Alzheimer's disease dementia risk in post-traumatic stress disorder: identification of common underlying mechanisms using rat models

by

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Preface

The research work described in this thesis was conducted by the candidate at the University of KwaZulu-Natal, Westville, South Africa, under the supervision of Professor Musa Mabandla.

The contents of this work have not been submitted in any form for any degree to another University, and where use has been made of the work of others, it is duly acknowledged in the text. The results reported are from investigations by the candidate.

Signed: Oluwaseun Samuel Faborode

Date: 02 December 2021

As the candidate's supervisor, I agree to the submission of this thesis.

Signed: Professor Musa Mabandla (Supervisor)

Date: 02 December 2021

Declaration

I, Oluwaseun Faborode, declare that:

1. The research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work
2. This dissertation has not been submitted in part or entirely for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information unless expressly acknowledged as being sourced from other persons.
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Signed 

Oluwaseun Samuel Faborode

Dedication

To my wife, son, and parents.

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List of Abbreviation

α	Alpha
β	Beta
° C	Degree Celsius
µ	Micro
µg	Microgram
µl	Microlitre
Aβ	Amyloid-beta
AD	Alzheimer's disease
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ANOVA	Analysis of variance
ARE	Antioxidant response element
BACE1	Beta-site amyloid precursor protein cleaving enzyme 1
BAX	Bcl-2-associated X protein
BIN1	Bridging integrator 1
cm	Centimetre
ATP	Adenosine triphosphate
C	Celsius
Ca^{2+}	Calcium ion
Caspase	Cysteine aspartic-specific proteases
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
COX-2	Cyclo-oxygenase 2
CS	Conditioned stimulus
CSF	Cerebrospinal fluid
CRF	Corticotrophin-releasing factor

EOAD	Early-onset Alzheimer's disease
EPM	Elevated plus maze
FAD	Familial Alzheimer's disease
FACS	Fluorescent-activated cell sorting
FCT	Fear conditioning test
FET	Fear extinction test
FITC	Fluorescein isothiocyanate
FKBP5	FK506 binding protein 51
Fmn2	Formin 2
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GSH	Glutathione
h	Hour
HDAC	Histone deacetylases
HPA	Hypothalamic-pituitary-adrenal
hrs	Hours
i.p.	Intraperitoneal
Keap1	Kelch-like ECH-associated protein 1
kDa	Kilodalton
kg	Kilogram
L	Litre
LOAD	Late-onset Alzheimer's disease
MAP-1	Modulator of apoptosis-1
MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
MDA	Malondialdehyde

mg	Milligram
min	Minute
miRNA	MicroRNA
mL	Millilitre
mm	Millimetre
mmol	Millimole
mol	Mole
MWM	Morris water maze
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
NADPH	Nicotinamide adenine dinucleotide phosphate
NBF	Neutral buffered formalin
NF-kB	Nuclear factor kappa-light-chain-enhancer
NFTs	Neurofibrillary tangles
NMDA	N-Methyl-D-aspartic acid
NOR	Novel object recognition
NR2B	NMDA Receptor Subtype 2B
NQO1	NAD (P) H: quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
OFT	Open field test
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFC	Prefrontal cortex
PSEN	Presenilin
PTSD	Post-traumatic stress disorder

ROS	Reactive oxygen species
SAD	Sporadic Alzheimer's disease
SD	Sprague-Dawley
SNP	Single nucleotide polymorphism
SEM	Standard error of mean
siRNA	Small interfering RNA
SOD	Superoxide dismutase
SPs	Senile plaques
SPS	Single prolonged stress
SSA	sub-Saharan Africa
TBI	Traumatic brain injury
TNF	Tumour necrotic factor
TREM2	Triggering receptor expressed on myeloid cells 2
US	Unconditioned stimulus
WHO	World Health Organisation
WMH	World Mental Health

Publications and Presentations

Published manuscript

1. Oluwaseun Samuel Faborode, Ernest Dalle, Musa Vuyisile Mabandla (2021): Exposure to footshock stress downregulates antioxidant genes and increases neuronal apoptosis in an Aβ(1–42) rat model of Alzheimer's disease (Neurochemistry International, Impact factor: 3.921). https://doi.org/10.1016/j.neuint.2021.105170
2. Trauma-like exposure alters neuronal apoptosis, Bin1, Fkbp5 and NR2B expression in an amyloid-beta (1–42) rat model of Alzheimer's disease (Neurobiology of Learning and Memory journal, Impact factor: 2.877). https://doi.org/10.1016/j.nlm.2022.107611
3. Inescapable footshocks induce molecular changes in the prefrontal cortex of rats in an amyloid-beta-42 model of Alzheimer's disease (Behavioural Brain Research, Impact factor: 3.332). https://doi.org/10.1016/j.bbr.2021.113679

Conference Presentations

1. Implication of amyloid-beta (1-42) lesioning on fear memory and anxiety in a rat model of posttraumatic stress disorder: School of laboratory medicine and medical sciences symposium, University of KwaZulu-Natal, South Africa. 18 Sept 2020. See Appendix III
2. Trauma-like exposure alters neuronal BIN1 and FKBP5 expression in the Amyloid-beta (1-42) rat model of Alzheimer's disease: AAPS-PSSA 2021 Congress (12 – 15 September 2021). See Appendix V
3. Exposure to footshock stress downregulates antioxidant genes and increases neuronal apoptosis in an Aβ(1-42) rat model of Alzheimer's disease: 15th SONA / 4th GNS Conference 2021 (27th - 30th October 2021). Appendix VI

Thesis Outline

The principal findings of this PhD research study have been compiled into articles and presented as a thesis by manuscript.

Chapter 1: Provides background information with a brief but deep review of selected topics relevant to the study. Study aims and objectives, hypotheses and potential benefits of this research are also highlighted.

Chapter 2: This chapter is the first manuscript that examines the effect of PTSD-like induction on redox homeostasis and cellular apoptosis in the amygdala and hippocampus of Aβ(1-42) lesion rat model of AD. This chapter is prepared in manuscript format and has been published in Neurochemistry International, Impact factor: 3.921. (https://doi.org/10.1016/j.neuint.2021.105170).

Chapter 3: This chapter is the second manuscript that examines the effect of PTSD-like induction on learning and memory outcomes in Aβ(1-42)-lesioned rats and how it is mediated by glutamate excitotoxicity and stress dysregulation. This chapter is prepared in a manuscript format and has been published in Neurobiology of Learning and Memory journal, Impact factor: 2.877. (https://doi.org/10.1016/j.nlm.2022.107611)

Chapter 4: This chapter is the third manuscript that provides information on changes in the prefrontal cortex associated with the effects of PTSD-like induction in an Aβ(1-42) lesion rat model of AD. This chapter is prepared in manuscript format and has been published in Behavioural Brain Research, Impact factor: 3.332. (https://doi.org/10.1016/j.bbr.2021.113679).

Chapter 5: This chapter provides a synthesis of the research findings and general conclusions. It also highlights recommendations for future studies.

Abstract

Post-traumatic stress disorder (PTSD) is a neuropsychiatric disorder that can develop from exposure to a trauma. Studies have shown that people who have PTSD are susceptible to developing dementia, mostly Alzheimer's disease (AD), suggesting common underlying risk factors in the comorbidity. Although several molecular pathways have been implicated in AD and PTSD, including oxidative stress, cellular apoptosis, synaptic dysfunction and stress dysregulation, the underlying neurobiological mechanisms linking AD and PTSD are less understood. This study, therefore, investigated the effect of trauma-like exposure in an amyloid-beta (Aβ) rat model of AD.

