blotting membrane was reduced in all animals (figure 4.4a). Quantitative analysis of the expression normalized with actin showed that there is an increasing trend in the relative expression of GluN1 is datumetine exposed animals compared to control (figure 4.4b). Increased relative expression of GluN1 in 1.0 mg Datumetine animals is significant (*p<0.05) compared to the control.

CamKII-α a C-terminus associated protein molecule of NMDAR which is important in information processing, storage and NMDAR functions was evaluated. Expression of CamKII-α was more in datumetine exposed animals to the control animals (figure 4.5a) with 0.25 mg Datumetine animals showing the highest level of expression. Relative expression of CamKII-α normalized with actin showed that exposure to datumetine significantly (**p<0.01, ***p<0.001) upregulated expression of CamKII-α (figure 4.5b). The level of increase was inversely correlated to the dose of datumetine exposure as animals exposed to 1.0 mg/kg body weight showed a reduction in the level of CamKII-α than 0.25 mg Datumetine animals (figure 4.5b).

CamKII-α is autophosphorylated at Thr286 by calcium influx upon NMDAR activation. Expression of pCamKIIα-T286 was present in all experimental animals (figure 4.6a). The band thickness was more in 0.25 mg Datumetine animals. Quantitative analysis of relative expression of pCamKIIα-T286 relative to actin indicates a declining trend in the relative expression of datumetine exposed animals (figure 4.6b). This reduced relative expression was significant

(*p<0.05) in animals exposed to 1.0 mg/kg body weight of datumetine compared with the control.

Cyclic AMP response element-binding (CREB) protein one of the important signalling molecules of NMDAR activation responsible for the initiation of transcription of proteins required for cell survival and synaptic plasticity was assessed. Expression of CREB was reduced in 1.0 mg Datumetine animals to the control and 0.25 mg Datumetine animals (figure 4.7a). Quantitative analysis of the relative expression to actin revealed significant (*p<0.05) reduction in 0.25 mg Datumetine animals compared to control with no significant difference between 1.0 mg Datumetine animals and control (figure 4.7b).

Brain-derived neurotrophic factor (BDNF), protein responsible neuronal survival and synaptic plasticity which is one of the end products of NMDAR signalling cascade process was checked. Expression of BDNF was reduced in 1.0 mg Datumetine animals than other groups (figure 4.8a). Quantitative analysis of relative expression of BDNF to actin showed a trend in the decline of relative expression of BDNF in datumetine exposed animals compared to control. This reduction was significant (**p<0.01) in animals exposed to 1.0 mg/kg of Datumetine.

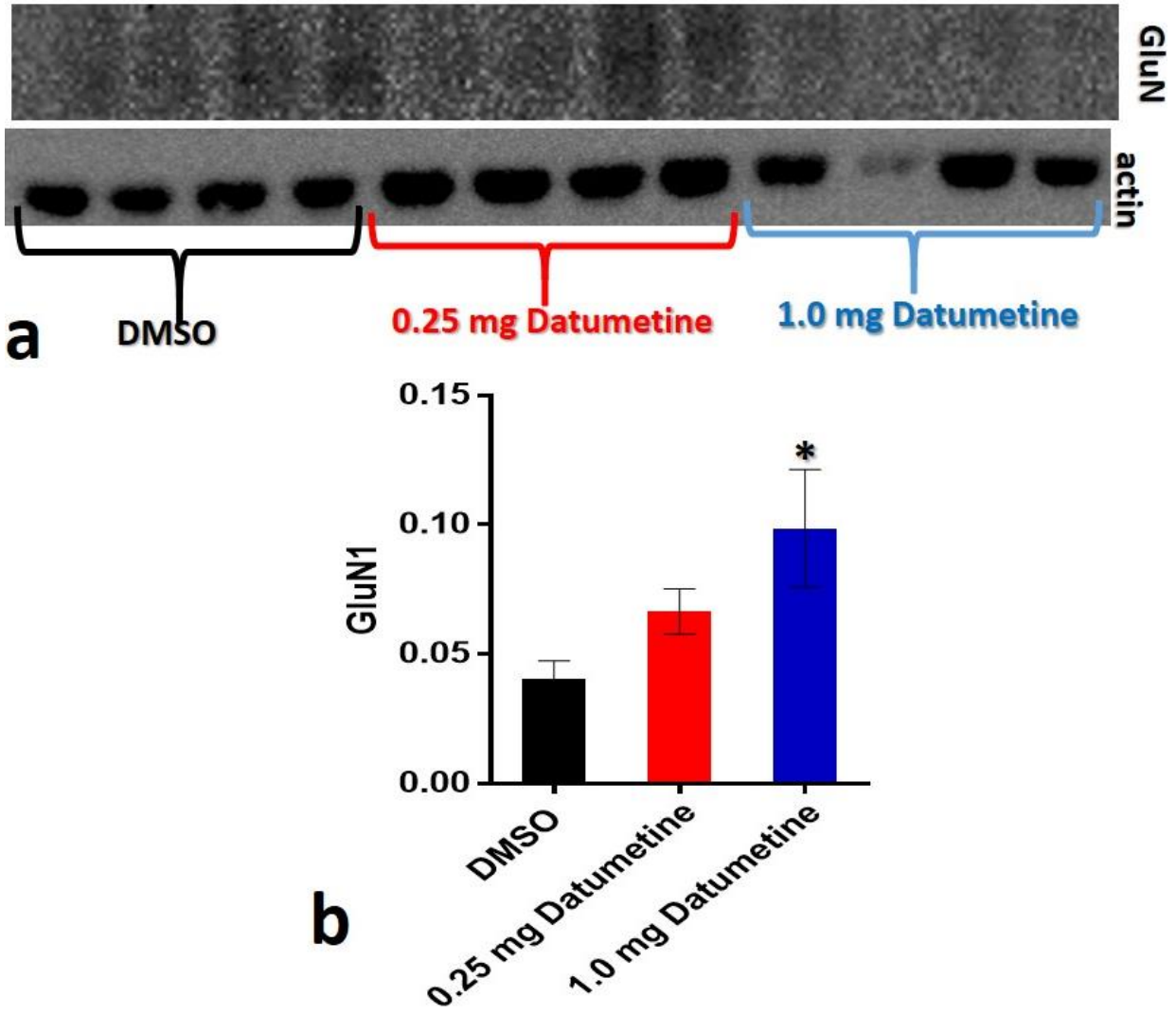


Figure 4.4: Results of GluN1 expression showing (a): representative immunoblots of GluN1 expression in the lysates of the hippocampus of experimental animals, and (b): quantification of relative GluN1 levels in the lysates of the hippocampus of experimental animals. (n=4 per group, *p<0.05 one-way ANOVA with Tukey post-hoc test). Exposure to datumetine upregulates GluN1 expression.

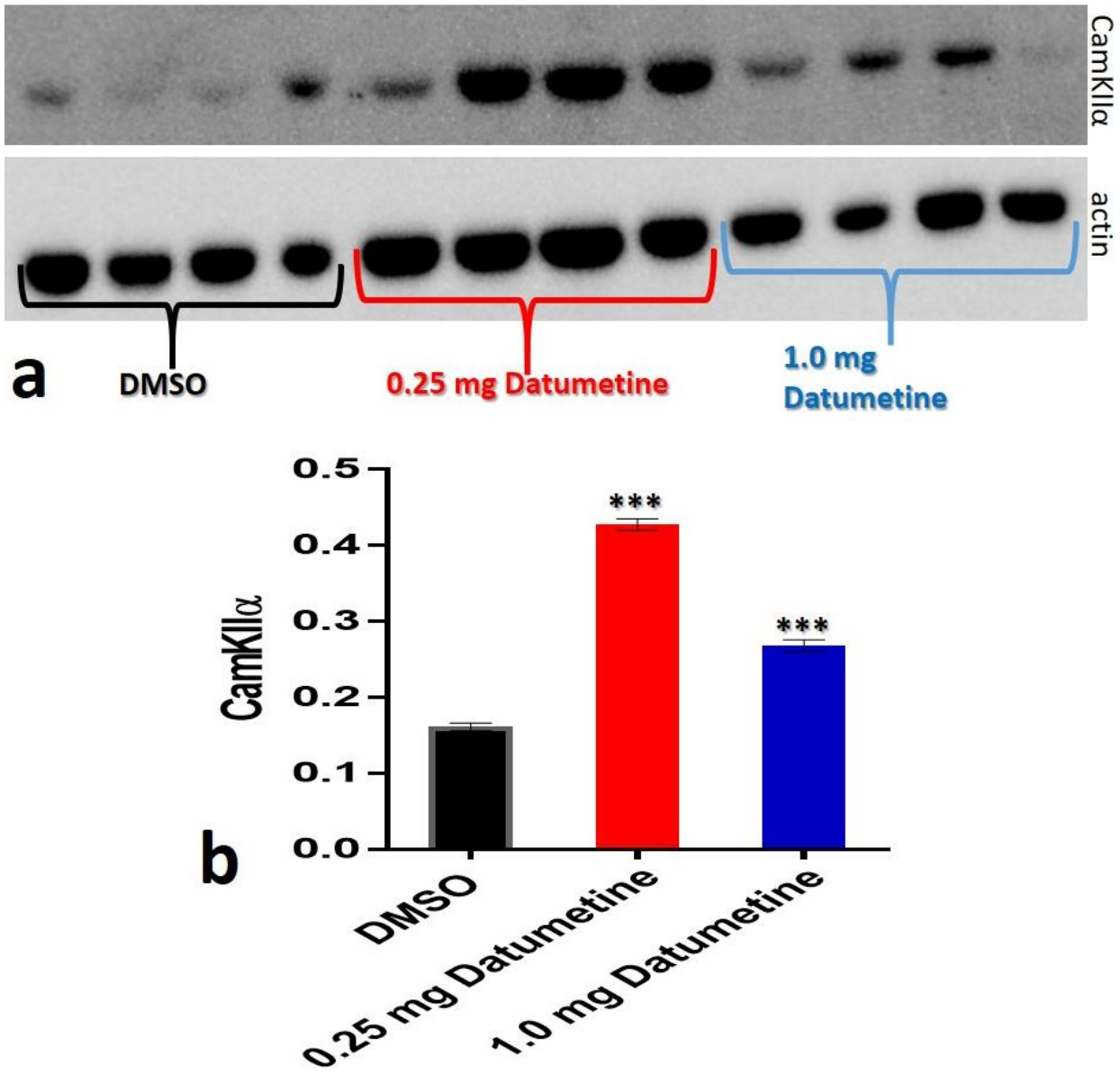


Figure 4.5: Results of CamKIIα expression showing (a): representative immunoblots of CamKIIα expression in the lysates of the hippocampus of experimental animals, and (b): quantification of relative CamKIIα levels in the lysates of the hippocampus of experimental animals. (n=4 per group, $^{**}p<0.01$, $^{***}p<0.001$ one-way ANOVA with Tukey post-hoc test). Exposure to datumetine significantly upregulates CamKIIα expression.

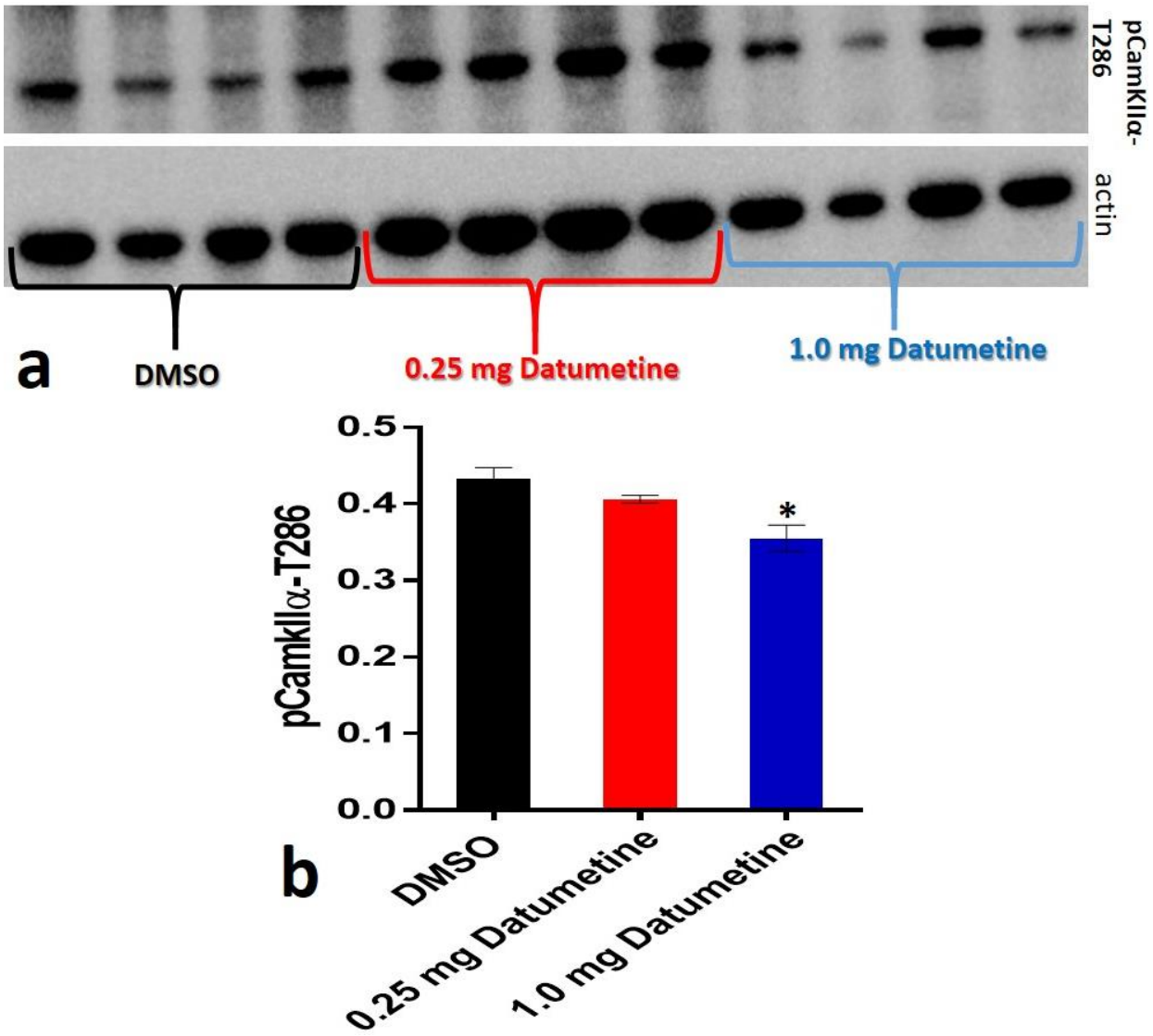


Figure 4.6: Results of pCamKIIα-T286 expression showing (a): representative immunoblots of pCamKIIα-T286 expression in the lysates of the hippocampus of experimental animals, and (b): quantification of relative pCamKIIα-T286 levels in the lysates of the hippocampus of experimental animals. (n=4 per group, *p<0.05 one-way ANOVA with Tukey post-hoc test). Datumetine reduced the expression of pCamKIIα-T286 in the hippocampus.

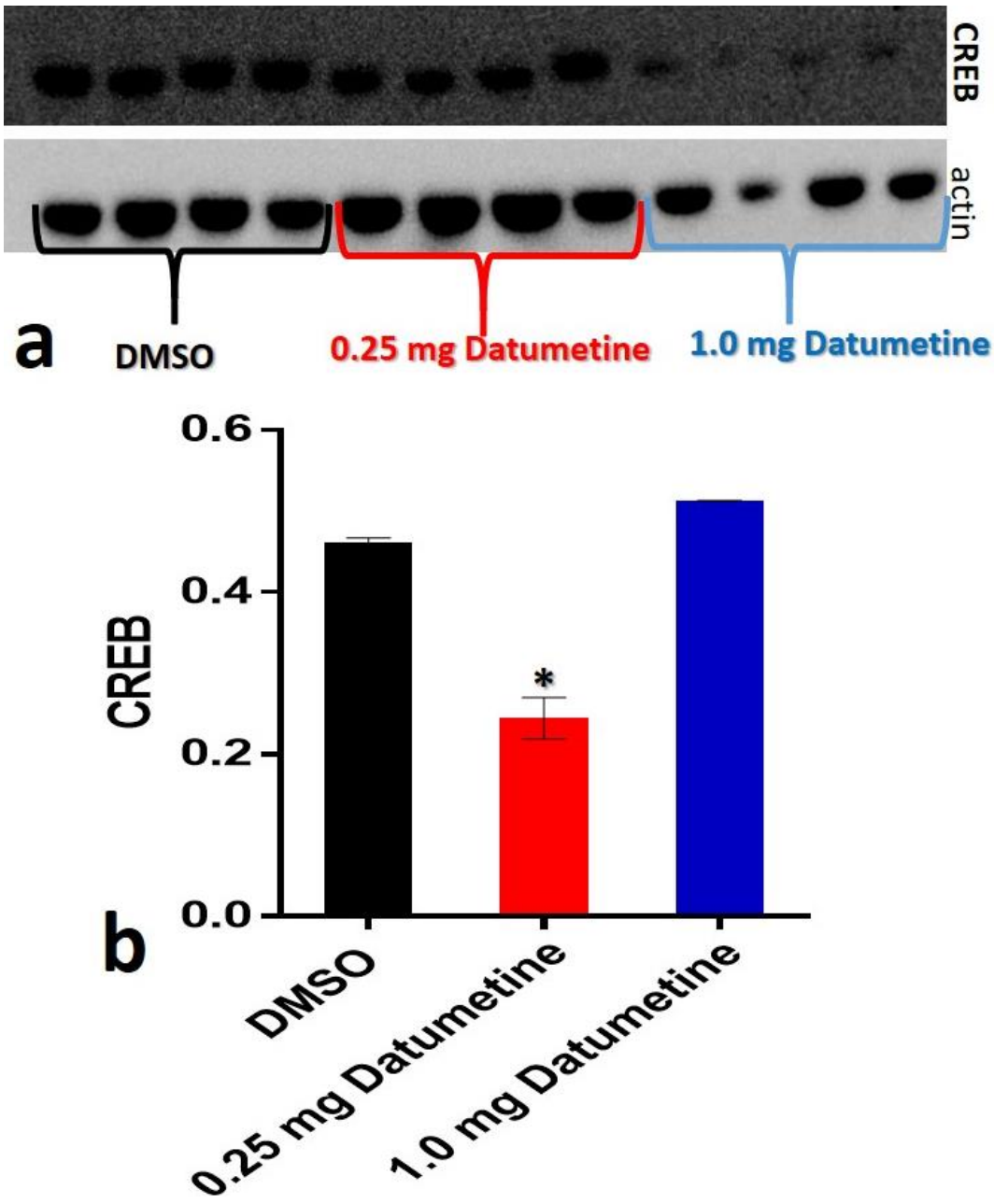


Figure 4.7: Results of CREB expression showing (a): representative immunoblots of CREB expression in the lysates of the hippocampus of experimental animals, and (b): quantification of relative CREB levels in the lysates of the hippocampus of experimental animals. (n=4 per group, *p<0.05 one-way ANOVA with Tukey post-hoc test). 0.25 mg Datumetine animals showed a significant reduction in hippocampal CREB expression.

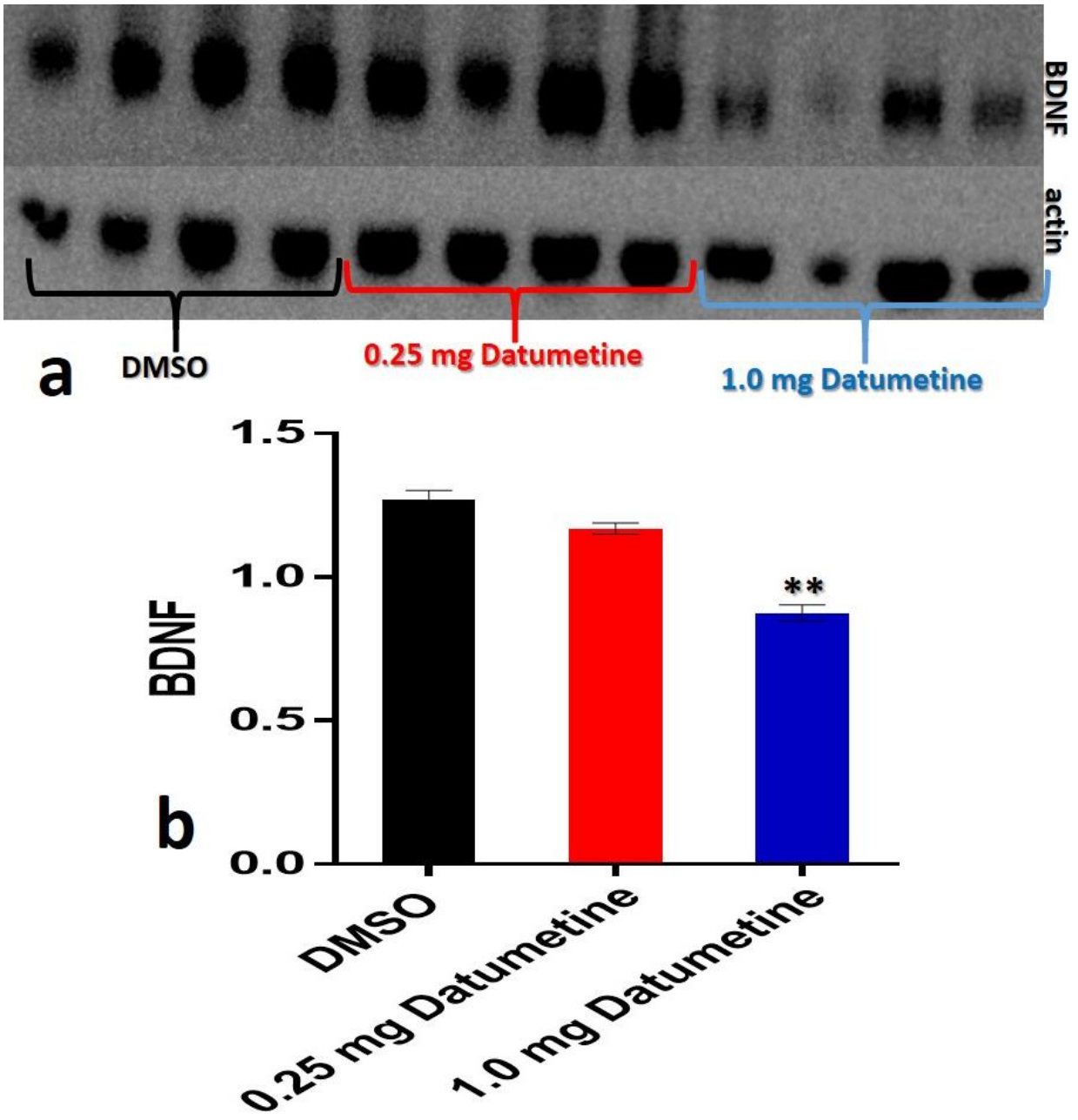


Figure 4.8: Results of BDNF expression showing (a): representative immunoblots of GluN1 expression in the lysates of the hippocampus of experimental animals, and (b): quantification of relative BDNF levels in the lysates of the hippocampus of experimental animals. (n=4 per group, $^{**}p<0.01$ one-way ANOVA with Tukey post-hoc test). Exposure to datumetine reduced the level of hippocampal BDNF.