Seventy-two adult male Sprague-Dawley rats were used throughout the study. The animals were randomly divided into four groups where they received either footshocks or Aβ(1-42) injection or were exposed to footshocks and Aβ(1-42) injection or remained naive. Following inductions, the animals were tested for cognitive, locomotor and anxiety-like behaviours. Thereafter, brain samples were collected for further neurochemical analyses.

Our results show that footshocks increased anxiety-like behaviour and impaired fear memory extinction in Aβ(1-42) lesioned rats. A combination of footshocks and Aβ(1-42) also reduced the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), NAD (P) H: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and increased the expression of Kelch-like ECH-associated protein 1 (Keap1) in the amygdala and hippocampus. Prior exposure to footshocks before Aβ(1-42) lesion caused a decrease in the number of crossing in the target quadrant of the Morris water maze test and reduced percentage alternation in the Y-maze test, indicating memory deficits. There was an interactive effect of footshocks and Aβ(1-42) lesion on the downregulation of BIN1 and the upregulation of NR2B in the hippocampus. There was also an interactive effect of footshocks and Aβ(1-42) lesion on the upregulation of FKBP5 in the hippocampus, amygdala, and PFC.

Our finding suggests that footshock stress can exacerbate AD-like pathology via dysregulated redox balance, BIN1 downregulation, FKBP5 and NR2B upregulation, and increased apoptosis in the brain of Aβ(1-42)-lesioned rats. These molecular changes were associated with increased anxiety, impaired fear extinction and memory deficits. These findings, therefore, suggest common molecular mechanisms in PTSD and AD.

Iqoqa

Isifo sengcindezi eba semva kwesehlakalo esibi i-post-traumatic stress disorder (i-PTSD) yisifo sokusebenzelana kwezinzwa nensebenzamqondo esidala wukwehlelwa yisehlakalo esibi. Ucwaningo selukhombise ukuthi abantu abane-PTSD basengcupheni yokuba nesifo sokukhohlwa, ikakhulukazi i-Alzheimer's (i-AD), okuchaza ukuthi izimo eziyingcuphe enkulu ekubeni nezifo eziyizimbelambela. Nakuba izindlelamigudu eziningi zamamolekhyuli zisoleka kwi-AD ne-PTSD, okufaka nengcindezi ye-oxidative, i-cellular apoptosis, i-synaptic dysfunction kanye nokungalawuleki kwengcindezi, izindlelamigudu zempiliswanozinzwa nomzimba ezixhumanisa i-AD ne-PTSD akuqondwa ngokuphelele. Ngakho-ke lolu cwaningo, luphenye umthelela wokubekeka endaweni ecisho ibe yisehlakalo esinzima kwimodeli yamagundane i-amyloid-beta (Aβ) ye-rat model of AD.

Amagundane amadala esilisa angamashumi ayisikhombisa nambili asetshenziswa kulolu cwaningo. Izilwane zehlukaniswa ngokungahleliwe zaba amaqembu amane lapho zanikwa khona ama-footshocks noma umjovo we-Aβ(1-42) noma ayengathola i-footshocks nomjovo we-Aβ(1-42) noma ahlale engazi. Ukulandela ukwethulwa, izilwane zahlolelwa ukuziphatha kwazo ngokomcabango, ukuhamba nokuba nexhala. Emva kwalokho, amasampula obuchopho aqoqwa ukuze aphinde acutshungulelwe amanyurokhemikhali.

Imiphumela yethu iveze ukuthi ama-footshocks enyuse ukuziphatha sakuba nexhala kanye nokuqeda ukukhumbula ukwesaba okugwegwile kuma-Aβ(1-42) onikwe amalesioned rats. Ingxubevange ye-footshocks ne-Aβ(1-42) iphinde yehlisa ukuvela kwe-nuclear factor erythroid 2-related factor 2 (Nrf2), i-NAD (P) H: i-quinone oxidoreductase 1 (NQO1), i-heme oxygenase-1 (HO-1), kanye nokuvela okusezingeni eliphezulu kwe-Kelch-like ECH-associated protein 1 (Keap1) kwi-amygdala ne-hippocampus. Ukusondelana nama-footshocks kwangaphambi kwe-Aβ(1-42) kwdala ukwehla esibalweni sokuhlanganayo kwi-target quadrant yokuhlolwa kwe-Morris water maze test nenguqunguquko yephesenti kwi-Y-maze test, okuveza ukushoda kokukhumbula. Kwaba nomthelela onokungenelana wama-footshocks kanye ne-Aβ(1-42) lesion ekulawulenikwehlisa i-BIN1 nasekulawulenikukhuphula i-NR2B kwi-hippocampus. Kwaphinde kwaba nomthelela ongenelanayo we-footshocks ne- Aβ(1-42) lesion ekulawulenikukhuphula i- FKBP5 kwi-hippocampus, i-amygdala, ne- PFC.

Esikutholile kuphakamise ukuthi ingcindezi ye-footshock ingabhebhezela isakhiwosifo esifuze i-AD nge-dysregulated redox balance, i-BIN1 downregulation, i-FKBP5 ne-NR2B upreguation, i-apoptosis ephezulu ebuchosheni be-Aβ(1-42)-ekuma-lesioned rats. Lezi zinguquko kumamolekhyuli zazihlotshaniswe nokwenyuka kwexhala, inqedakwesaba egwegwile nokulahlekelwa wukukhumbula. Ngakho-ke lokhu okutholakele, kuphakamisa izindlela ezejwayelekile zomumo wamamolekhyuli kwi-PTSD ne-AD.

1 CHAPTER 1: LITERATURE REVIEW

1.1 Background

Alzheimer's disease (AD) is a neurodegenerative disease that presents with a gradual loss of neurons in brain areas e.g. hippocampus, with a corresponding loss of cognitive functions ("2021 Alzheimer's disease facts and figures," 2021; Jack et al., 2013). AD has a huge emotional and economic burden on our society (Patterson, 2018; Winblad et al., 2016). It is also the world's leading form of dementia and has affected over 75 million people, with a predicted increase of 135 million by 2050 (Leidinger et al., 2013; Shi et al., 2018). Although AD is seen as a clinical condition, there is evidence that pathological changes can be detected years before the onset of clinical symptoms (Aisen et al., 2017; Jack et al., 2018). Therefore, the elucidation of the various risk factors responsible for the disease's onset and progression is relevant.

Alzheimer's disease is characterized by extracellular senile plaques formed as a result of toxic amyloid-beta (Aβ); and intracellular neurofibrillary tangles (NFTs), which are from aggregated hyperphosphorylated tau proteins (Winblad et al., 2016). Amyloid-beta peptides produced as monomers are relatively non-toxic until they aggregate into oligomers, forming amyloid plaques (Chasseigneaux and Allinquant, 2012; Roberts et al., 1994). Alzheimer's disease is of several types: the familial type which accounts for about 1% of total AD cases, which develops as a result of mutations in the amyloid precursor protein (APP), presenilin 1 (PSEN1), or PSEN2 genes (Ryman et al., 2014). This type of AD can be developed as early as age 20, although the average age is 46.2 years (Ryman et al., 2014). The early-onset Alzheimer disease (EOAD), which accounts for about 5% of total AD cases, usually develops before age 65 and is more common than Familial AD (Bettens et al., 2013). The other form of AD develops after the age of 65. Late-onset Alzheimer's disease (LOAD) has been associated with certain genetic risk factors, with the most notable being apolipoprotein E (APOE) gene (Hardy and Higgins, 1992) and bridging integrator 1 (BIN1) (Zhu et al., 2021). Most AD cases are sporadic with poorly understood aetiology (Forny-Germano et al., 2014).

Currently, there are no effective drugs to cure AD, hence a need to understand its pathophysiology (Huang et al., 2020). One of the reasons for the delayed development of effective treatment can be attributed to the complexity and multifactorial nature of the disease (Gulyaeva et al., 2017). Therefore, many preclinical studies have targeted a variety of processes in its pathogenesis and progression. Many include inhibiting plaque formation and apoptosis, synaptic plasticity, oxidative stress, mitochondrial damage, and alterations in the cell cycle regulatory mechanisms (Wojsiat et al., 2018; Zhao and Lukiw, 2018).

Post-traumatic stress disorder (PTSD) is a psychiatric disorder that results from exposure to a life-threatening trauma (Careaga et al., 2016). These events may include war, automobile accidents, sexual and physical abuse, and loss of a loved relative (Breslau et al., 1998). Symptoms relating to PTSD can be categorised into four clusters viz. 1) Intrusion/re-experiencing symptoms, 2) Avoidance symptoms, 3) Negative cognitions and mood, 4) Symptoms of hyper-arousal. (American Psychological Association (APA), 2013). The worldwide prevalence of PTSD differs due to the varying trauma exposures but affects about 4% of the world's population (2). Prevalence in Western Europe is estimated to be 1 – 2%, while it is 6 – 9% in North America (Kessler et al., 2010). In countries that have been exposed to extended periods of violence, such as war, the prevalence rate is just over 10% (Kessler et al., 2010). Rape accounts for the highest burden of PTSD globally (13.1%), followed by other sexual assault incidents (15.1%) while PTSD due to being stalked is at 9.8%. Trauma due to the unexpected death of a loved one, accounts for 11.6% (Kessler et al., 2017). In the South African population, the lifetime prevalence rate of PTSD is 2.3% for all ages (Herman et al., 2009). For ages between 18 and 34 years, the prevalence is 1.8% (Herman et al., 2009). The prevalence in South Africa has been mostly attributed to high levels of interpersonal violence due to socioeconomic disparities that lingered post-apartheid (Carey et al., 2004; Jewkes and Abrahams, 2002). In addition, the majority (> 70%) of the populace has experienced at least one potentially traumatic event (Atwoli et al., 2013).

Patients suffering from PTSD display extreme traumatic stress to an extent in which normal psychological defence mechanisms fail (Adshead et al., 2000). This failed defence mechanism results in several symptoms, including impaired socialisation, exaggerated anxiety, and impaired fear extinction (Goswami et al., 2013). Thus, a major therapeutic approach has been to repeatedly expose the patients to their fear memories, which has proven to reduce anxiety symptoms and aversive behaviours related to the fear (fear extinction) (Sananbenesi et al., 2007; Sotres-Bayon et al., 2006). The hippocampus, amygdala and prefrontal cortex are involved in the regulation of fear memories (Milad and Quirk, 2002; Petrovic et al., 2018). For instance, the hippocampus is involved in memory recall of safe episodes when exposed to fearful events (Kirkpatrick and Heller, 2014). Decreased hippocampal volumes have been observed in PTSD patients, but may be a risk factor rather than a consequence (Bonne et al., 2001). In addition, the prefrontal cortex has been implicated in its inhibitory role on the functions of the amygdala in fear regulation, allowing the prefrontal cortex to overwrite the initial fear, giving way for safe episodes to become persistent (Craske et al., 2008). Similarly, the perturbation of the HPA function has been implicated in PTSD pathology, as elevated CRF levels have been observed in PTSD patients (Stoppelbein et al., 2012). Dysregulated levels (high or low) of cortisol have also been shown to drive PTSD pathology (Speer et al., 2019; Stoppelbein et al., 2012; Van Den Heuvel et al., 2019). Despite the extensive studies on PTSD, reliable at-risk

biomarkers are unavailable (Zoladz and Diamond, 2013). It has been suggested that identifying target systems involved in fear extinction should constitute future research focus in understanding PTSD pathology (Kelmendi et al., 2016; Young et al., 2015).

1.2 Magnitude of the problem

In Africa, data showing the prevalence and incidence of AD and other related dementias are scarce compared to the Europe and Americas. This poses a significant limitation on the continental and global overview of AD and other related dementia. However, studies regarding AD are gradually emerging from Africa.

About a decade ago, an estimate of the prevalence of dementia in Africa was attempted but found only ten relevant studies to analyse. This study estimated the overall prevalence of dementia to be 2.4% in adults over 50 years. AD accounted for over 50% of the cause of dementia. Over 70% of this population live in sub-Saharan Africa (George-Carey et al., 2012).

A report by the Alzheimer's disease international in 2014 and 2017 estimated the prevalence of dementia in sub-Saharan Africa to be 7.2% and 6.38% of the population aged 60 years and above (Guerchet et al., 2017; Prince et al., 2014). A recent study sampling the aged population in rural Uganda estimated that 20% of the population with AD and related dementias (Mubangizi et al., 2020). The Ugandan estimate was higher than a South African (8%) study, utilising similar age distribution and screening tools (De Jager et al., 2017). A Tanzanian study using a high yielding screening tool (Full Community Screening Instrument for Dementia (CSID) and Diagnostic and Statistical Manual of Mental Disorders (fourth edition)) estimated a dementia prevalence of 6.4% among the old age population (Longdon et al., 2013). The high Ugandan estimate was attributed to certain limitations. For example, the screening tool used in the study (brief CSID) usually generate high prevalence estimates because it detects both early dementia and mild cognitive impairment (Mubangizi et al., 2020). In addition, participant self-reported data emerging from early and midlife exposure variables may be a source of bias because of possible poor memories (Mubangizi et al., 2020). Although these points are valid, the study still in some way contributes and validates the prevalence of AD and other dementias in Africa.

Over the years, the quality of dementia studies in Africa has improved. However, the evidence of risk factors is still weak, and more work on AD is required in the description of the studies (Akinyemi et al., 2021; Guerchet et al., 2017; Ojagbemi et al., 2021).

Recent global estimates on PTSD come from World Health Organisation's World Mental Health (WMH) surveys, which only included one national survey and one regional survey from sub-Saharan Africa (SSA) (Koenen et al., 2017). The low representation of data from countries in SSA is

consistent with the unavailability of data from SSA generally. (Baxter et al., 2013; Ng et al., 2014). WHO found that road traffic deaths (World Health Organization, 2008) and intimate partner violence and/or non-partner sexual violence are highest in Africa (Abrahams et al., 2014; WHO, 2013). Also, many countries that are more vulnerable to natural disasters are found in SSA (Hilft, 2017). SSA has been affected by war and armed conflicts, many of which have been going on for years (Marc, n.d.). In addition, violence and trauma arising from colonisation may contribute to the high rates of PTSD in SSA (Fanon, 2016).

Increased exposure to traumatic events in SSA is made worse by very low access to mental health care ("Mental health atlas," 2015; Saxena et al., 2007). Given that most of the countries in Africa are low-income or lower-middle-income countries, it is likely that many people with PTSD will not get treatment, which puts them at risk of developing other chronic conditions (Ng et al., 2020). The combination of increased exposure to traumatic events and lack of medical care ("Mental health atlas," 2015; Saxena et al., 2007), may eventually lead to an increased burden of PTSD in SSA (Bromet et al., 2017; Charlson et al., 2016). For a review, see (Ng et al., 2020).