4.5 Increased Gliosis in the Hippocampus of Datumetine Exposed Animals

Part of brain response to insult is the activation of microglia and astrocyte. Hippocampal slices were stained using DAPI indicated in blue for cellular nuclei (first column in figure 4.9 and 4.10). Nuclei presence was seen in all the regions of the hippocampus which is more populated in the granular cell layer (GCL) of the cornuammonis (CA) regions and dentate gyrus (DG) as seen in all experimental groups.

Matured neurons were further stained to check for neural loss using anti-NeuN indicated in green (second column in figure 4.9 and 4.10). Animals in the DMSO/control group showed the presence of clustered matured neurons in all CA and DG regions of the hippocampus. Scattered presence of matured neurons was also evident in the molecular layer (ML) of the hippocampus (Upper panel). Clustered presence of matured neurons is also evident in the GCL of CA and DG regions of the hippocampus in animals exposed to 0.25 mg/kg datumetine (middle panel). These animals showed sparse and little presence of matured neurons in the ML of the hippocampus. The lower panel represents animals administered 1.0 mg/kg datumetine. There is also the presence of matured neurons in the GCL in both CA and DG regions of the hippocampus. Although, like 0.25 mg Datumetine animals, 1.0 mg Datumetine animals also showed reduced expression of matured neurons in the ML of the hippocampus.

Activated microglia were checked using anti-CD11b showed in red (third column in figure 4.9). For the control animals (upper panel), expression of microglia is seen in the ML and areas beneath the GCL of DG of the hippocampus. 0.25 mg Datumetine animals (middle panel) showed similar expression of microglia to control animals, the intensity was more than the control animals. 1.0 mg Datumetine animals (lower panel) showed microglia expression in the

CA regions together with the ML of the hippocampus. Presence of microglia in 1.0 mg Datumetine animals were also seen in the regions beneath GCL of the DG.

Astrocytic profile was also assessed in the hippocampus of experimental animals using ant-GFAP (third column figure 4.10). Expression of GFAP positive cells in the control animals was more evident in the ML and part of DG of the hippocampus (upper panel). Middle panel, representative of 0.25 mg Datumetine animals showed GFAP positive expression in areas like the control animals (i.e. ML and part of DG of the hippocampus). Animals exposed to 1.0 mg/kg of datumetine showed expression of GFAP more in CA3 region, DG and ML of the hippocampus (lower panel).

The last column showed the merged image of all horizontal panels showing the relationship of each immunopositive cells.

Quantification of the fluorescent intensity was evaluated to predict the number of immunopositive cells. This was done for NeuN and CD11b positive cells. NeuN positive cells showed a significant (*p<0.05) decline in animals exposed to datumetine compared to control (table 4.1) indicating loss of neurons. While CD11b positive cells showed a significant (*p<0.05) increase in animals exposed to datumetine compared to control (table 4.1) indicating microgliosis and activation.

Quantification of the fluorescent intensity from astrocyte stained slides also showed a decline in NeuN positive cells with 1.0 mg datumetine animals showing significant (*p<0.05) (table 4.1). Like quantification observed from CD11b positive cells, GFAP positive cells also showed significant (*p<0.05) increase in the GFAP intensity compared to control (table 4.1).

Figure 4.9: Representative photomicrograph of the hippocampus of experimental animals showing nuclei (blue), neurons (green), microglia (red) and merged. Exposure to datumetine reduced expression of neurons in the hippocampus whereas microglia expression was increased.

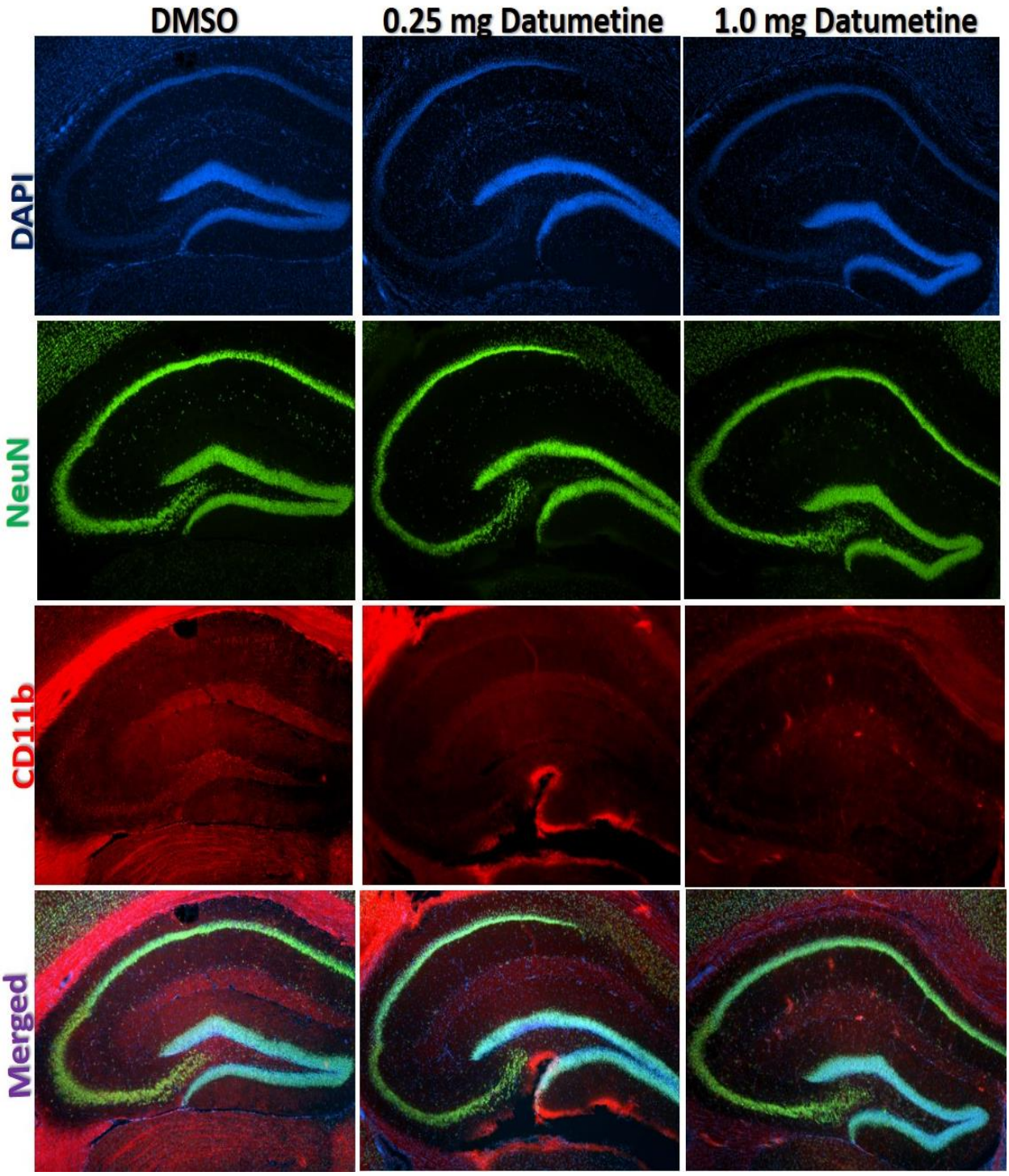

DMSO
0.25 mg Datumetine
1.0 mg Datumetine
DAPI
NeuN
CD11b
Merged

Figure 4.9

Figure 4.10: Representative photomicrograph of the hippocampus of experimental animals showing nuclei (blue), neurons (green), astrocyte (red), and merged. Exposure to datumetine reduced hippocampal neurons with increased astrocytes expression.

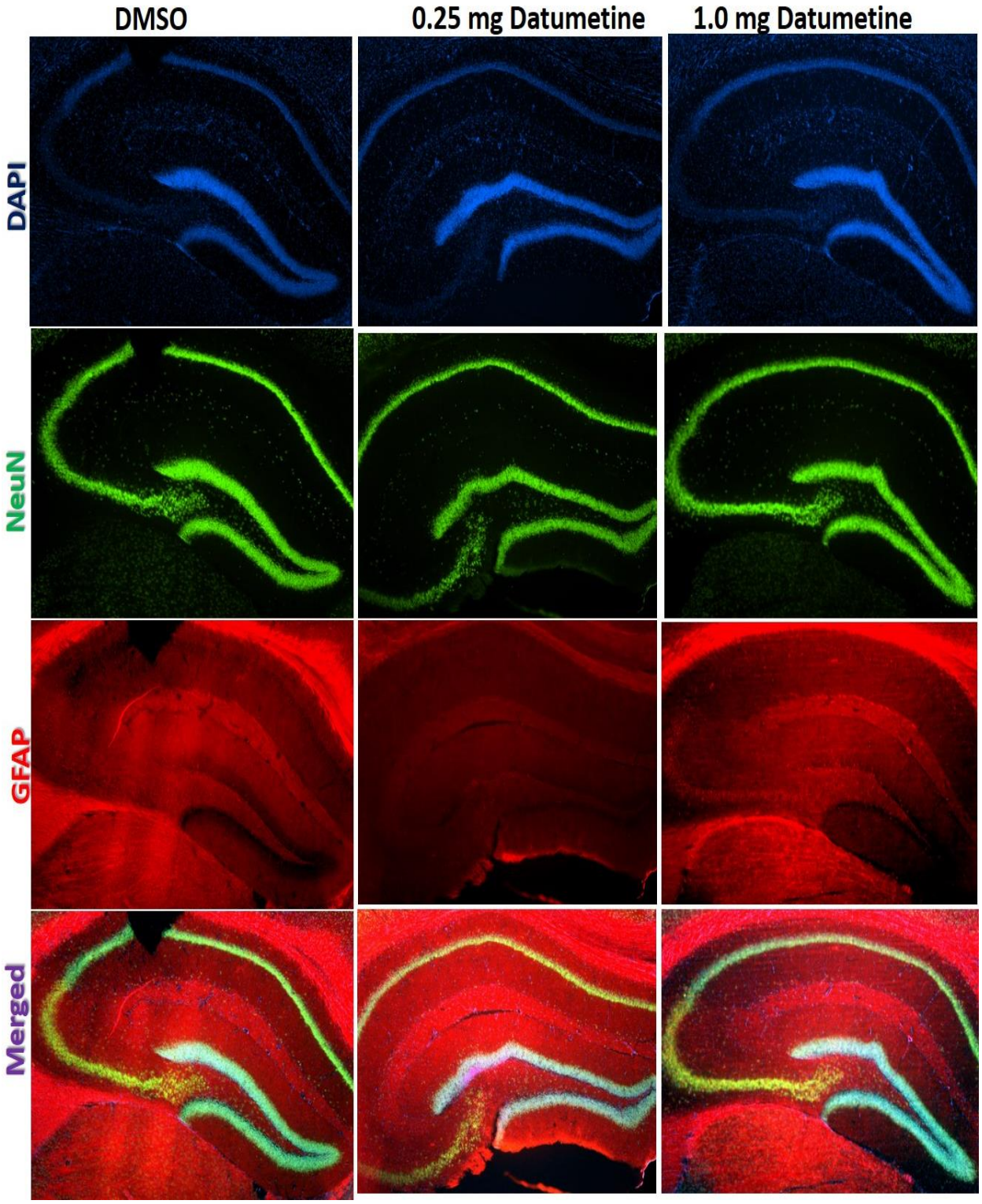

DMSO
0.25 mg Datumetine
1.0 mg Datumetine
DAPI
NeuN
GFAP
Merged

Figure 4.10

4.6 Increased activity of Excitatory and Inhibitory Neurons in Datumetine Exposed Animals

Since it was hypothesized that datumetine interacts with NMDAR, the activity of glutamate neurons that release glutamate neurotransmitter in the brain was assessed. Glutamate neuron was checked using vesicular glutamate transporter antibody (anti-vGlut1) indicated in red together with DAPI for nucleus stain (figure 4.11).

All experimental animals showed the presence of nuclei indicated by the blue colour in all areas of the hippocampus (first column figure 4.11). Middle column showed the expression of vGlut1 indicated in red. Control animals showed expression of vGlut1 in the CA and DG regions of the hippocampus with little expression in the ML of the hippocampus (upper panel in the middle column of figure 4.11), similarly, 0.25 mg Datumetine animals showed increased expression of vGlut1 in the ML of the hippocampus together with the CA and DG regions of the hippocampus (middle panel of middle column). Expression of vGlut1 was more in animals exposed to 1.0 mg/kg Datumetine (lower panel of middle column), the expression was observed in all regions of the hippocampus.

Quantification analysis of vGlut1 intensity revealed significant ($*p<0.05$) increase in animals exposed to datumetine compared to control (table 4.1). This indicating increased activity of glutamate neurons. No significant difference between the datumetine exposed animals.

The inhibitory system wasalso evaluated by checking for the major inhibitory neurotransmitter (gamma-aminobutyric acid - GABA) in the animal's hippocampus. This was achieved by using anti-GABA (red) and DAPI (blue) (figure 4.12). Nuclei presence was seen in all groups of experimental animals (first column figure 4.12). GABA expression was observed sparsely in the

CA region of the hippocampus in the control animals (upper panel, middle column figure 4.12). 0.25 mg Datumetine animals showed expression of GABA in all regions of the hippocampus (middle panel, middle column figure 4.12). 1.0 mg Datumetine animals also showed increased expression of GABA in all regions of the hippocampus (lower panel, middle column figure 4.12).

Quantification of GABA intensity was evaluated (table 4.1), animals exposed to datumetine showed significant (*p<0.05) increase in GABA intensity than the control animals. No significant difference was observed between the datumetine groups.

Figure 4.11: Representative photomicrograph of the hippocampus of experimental animals showing nuclei (blue), and glutamate neurons (red) and merged. Datumetine exposure increases the activity of glutamate neurons.

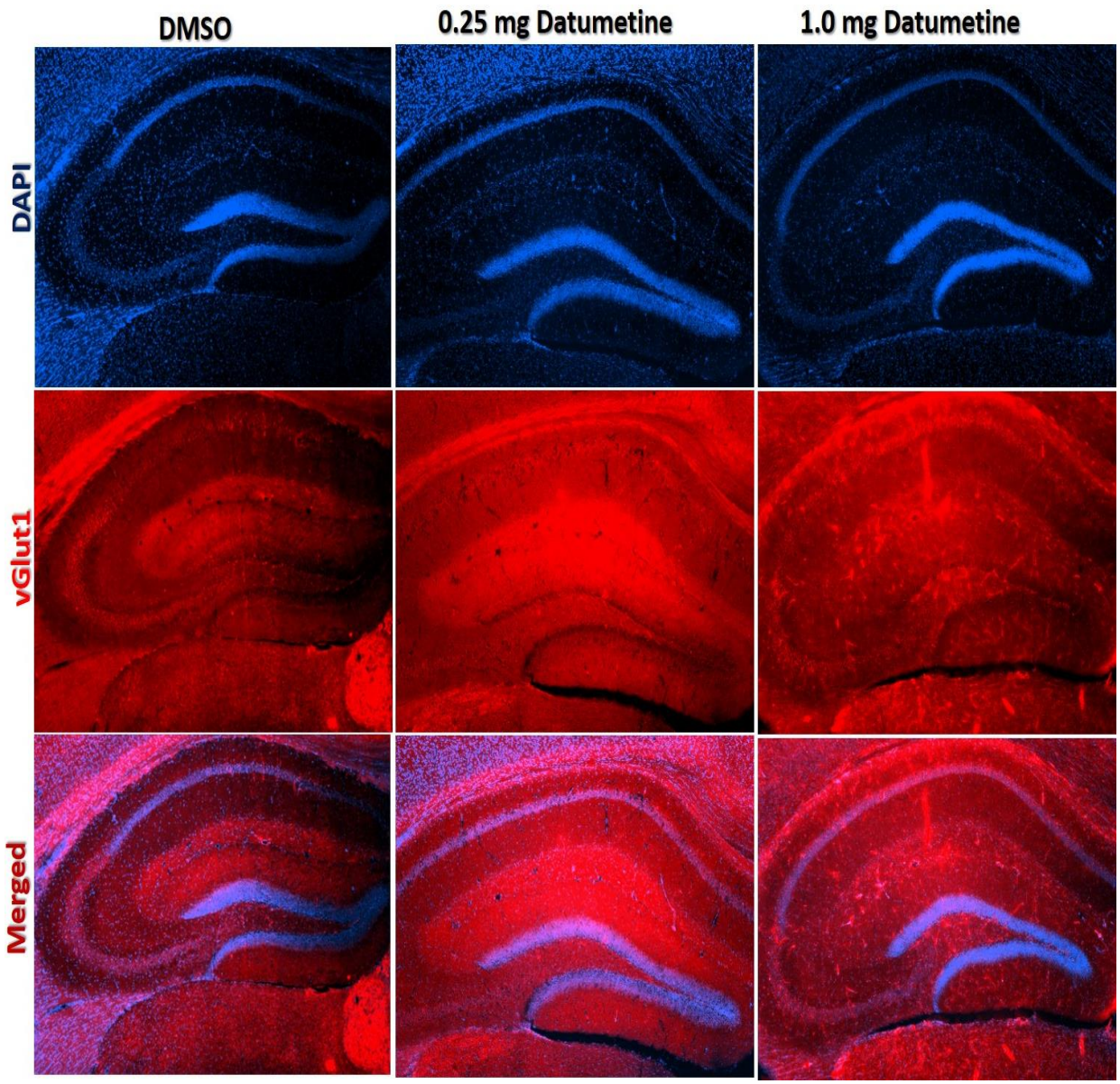


Figure 4.11

Figure 4.12:Representative photomicrograph of the hippocampus of experimental animals showing nuclei (blue), GABAergic neurons (red) and merged. Datumetine exposure significantly increased hippocampal GABA neurons activity.

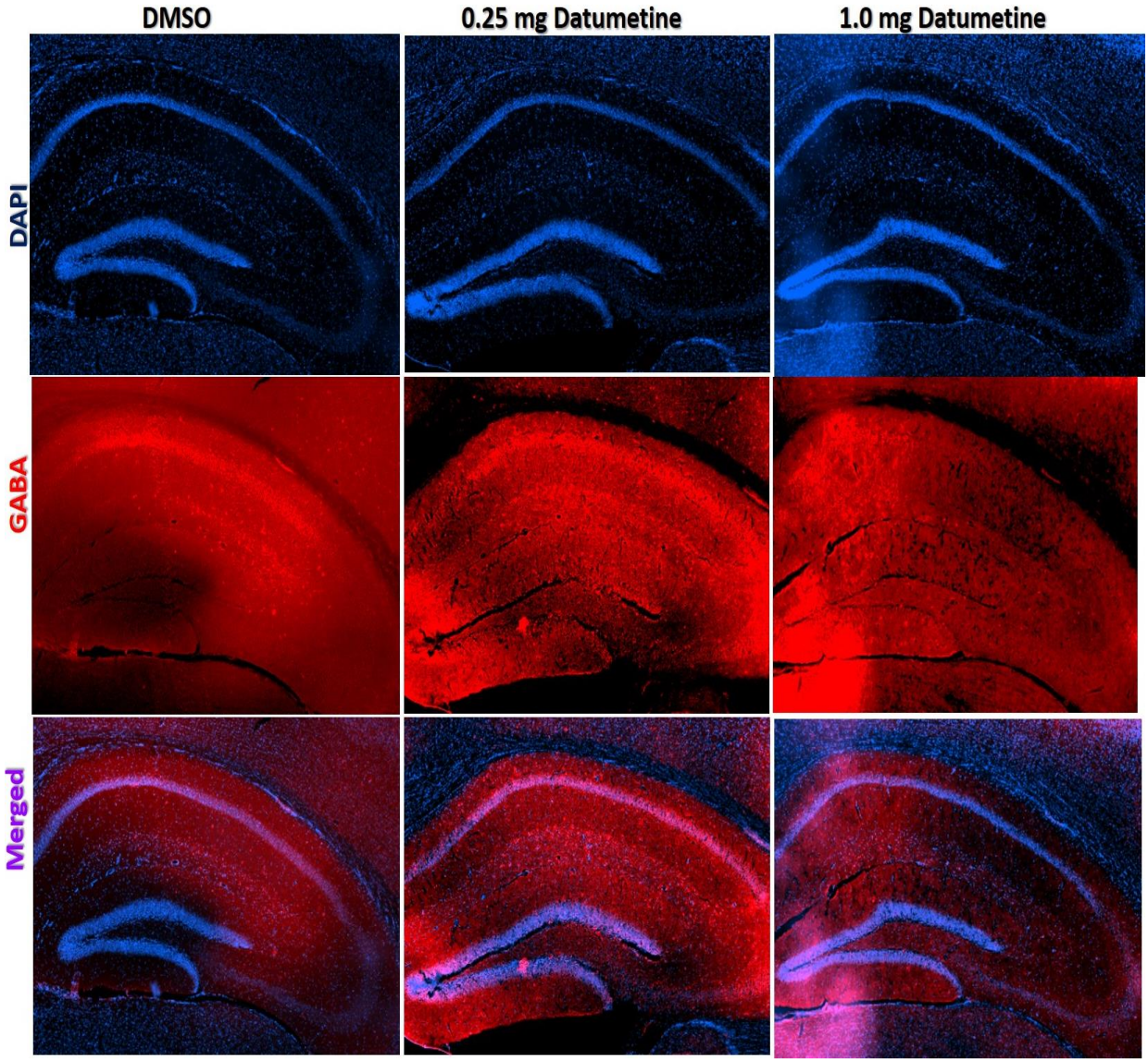


Figure 4.12

4.7 Hippocampal Neuronal Subtypes Activity in Datumetine Exposure

Status of other neuronal subtypes (cholinergic, dopaminergic and serotonergic) was assessed in the hippocampus of experimental animals (figure 4.13-15). The cholinergic activity was evaluated by staining for alpha-7 cholinergic receptors (CHRNA7) indicated with red and DAPI for nuclei indicated in blue (figure 4.13).

Nuclei expression was observed in all regions of the hippocampus of experimental animals indicated by the blue colouration (first column figure 4.13, 4.14 and 4.15). Expression of CHRNA7 was observed in the CA region of the hippocampus together with the ML (upper panel, middle column, figure 4.13). 0.25 mg Datumetine animals showed CHRNA7 expression in the CA region and ML of the hippocampus which is more than that of the control animals (middle panel, middle column, figure 4.13). Similarly, 1.0 mg Datumetine animals had CHRNNA7 expression in all CA and ML regions of the hippocampus (lower panel, middle column, figure 4.13). Quantification of CHRNA7 fluorescence was evaluated (table 4.1), exposure to datumetine significantly ($*p<0.05$) increased the expression of CHRNA7 than the control.