1.3 PTSD as a risk factor for dementia

It has been shown that stress due to PTSD can increase susceptibility to developing other pathologies such as AD (Bao et al., 2008; Stuller et al., 2012). More so, several reports have shown that people with PTSD are twice as likely to develop dementia, including AD, but the mechanism underlying this comorbidity is largely unclear (Burri et al., 2013; Stilling et al., 2014; Yaffe et al., 2010). One of the hypotheses linking PTSD to dementia arises from the evidence showing that dysregulation of the HPA axis is implicated in PTSD, which may also result in dementia (Herbert et al., 2006; Yehuda, 2002). The association between PTSD and memory deficits may reflect the inverted U-shaped curve of the relationship between glucocorticoid levels and cognitive performance (Yehuda et al., 2005). However, it may also reveal the complex nature between trauma and disturbance to HPA axis functioning. Changes in cognitive function may result from the previously increased cortisol levels experienced at the time of trauma leading to an overall dysregulation of the HPA axis (Milligan Armstrong et al., 2021) (Figure 1). Similarly several preclinical data has shown that prolonged stressors can exacerbate AD-like pathologies in animal models (Carroll et al., 2011; Rothman et al., 2012). In addition, several medical comorbidities have been suggested to be involved in the PTSD link to AD, including traumatic brain injury (TBI) and depression (Hoge et al., 2008; Yaffe et al., 1999).

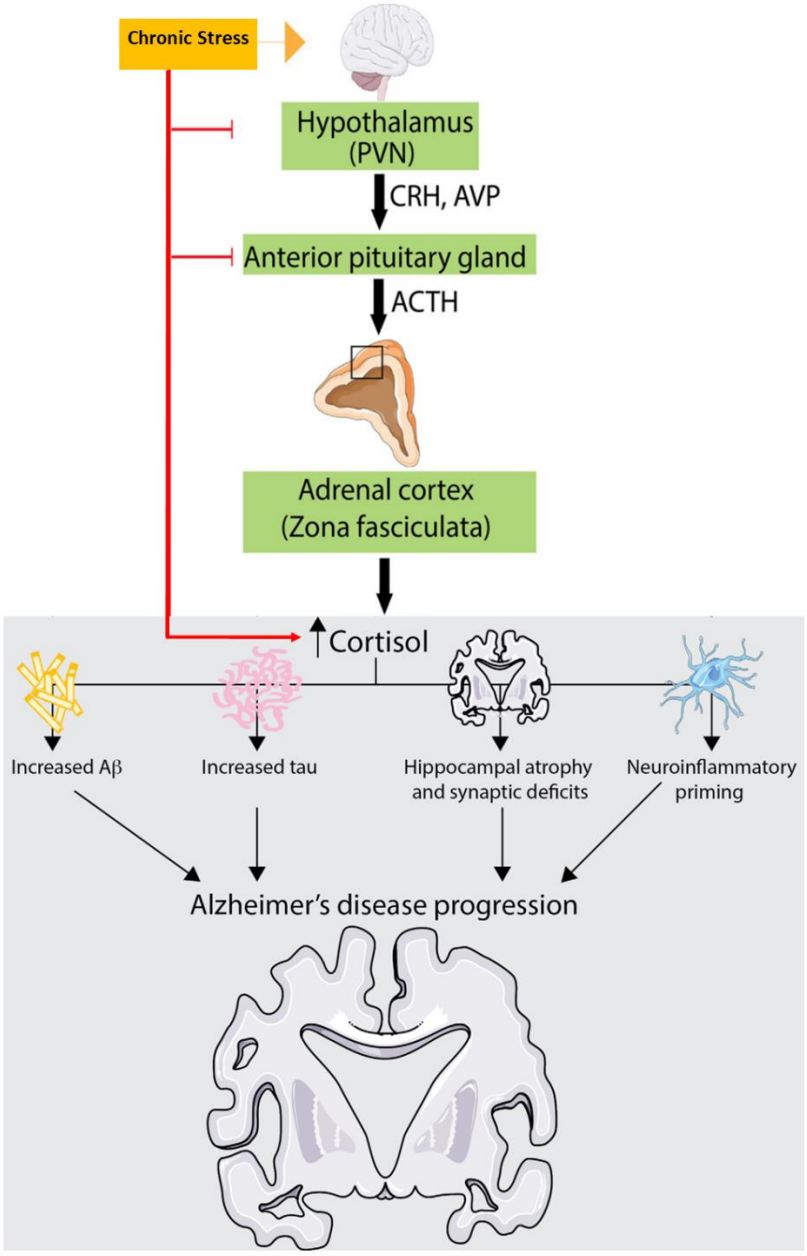

Figure 1. Underlying mechanisms linking chronic stress, hypothalamus–pituitary–adrenal (HPA) axis and Alzheimer's disease (AD) progression. (Adapted from (De Kloet et al., 2005; Milligan Armstrong et al., 2021)).

Although there is evidence to support the involvement of stress in AD, the link between PTSD and AD remains elusive. Therefore, investigating stress-cognition linkages using neurobehavioral and molecular approaches can prove helpful in understanding the common underlying mechanisms relating to the comorbidity (Greenberg et al., 2014).

A few studies have elucidated the molecular link between PTSD and AD. For example, Justice and co-workers showed that acute and chronic PTSD-like induction led to an increase in Aβ production

(Justice et al., 2015). Elevated Aβ, in turn, accelerates AD pathogenesis which can be exacerbated by acute stress exposure (Carroll et al., 2011; Justice et al., 2015). Justice and co-workers showed that the expression of APP and PSEN1 exacerbated neurobehavioral and endocrine responses following PTSD-like induction. In turn, the PTSD-like induction increased Aβ circulating in the CSF mediated by CRF-1 pathway in the HPA axis (Justice et al., 2015). They also found that Aβ can lead to hyperexcitation of the CRF neurons, therefore suggesting molecular mechanisms by which AD-like pathology can interact with PTSD-like conditions (Justice et al., 2015). Another review investigating the bidirectional associations between AD and PTSD revealed that the development of PTSD in mid-life can induce AD in late-life via hypometabolism in the brain areas associated with the diseases, including the hippocampus and cortex (Ridge et al., 2013).

Although genetic factors between AD and psychiatric disorders have been studied, there is not enough evidence suggesting common molecular mechanisms for AD and PTSD (Brady et al., 2000; Gibson et al., 2017). Agis et al. sought to provide evidence of molecular mechanisms linking AD and PTSD (Agís-Balboa et al., 2017). They studied the role of the Formin 2 (Fmn2) gene, previously screened as member genes dysregulated in the hippocampus of ageing mice (Peleg et al., 2010; Schuh, 2011). Fmn2 is a gene involved in actin dynamics and synaptic functions, and its downregulation leads to cognitive deficits both in mice and humans (Almuqbil et al., 2013; Law et al., 2014; Peleg et al., 2010). Agis et al. showed that the loss of Fmn2 led to increased PTSD-like responses and impaired synaptic plasticity in mice. They also found that the Fmn2 knock-out mice developed an increasing age-related memory deficit. They, therefore, concluded that Fmn2 is implicated in both AD and PTSD, suggesting a link between these two diseases (Agís-Balboa et al., 2017).

Furthermore, the MS4A (Membrane Spanning 4-Domains A) is a family of genes that code for transmembrane proteins involved in signal transduction and cell activation, and are largely expressed in the glial cells (Pimenova et al., 2018). MS4A6A gene is a member of the MS4A gene family, located on chromosome 11. A recent study using human datasets, found an association for the MS4A6A. They demonstrated that MS4A6A is a common genetic signature for AD and PTSD and suggested that immune response is a common pathway for PTSD and AD (Lutz et al., 2020).

1.4 Role of oxidative stress in PTSD and AD

Oxidative stress results from an imbalance between oxidants and antioxidants in favour of pro-oxidants (Sies et al., 2017). Oxidative stress has been implicated in neurodegeneration (Dasuri et al., 2013). It has been hypothesized that AD progression is a result of redox imbalance because oxidative stress markers have been found early both in the brain and the plasma of humans and in animal models of AD (Collin et al., 2018; Tönnies and Trushina, 2017; Youssef et al., 2018). Similarly, Aβ and tau pathology induces neuronal damage via increased oxidative stress (Martin et al., 2017).

Similarly, several reports have implicated oxidative stress in neuropsychiatric disorders, including PTSD (Bremner, 2006; Hassan et al., 2016; Nakao et al., 2021). Preliminary clinical evidence for the involvement of oxidative stress in the pathophysiology of PTSD comes from cross-sectional studies that have found significant differences in blood antioxidant enzyme concentrations and oxidative stress-related gene expression between PTSD patients and controls (Miller et al., 2018). Other studies also point to the presence of depleted levels of glutathione transferases (which interact with GSH to detoxify pro-oxidant molecules) in the pathophysiology of PTSD (Miller et al., 2018). (For review, see (Miller et al., 2018)). The Nrf2 signalling has also been shown to be involved in the defence mechanisms against ROS in psychiatric conditions (Bhandari et al., 2021).

At the cellular level, oxidative stress is regulated via the interaction of nuclear factor erythroid 2-related factor 2 (Nrf2) with Kelch-like ECH-associated protein 1 (Keap1), thereby highlighting Nrf2 as a key target in AD treatment (Dinkova-Kostova and Talalay, 2008; Itoh et al., 1999). Nrf2 acts as a transcription factor that regulates the expression of antioxidant enzymes found in many body cells and tissues (Nguyen et al., 2009). At the basal level, Nrf2 is found inactive in the cytoplasm of the cell, coupled with Keap1. This binding has been shown to make Nrf2 vulnerable to degradation (Zhuang et al., 2017). However, in the presence of reactive oxygen species, Nrf2 unbinds from Keap1, translocates into the nucleus, where it binds with antioxidant response element (ARE) (Jiang et al., 2016). This coupling stimulates the transcription of ARE genes leading to the production of antioxidant enzymes and molecules in the cell (Zhuang et al., 2017).

1.5 Apoptotic dysregulation in PTSD and AD

Apoptosis is commonly called programmed cell death as it defines how cells progress towards death following stimuli (Kataoka and Tsuruo, 1996). Although several stimuli can initiate apoptosis, it is coordinated by a few molecules called caspases (Álvarez et al., 2011; Leong et al., 2020). Apoptosis can occur via two pathways: (1) the mitochondrial or intrinsic apoptosis pathway; and (2) the death receptor-mediated or extrinsic apoptosis pathway. The extrinsic pathway involves the activation of procaspase 8 or 10 (Álvarez et al., 2011). Caspase 8 activation further leads to either caspase 3 activation or indirectly cleaving BH3, which after relocating to the mitochondria, stimulate the release of cytochrome-c and eventual caspase-3 activation leading to cell death (Álvarez et al., 2011; Siegel, 2006). On the other hand, the intrinsic apoptotic pathways can be initiated by various intracellular conditions like oxidative stress, dysregulated calcium ion influx or other conditions that induce the permeabilization of the mitochondria outer membrane (Figure 2) (Galluzzi et al., 2012; Kanamaru et al., 2015). The convergence of the intrinsic and extrinsic pathways is the activation of caspase-3, also called the execution caspase (Sun et al., 2011). Caspase-3 activation causes DNA fragmentation,

chromatin condensation, and disintegration of cellular components into apoptotic bodies that are further lysed for removal from the cell (Elmore, 2007; Gervais et al., 1999).

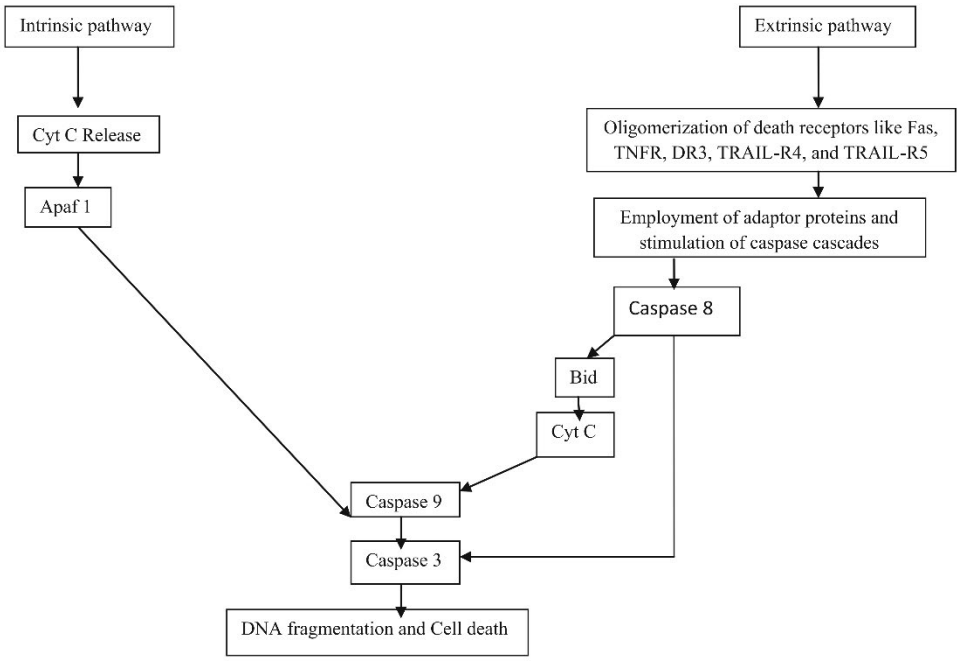

Figure 2. Intrinsic and extrinsic pathway of apoptosis (Obulesu and Lakshmi, 2014)

AD brains experience significant neuron loss, which likely contributes to the progressive decline of memory and cognitive functions (Silva et al., 2012). In addition, oxidative stress, Aβ and tau pathologies are also hallmarks of the pathophysiology of AD (Obulesu et al., 2011; Szewczyk, 2013). The aggregation and deposition of Aβ are considered critical to the development of AD (Selkoe, 2002; Selkoe and Hardy, 2016). The most common isoforms of Aβ are Aβ(1-40) and Aβ(1-42). Although Aβ(1-40) is the predominant species, Aβ(1-42) is more fibrillogenic and neurotoxic (Yamaguchi et al., 2013), suggesting that Aβ(1-42) plays a critical role in the initiation of plaque formation and AD onset. Evidence shows that Aβ forming plaques induce progressive loss of neurons and synapses (Mucke and Selkoe, 2012). Aβ(1-42) is known to form “amyloid channels” that facilitate increased calcium ion influx into the cell, leading to neurotoxicity-induced apoptosis by activating the cytochrome-c and caspase pathways (Aleardi et al., 2005; X.-J. J. Han et al., 2017; Heo et al., 2002; Lee et al., 2016; Lin et al., 2001). The primary phenotypic characteristics of AD is the gradual decline in cognitive abilities resulting from neuronal loss due to apoptosis (Silva et al., 2012). It has also been shown that AD brains display increased DNA fragmentation, a marker of apoptosis (Colurso et al., 2003).