Dopaminergic neuron activity was also evaluated using an antibody against tyrosine hydroxylase (anti-TH) (figure 4.14). Control animals showed TH expression mainly in the ML of the hippocampus with sparse expression in the CA1 region (upper panel, middle column figure 4.14). More expression of TH was observed in the ML of the hippocampus in 0.25 mg Datumetine animals (middle panel, middle column figure 4.14). Similarly, 1.0 mg Datumetine animals showed increased expression of TH in the ML of the hippocampus (lower panel, middle column, figure 4.14). Immunofluorescence quantification also showed that TH intensity was

more in animals exposed to datumetine (table 4.1). This increased intensity was significant (*p<0.05) in datumetine exposed animals than the control.

Serotonergic neuronal activity was assessed by staining for tryptophan hydroxylase 1(TPH-1) shown in red (figure 4.15). Control animals (upper panel, middle column, figure 4.15) showed TPH-1 expression sparsely in the CA region and ML of the hippocampus. The animals also showed high expression of TPH-1 in the DG of the hippocampus. The middle panel representing animals of 0.25 mg Datumetine group showed little expression of TPH-1 in the CA region and ML of the hippocampus with no expression at the DG area. Animals exposed to 1.0 mg/kg datumetine showed expression of TPH-1 sparsely in the DG region and scattered expression at the ML and CA region of the hippocampus (lower panel, middle column figure 4.15). Fluorescence quantification of TPH-1 expression showed significant (*p<0.05) decrease in datumetine exposed animals compared to the control (table 4.1).

Figure 4.13: Representative photomicrograph of the hippocampus of experimental animals showing nuclei (blue), cholinergic neurons (red) and merged. Datumetine exposure increases cholinergic activity in the hippocampus of experimental animals.

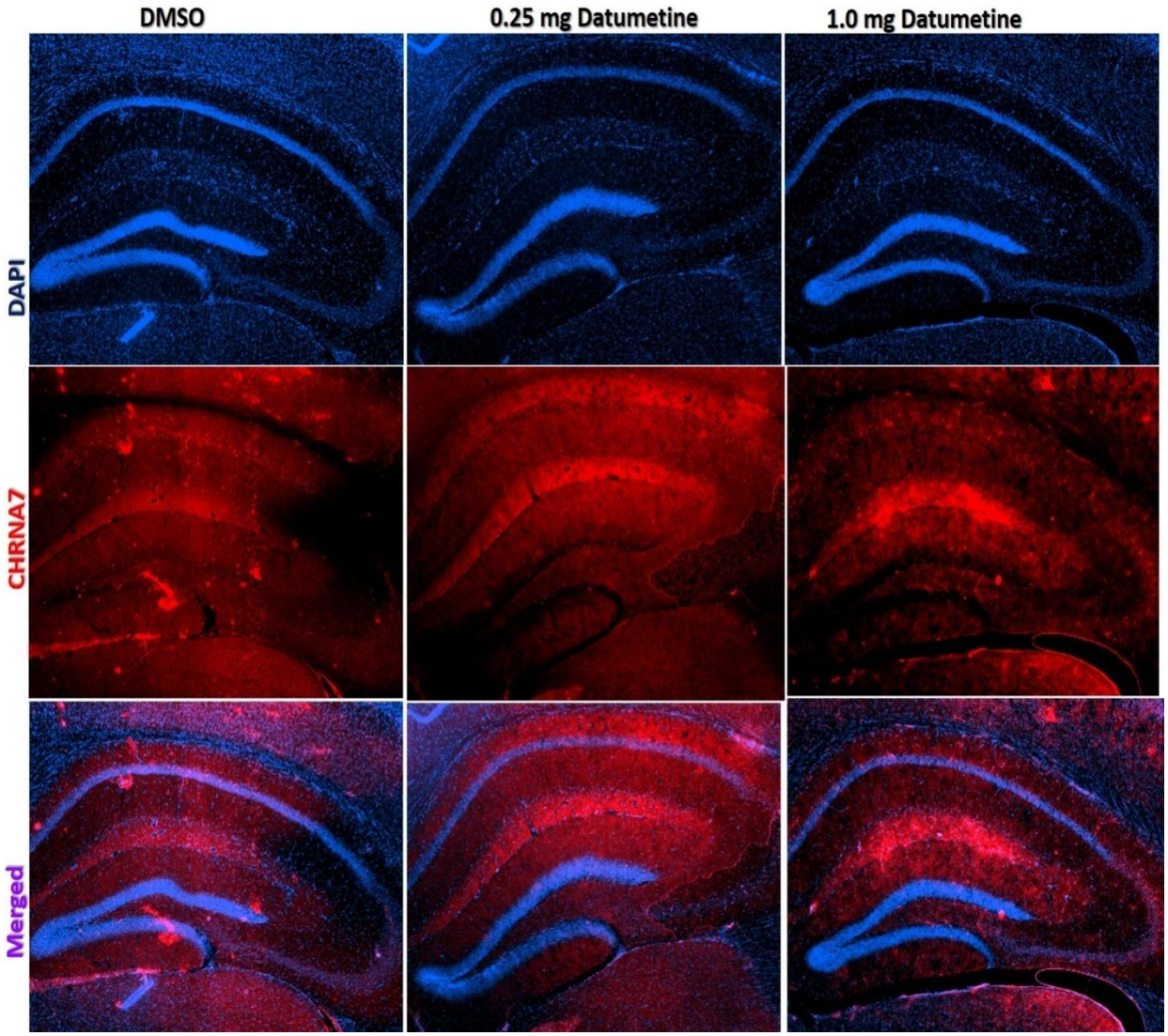


Figure 4.13

Figure 4.14: Representative photomicrograph of the hippocampus of experimental animals showing nuclei (blue), dopaminergic terminals (red) and merged. The dopaminergic activity was increased in animals exposed to datumetine.

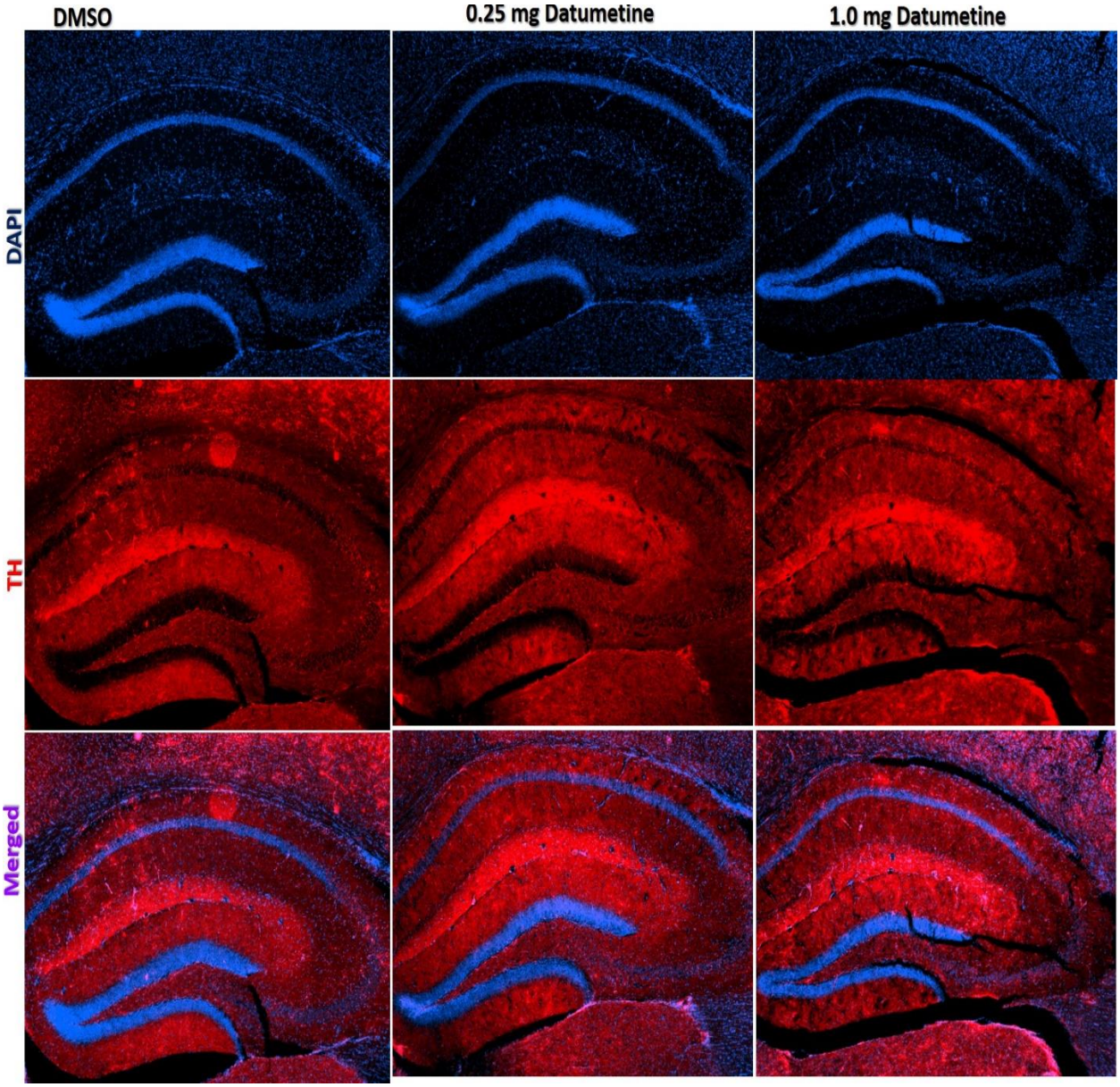


Figure 4.14

Figure 4.15: Representative photomicrograph of the hippocampus of experimental animals showing nuclei (blue), serotonin neurons (red) and merged. Datumetine exposure induces loss of serotonergic neurons in the hippocampus.

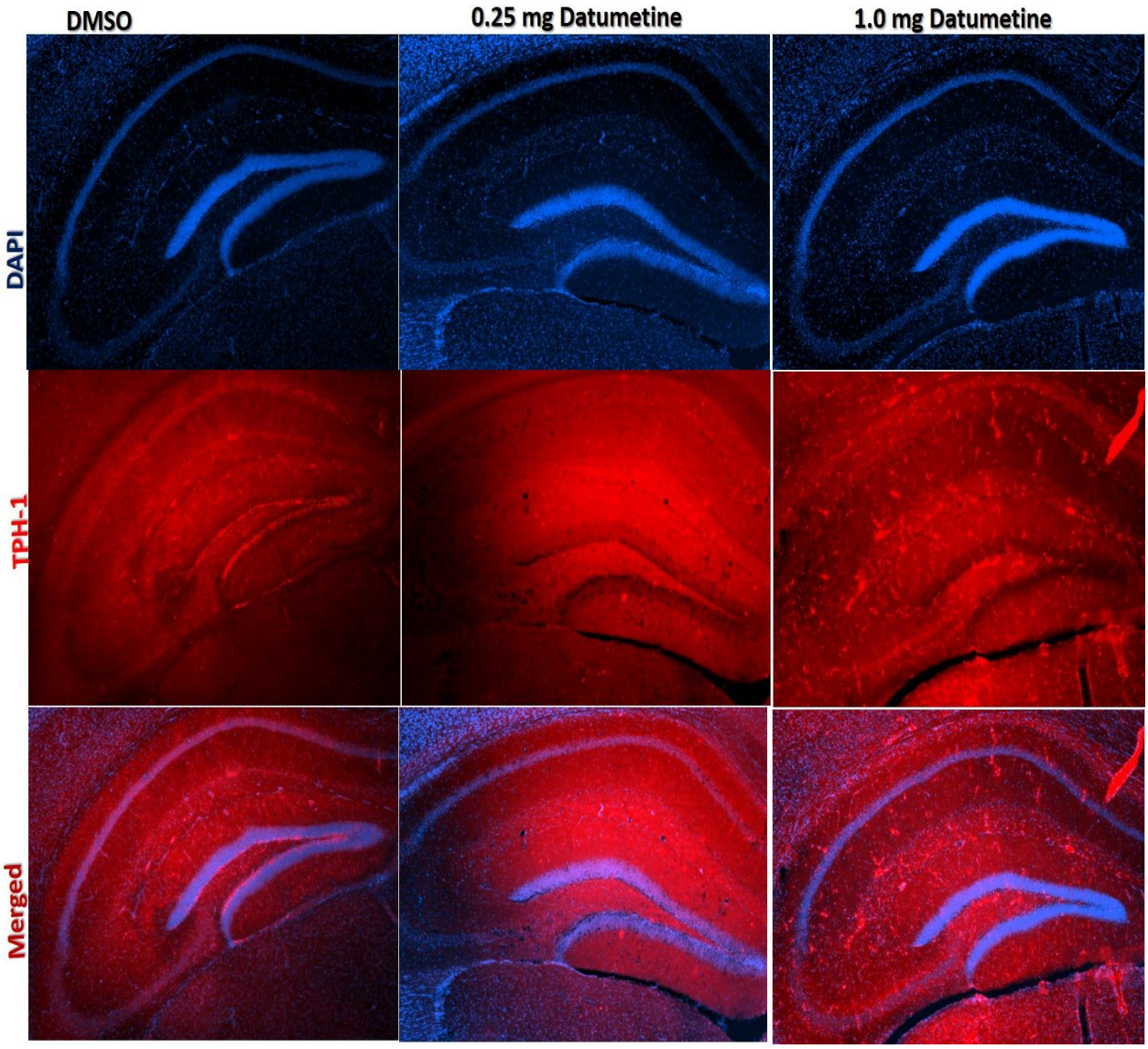


Figure 4.15

Table 4.1: Quantification of immunofluorescence staining intensity

Fluorescence Quantification	**DMSO**	**0.25 mg Datumetine**	**1.0 mg Datumetine**
NeuN	708.17 ± 36.38	519.44 ± 3.06*	457.295± 6.23*
CD11b	1140.01 ± 48.60	1335.79 ± 13.47*	1303.48 ± 52.78*
GFAP	1107.38 ± 36.54	1619.57 ± 118.68*	1591.63 ± 83.34*
vGlut1	378.10 ± 36.39	1561.00 ± 101.30*	1646.00 ± 41.75*
GABA	626.90 ± 10.43	957.40 ± 2.00*	907.30 ± 27.97*
CHRNA7	649.40 ± 16.96	1181.00 ± 115.50*	885.00 ± 13.92*
TH	801.80 ± 73.44	1136.00 ± 12.21*	1022.00 ± 6.51*
TPH-1	1368.00 ± 64.52	791.60 ± 98.15*	953.70 ± 22.28*

n=10 slices per group, *p<0.05 one-way ANOVA with Tukey post-hoc test

4.8 Datumetine Induces Loss of Neural Connections

Expansion microscopy technique was done to check for neural connections in the hippocampus. This was achieved by staining for neurofilament by immunofluorescence which serves as the structural protein of neurons in the hippocampus of experimental animals, and then expanding the slices to increase the magnification and clarity.

In the CA1 region of the hippocampus (figure 4.16), Neurofilament indicated in red was well expressed and seen to form a network around the nucleus (blue) which form like a lattice arrangement creating a whole occupied by the nucleus (4.16a). 0.25 mg Datumetine animals, neurofilament was poorly expressed (4.16b). The animals also showed an irregular arrangement of the neurofilament with no lattice arrangement observable. Similarly, 1.0 mg Datumetine animals showed reduced expression of neurofilament (figure 4.16c) together with poor lattice arrangement.

Similar observations were seen in the CA2 region of the hippocampus of experimental animals (4.17). Control animals showed normal expression of neurofilament, orderly arranged to form a network around the nucleus (blue) (figure 4.17a), and lattice arrangement is also evident. CA2 expression of neurofilament was also evident in 0.25 mg Datumetine animals (figure 4.17b) though reduced to control animals. The filaments are seen to be arranged to form a network around the nucleus. 1.0 mg Datumetine animals expressed scanty neurofilament in their CA2 region of the hippocampus (4.17c). Little neurofilament observed were seen to be irregularly arranged in this group of animals.

Likewise, in the CA3 region of the hippocampus (figure 4.18), Control animals showed normal expression of neurofilament that is orderly arranged around the nucleus (figure 4.18a). Animals

exposed to 0.25 mg/kg Datumetine showed less expression of neurofilament which are irregularly arranged (figure 4.18b). 1.0 mg Datumetine animals showed very little expression of neurofilament which are irregularly arranged (figure 4.18c).

The DG of the hippocampus was also observed (figure 4.19). Neurofilament expression was well evident in the ML part of DG of the hippocampus and are seen to be oriented in one direction in the control animals (figure 4.19a). 0.25 mg Datumetine animals also showed expression of neurofilament in the ML part of DG (figure 4.19b), the neurofilament was not seen to be arranged in orientation and they appear marshy. Like 0.25 mg Datumetine animals, 1.0 mg Datumetine animals also showed reduced expression of neurofilament in the ML part of DG (figure 4.19c). The neurofilament observed are not aligned in a direction and appear marshy.

Figure 4.16: Expansion microscopy images of the cornuammonis 1 (CA1) region of the hippocampus stained for neurofilament (red) and nuclei (DAPI-blue) (x40 obj). (**a**) DMSO (**b**) 0.25 mg Datumetine (**c**) 1.0 mg Datumetine. Datumetine induces loss of neurofilament in the hippocampus.

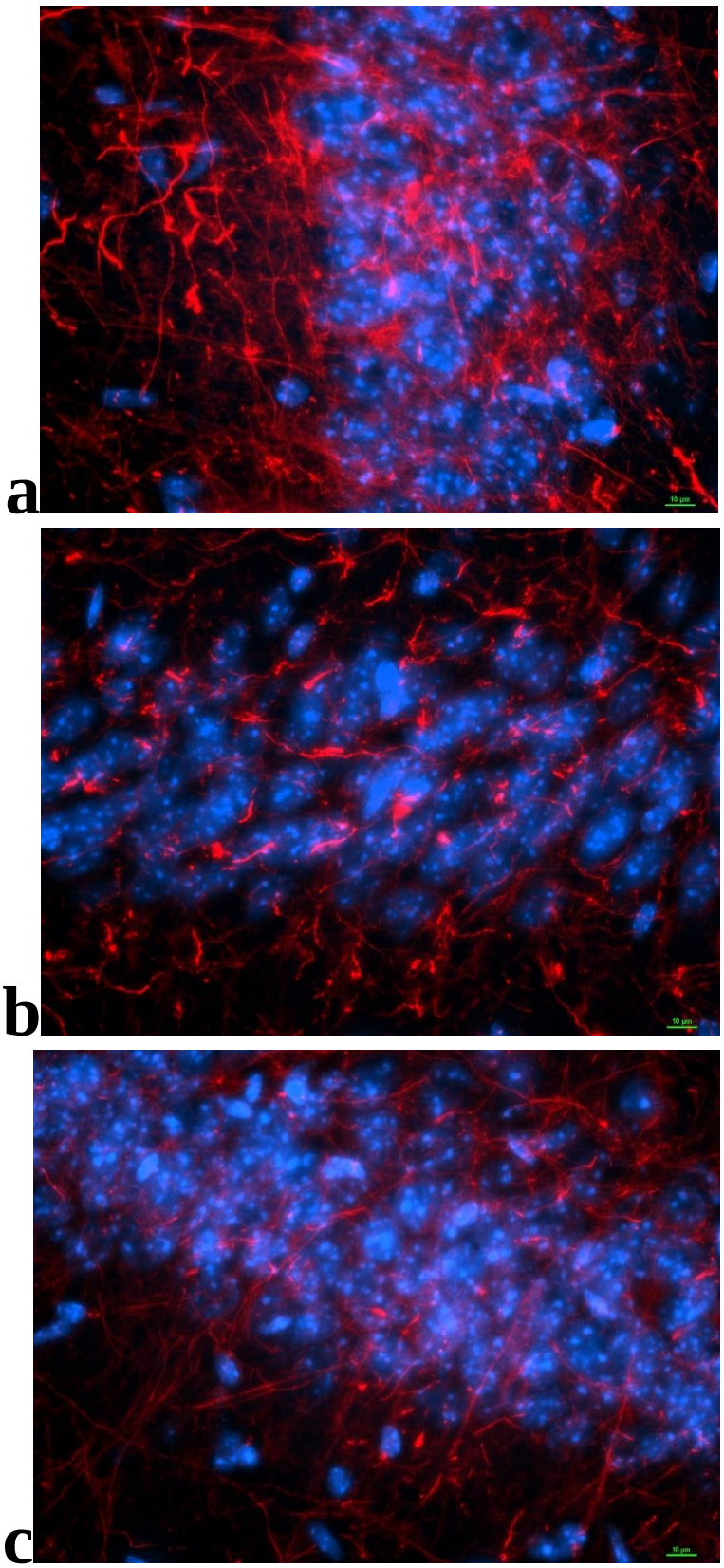


Figure 4.16

Figure 4.17: Expansion microscopy images of the cornuammonis 2 (CA2) region of the hippocampus stained for neurofilament (red) and nuclei (DAPI - blue) (x40 obj). (**a**) DMSO (**b**) 0.25 mg Datumetine (**c**) 1.0 mg Datumetine. Datumetine induces loss of neurofilament in the hippocampus.