There is evidence of increased cellular apoptosis following PTSD-like induction. For instance, several hallmarks of apoptosis have been observed in the amygdala and prefrontal cortex of rodents following

single prolonged stress (SPS) (Han et al., 2015; Wen et al., 2016). Glutamate is thought to play a vital role in the pathogenesis of PTSD (Kelmendi et al., 2016) for several reasons: glutamate underlies synaptic plasticity and memory formation (Lamprecht and LeDoux, 2004), stress significantly impact glutamate transmission (Popoli et al., 2012), and the glutamate receptor antagonist ketamine may have efficacy in treating PTSD (Feder et al., 2014). An imbalance between the levels of glutamate and GABA has also been shown to favour neuronal apoptosis in the hippocampus of rats following stress exposure (Gao et al., 2014). As shown by previous studies, three components are thought to be involved in the apoptosis pathway in PTSD (Li et al., 2010; Wan et al., 2016). Firstly, following varying apoptotic signals, membrane potential can be affected by genetic or non-genetic cortisol activity (Falkenstein and Wehling, 2000). For the non-genetic activity, stress stimulates cortisol release, which activates glucocorticoid receptors (GR). This activated GR then couples to the mitochondria, altering its membrane potential. On the other hand, the genetic activity involves GRs binding to specific molecules (for instance, heat shock protein 70), which then translocate to the nucleus, where it initiates transcriptional activities (Jia et al., 2018). GR activation can then cause an increase in BAX, which translocates to the mitochondria, where it couples with MAP-1, leading to changes in mitochondria membrane potential, which in turn, cause a release of cytochrome-c and a subsequent caspase activation leading to cell death (Lindsten et al., 2005; Tan et al., 2005).

1.6 The role of synaptic transmission in PTSD and AD

Synapses form a fundamental communication unit between neurons, and their dysfunction is implicated in several neurological disorders (Fu and Ip, 2017; Maiti et al., 2015). Previous reports have shown that loss of synapses in AD leads to cognitive deficits (De Wilde et al., 2016; Forner et al., 2017; Querfurth and LaFerla, 2010). Aβ and tau are also implicated in the pathogenesis of AD as they are known to act at the synapses (Forner et al., 2017), leading to synaptotoxicity (Sheng et al., 2012). The accumulation of Aβ at the presynapse is due to the accumulation of BACE1, which cleaves the APP to form Aβ (Ye et al., 2017). The accumulation of Aβ at the synapse consequently disrupts basic presynaptic functions, including axonal transport, synaptic vesicle cycling, and neurotransmitter release (Chen et al., 2019). Aβ oligomers target the postsynaptic compartment of excitatory synapses with high affinity leading to synaptic dysfunction by forming complexes with several plasma membrane molecules (Koffie Robert M et al., 2009). Although Aβ oligomers have been implicated in synaptic dysfunction, the Aβ-synapse specificity is still unclear (Chen et al., 2019).

ApoE ε4 is the single most important genetic factor associated with an increased risk of developing AD (Fenoglio et al., 2018; Harold et al., 2009). However, ApoE ε2 is associated with a reduced risk of developing AD but with an increased risk of developing more severe memories in a PTSD condition (Johnson et al., 2019). The second risk gene associated with LOAD pathology is the Bridging

Integrator 1 (BIN1) gene (Lee et al., 2011; Naj et al., 2011). BIN1 belongs to the Bin/Amphiphysin/Rvs family of adaptor protein family that is involved in the regulation of membrane dynamics and synaptic vesicle endocytosis (De Rossi et al., 2019; Prokic et al., 2014). BIN1 is localized at presynaptic neurons, and its downregulation in cortical neurons has been shown to increase Aβ production (Miyagawa et al., 2016). Studies have shown that the downregulation of neuronal BIN1 impaired APP and BACE1 sorting into vesicles, therefore increasing the contact between APP and BACE1, leading to increased Aβ production (Ubelmann et al., 2017).

Furthermore, AD pathology characterised by Aβ plaque formation and subsequent synaptic dysfunction is also associated with glutamate excitotoxicity (Zhang et al., 2016). The N-methyl D-aspartate receptors (NMDARs) belongs to a family of ligand-gated glutamatergic receptors that play critical roles in synaptic transmission and cognition (Zhang et al., 2016). Increased glutamate causes overactivation of NMDA receptors, causing an increase in calcium ion influx into the cell. Excessive calcium influx disrupts mitochondrial membrane permeability, leading to cytochrome-c release, increased ROS generation and eventually, cellular apoptosis (Ferreira et al., 2012; Kamat et al., 2013). NMDARs are localized on both pre- and postsynaptic neurons. While presynaptic NMDARs are involved in synaptic transmission and plasticity, they act as detectors for short- and long-term synaptic plasticity (Duguid, 2013). Aβ induces glutamate release, which in turn cause overactivation of neuronal NMDA leading to synaptic loss (Talantova et al., 2015). In addition, Aβ oligomers have been shown to impair NMDARs-dependent long-term potentiation at the hippocampus synapses (Danysz and Parsons, 2012). NMDAR activation also induces elevated Aβ production (Zhang et al., 2016).

Several studies have also implicated increased extracellular glutamate in PTSD pathogenesis (Nishi et al., 2015; Perez-Garcia et al., 2019; Prasad and Bondy, 2015). Glutamate activity in PTSD is mediated by increased CRF and a subsequent glucocorticoid production induced by stress (Nair and Ajit, 2008). An increase in glucocorticoid leads to elevated glutamate release and eventual NMDAR overactivation (Nair and Ajit, 2008). These changes can induce neuronal cell death, which impacts cognitive functions associated with PTSD (Harvey et al., 2004; Nair and Ajit, 2008). In addition, it was observed that neuronal cell death correlated with an imbalance between glutamate and GABA, and overexpression of NMDAR in the amygdala and hippocampus impaired synaptic functions (Gao et al., 2014; F. Han et al., 2017; Yamamoto et al., 2008).

The HPA axis plays a critical role in stress regulation (Gaffey et al., 2016). Stress stimulates the release of Corticotrophin-releasing hormone (CRH), which stimulates the release of Adrenocorticotropic Hormone (ACTH), which then causes the release of cortisol (humans) or corticosterone (rodents) (Vale et al., 1981). The release of glucocorticoids activates glucocorticoid receptors (GRs) which also provide negative feedback inhibition to regulate the activities of the HPA

axis (Reul and De Kloet, 1985). Stress-induced HPA axis dysregulation is a characteristic feature in people susceptible to developing neuropsychiatric disorders (Yehuda et al., 2004). This dysregulation occurring at any stage can induce acute and chronic negative outcomes in the brain, including apoptosis, synaptic dysfunction, and cognitive deficits(Arango-Lievano and Jeanneteau, 2016; Hori et al., 2010; Yehuda et al., 2004).

Glucocorticoid receptors are transcription factor and act in concert with FK506 binding protein 51 (FKBP5) to regulate GRs sensitivity (Binder, 2009; Grad and Picard, 2007; Touma et al., 2011). FKBP5 regulates stress by reducing the binding affinity of glucocorticoids to GRs (reduced GR sensitivity), thereby preventing glucocorticoid receptor translocation into the nucleus of the cell (Binder, 2009). The cells of the hippocampus and amygdala have a high expression of GRs, which are involved in regulating responses to stress and trauma exposure (Greenberg et al., 2014; Videlock et al., 2008). FKBP5 not only regulates GR function but has also been shown to be involved in glutamatergic neurotransmission and synaptic plasticity (Blair et al., 2019; Rein, 2016). In fact, there is evidence that GRs regulate NMDA receptor subtype 2B function (de Kloet et al., 1998; Huang et al., 2010).

FKBP5 knock-out mice exhibit age-related neuropsychiatric phenotypes (O'Leary et al., 2011). HPA axis dysregulation increases Aβ accumulation leading to an upregulation of FKBP5 with age in animal models of AD (Blair et al., 2013; Brendel et al., 2015). In particular, reports have shown a relationship between trauma exposure, FKBP5 and PTSD (Segman et al., 2005; Yehuda, 2009). Further evidence also found a link between FKBP5 genetic risk and GR resistance, suggesting that people with certain FKBP5 risk alleles could be vulnerable to developing PTSD (Mehta and Binder, 2012; Young et al., 2018). These findings suggest that FKBP5 may be a viable biomarker for PTSD (Harms et al., 2017; Young et al., 2018).