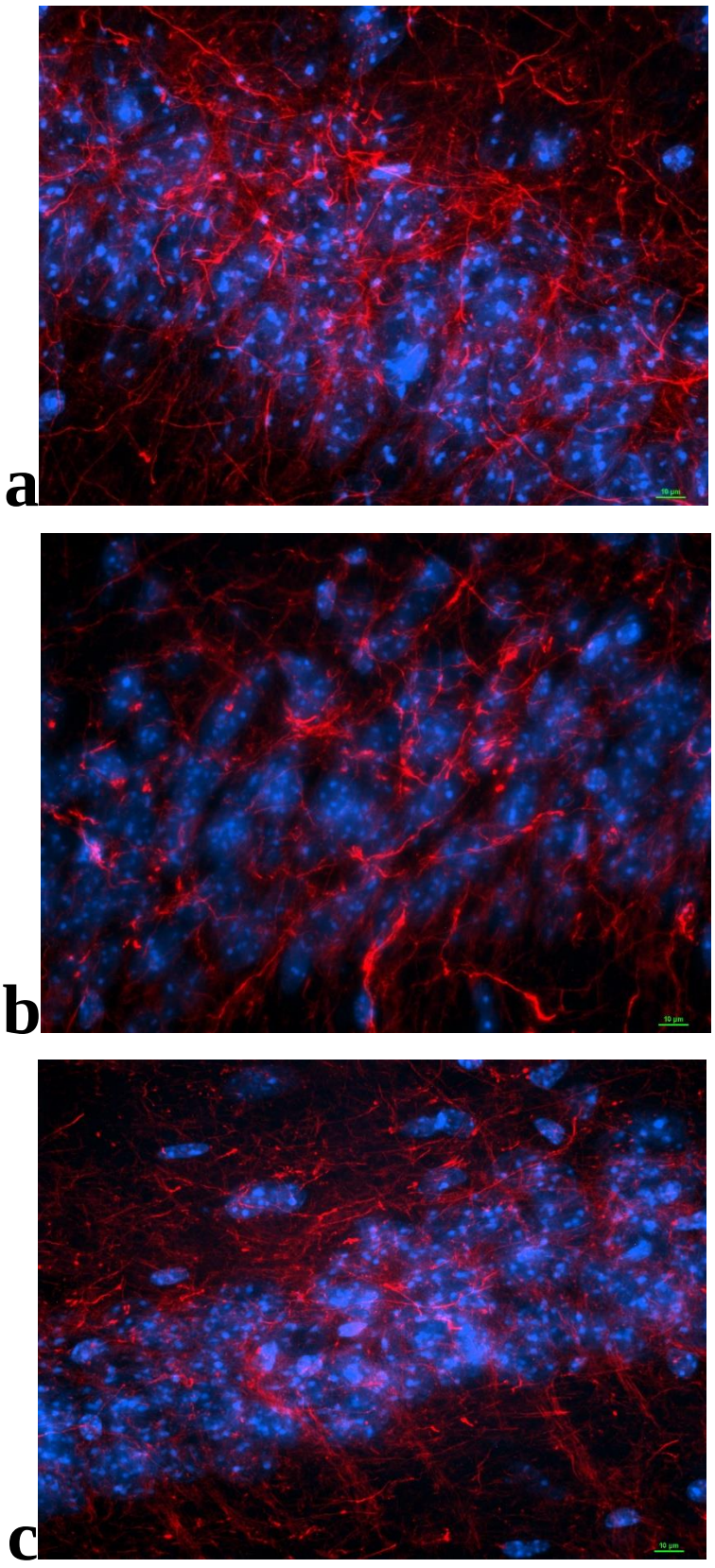


Figure 4.17

Figure 4.18: Expansion microscopy images of the cornuammonis 3 (CA3) region of the hippocampus stained for neurofilament (red) and nuclei (DAPI - blue) (x40 obj). (**a**) DMSO (**b**) 0.25 mg Datumetine (**c**) 1.0 mg Datumetine. Datumetine induces loss of neurofilament in the hippocampus.

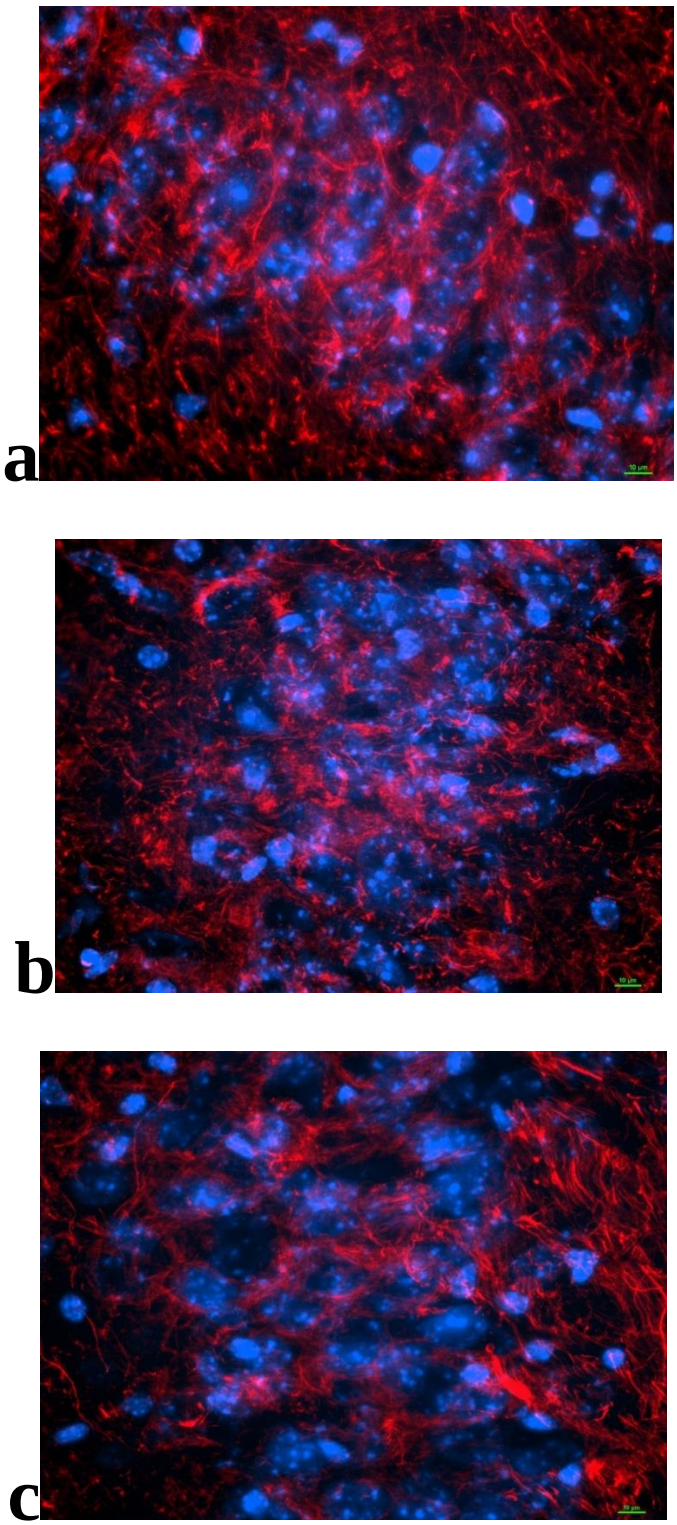
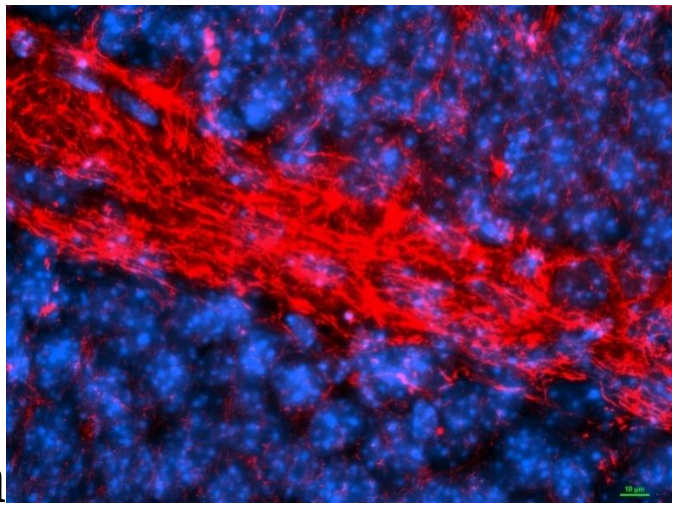


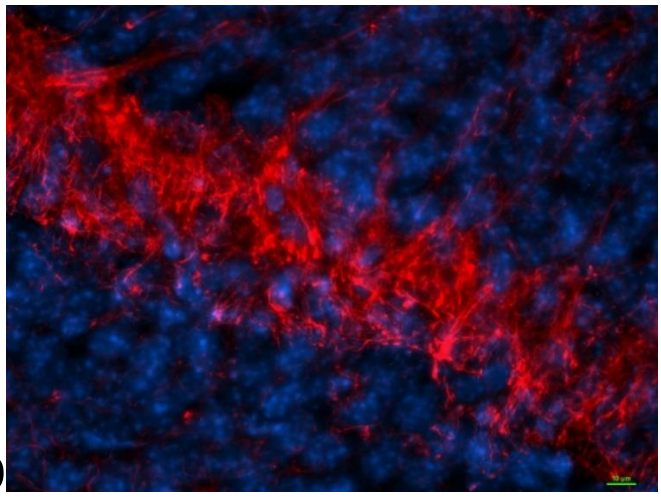
Figure 4.18

Figure 4.19: Expansion microscopy images of the dentate gyrus (DG) region of the hippocampus stained for neurofilament (red) and nuclei (DAPI - blue) (x40 obj). (**a**) DMSO (**b**) 0.25 mg Datumetine (**c**) 1.0 mg Datumetine. Datumetine exposure induces loss of neurofilament in the hippocampus.

a



b



c

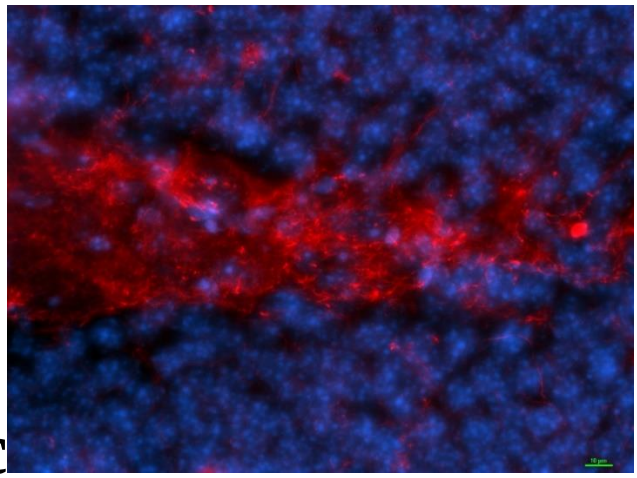


Figure 4.19

4.9 Datumetine Altered Synaptic Morphology with Synaptic Loss

Electron microscopy technique was done to check for viable synapse in the hippocampus. Viable synapse was identified by the presence of pre- and post-synaptic membrane, postsynaptic density (PSD) and presynaptic vesicles (figure 4.20). The number of viable synapses was reduced in animals exposed to datumetine; this effect was more in animals exposed to 1.0 mg/kg body weight of datumetine.

PSD thickness was measured in viable synapse identified in the micrograph of the experimental animals. Datumetine significantly (*p<0.05) increased the thickness of PSD in the hippocampus of experimental animals compared to control with 1.0 Datumetine animals having the highest PSD thickness (table 4.2).

The number of presynaptic vesicles identified in the viable synapse was observed to reduce significantly (*p<0.05) in datumetine exposed animals compared to control with 1.0 Datumetine animals with the least count (table 4.2).

Figure 4.20: Representative micrograph of showing viable synapse of the hippocampus. (PSD – postsynaptic density; SV – synaptic vesicles). Datumetine exposure alters hippocampal synaptic morphology of the mice.

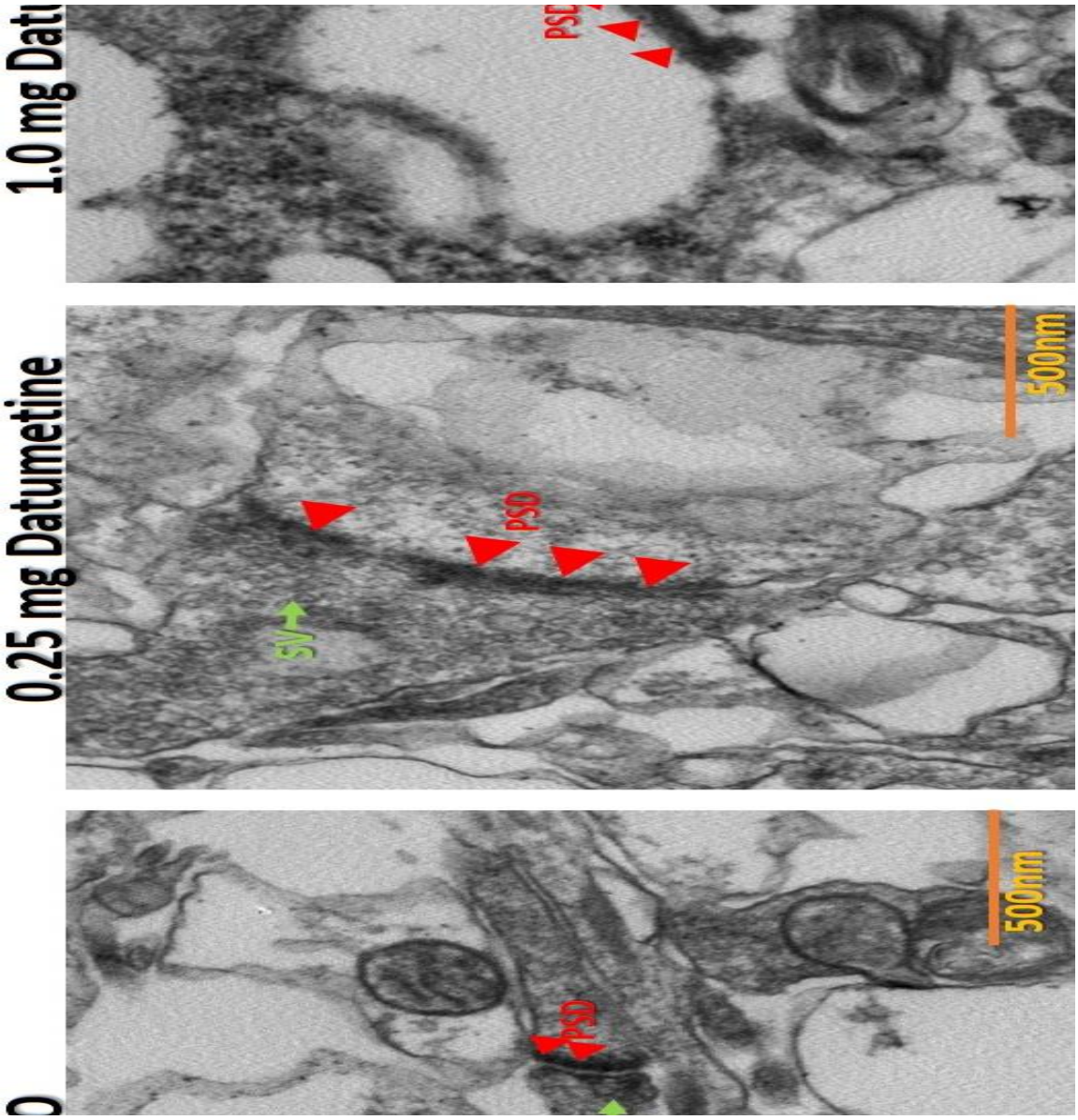


Figure 4.20

Table 4.2: Quantification of postsynaptic density and synaptic vesicles.

Synaptic Profile	**DMSO**	**0.25 mg Datumetine**	**1.0 mg Datumetine**
PSD (nm)	37.86 ± 2.23	57.49 ± 4.16*	101.6 ± 1.38*

SV	44.3 ± 6.18	22.57 ± 3.19*	9.00 ± 6.00*

n = 20 sections per group *p<0.05 one-way ANOVA with Tukey post-hoc test

4.10 Datumetine Prolonged Firing Activity of Bursty Hippocampal Neurons

To investigate the effect of datumetine on NMDAR electrical activity, datumetine was infused to anaesthetized animals with an implanted electrode. Infusion of 0.1 mg/kg datumetine prolong the firing activity of the hippocampal neurons (see figure 4.21). Before the infusion of the drug, the

bursts activity occurred in a few milliseconds, but after the datumetine was infused to the animals the burst activities increased to a few seconds. The interspike interval histogram (ISIH) for single units was used to classify the burst firing into bursty, tonic and irregular based on the shape of their waveforms and firing properties (figure 4.22). Burst firing is the characteristics of hippocampal glutamate neurons. These were then analysed to get the burst properties.

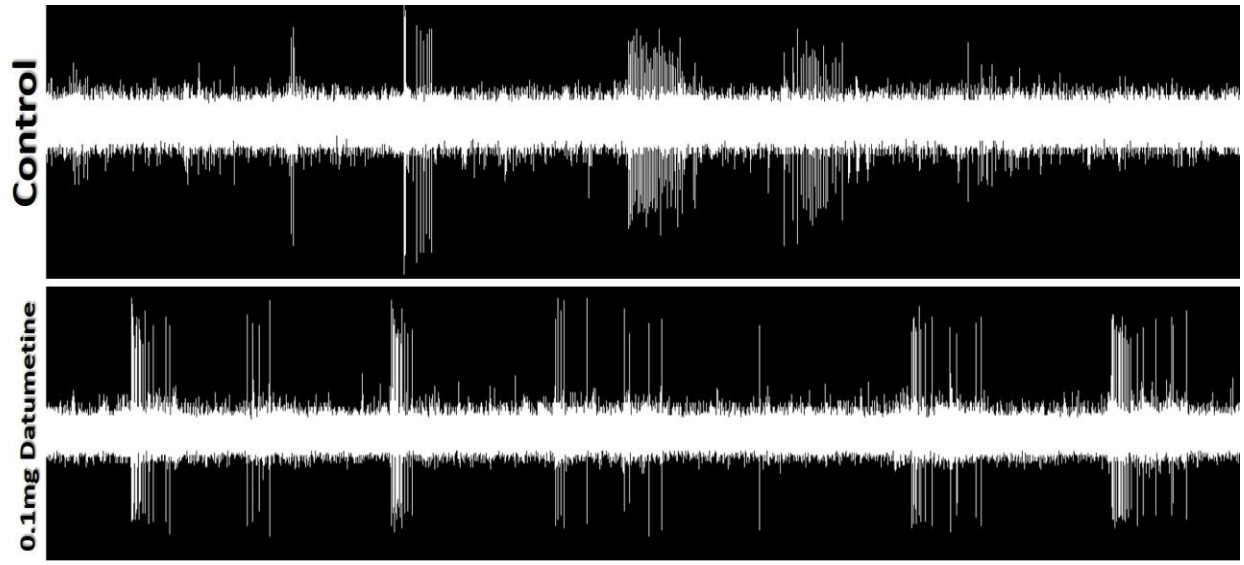


Figure 4.21: Representative diagram showing the spike train of experimental animals during recording. Both groups of animals showed burst activity with different characteristics. Datumetine increased the rate of burst activity in the hippocampus.

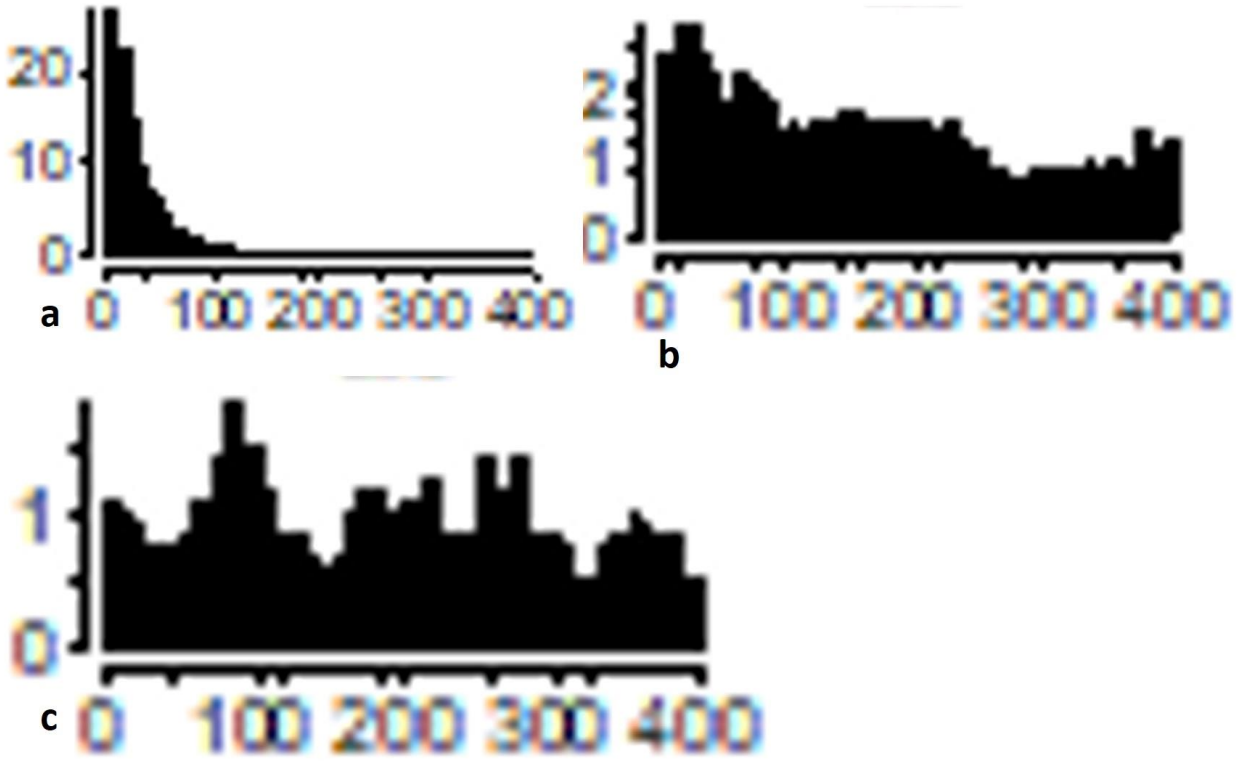


Figure 4.22: Representative inter-spike histogram of experimental animals used to divide the group of neurons. (a) bursts firing; (b) tonic firing; and (c) irregular firing.

4.11 Datumetine Alters Spikes Properties of Bursts Neurons

Spontaneous evoked neural spikes in the CA1-DG field were analysed to determine the impact of datumetine on NMDAR electric current. The time interval between spikes (ISI) which represents neural refractoriness was significantly (*p<0.05) increased in animals infused with 0.1 mg/kg datumetine compared to control (figure 4.23). The degree of distribution of spikes was measured using C_V2 analysis. Values less than one showed increase regularity in firing while values greater than one indicates irregular firing activity. Bursts neurons from the experimental animals showed that the spikes were of Poisson process in datumetine as they have values greater than one while that of control is less than one (figure 4.24). Statistical analysis showed that datumetine significantly (*p<0.05) increases the irregularity of spikes distribution compared to the control.

Individual action potential activity in the representation of spikes was significantly reduced in datumetine infused animals (*p<0.05) compared to control (figure 4.25). The number of spikes in a second termed as the frequency of spikes was significantly reduced (**p<0.01) compared to control (figure 4.26) indicating spike adaptation.