1.7 Animal models of Alzheimer's Disease

Although developing an animal model with AD pathology is not easy due to the complexity of the human brain and an unclear pathology of the disease, transgenic animals have been used extensively to study AD as they model important aspects of the disease (De Felice and Munoz, 2016; Orta-Salazar et al., 2016; Sasaguri et al., 2017). However, there has been a limitation in the translation of findings to clinical benefits because of the demerits of the artificial nature of generating transgenic animals (Kasza et al., 2017). Therefore, an ideal AD model should exhibit several aspects of the disease, including its aetiology and a time-dependent progression of the pathology, which involves similar structures and cells, alike in human pathology (Kasza et al., 2017).

AD pathogenesis is characterised by the formation and accumulation of Aβ oligomers, and Aβ oligomers have been shown to induce synaptic loss (Kasza et al., 2017; Selkoe, 2008; Shankar and Walsh, 2009). Consequently, Aβ peptides have been extensively used to model AD, usually injected intrahippocampally or intracerebroventricularly (ICV) into mice or rats (Balducci et al., 2010; Facchinetti et al., 2018; Kim et al., 2016). The direct infusion of oligomeric Aβ-42 into the brain of rodents provides an excellent in vivo model of AD amyloidopathy (Baleriola et al., 2014), which produces critical aspects of the AD pathology, including neuronal apoptosis, inflammatory responses, oxidative stress and ultimately cognitive dysfunction (Facchinetti et al., 2018; Luo et al., 2020; Zare et al., 2015). More importantly, this method offers adequate manipulation of Aβ peptide levels in a way that prevents any compensatory effects that may be found in a transgenic model (Jean et al., 2015).

1.8 Animal models of Post-traumatic stress disorder

Fear is necessary for survival, as it allows individuals to perceive and respond to threats adequately. However, exaggerated fear responses can precipitate neuropsychiatric conditions, including PTSD. A characteristic feature of PTSD is exaggerated fear response to mild trauma, particularly one that reminds the individual of the initial traumatic experience and a possibility of developing new fears (Dykman et al., 1997; S. Fanselow, 1984). In rodents, fear is measured as freezing, a behaviour that is considered relevant and an ethologically valid indicator of fear in humans and rodents (Fanselow and Bolles, 1979; S. Fanselow, 1984).

Previous reports elucidating the fundamental mechanisms of PTSD have employed several types of stressors, including single prolonged stress, predator stress, social defeat stress, and footshocks (Adamec et al., 2006; Daskalakis and Yehuda, 2014; Jia et al., 2018; Koolhaas et al., 2013; Zoladz et al., 2008). Among these, footshock is the most common method of stress exposure because it is uncontrollable, unpredictable and a single exposure can induce long-lasting symptoms (Bali and Jaggi, 2015). The electric footshock model involves a physical stressor with emotional aspects, and it is regarded as one of the best models to induce PTSD in rodents (Philbert et al., 2011; Siegmund and Wotjak, 2007). Other significant merits of footshocks include (1) it offers adequate manipulation of the delivery strategy, a concise number of shocks and current intensity, length of exposure and inter-shock interval; (2) the context can be easily replicated; (3) the changes can be adequately adjusted to suit the desired context; (4) in comparison to restraint stress, habituation is minimized (Siegmund and Wotjak, 2007). The stress-enhanced fear learning (SEFL) paradigm is a robust PTSD model based on footshock stress that uses some aspects of the Pavlovian fear conditioning (Perusini and Fanselow, 2015). In contrast, it produces sensitized fear response far greater than basic conditional fear responses, and it represents the exaggerated fear responses following intense trauma seen in PTSD patients (Perusini and Fanselow, 2015; Rau et al., 2005). The SEFL can induce increased anxiogenic

behaviour and glucocorticoid dysregulation (Perusini et al., 2016; Poulos et al., 2015). Thus, the SEFL proves to be a powerful tool in elucidating the mechanisms underlying PTSD pathology.

1.9 Rationale of the study

Causal links between PTSD and AD, although suggested, have not been established (Greenberg et al., 2014). Current evidence suggests that PTSD and AD have a bidirectional relationship (Desmarais et al., 2019). Studies of biological mechanisms associated with shared physiopathology between PTSD and AD are needed (Lutz et al., 2020). In addition, the scarce data elucidating the common susceptibility factors have led the mechanism involved in this risk susceptibility to being poorly understood. Therefore, it has become increasingly important to explore more mechanisms to help understand biological factors involved in the risk of developing AD after developing PTSD. Understanding the common susceptibility factors of PTSD associated with AD may provide new therapeutic approaches to treating PTSD and preventing the risk of developing AD.

1.10 Aim of the study

As the incidence and prevalence of AD are greater with PTSD, it is not clear if it is due to a common risk factor underlying PTSD and AD. Therefore, this study aimed to elucidate the effects of trauma-like exposure in an amyloid-beta (1-42) rat model of AD to identify common susceptibility factors underlying the pathophysiology of PTSD and AD.

1.11 Study objectives

To evaluate the effect of trauma-like exposure on amyloid-beta (1-42)-induced:

- learning and memory, and anxiety-like behaviours in rats;
- regulation of redox homeostasis in the brain of rats via the expression of antioxidant genes; and
- stress dysregulation, glutamate excitotoxicity and neuronal apoptosis in the brain of rats.

1.12 Clinical Implications

The overall approach used in this study has the potential to provide biologically relevant information about shared pathophysiology between AD and PTSD. The shared aetiology establishes a molecular phenotype that may extend to and explain, at least in part, some of the heterogeneity of the neuropsychiatric responses in AD, therefore providing therapeutic understanding in the treatment of PTSD while also preventing the risk of developing AD.

1.13 References

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Prologue

Chapter 2 of this study elucidated the effect of PTSD-like induction on redox homeostasis and cellular apoptosis in the amygdala and hippocampus of Aβ(1-42)-lesioned AD rat model. This chapter also examines the association of these molecular changes with anxiogenic and fear memory responses. This chapter is prepared in a manuscript format and has been published in the journal Neurochemistry International, Impact factor: 3.921. https://doi.org/10.1016/j.neuint.2021.105170. See Appendix IV.

2 **CHAPTER 2**

Exposure to footshock stress downregulates antioxidant genes and increases neuronal apoptosis in an Aβ(1-42) rat model of Alzheimer's disease

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Abstract

Post-traumatic stress disorder (PTSD) is a neuropsychiatric disorder that develops from exposure to trauma, mostly when normal psychological mechanisms fail. Studies have shown that people who have PTSD are susceptible to developing dementia, mostly Alzheimer's disease (AD), suggesting common underlying risk factors in the comorbidity. However, data elucidating links between these conditions is scarce. Here we show that footshock stress exacerbates AD-like pathology. To induce a trauma-like condition, the rats were exposed to multiple intense footshocks followed by a single reminder. This was followed by bilateral intrahippocampal lesions with amyloid-beta (Aβ) (1-42), to model AD-like pathology. We found that footshocks increased anxiety behavior and impaired fear memory extinction in Aβ(1-42) lesioned rats. We also found a reduced expression of nuclear factor erythroid 2-related factor 2 (Nrf2), NAD (P) H: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and an increased expression of Kelch-like ECH-associated protein 1 (Keap1) in the amygdala and hippocampus. Furthermore, oxidative stress level was sustained, which was associated with increased apoptosis in the amygdala and hippocampus.

Our finding suggests that AD-like pathology can induce oxidative changes in the amygdala and hippocampus, which can be exaggerated by footshock stress.