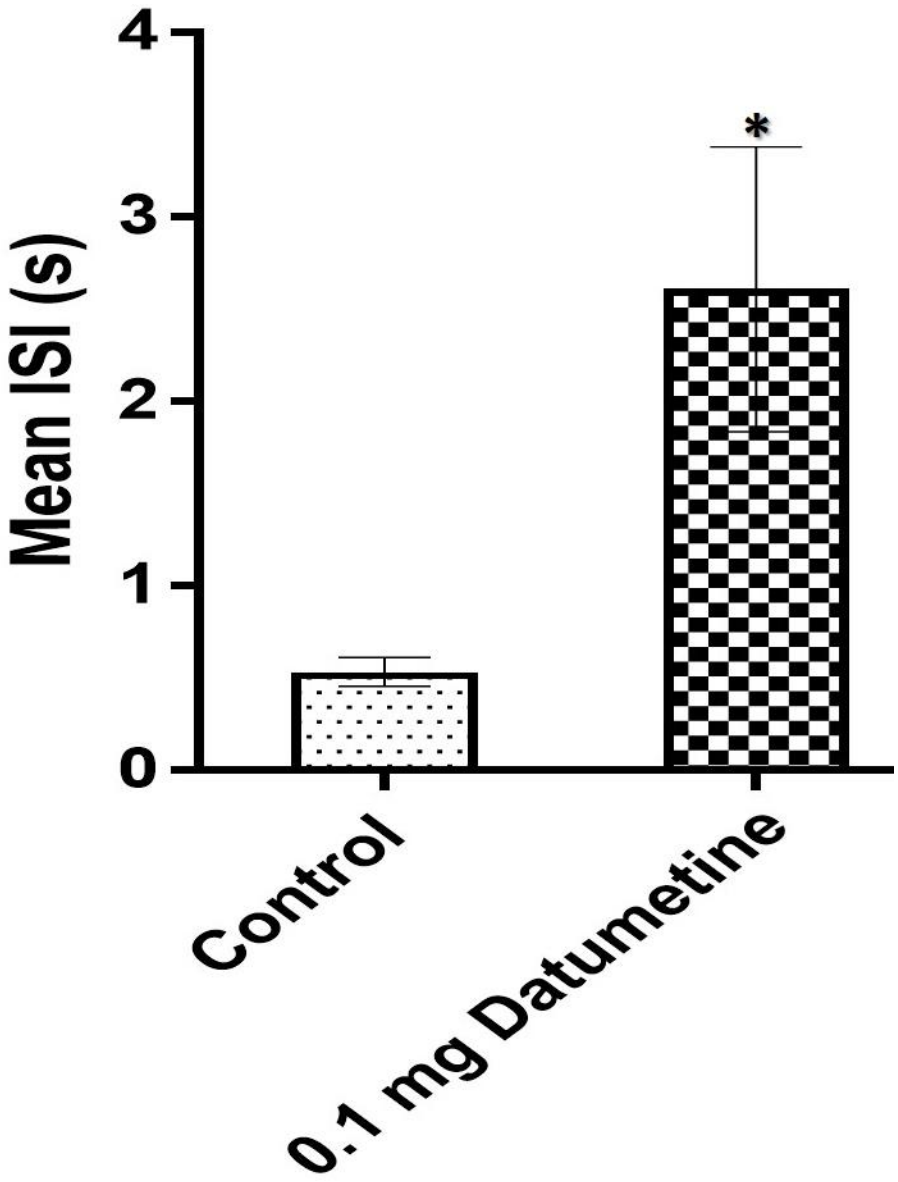


Figure 4.23: Bar chart showing the interspike interval of bursts neurons in the hippocampus. Animals infused with datumetine showed significant increase in ISI compared to control (*p<0.05)

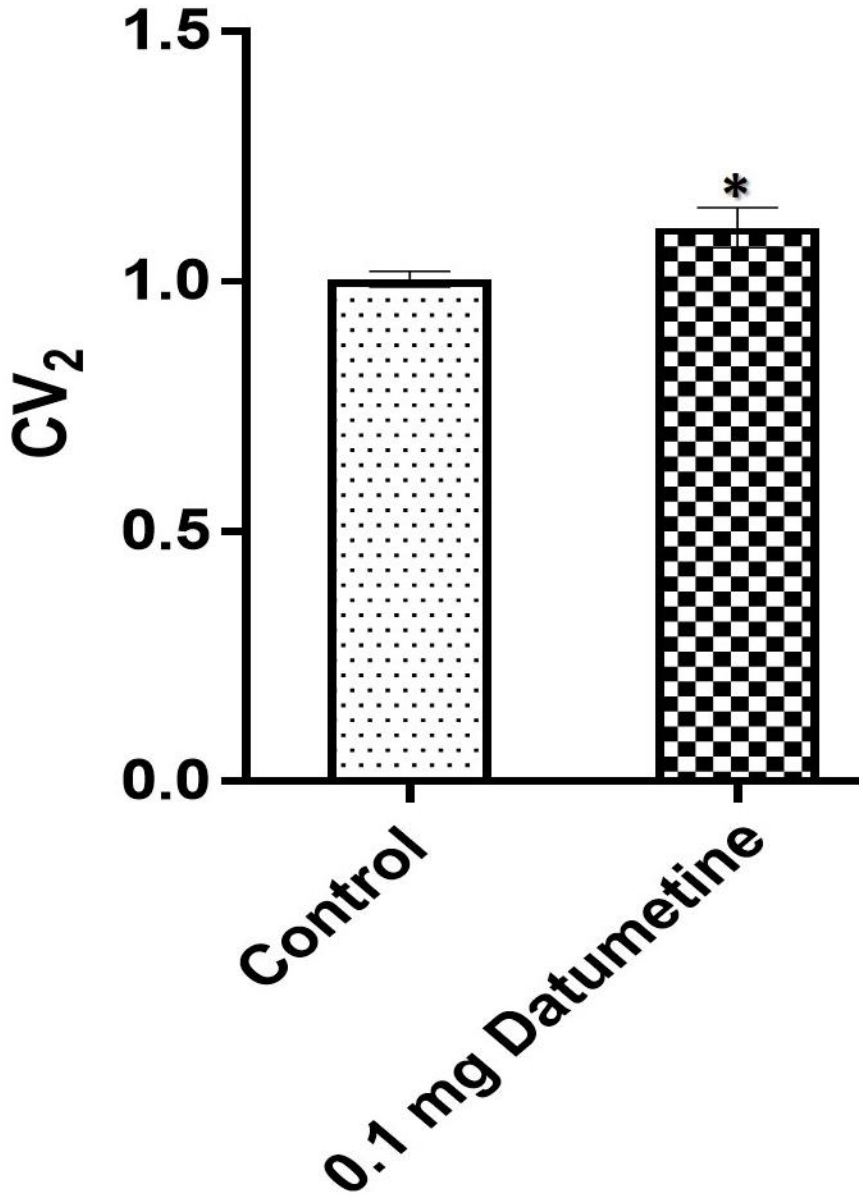


Figure 4.24: Graph showing the $C_V2$analysis of the bursty neurons in the hippocampus. Datumetine significantly increased the $C_V2$analysis compared to control (*p<0.05).

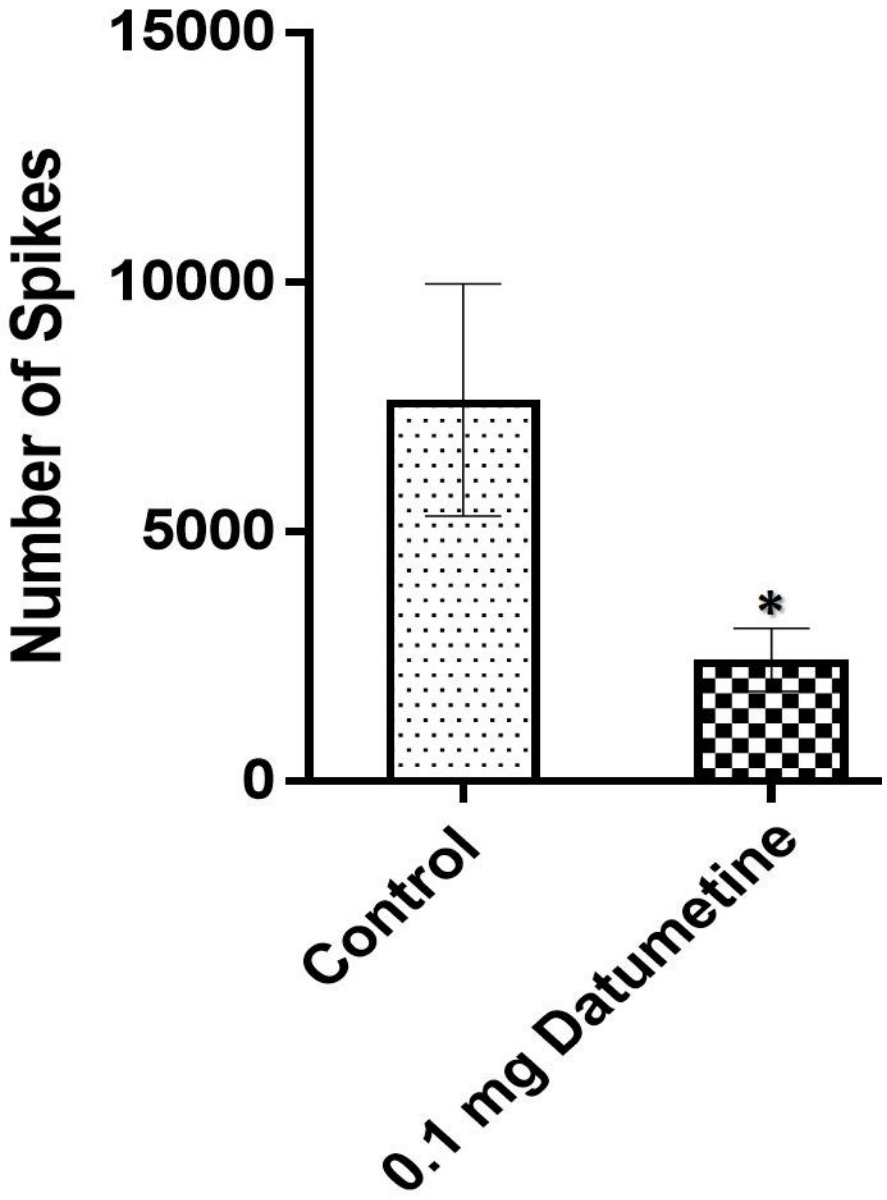


Figure 4.25: Graph showing the number of spikes in bursts of hippocampal neurons during the record session. Datumetine significantly reduced the number of spikes (*p<0.05).

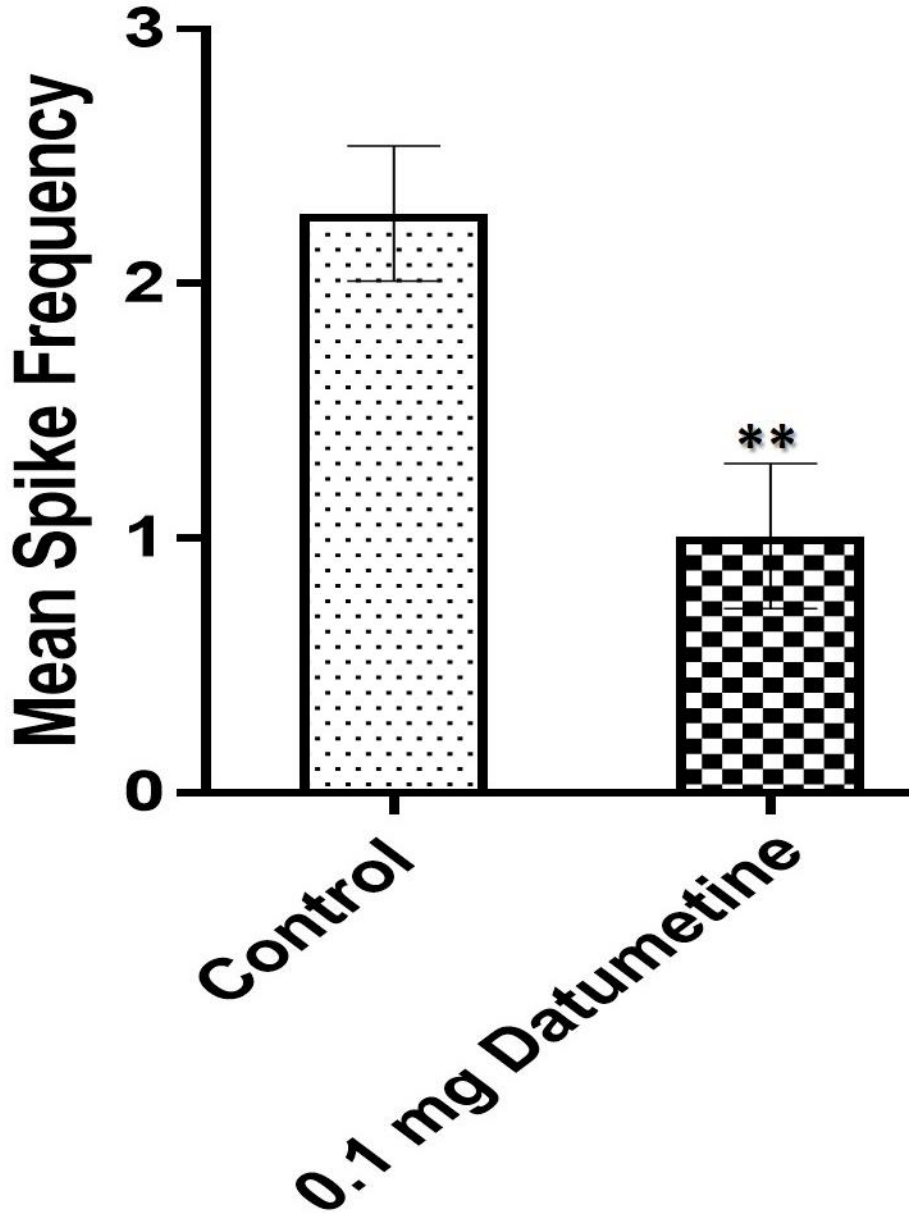


Figure 4.26: Graph of spikes frequency of bursts neurons in the hippocampus of experimental animals. Datumetine significantly reduced the frequency of spikes in the burst's neurons (**p<0.001).

4.12 Datumetine Increased Burst Activities of Bursts Neurons

Burst is defined as repeated action potential with a 0.5 ms interval. The number of bursts was found to increase in animals infused with datumetine than the control (figure 4.27) during the recording session. This increase was not statistically significant. The number of bursts activity per minute was significantly reduced (*p<0.05) in animals infused with 0.1 mg/kg datumetine (figure 4.28) than control.

The average time interval between each burst termed as the mean inter-burst interval (IBI) was found to be significantly (*p<0.05) increased in datumetine infused animals than the control (figure 4.29). Burst duration was increased in datumetine infused animals than the control (figure 4.30). No statistical significance was observed in the duration of the burst.

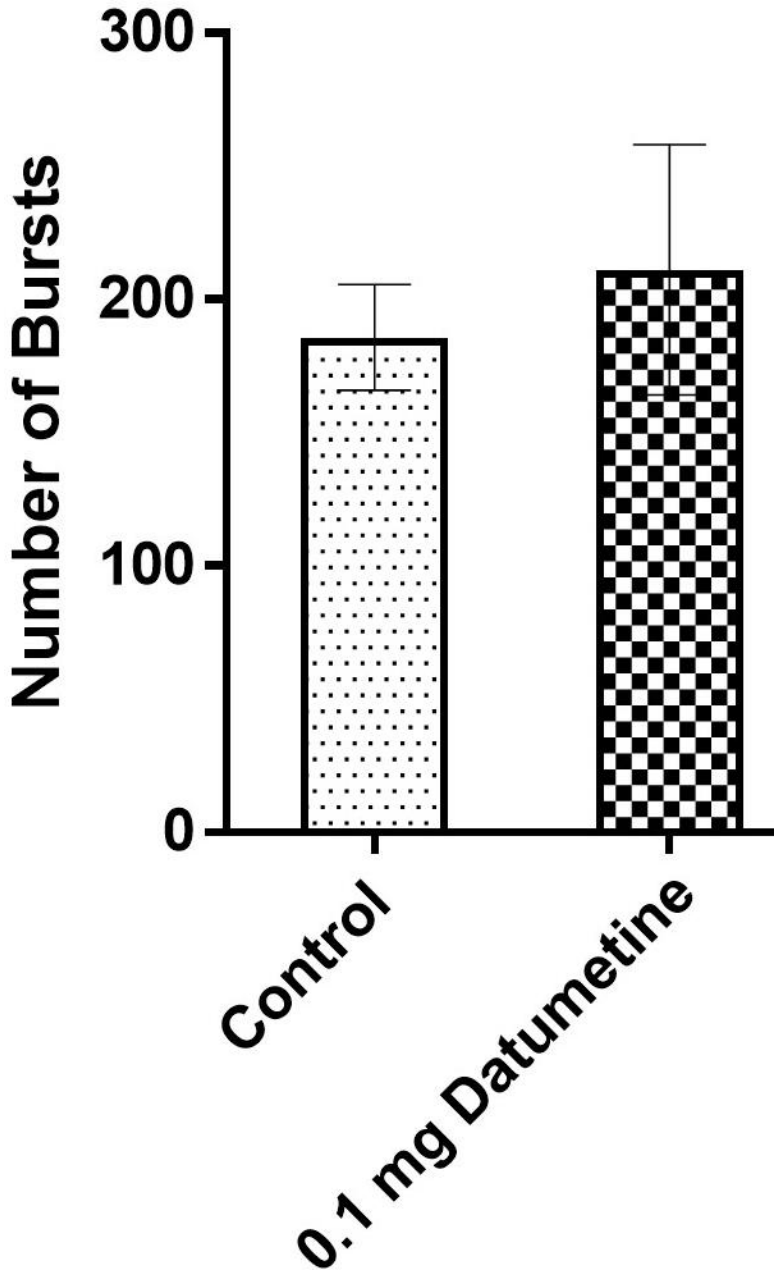


Figure 4.27: Graph showing the total number of bursts during the record session. Datumetine increased the number of bursts than the control but not statistically significant.

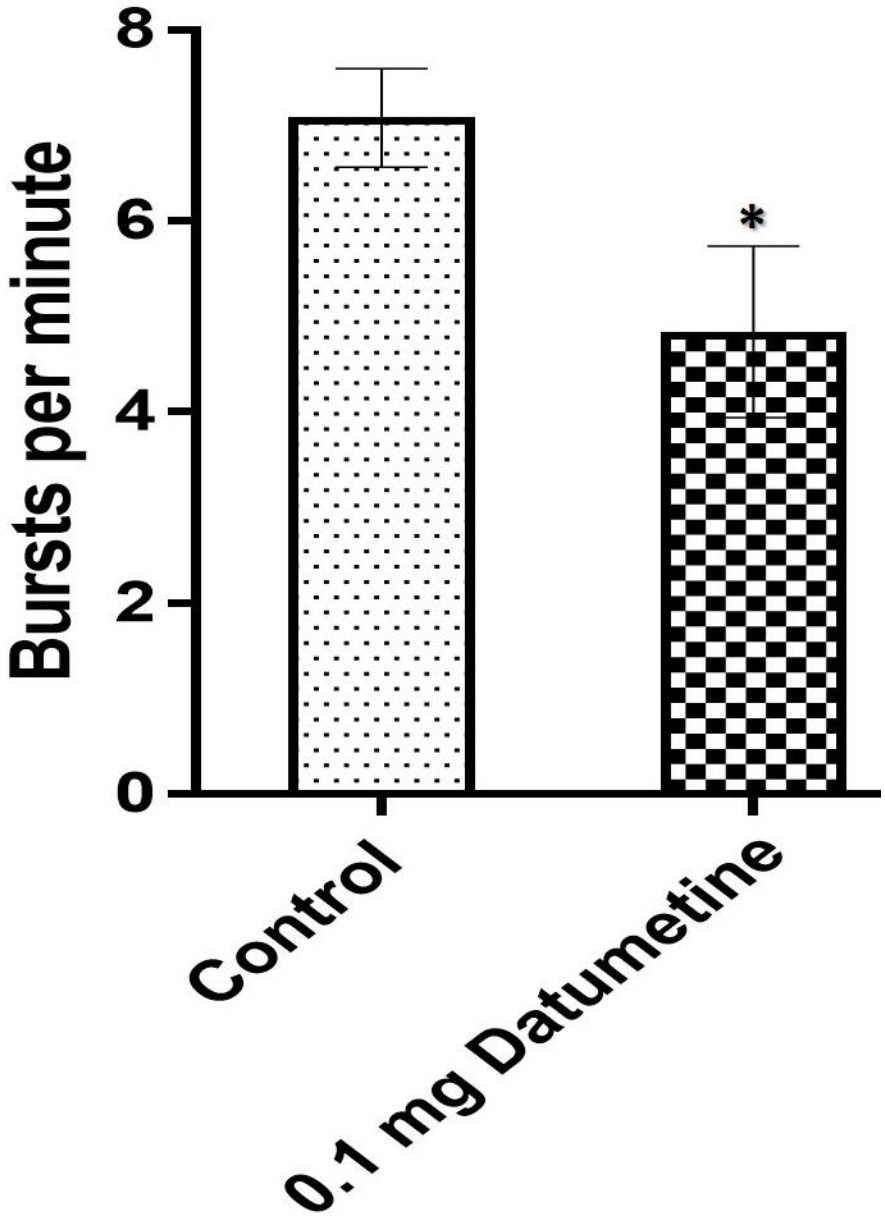


Figure 4.28: Graph showing the rate of bursts. Datumetine significantly (*p<0.05) increased the rate of bursts per minute than the control.

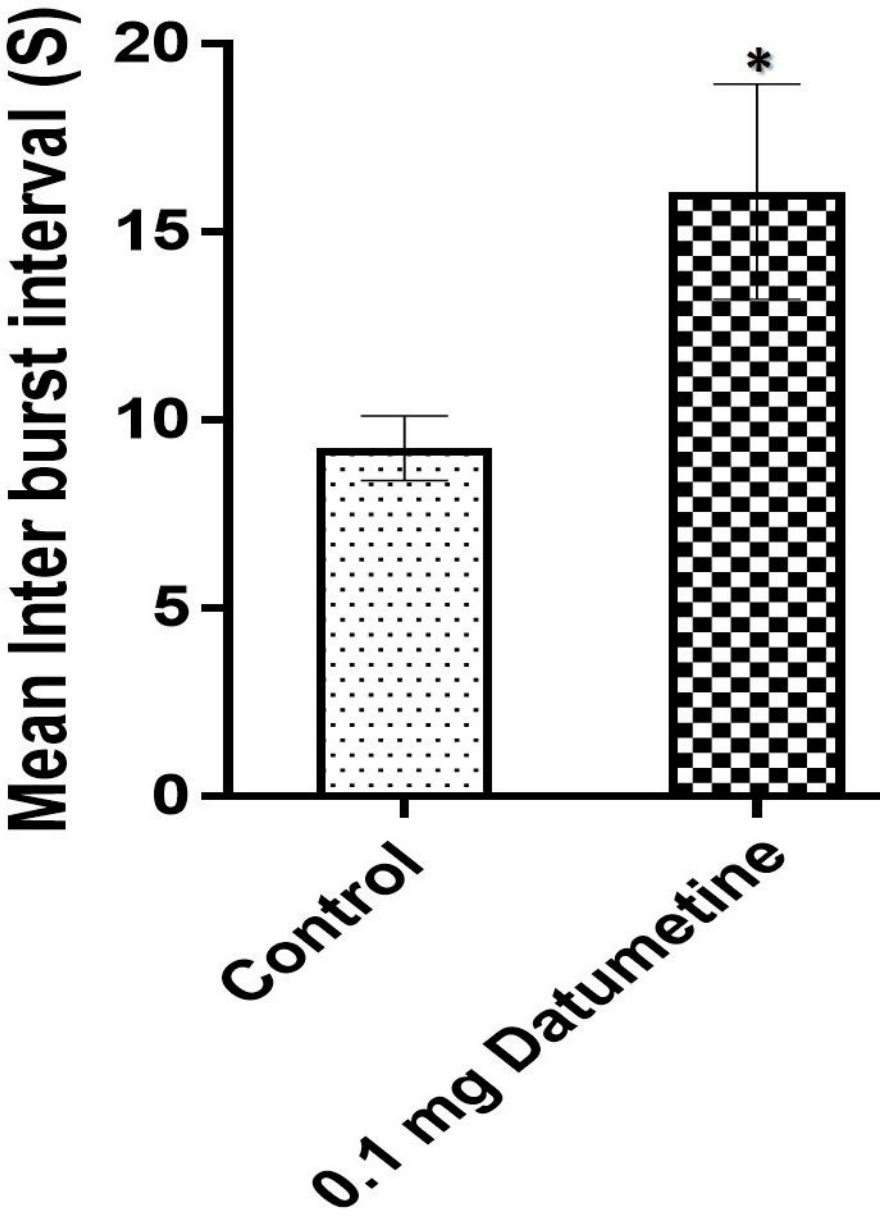


Figure 4.29: Graph showing the mean duration between bursts (interburst interval). Datumetine showed a significant increase compared to control (*p<0.05).

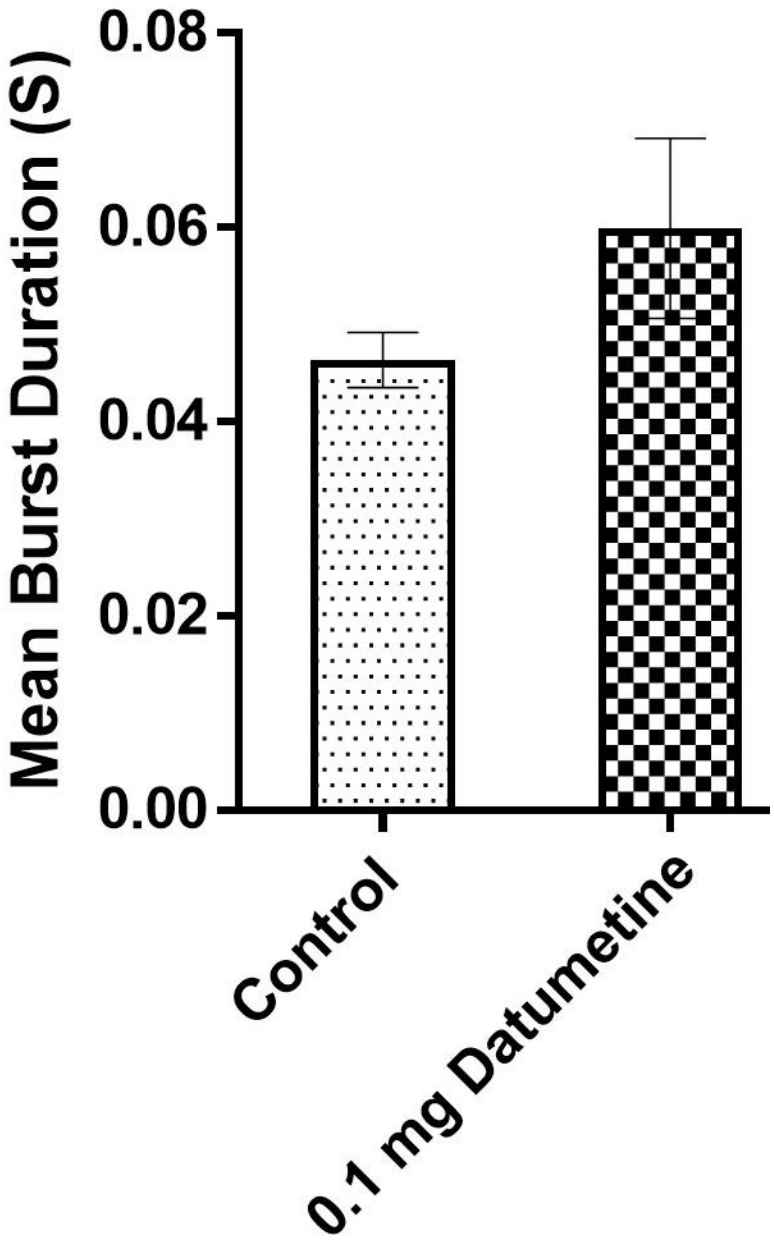


Figure 4.30: Graph showing the average burst duration of experimental animals. Datumetine prolonged burst duration though not significant to control.

CHAPTER FIVE

DISCUSSION

5.0 Introduction

Datura plant abuse is becoming popular among the youth's population in Nigeria (Moses, 2010). The effects of datura plant are usually attributed to its anticholinergic activity (Soni*et al.,* 2012) and its prolonged use has been reported to induce dementia (Khanra*et al.,* 2015). Anticholinergic properties of datura have been attributed to tropane alkaloids present in the plant (Yussuf, 1991; Donatus and Ephraim, 2009).

Recent reports have shown other effects of the plant on biological systems which are not due to cholinergic effects. Gupta *et al.* (2010) reported the anti-inflammatory effects of the plant which is like diclofenac sodium. The anti-bacterial effect of the plant has also been reported where it was shown to be potent against gram-positive bacteria cells (Takhi and Ouinten, 2011) and germicidal effects (Kurnal and Yalcin, 2009). Effects of the plant againstviruses have also been documented (Sharma *et al.,* 2009) as well as its wound healing potentials (Shanmuga*et al.,* 2002). Analgesic (Khalili *et al.,* 2004), antioxidant (Sreenivasa*et al.,* 2012) sedative (MalamiandAlhassan, 2014), and hypoglycaemic (Murthy *et al.,* 2004) effect has also been reported.

Most other reports on datura plants showing other effects apart from cholinergic effects have attributed it to other phytochemicals like flavonoids present in the plant extract (Malami and Alhassan, 2014; Babalola *et al.,* 2015) while others just allude to the fact of the plant affecting

but unable to identify the compound responsible (Murthy *et al.,* 2004; Al-Snafi, 2017), indicating that more needs to be explored about the plant.

With the advent of ligand docking technology, phytochemicals present in the plant were able to be simulated against receptor of interest (NMDAR) to show which of the compound is partly responsible for memory loss seen in datura abusers and the possible mechanisms.

5.1 Datumetine Binds With NMDAR

Datura plant has been reported to possess other compounds apart from the major alkaloid that has been previously identified (Khaleque*et al.,* 1974; Manickam *et al.,* 1993; Pan *et al.,* 2007; Yang *et al.,* 2007; Kuang*et al.,* 2008; Kiruthika and Sonaraj, 2011; Yang *et al.,* 2014a, b; Han *et al.,* 2015). Recent studies have reported other biological effects of the plant which has not been attributed to any specific compound in the plant (Murthy *et al.,* 2004; Al-Snafi, 2017). Since recreational users of the plant don't remember anything during the intoxication process, it was hypothesized that the plant may contain compounds that affect hippocampal functioning.

Docking has been a veritable tool in screening a large library of compounds against the protein of interest to show their interaction (Kitchen *et al.,* 2004; Gupta *et al.,* 2018). Docking result revealed that datumetine forms a best binding fit to NMDAR, a major ionic receptor in hippocampal functioning (Lambert and Jones, 1989; 1990; Tóth, 2010). Indicating datura not only have anticholinergic properties (Yussuf, 1991; Donatus and Ephraim, 2009) but can mediate NMDAR activities. Datumetine was simulated against other ionic receptors in the brain and it showed no binding fits with others, indicating that it is specific for NMDA receptor only.

Tang and Chen (2015) have used this method of docking to screen different compounds present in some traditional medicine for tyrosinase inhibitors. They found that compound with best binding fits was able to modulate tyrosinase activity showing that ligand docking is a veritable tool in screening compounds for biological activities. In their study, datumetine was part of the compound simulated and it was found to show very weak interaction with tyrosinase enzyme (Tang and Chen, 2015). Although docking has been promising in the field of science to screen phytochemicals, docking result does not show the biological implication of the binding/interaction (Chen, 2015). Hence, leading to our further research to show the biological implication of datumetine-NMDAR interaction.

5.2 Datumetine Effects on Short-Term Working Memory

To ascertain the biological effect of datumetine-NMDAR interaction, datumetine was administered to C57BL/6 mice. It was observed that datumetine induces a seizure in the animals, which suggest positive enhancement of NMDAR activity (Hackos and Hansen, 2017). This indicated that datumetine can act as NMDAR agonist since it can bind at the orthostatic binding site of NMDAR as revealed from the docking results. Seizure usually arises as a result of hyperactivity of excitatory neurotransmission or reduced activity of inhibitory neurotransmission (Ghasemi and Schacter, 2011). Seizure activity observed in these animals is more of enhanced NMDAR activity rather than reduced inhibition as datumetine will not bind to GABA receptors. Seizure activity is well correlated with the agonistic effect of NMDAR activity as reported by Rodriguez-Munoz *et al.* (2018). The authors showed that injection of NMDAR agonist in the ventricles of rodents induces seizure (Rodriguez-Munoz *et al.* 2018). Seizures experienced in this experiment is like reports obtained when synthetic NMDA is administered to animals evident by reports of Moreau *et al.* (1989) and Mathis and Ungerer (1992). Activation of NMDAR is also

implicated in Rett syndrome (Balakrishnan and Mronov, 2018a), which is characterised by seizure activity (Balakrishnan and Mronov, 2018b,c).

NMDAR Positive allosteric Modulators (PAMs) which are compounds that indirectly enhance the activity of NMDAR have been identified (Hackos and Hanson, 2017). These set of compounds are proposed not to possess overexcitation potentials that are inherent of agonist (Hackos and Hanson, 2017). Although docking results showed that datumetine can also bind to NMDAR at the allosteric binding site, seizures are not associated with allosteric modulation of NMDAR (Zhu and Paoletti, 2015). Different classes of PAMs have been identified with a different mode of action since PAMs will not enhance receptor activity without the presence of agonist/ ligand (Conn *et al.,* 2009). The seizure observed in this study is different from what has been reported about PAMs. Hemelikova*et al.* (2019) reported that lectins increase the sensitisation of NMDAR to synthetic NMDA but they did not observe any seizure or adverse effect in the animals. Tacrine, another compound that indirectly modulates NMDAR activity does not induce any seizure activity in rodents (Horak*et al.,* 2017).

To test the memory deficit hypothesis, datumetine exposed animals were assessed on memory paradigm using NOR and Y-maze after long term exposure. The hippocampal-dependent task of Y-maze was significantly impaired in animals exposed to a high dose of datumetine. NOR result indicates memory decline in all animals exposed to datumetine. Memory decline has been attributed to datura use (Olawepo*et al.,* 2017) although some authors attributed this effect to the anticholinergic properties of the plant (Abena*et al.,* 2004; Babalola *et al.,* 2013; Deepa *et al.,* 2014; Babalola *et al.,* 2015; Etibor*et al.,* 2015). The results showed that not only the tropane alkaloids are responsible for the memory deficit seen in datura intoxication but datumetine also plays a major role. NMDAR agonists are reported to enhance long term potentiation (LTP)

(Collingridge*et al.,* 2010; Huganir and Nicoll, 2013) leading to enhance memory (Zito and Scheuss, 2009). Unlike what has been reported about NMDAR agonist, this observation showed impaired memory formation. Administration of $_{D}$-cycloserine, a partial NMDAR agonist was able to reverse memory deficit observed after chronic NMDAR inhibition (Vishnoi*et al.,* 2015), this disparity may be based on the mechanism of action of the drug as $_{D}$-cycloserine acts by binding to glycine binding sites without excitotoxicity (Ressler *et al.,* 2004; Ledgerwood*et al.,* 2005), unlike datumetine that may replace glutamate at its binding site. Another possibility for the disparity may be the dose exposure and that in their work NMDAR was previously blocked. Memory loss is well associated with neurotoxicity (Papouin*et al.,* 2012). Memory impairment due to NMDAR excitotoxicity has been reported (Canu*et al.,* 2014). Overactivation of NMDAR is also associated with the induction of long-term depression (LTD) (Szydlowska and Tymianski, 2010) which has been implicated in memory deficit (Manahan-Vaughan *et al.,* 2008). Unlike some reports of improved memory due to NMDAR agonist exposure (Vishnoi*et al.,* 2015), datumetine induced memory deficit may be due to hyperactivation of NMDAR leading to excitotoxicity.

5.3 Datumetine Alters NMDAR Signalling

NMDAR activation allows for Ca^{2+} influx to mediate diverse intracellular signalling (Traynelis*et al.,* 2010). Since datumetine is suspected to enhance NMDAR activity, signalling pathway of NMDAR is expected to be favoured. Long term exposure to datumetine increased the expression of GluN1. This increase was attributed to response in altered excitatory neurotransmission (Gan *et al.,* 2013). Increase GluN1 expression has been reported to be due to increasing NMDAR activity (Gan *et al.,* 2013) which is consistent with the previous observation of seizure earlier reported. This observation is like what is observed in chronic alcohol exposure (Trevisan*et al.,*

1994; Roberto and Siggins,2006). Although alcohol is reported to enhance inhibitory transmission in the brain (Goodwani*et al.,* 2017), chronic alcohol exposure is showed to enhance NMDAR activity (Chen *et al.,* 2011; Alasmari*et al.,* 2018). Similarly, neuropsychiatry disorders that are characterised with hyperactivity of NMDAR (e.g. stroke, ischaemia, Rett syndrome and mood disorders) revealed increased expression of NMDAR in post mortem brain samples (Hashimoto *et al.,* 2007; Lan *et al.,* 2009; Sanacora*et al.,* 2012) and preclinical studies have indicated that NMDAR antagonist alleviates the symptoms (Trullas and Skolnick, 1990; Paul and Skolnick, 2003) alluding to the fact that hyperactivation of NMDAR is part of the aetiology of these disorders. This observation is contrary to the report ofCristino*et al.* (2015), which showed that indirectly increasing NMDAR activity by knocking out gene coding for $_{D}$-aspartate oxidase reduces GluN1 expression in the hippocampus with increased GluN1/AMPAR ratio. This disparity may be in part due to the overall altered glutamate homeostasis observed in their study.

CamKII-α is implicated in NMDAR signal and memory encoding (Coultrap and Bayer, 2012). It is a holoenzyme that is autophosphorylated by NMDAR activation (Malenka and Bear, 2004) and is reported to be responsible for the bidirectional effects of NMDAR signalling (Barlett and Wang, 2013). Datumetine significantly increased the expression of CamKII-α which is consistent with GluN1 increased expression. Chronic expression of CamKII-α is neurotoxic (Barlett and Wang, 2013). Reports from psychosocial stress research which is due to hyperfunction of NMDAR (Fontella *et al.,* 2004) showed reduced basal CamKII and phosphorylated form (Gerges *et al.,* 2004) this was attributed to dysregulation of NMDAR activity and modification of downstream molecules (Vasquez *et al.,* 2014). Increased NMDAR activity was also implicated in opioid-induced hyperalgesia (OIH) (Lee *et al.,* 2011), which was also associated with

increased Cam\KII activity as reported by Qi *et al.* (2019) that blocking CamKII activity reverses OIH in rodents. Increased CamKII expression due to enhanced NMDAR activity is also reported in chronic alcohol consumption (Renteria *et al.,* 2017) and is well correlated to alcohol-drinking behaviours (Easton *et al.,* 2013).

Although,CamKII-α is tightly regulated by autophosphorylation upon NMDAR activation through Ca^{2+} (Gardoni*et al.,* 1999). Level of pCamKII-α-T286 was reduced in animals exposed to datumetine. CamKII-α is associated with GluN2A subunit of NMDAR (Gardoni*et al.,* 2001) upon NMDAR activation influx of Ca^{2+} ions lead to its phosphorylation at Thr286 (Coultrap and Bayer, 2012; Lisman *et al.,* 2014; Coultrap*et al.,* 2014). This phosphorylated state is independent of Ca^{2+} (Kennedy, 2010; Coultrap*et al.,* 2014) and is associated more to GluN2B (another subunit of NMDAR) (Barria and Malinow, 2005). With increased activity of NMDAR, it will be expected that CamKIIα will be autophosphorylated at Threonine286 which will further affect NMDAR signalling independent of Ca^{2+} influx (Miller and Kennedy, 1986). This result showed reduced expression of pCamKIIα-T286 familiar to what was reported in chronic stress state where dysregulation of NMDAR-CamKII signalling was implicated (Gerges *et al.,* 2004; Calabrese *et al.,* 2012). One possible mechanism for this scenario is the activation of calcineurin, another calcium buffer protein which is reported to have more affinity for Ca^{2+} than CamKIIα (Li *et al.,* 2012; Rumi-Masante*et al.,* 2012). Activated calcineurin activates protein phosphatase-1 (PP1) to desensitise phosphorylated CamKIIα (Bradshaw *et al.,* 2003). In a model of chronic pain reported by Qi *et al.* (2019) remifentanil-induced postoperative hyperalgesia (RIPH) enhances NMDAR activity elevating the level of pCamKIIα-T286. Although the authors found no changes in the level of basal CamKIIα, the disparity in the observation may be due to method of evaluating the level of expression as the authors normalise the level of pCamKIIα-T286

immunoblot with that of CamKIIα immunoblot, unlike this study where all immunoblots were normalised with actin. Reduced expression of pCasmKIIα-T286 has been reported in compounds that indirectly modulate NMDAR activity through serotonin and opioid pathways (Rodriguez-Munoz *et al.,* 2018). This, the author suggested was necessary to reduce the overactivation of NMDAR. Increased expression of pCamKIIα-T286 recorded with increased NMDAR is of short term and well correlated to LTP induction (Malenka and Bear, 2004;Buard*et al.,* 2010;Ashpole and Hudmon, 2011; Barlett and Wang, 2013). This study is of prolonging datumetine exposure which overstimulates NMDAR for a long time. This overactivation may have stimulated the activity of calcineurin seen in chronic stress (Gerges *et al.,* 2004; Calabrese *et al.,* 2012) thereby shutting off the NMDAR/CamKIIα signalling pathway.

Activation of NMDAR downstream signalling pathway is the activation of CREB (Papadia*et al.,* 2005; 2008;Leveille*et al.,* 2008; 2010;Hardingham and Bading, 2010;Zhang *et al.,* 2011). CREB is also activated by way of phosphorylation by pCamKIIα-T286 (Deisseroth*et al.,* 1998). Consistent with the observation in the expression of pCamKIIα-T286, datumetine reduced the expression of CREB. Activation states of NMDAR have been reported to regulate CREB activity (Sun *et al.,* 2018). Moderate activation of NMDAR increased CREB activity while overactivation reduced it (Valera *et al.,* 2008). Different subunit of NMDAR has also been reported to differentially regulate the activity of CREB (Sun *et al.,* 2018). GluN2A activation enhances CREB activity (Hardingham *et al.,* 2002; Terasaki*et al.,* 2010; Sasaki *et al.,* 2011; Zhou *et al.,* 2013) while GluN2B transiently phosphorylates CREB and prolong activation reduces CREB activity (Hardingham *et al.,* 2002; Valera *et al.,* 2008; Luo *et al.,* 2011). Reduction in CREB expression observed is also related to what was reported in the state of both

synaptic and extrasynaptic NMDAR activation (Zhou *et al.,* 2013). Consistent with earlier results reduced CREB activity is implicated in state of NMDAR overactivation (Zhou *et al.,* 2013).

In normal NMDAR signalling, activation of CREB translocates to the nucleus to increase the expression of some genes responsible for synaptic plasticity and cell survival (Deisseroth*et al.,* 1998; Tao *et al.,* 2002; Chen *et al.,* 2003; Martinowich*et al.,* 2003; Tian *et al.,* 2010). One of the pro-survivals and synaptic plasticity gene code for BDNF (Zhou *et al.,* 2013). Consistent with the previous signalling pathway molecule, datumetine reduced BDNF expression in exposed animals compared to control. NMDAR subunit differentially regulates BDNF activity (Sun *et al.,* 2018). Chen *et al.* (2007) reported that GluN2A upregulate BDNF activity in moderate and overactivation state whereas GluN2B upregulate BDNF activity in moderate activation state and downregulate BDNF activity in overactivation state (Zhou *et al.,* 2013). Most significant reduction of BDNF is seen in 1.0 Datumetine animals, this suggests overactivation of NMDAR by datumetine correlating with the conclusion by Zhou *et al.* (2013) that activation of both synaptic and extrasynaptic NMDAR regulates BDNF expression differentially based on activation state. Impaired BDNF has been implicated as part of the pathophysiology of stress (Vasquez *et al.,* 2014).In a study of acute restraint stress, mRNA of BDNF was reduced in the hippocampus (Murakami *et al.,* 2005) and chronic restraint stress reduced BDNF protein expression (Xu *et al.,* 2004). As earlier pointed out that chronic stress enhances NMDAR activity with altered signalling, our results mimic what is observed in a chronic state of stress as reported by Li *et al.* (2013). Although, increased BDNF expression has also been reported in some chronic stress model when applied for 10 days (Nair *et al.,* 2007) and mild stress at two weeks (Xiao *et al.,* 2011) while Gronli*et al.* (2006) and Allaman*et al.* (2008) observed no changes. Most reports that recorded low expression of BDNF in chronic stress estimate total hippocampal

BDNF (Vasquez *et al.,* 2014) like in this study as well. Unlike studies that estimate BDNF expression at different regions of the hippocampus which reported different pattern of expression in each region (Gronli*et al.,* 2006; Park *et al.,* 2006).

BDNF has also been implicated in excitotoxicity observed in hypoxia (Das *et al.,* 2018). Exogenous supplements of BDNF protects neurons against hypoxia-induced death (Kume*et al.,* 1997; Jain *et al.,* 2013). Like this observation, exposure to hypoxia reduced BDNF expression and increase the expression of NMDAR (Das *et al.,* 2018). The authors attributed the reduced expression of BDNF observed to be due to increasing the activity of GluN2B subunit of NMDAR as they have earlier reported increased activity of NMDAR in hypoxic condition (Hota*et al.,* 2008a,b). Although the specific subunit of datumetine binding to NMDAR is not known, whether it requires the presence of glutamate at the synapse for its activity. One possible explanation for the effect of datumetine seen maybe that datumetine compete with glutamate for binding at the synapse leading to lateral diffusion of either glutamate or datumetine to activates extrasynaptic NMDAR. Extrasynaptic NMDAR activation has been associated with NMDAR induced cell death (Luo *et al.,* 2011).

5.4 Datumetine Alters Hippocampal Morphology

Using immunofluorescence technique, the effect of long term datumetine exposure was assessed in the hippocampus of the animals. Increased activity of glial cells (both astrocyte and microglia) was observed in animals exposed to datumetine. Increased activity of these glial cells has been attributed to injury to the brain (Pekny *et al*., 1999; Sofroniew and Vinters, 2010; Gorina *et al*., 2011; Zamanian *et al*., 2012; Paintlia *et al*., 2013), neuroinflammation (Takeuchi, 2013; Colombo and Farina, 2016; Popichak*et al.,* 2018), synaptic pruning (Paolicelli*et al.,* 2011;

Li *et al.,* 2012) and excitotoxicity (Matute*et al.*, 2002; Rossi *et al.,* 2004; Kauppinen and Swanson, 2007). Previous reports have shown that moderate activation of NMDAR is essential for cell survival, synaptic plasticity and LTP necessary for learning and memory (Bliss and Collingridge, 1993, Malenka and Nicoll, 1999; Martin *et al.,* 2000; Lu *et al*., 2001; Park *et al.*, 2004; Collingridge*et al.,* 2010; Traynelis*et al.*, 2010; Ahmad *et al.,* 2012; Papouin*et al*., 2012; Iacobucci and Popescu, 2017) whereas overactivation of NMDAR has also been implicated in cell death (Rothman and Olney, 1986; Choi *et al.,* 1988; Tymianski, 1996; Villmann and Becker, 2007; Szydlowska and Tymianski, 2010; Milnerwood*et al.,* 2010; Xia *et al.,* 2010). Studies on neurodegeneration diseases in rodents have also implicated impaired NMDAR function as part of the pathophysiology of the disease progression (Paoletti*et al.,* 2013). NMDAR hypofunction has been recorded in schizophrenia (Javitt and Zukin, 1991; Krystal *et al.,* 1994; Akbarian*et al.,* 1996; Mohn*et al.,* 1999; Bitanihirwe*et al.,* 2009; Moghaddam and Javitt, 2012; Zhou and Sheng, 2013; Hackos and Hanson, 2017) while NMDAR overactivation were reported in Alzheimer's disease (AD) (Li *et al.,* 2009; 2011; Klyubin*et al.,* 2011), stroke (Bullock *et al.,* 1998; Hackos and Hanson, 2017;), Huntington's disease (Fan *et al.,* 2007; Okamoto *et al.,* 2009; Milnerwood*et al.,* 2010; Puddifoot*et al.,* 2012), pain (Wu and Zhuo, 2009; Niesters and Dahan, 2012) and depression (Berman *et al.,* 2000; Zarate *et al.,* 2006). The major hallmark of all these neurodegenerative diseases is the loss of neurons in a different part of the brain implicating different mechanisms (Biglan*et al.,* 2013; Tabrizi *et al.,* 2013; De Strooper and Karran, 2016).

This study implicates NMDAR hyperfunction as part of the effect of datumetine on NMDAR which predisposes the neurons to undergo neurotoxicity as pointed earlier. The in vivo cellular response to excitotoxicity/traumatic insult to the brain is the activation of glial cells majorly astrocytes (Pekny and Pekna, 2016; Turlejski*et al.,* 2016; van Dijk *et al.,* 2016; Khakh*et al.,*

2017; Shinozaki *et al.,* 2017; Bisht *et al.,* 2018; Chun and Lee, 2018;Lee *et al.,* 2018; Ahmad *et al.,* 2019; Cheng *et al.,* 2019; Subhramanyam*et al.,* 2019). Astrocytes act by changing the metabolism of the neurons initiating their cell death cascades (Simard and Nedergaard, 2004; Pellerin *et al.,* 2007; Rouach*et al.,* 2008; Suzuki *et al.,* 2011; Han *et al.,* 2013) whereas microglia acts by phagocytosing the neural debris (Takahashi *et al.,* 2005; Wake *et al.,* 2009; Hristova*et al.,* 2010; Paolicelli*et al.,* 2011; Parkhurst *et al.,* 2013; Schafer *et al.,* 2013; Squarzoni*et al.,* 2014; Reemst*et al.,* 2016) and also initiating synaptic denervation by engulfing the synapse (Araujo and Cotman, 1992*;* Frade and Barde, 1998; Ueno *et al.,* 2013). Astrocytes also take part in the formation of the synapse (Freeman, 2015; Allen and Eroglu, 2017) and it has been reported that they also express NMDAR on their membrane (Tanaka *et al.,* 1997; Yang *et al.,* 2009). Since datumetine is expected to bind with NMDAR, datumetine may also bind with the receptors on the glia cells to mediate diverse cellular response in the astrocytes (Djukic*et al.,* 2007; Sibille*et al.,* 2014; Tani*et al.,* 2014) which may also affect neural functions.

Increased activity of glia cells is well reported in animal models of neurodegeneration which hyperactive NMDAR has been implicated (Pekny and Pekna, 2016; Turlejski*et al.,* 2016; Khakh*et al.,* 2017; Shinozaki *et al.,* 2017; Liddelow and Barres, 2017; Ni *et al.,* 2018; Arranz and De Strooper, 2019; Cheng *et al.,* 2019; Simon *et al.,* 2019; Subhramanyam*et al.,* 2019). Increase activated glial cells has been well reported in AD. Activated microglia are usually found near amyloid plaque (Perlmutter *et al.,* 1992; Carpenter *et al.,* 1993; Mawuenyega*et al.,* 2010) which further activates the inflammatory response of the glia to produce more plaques (Nagai *et al.,* 2001; Stewart *et al.,* 2010; Kummer*et al.,* 2011). Increased glial activity is also implicated in the progression of AD (Harris *et al.,* 2012; Asai*et al.,* 2015; Maphis*et al.,* 2015). Stroke which is another neurodegenerative condition where NMDAR hyperfunction is implicated recorded

increased glia activity (van Dijk *et al.*, 2016). Using different models of haemorrhagic stroke, increased glia response has been documented in cisterna magna injection (Matz *et al.*, 1996a,b; Turner *et al.*, 1999; Widenka*et al.*, 1999; Nieuwkamp*et al.*, 2009; Zhang *et al.*, 2011; Hanafy, 2013; Plog*et al.*, 2014; Sun *et al.*, 2014; Zhang *et al.*, 2015), endovascular perforation (Greenhalgh *et al.*, 2012a,b; Luo *et al.*, 2010; Kooijman*et al.*, 2014; Uekawa*et al.*, 2014; Schneider *et al.*, 2015; Zhang *et al.*, 2015a;b), subarachnoid vein transection (Smithason*et al.*, 2012), unilateral blood injection (Simard *et al.*, 2012; Kwon *et al.*, 2015).All reports point to the fact that increased glia response further increase disease burden. Using the model of ischemic stroke by exposing neurons to hypoxia. Turlejski*et al.* (2016) also showed that there is increased glial activity in animals exposed to hypoxia. Similarly, in rodent spinal cord injury which is a model used for neuropathic pain research (Ishola *et al.*, 2019) increased activity of glial cells was recorded andsuggested to be responsible for the migration of macrophages to the site of injury (Cheng *et al.*, 2019). Dysfunction of glia cells is also documented in Huntington's disease (Khakh*et al.*, 2017). Accumulation of mutant huntingtin protein (mHTT) is shown to be present in glia cells (Shin *et al.*, 2005). Glia cells were reported to increase by two-fold during the progression of the disease (Hedreen and Fostein, 1995; Faideau*et al.*, 2010) and implicated in the progression of the disorder (Bradford *et al.*, 2010; Faideau*et al.*, 2010; Parsons *et al.*, 2016).

Different mechanism has been implicated to be responsible for the increased glia activity observed in neurodegenerative diseases (Pike *et al.*, 1994; Le *et al.*, 2001; Wyss-Coray*et al.*, 2003; Allaman*et al.*, 2010; Stewart *et al.*, 2010; Carty and Bowie,2011; Colangelo *et al.*, 2014; Ben Haim *et al.*, 2015; Peng *et al.*, 2015; Yu and Richard, 2015; Cai *et al.*, 2016; Shinozaki *et al.*, 2017). Since glial cells possess neurotransmitter transporter (Tanaka *et al.*, 1997; Yang *et al.*, 2009; Gorshkov*et al.*, 2018) and they can also release some compounds referred to as

gliotransmitters (Araque*et al.*, 2014; Chun and Lee, 2018; Gorshkov*et al.*, 2018),the observation from this study suggests that datumetine increases NMDAR activity in the neuron which initiates glia response to protect the neurons as shown in other reports that glia response to injury at acute phase helps to protect further damage of neurons (Sofroniew and Vinters, 2010; Clarke and Barres, 2013; Schousboe*et al.*, 2013; Shinozaki *et al.*, 2017; Chun and Lee, 2018). Since the administration of datumetine was for two weeks, there may be a shift of glia response from been protective to initiate further damage as either a response to datumetine activity or breakdown of crosstalk between neurons and glia cells (Cardona *et al.*, 2006; Lyons *et al.*, 2007; Walker *et al.*, 2009; Bhaskar *et al.*, 2010; Sheridan and Murphy, 2013; Holtman*et al.*, 2015; Keren-Shaul*et al.*, 2017; Krasemann*et al.*, 2017; Simon *et al.*, 2019).

On the contrary, increased activity of glial cells is also evident in the neurodegenerative disease where hypofunction of NMDAR is implicated (Volk, 2017; Subhramanyam*et al.*, 2019). This disparity indicates that NMDAR activity is not the major mechanism involved in glia activation, but usually a response to neural insult.

Since datumetine is shown to potentiate NMDAR activity, to investigate the changes in the excitatory terminals, glutamatergic presynaptic terminals were investigated with fluorescence staining of vGlut1. Long term exposure to datumetine significantly increased the expression of vGlut1 than the control. This indicates increased activity of glutamate transmission (Wojcik *et al.*, 2004; Reimer, 2009; Reimer and Voglmaier, 2014). vGlut1 is responsible for actively transporting and storing of glutamate at the presynaptic terminals (Maycox and Jahn, 1990; Fremeau*et al.*, 2004; Reimer and Edwards, 2004; Moriyama and Yamamoto, 2004; Omote and Moriyama, 2013; Omote*et al.*, 2016; Moriyama *et al.*, 2017). Increased expression of vGlut1 showed that there is a high accumulation of glutamate at the presynaptic terminals indicating

enhanced glutamate transmission (Stuber *et al.,* 2010). Unlike previous results, Brancato*et al.* (2017) reported reduced expression of vGlut1 in female mice with no observable changes in the male after exposure to sub-chronic variable stress. Although as pointed earlier, increased activity of NMDAR is part of the pathophysiology of stress, the disparity in the expression of vGlut1 may be due to brain areas as they reported reduced expression in the nucleus accumbens (Brancato*et al.,* 2017), hence hippocampal changes using the same model is needed to ascertain if the part of the brain responds differently to the modelled stress. Chronic stress which also implicates hyperactivity of NMDAR, chronic unpredictable stress (CUS), a model of chronic stress reported inducing low expression of vGlut1 in the prefrontal cortex (PFC) of mice (Yu *et al.,* 2018). The substance that increases the level of vGlut1 in the PFC has been shown to play a critical role in alleviating the symptoms of depression (Voleti*et al.,* 2013; Chowdhury *et al.,* 2017; Wohleb*et al.,* 2017; Yu *et al.,* 2018). Scopolamine, another alkaloid found in *Datura metel* is reported to exhibits anti-depressant effect by increasing the expression of vGlut1 in mice (Yu *et al.,* 2018), this result was more dependent on the interaction of AMPAR than NMDAR (Yu *et al.,* 2018). Unlike previous reports mentioned, increased expression of vGlut1 was recorded in PFC and hippocampus of offspring of animals exposed to stress in utero (Cao *et al.,* 2018). Exposure of animals to intrauterine stress is reported to induce depression in the animals at adult (Cai *et al*., 2007; 2008; Maccari and Morley-Fletcher, 2007; Sun *et al*., 2013; Zhang *et al*., 2013). The increased expression of vGlut1 observed in the animals exposed to stress in utero showed that glutamate transmission is implicated in depression (Cao *et al.,* 2018). The disparity in the level of vGlut1 observed in the two models of stress showed that different stress model induces different neurobiological processes.

AD which is also characterised by increased NMDAR activity showed reduced expression of vGlut1 in the hippocampus (Rodriguez-Perdigon*et al.,* 2016) contrary to the present observation in the hippocampus. Since increased NMDAR activity is implicated it will be suggested that vGlut1 will increase, this disparity may be attributed to neurodegeneration changes occurring in the AD brain. Another possible mechanism is the mode of AD induction since the authors used a high-fat diet. They alluded the changes in their observation to metabolic shift from glucose since glucose is necessary for the formation of vGlut1 (Ikemoto*et al.,* 2003; Rodriguez-Perdigon*et al.,* 2016). Stroke which also induces increased activity of NMDAR showed reduce expression of vGlut1 reactivity in the CA1 region of the hippocampus of gebrianimals (Chen *et al.,* 2018). Although treatment with an antioxidant significantly improved the reduced expression of vGlut1 observed (Chen *et al.,* 2018), this is different to this observation, although the authors specifically focussed on the CA1 region whereas all regions of the hippocampus were quantified in this study indicating that different regions may respond to or undergo different changes during insult to the brain. Low level of vGlut1 was also recorded in primary sensory neurons in the cortex of sciatic nerve ligation model of neuropathic pain (Brumovsky*et al.,* 2011; Malet*et al.,* 2013) which also implicates increased activity of NMDAR. Increased vGlut1 expression is also reported in aged animals with impaired memory (Menard *et al.,* 2015). The authors reported reduced expression of glutamate receptors and attributed impaired memory observed to the imbalance between the presynaptic glutamate terminals and postsynaptic receptors (Menard *et al.,* 2015).

Contrary to popular opinion that vGlut1 increases when activity is suppressed and decrease when activity is increased (Seal, 2016), the results from this work showed altered NMDAR activity which is like increased NMDAR activity and increased vGlut1 expression. This disparity may be

in part as a result of neural loss in neurodegenerative disorders (Biglan*et al.,* 2013; Tabrizi *et al.,* 2013; De Strooper and Karran, 2016) been compensated with hypersensitive receptor as indicated as part of changes in neuropathic pain (Ishola *et al.,* 2019).

Brain normal functioning is based on the balance of activity of all the neurotransmitter system (Fuster and Bressler, 2015). Perturbation of one neurotransmitter system directly or indirectly affects another neurotransmitter system. To further assess the effect of datumetine on brain neurotransmitter system, fluorescence staining of other neural subtypes was done. Increased hippocampal excitation is well correlated to increase GABA activity (Xue*et al.,* 2011; Yao *et al.,* 2018). Together with the increased expression of vGlut1 expression of GABA too was increased in animals exposed to datumetine. Since ligand docking result showed that datumetine is specific for NMDAR binding, increased level of GABA will be as a result of increased NMDAR activity as it is well established that increased NMDAR activity leads to increase GABA release(Xue*et al.,* 2011; Yao *et al.,* 2018). Fattorini*et al.* (2019) reported the presence of mixed synapses that co-release both glutamate and GABA in the mouse cortex. Studying the characteristics of this synapses, it was found that properties of the mixed synapses are like that of single synapse with only NMDAR activity showing reduced activity in the mixed synapse than single synapse (Fattorini*et al.,* 2019). The authors failed to explain the regulatory mechanism guiding the release and the signalling response at the postsynaptic channels. Like the observation recorded in this work, administration of NMDAR agonist is shown to increase the level of GABA in cortical neurons (Drejer and Honore, 1987). This observation is reported to be mainly in part on intracellular Ca^{2+} concentration (Lindefors*et al.,* 1997). Release of GABA in the cortex is under the control of NMDAR activity (Drejer and Honore, 1987; Qin *et al.,* 1994; Laprade and Soghomonian, 1995), the mechanisms behind this regulation is still sceptical, the possible

mechanism is the presence of glutamate receptors on the GABAergic neurons or plastic changes in the brain as a countermeasure to increase excitatory transmission (Bavelier *et al.,* 2010; Le Magueresse and Monyer, 2013).

Unlike the report of NMDAR, acute stress enhanced GABAergic activity while chronic stress reduced GABA activity in the hippocampus (Hu *et al.,* 2010). In chronic variable stress exposure for fourteen days, increased excitation and inhibitory activity were recorded (McKlveen*et al.,* 2016) like what was observed in the experiment. Similarly, contracting observations have been documented. Reduced GABAergic activity is recorded in chronic mild stress (Czéh*et al.,* 2018), chronic isolation tests (Todorović*et al.,* 2019), chronic unpredictable stress (Banasr*et al.,* 2017), social defeat test (Veeraiah*et al.,* 2014) which increased NMDAR is correlated. Major depressive disorders (MDD) is linked to altered GABA activity (Ma *et al.,* 2016).

Datumetine increased the activity of cholinergic neurons. Cholinergic transmission positively regulates the activity of NMDAR (Zweifel*et al.,* 2008; Oswald *et al.,* 2015). Similar to what was observed in the memory tests, increased cholinergic activity is reported in patients with mild cognitive impairment (Ikonomovic*et al.,* 2003). On the contrary, the level of cholinergic transmission is reduced in the hippocampus of rodents induced with vascular dementia (Cao *et al.,* 2016) which is positively correlated to memory decline. In this study, nAChR-α7 was used to identify cholinergic terminals. nAChR-α7was reported to enhance glutamate transmission (Koukouli and Maskos, 2015). nAChR-α7 was shown to directly control the gating of Ca^{2+} and upon activation can induce local depolarisation which leads to glutamate release (Zhong *et al.,* 2008; 2013). This process occurs presynaptically, while at the postsynaptic channel, nAChR-α7 induces glutamate release by amplification of weak signals (Ji and Dani,2000; Ji *et al.,* 2001). It has been reported that nAChR-α7 and NMDAR directly interact in the hippocampus (Li *et al.,*

2012). This activity of nAChR-α7 may explain the increased level of vGlut1 observed in the treated animals.

The activity of glutamate transmission and nAChR-α7 activity areclosely related. Like the results obtained from this experiment, nAChR-α7 knock out mice showed reduced glutamate synapse (Koukouliand Maskos,2015), whereas activation of nAChR-α7 by nicotine increased glutamate synapse in cell culture (Lozada *et al.,* 2012). The interaction of glutamate and acetylcholine system is not unidirectional, blocking of NMDAR activity reduced the effects of nicotine (Liechti*et al.,* 2007; Kenny *et al.,* 2008; D'Souza and Markou, 2011). Increased activity of nAChR-α7 is observed in AD which also showed aberrant NMDAR activity (Wang *et al.,* 2000a,b). Aβ is reported to colocalise with nAChR-α7 in the hippocampus and can activate it leading to excess glutamate-induced toxicity (Pirttimaki*et al.,* 2013; Talantova*et al.,* 2013). Similarly, schizophrenia which is characterised by reduced NMDAR activity also showed reduced nAChR-α7 activity as blocking of nAChR-α7 showed similar symptoms (Mansvelder and McGehee, 2000; Bencherif*et al.,* 2012). Potentiating of nAChR-α7 have shown promising results in the treatment of schizophrenia (Preskorn, 2014; Deardorff *et al.,* 2015; Van Goethem*et al.,* 2015).

In a study conducted by Sing *et al.* glutamate and dopamine transmission is reported to be interrelated in which NMDAR activity modulate dopamine-1 (D1) receptor activity bidirectionally (Sing *et al.,* 1990). They showed that increasing the activity of NMDAR increased dopamine transmission and signalling mediated by D1 receptors in the striatum and substantia nigra (Singh *et al.,* 1990). Hippocampus receives dopaminergic inputs from the ventral tegmental area (VTA) (Lisman and Grace, 2005; McNamara, 2014; Rosen, 2015; Weitemier and McHugh, 2017) and is proposed that these inputs play a role in addiction and

memory (Lisman and Grace, 2005; McNamara and Dupret, 2017). In this study, datumetine which is proposed to enhance the activity of NMDAR also showed increased expression of dopaminergic terminals. Studies have shown the presence of different dopamine receptor subtypes in the hippocampus (Ishola *et al.,* 2018). This observation may be inpart due to the modulation of dopamine activity mediated by D1 as reported by Singh *et al.,* (1990). Dopamine-2 (D2) receptor which is another subtype of dopamine receptor usually mediates the control of movement (Ogundele*et al.,* 2015; Bankole *et al.,* 2015; Ishola *et al.,* 2015, 2018) and is found mainly on medium spiny interneurons (Ishola *et al.,* 2018). NMDAR activity is reported to have an antagonistic effect on dopaminergic activity mediated by D2 receptors (Amalric*et al.,* 1994).

Exposure to psychostimulants which enhance dopamine release has been reported to be mediated in part by NMDAR (Kalivas, 1995). Blocking of NMDAR in the VTA is reported to attenuate the enhanced dopamine release and behavioural changes seen in animals exposed to psychostimulants (Singh *et al.,* 1990; Kalivas and Alesdatter, 1993). The interaction of glutamate and dopamine transmission system in the brain is not clear cut. Contrary to the result obtained, studies done on mice striatum showed that blocking NMDAR activity enhances locomotor activity similar to D2 agonist treatment (Mele *et al.,* 1996) this action was mediated by D2 receptor activity while they also found out that memory impairment observed by blocking NMDAR was reversed by D1 agonist implicating the role of D1 receptors in memory formation (Mele *et al.,* 1996). Although locomotor activity was not assessed in this experiment, and the striatum of the animals was not assessed, future studies need to examine the effects datumetine enhancement of NMDAR may have on the D1 receptor in the hippocampus as well as the behavioural implication.

Studies done on the prefrontal cortex (PFC) showed that blocking NMDAR activity increases the concentration of dopamine (Lindefors*et al.,* 1997). Contrary to findings obtained in this work, the disparity may be due to the method used to quantify dopaminergic terminals. The authors used microdialysis technique to assess extracellular concentration whereas immunofluorescence staining for enzymes responsible for dopamine production was used in this study. Also, the study is on PFC different from hippocampus evaluated in this study, although, repeated treatment with NMDAR antagonist fails to achieve the same result (Lindefors*et al.,* 1997). A similar observation of increased dopamine release to NMDAR blocking was also obtained in the rodent striatum (Peeters*et al.,* 2002). In a study by Rios Valentim Jr *et al.* transient activation of D1 receptors in the medial PFC enhance glutamate transmission whereas overactivation of D1 receptors reduced glutamate transmission (Rios Valentim Jr *et al.,* 2009). The reason for this is because D1 receptors are present on both excitatory pyramidal and inhibitory interneurons (Rios Valentim Jr *et al.,* 2009) and transient stimulation of D1 receptors acton excitatory pyramidal neurons which aid glutamate release whereas overactivation of D1 receptors activates the interneurons hereby affecting glutamate release (Goldman-Rakic*et al.,* 2000; Rios Valentim Jr *et al.,* 2009). A similar organisation is not yet documented in the hippocampus, but inferring from the above statement, it may indicate that datumetine activating NMDAR leads to increase dopaminergic transmission which may then activates both D1 and D2 type of dopamine receptors leading to impaired memory.

Serotonin transmission bidirectionally modulates NMDAR functions (Maura *et al.,* 2000; Yuen *et al.,* 2005). In this study, datumetine reduces the expression of serotonergic terminals which is more profound in the DG of the hippocampus. Consistent with the present observation increased serotonergic transmission is well correlated with NMDAR blocking. Acute exposure of ketamine

which is an antagonist of NMDAR increases the cortical level of serotonin (Smith *et al.,* 1981; Lindefors*et al.,* 1997) similar observation was also recorded in the frontal cortex of rat after MK-801 (NMDAR antagonist) acute exposure (Loscher and Honack, 1992). The interplay of NMDAR and serotonin is also showed to be contradictory in the periaqueductal grey (PAG) mater (Moraes*et al.,* 2008). Similar to what was observed in this study, serotonin type 1-A (5-HT_{1A}) receptors which modulate fear behaviour in rodents (Griebel, 1995; Setem*et al.,* 1999) when activated makes the animal anti-aversive (Deakin and Graeff, 1991; Lovick*et al.,* 2000; Graeff, 2002) was counteracted when NMDAR agonist was exposed to the animals (Schmitt *et al.,* 1995; De Souza *et al.,* 1998; Carobrez*et al..,* 2001; Moraes*et al.,* 2008). Similarly, a study was done on the hippocampus to evaluate the level of neurotransmitter transporters concerning memory formation and exposure to amnesic agents revealed that in memory formation hippocampal glutamate, GABA and serotonin transporters were downregulated, furthermore, an amnesic agent used impairs both long- and short-term memory and also downregulates hippocampal serotonin transporter (Tellez *et al.,* 2012).

5.5 Datumetine Alters Hippocampal Synapse

Increased excitatory transmission leads to synaptic pruning (Henson *et al.,* 2017; Inquimbert*et al.,* 2018) to regulate brain activities. The neural connection was assessed using expansion microscopy techniques stained for neurofilament. Datumetine induces loss of neural connections in all regions of the hippocampus. This may be due to the increased excitatory transmission (Sattler *et al.,* 1999; Dong *et al.,* 2009; Zhou *et al.,* 2013) induced by datumetine. Glia cells also play a major role in synaptic pruning (Paolicelli*et al.,* 2011; Li *et al.,* 2012), as stated earlier datumetine exposure increased the activities of glia. This together showed that prolong exposure of datumetine increased NMDAR activities leading to hyperexcitation in the hippocampus.

Electron microscopy studies on the synapse revealed that datumetine exposed animals showed a reduction in the number of viable synapses with 1.0Datumetine animals showing the great reduction than the control animals. This showed that overactivation of NMDAR leads to synaptic loss (Talantova*et al.*, 2013; Zhou *et al.*, 2013; Lewerenz and Maher, 2015). The postsynaptic density was thicker in datumetine exposed animals with a great reduction in presynaptic vesicles. Chemical neurotransmission is enhanced by the release of synaptic vesicles (Trkanjec and Demarin, 2001; Ikeda and Bekkers, 2009) which are tightly regulated by re-uptake back to the presynaptic neurons (Piedras-Rentería*et al.*, 2004; Dickman *et al.*, 2012; Davis and Műller, 2015). Datumetine greatly reducing the number of synaptic vesicles showed that either reuptake of the vesicles is altered, or rate of production is not balanced with the rate of release (Wang *et al.*, 2016; Li and Kavalali, 2017). Another possible explanation may be that NMDAR binding with datumetine increases the affinity of presynaptic NMDAR for glutamate thereby increasing the release of neurotransmitters (Reimer *et al.*, 1998; Takamori, 2016).

5.6 Datumetine Alters Hippocampal Electrical Activities

After confirmation of datumetine-NMDAR interaction, the functional effect corresponding to NMDA receptor Ca^{2+} current in the hippocampus (CA-DG field) when bound with datumetine was investigated. Based on the firing properties of the neuron, bursts neurons activities were further characterized. Datumetine prolongs the duration of the firing rate between spikes. This showed that the opening of NMDAR channels was extended indicating prolong refractory period. NMDARs at the postsynaptic terminals, when bound to glutamate in the presence of magnesium allow Ca^{2+} influx into the neuron and K^{+} efflux leading to depolarization in few milliseconds (Song *et al.*, 2017). It is speculated here that the prolonged ISI occurs when

datumetine bounds to NMDAR which keeps the channel open for more duration of time allowing for more ion exchange till either the receptor is desensitized or datumetine is metabolized.

The prolong refractory period of datumetine is further evident by the reduction in the number of spikes and frequency of spikes recorded in the burst firing neuron population. Sabatier *et al.* (2004), reported that prolong ISI is due to Ca^{2+} rush leading to hyperpolarising afterpotential (HAP) effect. Reduced mean spike frequency has been reported to be adaptation changes of the membrane to overstimulation (Benda and Tabak, 2014). This change is reported to be due to either HAP (Sah, 1996), excitation and inhibitory interaction (Sutherland *et al.*, 2009) or receptor fatigue (Benda and Tabak, 2014). The regularity of firing which was represented by the CV_2analysis showed that datumetine alters the regularity of firing of hippocampal neurons. Hippocampal neurons fire in synchrony correlating input with output (McNaughton, 1980; Tóth, 2010), while datumetine altering this synchrony may increase error in the hippocampal behavioural task as reported in other literature (Lu *et al.*, 2001; Lau *et al.*, 2009; Lűscher and Malenka, 2012; Park *et al.*, 2014).

Burst neuron showed an increase in the number of burst activities together with the duration of burst after datumetine treatment. This showed that datumetine act as a positive potentiator of the NMDAR by increasing the NMDAR's activity. Datumetine prolongs the interval between bursts and reduces the rate of burst occurrence. This is in line with the prolong refractory period induced by datumetine binding.

CHAPTER SIX

6.1 Summary

This work was designed to identify the compounds present in *Datura metel* plant that is partly responsible for memory loss observed in its users. Computer software was used to screen compounds identified and isolated from Datura plant against NMDAR which is responsible for memory formation and processing. Datumetine shows the highest binding fit among the compounds screened and the biological implication of datumetine-NMDAR interaction was evaluated in mice. Administration of datumetine to mice induces seizures in the animals and prolong treatment leads to memory deficit with altered NMDAR activity in the hippocampus. Prolong datumetine exposure also induces excitotoxicity and altered brain neurotransmitters

system in the hippocampus. Neural connections and spine morphology were altered, and the electrical firing of hippocampal neurons was prolonged due to datumetine exposure.

6.2 Conclusion

This study showed that datumetine binds to NMDAR to modulate its functions and prolong exposure leads to increased activity of glutamate transmission leading to memory deficit as a result of altered NMDAR signalling due to excitotoxicity and synaptic loss. Datumetine also alters the electrical activity of NMDAR by delaying its desensitization.

6.3 Contributions to Knowledge

The results from this study have shown the potential of *Datura metel* to modulate NMDAR through the action of datumetine on hippocampal NMDAR. This has also added to the body agents that can act as modulators of NMDAR.

6.4 Recommendations

I. Abuse of *Datura metel* plants should be discouraged.

II. More studies need to be done on other compounds present in the plant to ascertain their health implications.

III. Effect of datumetine should be studied on other parts of the brain and body systems

6.5 Suggestions for Future Research

Although this research work showed the adverse effects of prolonged datumetine exposure on the hippocampus functions. Further studies should be done to show;

1. Does datumetine affect all NMDAR subtypes functions

2. Is there be any physiological effect differences if datumetine binds with NMDAR at its orthostatic binding site or the allosteric binding site or both
3. Does datumetine replaces glutamate or still requires glutamate binding for its activity on NMDAR
4. Can low dose of datumetine confers NMDAR synaptic plasticity in the brain
5. Is there any therapeutic potential of datumetine in a situation where there is NMDAR hypofunction?

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Appendices

Appendix 1: Ethical Approval Document

UNIVERSITY OF ILORIN, ILORIN, NIGERIA
UNIVERSITY ETHICAL REVIEW COMMITTEE

Vice-Chancellor: Prof. A.G. Amball
DVM (ABU), M.V. Sc., Ph.D (Liverpool, UK),
MVCN, MCVSN, MNVMA, FCVSN
Registrar: Mr. E.D. Obafemi
B.A (Ilorn), Cert. Public Information (Kaduna),
MNIPR

P.M.B. 1515, Ilorin
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Website: ethicalreview.unilorin.edu.ng
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Our Ref: UIL/UERC/07/465A044

Date: 14th June, 2018

Protocol Identification Code: UERC/BMS/108
UERC Approval Number: UERC/ASN/2018/1277

EFFECTS OF DATUMETINE ON HIPPOCAMPAL CYTOARCHITECUTURE AND FUNCTIONS IN MALE WISTAR RATS

Name of applicant/Principal Investigator:	**ISHOLA**, Azeez Olakunle
Address of Applicant:	Department of Anatomy Faculty of Basic Medical Sciences University of Ilorin, Ilorin
Type of Review:	Full Committee Review
Date of Approval:	14/06/2018

Notice of Full Committee Approval

I am pleased to inform you that the research described in the submitted proposal has been reviewed by the University Ethical Review Committee (UERC) and given full Committee approval.

This approval dates from 14/06/2018 to 13/06/2021, and there should be no participant accrual or any activity related to this research to be conducted out side these dates.

You are requested to inform the committee at the commencement of the research to enable it appoints its representative who will ensure compliance with the approved protocol. If there is any delay in starting the research, please inform the UERC so that the dates of approval can be adjusted accordingly.

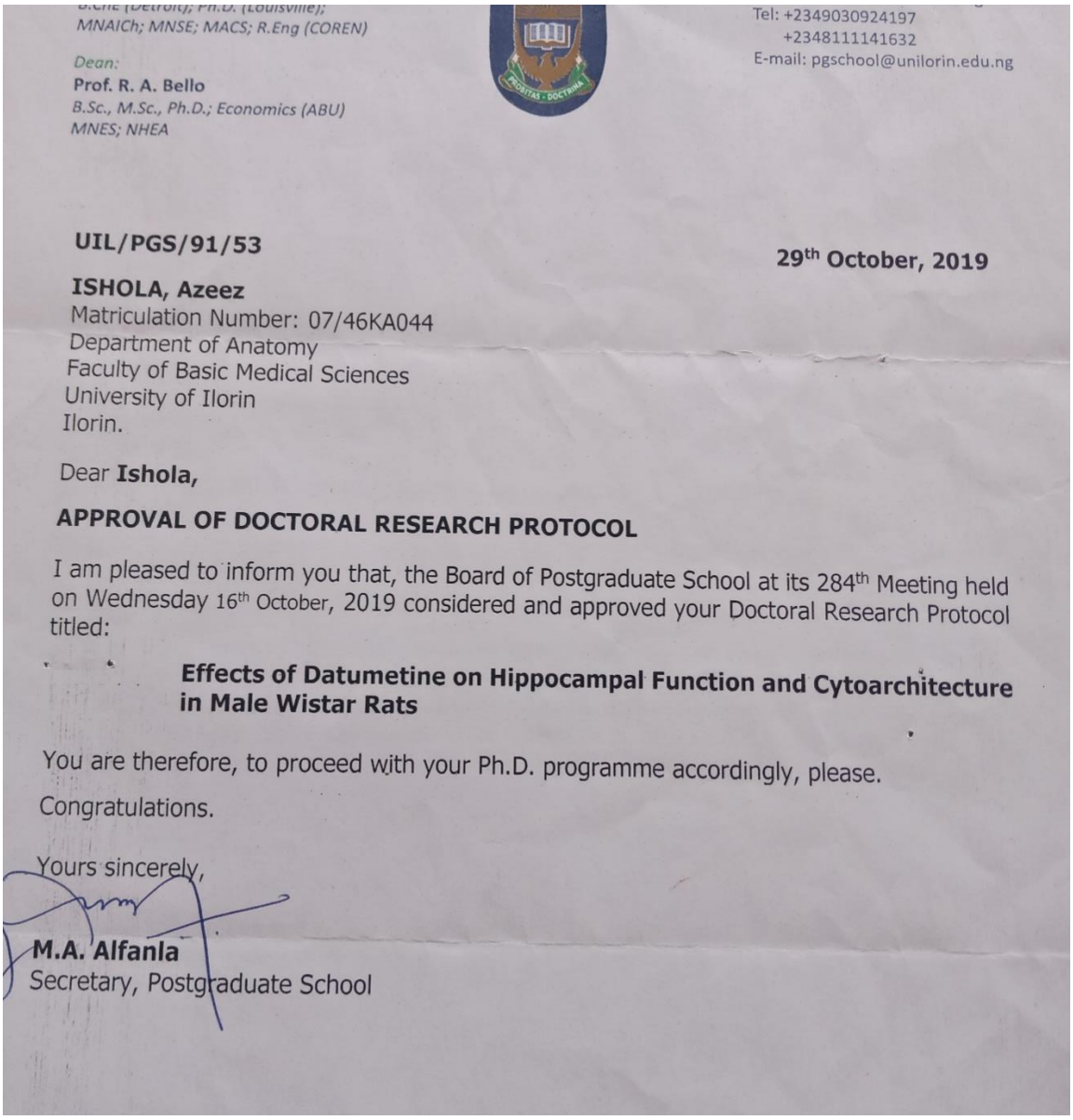
The UERC requires you to comply with all institutional guidelines and regulations and ensure that all adverse events are reported promptly to the UERC. No changes are allowed in the research without prior approval by the UERC. Please note that the UERC reserves the right to conduct monitoring/oversight visit to your research site without prior notification.

Thank You.

Ismaila Isah
For: University Ethical Review Committee

"...if it's not ethical, it's not scientific, if it's not scientific it's not ethical"

Appendix 2: Protocol Approval Document



MNAICh; MNSE; MACS; R.Eng (COREN)

Dean:
Prof. R. A. Bello
B.Sc., M.Sc., Ph.D.; Economics (ABU)
MNES; NHEA

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UIL/PGS/91/53

29th October, 2019

ISHOLA, Azeez
Matriculation Number: 07/46KA044
Department of Anatomy
Faculty of Basic Medical Sciences
University of Ilorin
Ilorin.

Dear **Ishola,**

APPROVAL OF DOCTORAL RESEARCH PROTOCOL

I am pleased to inform you that, the Board of Postgraduate School at its 284th Meeting held on Wednesday 16th October, 2019 considered and approved your Doctoral Research Protocol titled:

Effects of Datumetine on Hippocampal Function and Cytoarchitecture in Male Wistar Rats

You are therefore, to proceed with your Ph.D. programme accordingly, please.

Congratulations.

Yours sincerely,

M.A. Alfanla
Secretary, Postgraduate School

Appendix 3: Ligand docking result for NMDAR allosteric site

```
Date 22.02.2017,                                                    *
Originator: OlubiyiOlujide, PharmChem, OAU.                        *
Binding free energy scanning of selected constituents of Datura spp  *
Molecular target: NMDAR (allosteric binding site)                   *
Method: Virtual screening (AutoDockVina)                           *
Ref: Mr Ishola Azeez, Anatomy, ABUAD                                *
**********************************************************************
Natural product                        |      Delta_G(binding)     *
**********************************************************************
datumetin                              |      -8.3                 *
3-phenylacetoxy-6-7-epoxynortropane    |      -8.3                 *
aponoratropine                         |      -8.0                 *
apoatropine                            |      -7.9                 *
3B-phenylacetoxytropane                |      -7.9                 *
3A-phenylacetoxytropane                |      -7.7                 *
3tigloyloxy6propionyloxytropane        |      -7.5                 *
```

3-tigloyloxynortropane	-7.2	
aposcopolamine	-7.1	
3Btigloyloxy6Bacetoxytropane	-7.1	
3hydroxy6tigloyloxytropane	-7.0	
3Atigloyoxy6Bacetoxytropane	-7.0	
3A-tigloyloxytropane	-7.0	
norscopolamine	-6.9	
aponorscopolamine	-6.9	
3B-tigloyloxytropane	-6.9	
3Btigloiloxy6Bhydroxytropane	-6.9	
3isovaleryloxy6hydroxytropane	-6.7	
3hydroxy6methylbutyryloxytropane	-6.7	
3Bhydroxy6Btigloyloxytropane	-6.7	
3Atigloyloxy6Bisobutyryloxytropane	-6.6	
3-hydroxy6isobutyryloxytropane	-6.5	
N-methylscopolamine	-6.4	
3isobutyryloxy6hydroxytropane	-6.4	
3-propionyloxy-6-hydroxytropane	-6.2	
3-2methylbutyryloxytropane	-6.2	
6-hydroxyacetoxytropane	-6.0	
atropine	-5.9	
3hydroxyacetoxytropane	-5.9	
3-acetoxytropane	-5.7	
3-6-diacetoxytropane	-5.5	
3Atigloyloxy7Bisobutyryloxytropane	-5.3	
hyoscyamine	-5.2	
tropinone	-5.1	
scopoline	-5.1	
scopolamine	-5.1	
scopine	-5.0	
pseudotropine	-5.0	
3-6-dihydroxytropane	-5.0	
tropine	-4.8	

Appendix 4: Ligand docking result for NMDAR orthostatic site

Date 22.02.2017,
Originator: OlubiyiOlujide, PharmChem, OAU.
Binding free energy scanning of selected constituents of Datura spp
Molecular target: NMDAR (orthostatic binding site)
Method: Virtual screening (AutoDockVina)
Ref: Mr Ishola Azeez, Anatomy, ABUAD

Natural product	Delta_G(binding)
apoatropine	-8.8
datumetin	-8.6
aponoratropine	-8.6
3A-phenylacetoxytropane	-8.4
3-phenylacetoxy-6-7-epoxynortropane	-8.1
3B-phenylacetoxytropane	-8.0
norscopolamine	-7.8

aposcopolamine	-7.8	*
aponorscopolamine	-7.7	*
3tigloyloxy6propionyloxytropane	-7.6	*
3Atigloyloxy6Bisobutyryloxytropane	-7.4	*
3Btigloyloxy6Bacetoxytropane	-7.3	*
3hydroxy6tigloyloxytropane	-7.2	*
3B-tigloyloxytropane	-7.2	*
3Atigloyoxy6Bacetoxytropane	-7.2	*
3Atigloyloxy7Bisobutyryloxytropane	-7.2	*
3-2methylbutyryloxytropane	-7.1	*
3Bhydroxy6Btigloyloxytropane	-7.0	*
3hydroxy6methylbutyryloxytropane	-6.8	*
3Btigloiloxy6Bhydroxytropane	-6.8	*
3isovaleryloxy6hydroxytropane	-6.7	*
3-hydroxy6isobutyryloxytropane	-6.6	*
atropine	-6.5	*
3isobutyryloxy6hydroxytropane	-6.5	*
N-methylscopolamine	-6.1	*
3-propionyloxy-6-hydroxytropane	-6.1	*
scopolamine	-6.0	*
hyoscyamine	-6.0	*
6-hydroxyacetoxytropane	-6.0	*
3hydroxyacetoxytropane	-6.0	*
3-tigloyloxynortropane	-5.9	*
3A-tigloyloxytropane	-5.9	*
3-acetoxytropane	-5.9	*
3-6-diacetoxytropane	-5.8	*
scopoline	-5.7	*
tropinone	-5.2	*
3-6-dihydroxytropane	-5.1	*
scopine	-5.0	*
pseudotropine	-5.0	*
tropine	-4.9	*

Appendix 5: First page of published article from the project work

DRUG AND CHEMICAL TOXICOLOGY
https://doi.org/10.1080/01480545.2020.1776315

ORIGINAL ARTICLE

Datumetine exposure alters hippocampal neurotransmitters system in C57BL/6 mice

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ABSTRACT

Our previous study showed that datumetine modulates NMDAR activity with long term exposure leading to memory deficit and altered NMDAR signaling. We aim to explore the neurotransmitters perturbations of acute datumetine-NMDAR interaction. Fifteen C57/BL6 mice were used for the study, they are divided into three groups of 5 animals each. Animals were administered DMSO (DMSO/Control), 0.25 mg/kg body weight of datumetine (0.25 Datumetine) and 1 mg/kg bodyweight of datumetine (1.0 Datumetine) intraperitoneally for 14 days. At the end of treatment, animals were euthanized in isoflurane chamber, perfused transcardially with 1XPBS followed by PFA. Immunofluorescence procedure was done to check the distribution of neurons, astrocytes, microglia and major neuronal subtypes in the hippocampus. Expansion and electron microscopy techniques were used to assess the condition of the synapses. Quantitative data were expressed as: mean±SEM and analyzed using ANOVA with Tukey post hoc using $p < 0.05$ as significant. Datumetine increased the expression of CD11b, GFAP, vGlut1, GABA, CHRNA7 and TH while expression of TrPH and NeuN were reduced in the hippocampus compared to control animals. Synaptic loss was evident in datumetine exposed animals with reduced synaptic vesicles accompanied by a thickness of postsynaptic density than that of control animals. This study concludes that acute datumetine exposure alters hippocampal neurotransmitter systems.

ARTICLE HISTORY
Received 6 March 2020
Revised 20 May 2020
Accepted 25 May 2020

KEYWORDS
Datumetine; NMDAR; excitotoxicity

1. Introduction

Datura metel is a *Solanaceae* plant which contains different alkaloids (Prasad and Gowda, 2005). It is used locally in the treatment of asthma, bronchodilation, glaucoma, and antibiotics (Hilal *et al.* 2014) and cancer treatment (Devi *et al.* 2011). Recreation use of Datura plant is increasing among adolescents (Moses 2010). World drug report as produced by the United Nations Office of Drug and Crimes claimed that datura plant is one of the new psychoactive substance (NPS) recorded in Africa (United Nations Office on Drugs and Crime (UNODC), 2017, 2018).

In Nigeria, 27 thousand people had been reported to be exposed to the use of datura with 0.03 prevalence rate (United Nations Office on Drugs and Crime (UNODC) 2018). Despite this number, deaths have been reported in the national dailies where datura extract was added to the alcoholic drinks to increase intoxication. Long term use of the plant has been reported to induce psychosis in humans (Khanna *et al.* 2015). Recreational users of datura suffer memory loss during the phase of intoxication, this led to the hypothesis that datura may possess compound(s) that affect hippocampal functioning.

In our previous study, we found that datumetine which is one of the alkaloids found in *Datura metel* has the ability to bind with NMDA receptor at both its orthosteric and allosteric binding sites, alters NMDAR calcium current *in vivo* and long-term exposure induces memory deficit and altered NMDAR signaling in mice (Ishola *et al.* 2020). Our study is the first to show the biological effects of datumetine on animal which we proposed to be in part responsible for memory loss observed in *Datura metel* abusers. In this present study, we aim to further characterize the effects of acute datumetine exposure on the hippocampal neurotransmitter system *in vivo*.

2. Materials and methods

2.1. Ethical approval

Ethical approval was gotten from the University of Ilorin Ethical Review Committee (UERC) with approval number UERC/ASN/2018/1277 and protocol identification code UERC/BMS/108.

2.2. Purchase and preparation of datumetine

Datumetine (CAS No.: 67078-20-0) 98% purity was purchased from ChemFaces Biochemical Company (China). The compound was dissolved in dimethyl sulfoxide (DMSO) to achieve 1 mg/mL stock solution stored at 4°C. It was then serially diluted based on the dosage of animals for working solution (kept at room temperature) to administer to animals.

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Supplemental data for this article is available online at here.