Keywords: Alzheimer's disease;post-traumatic stress disorder;apoptosis;fear extinction; amyloid-beta;oxidative stress

2.1 Introduction

Post-traumatic stress disorder (PTSD) is a fear-based psychological disorder that results from exposure to traumatic events (1). It is characterized by the sufferer's inability to dissociate a previous adverse event from

a perceived new threat. The ability to differentiate between threats occurring at different time points requires cognitive flexibility and extinction learning (2). In humans, increased stress responses found in PTSD are associated with neuropsychiatric dysfunctions and increase vulnerability to other diseases, including Alzheimer's disease (AD) (3–5). Coincidentally, studies have shown that people with PTSD have twice the risk of developing dementia, mostly AD (6–9). However, the underlying mechanisms are not well understood. It has also been shown that prolonged stress increased amyloid plaque formation and amyloid-beta (Aβ) release in AD animal models (10–12). Aβ peptides also influenced PTSD-like responses via the corticotropin-releasing factor 1 receptor activation, suggesting that PTSD-like trauma can exacerbate AD dementia, increasing PTSD responses (13). Also, the genetic mutation of Formin 2, a gene associated with synaptic transmission and memory function, was found to affect hippocampal plasticity, leading to fear memory impairment in AD animal models (14–17). Although stress has been implicated in dementia, the link between PTSD and AD remains unclear (18–20). Recent studies have shown that oxidative stress plays a critical role in neuronal dysfunction and neurodegeneration (21). Similarly, oxidative stress is involved in stress-induced pathologies (22). The nuclear factor erythroid 2-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) pathway regulates oxidative stress involved in AD pathology (23,24), as well as psychiatric disorders (25). In this study, we tested if footshock stress could exacerbate fear and anxiety-like behaviors in Aβ(1-42) rat AD model. We also aimed to determine if footshocks stress would exaggerate the activities of Nrf2-Keap1 and its downstream antioxidant genes in the hippocampus and amygdala of Aβ(1-42) rat AD model.

2.2 **Materials and methods**

Animals

Male Sprague Dawley rats (10 weeks old) were used for this study. All protocols were approved by the Animals Research Ethics Committee of the University of Kwazulu-Natal, with the number AREC/005/019D. Rats were housed under standard conditions (Temperature, 21±2°C; humidity, 55–60%; lights on at 07:00 and off at 19:00 hrs). Food and water were provided to the animals ad libitum. Rats were randomly divided into four groups as follows: (1) Control: received no footshock; vehicle infusion (sham surgery), n=8 (2) Shock: received footshocks; vehicle infusion (sham surgery), n=8 (3) Aβ(1-42): received no footshocks; amyloid-beta (1-42) infusion, n=8 (4) Shock+Aβ(1-42): received footshocks; amyloid-beta (1-42) infusion, n=8.

Experimental design:

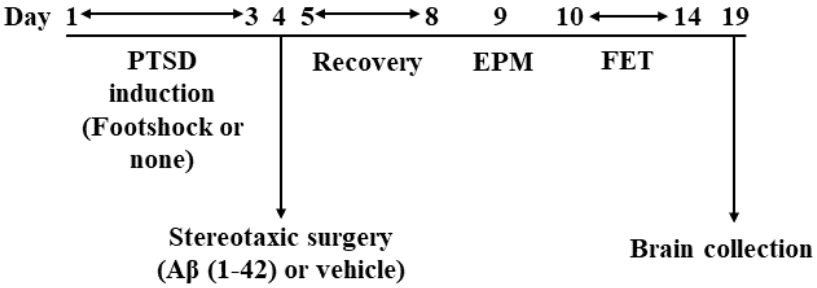

Figure 1. Experimental timeline.

On day 1, animals received footshocks or nothing and were tested for freezing over the next two days. Twenty-four hours after, they received a bilateral intra-dCA1 infusion of Aβ(1-42) or vehicle solution. After five days of recovery, animals were tested on the elevated plus maze (EPM), followed by another five days for fear extinction test (FET). Animals were euthanized on day 19 (2 weeks post-lesion).

Animal model of Post-traumatic stress disorder

A post-traumatic stress disorder-like condition was induced using the stress-enhanced fear learning protocol (26,27). The procedure took three days. On day 1, each animal was placed in the conditioning chamber (Context A), and 15 non-cued footshocks of 1mA for 1second were administered over 90 minutes, after which the freezing behavior was measured for 5 minutes (fear to trauma). On day 2, the animal was placed in a new conditioning chamber (Context B), allowed to habituate for 3 minutes, and the freezing behavior was measured (fear generalization). Following the habituation, the animal received a single footshock. On day 3, Context B was repeated without footshock, and the freezing behavior was measured over 5 minutes (fear to mild shock). The sham-treated animals were also placed in the conditioning chambers for the prescribed duration without the footshocks. Freezing behavior was reported as the percentage time the animal stopped moving (except for respiration) for the observation period (26,28).

Animal model of Alzheimer's disease

Intrahippocampal Aβ(1-42) lesion as a model of Alzheimer's disease has been widely used and validated (29–32). Animals were deeply anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg) via the intraperitoneal route. Complete anesthesia was determined by the loss of the toe pinch reflex (30). Animals were then placed on the stereotaxic apparatus (David Kopf Instrument, Tujunga, USA) and lesioned bilaterally with amyloid-beta (1-42) peptide (Thermo Fisher Scientific, USA) using a 25G Hamilton syringe. 5 µl of Aβ(1-42) was infused at a rate of 1 µl/min into each side of the brain targeting the hippocampus using the following coordinates from bregma: Anteroposterior (AP) = −4.8 mm; mediolateral (ML) = ±3.4 mm; dorsoventral (DV) = −3.0 mm) (33). After the infusion, the needle was left in place for 2 minutes to allow for proper diffusion of the peptide. After removing the needle, the incision was sutured, disinfected, and the animal was placed under a heating lamp as it recovered from the anesthesia. Following recovery, the animals

were injected with temgesic (0.05 mg/kg, subcutaneously) before returning to their home cages. Sham-treated animals were infused with the same amount of phosphate-buffered saline (PBS).

Elevated plus maze test (EPM)

The animals were further assessed for anxiety-like behavior using the elevated plus maze apparatus. Beyond its use as a standard model in screening anxiolytic compounds, the EPM is also used to study effects on brain areas, such as the amygdala and hippocampus, involved in anxiety behavior (34,35). The apparatus consists of two closed arms, and two open arms elevated 60 cm above ground level (36). The test entailed placing the animals in the center of the maze facing the open arm, and anxiety-like behavior was measured by the number of entries and time spent in the closed arms (37,38). Entry was defined as all four paws being positioned inside the arm. A video camera placed above the apparatus to eliminate human interference was used to record the test.

Fear extinction test

Animals were returned to the shock chamber daily for 5 days without exposing them to the shock stimulus. Freezing behavior was measured over 5 minutes (39).

Sample collection

At the end of the behavioral testing, animals were decapitated using a sharp guillotine. The whole brain was quickly removed, rinsed in cold 0.9% saline. The hippocampus and amygdala were dissected on a cold glass plate, transferred into Eppendorf tubes, snapped frozen in liquid nitrogen, and stored in a biofreezer at -80°C until analyses.

Oxidative stress profile

The extent of lipid peroxidation (using malondialdehyde (MDA) as a marker) was measured as per the protocol proposed by Dahle and colleagues (40). Catalase (CAT) was measured as described by Sinha (41), and the concentration of superoxide dismutase (SOD) was estimated using a commercially available ELISA kit (Elabscience, Wuhan, China). Glutathione (GSH) was measured by a reaction of the supernatant and 5,5-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent). Protein measurement was done according to the kit manufacturer's protocol (BioRad, CA, USA). All absorbance measurements were read using a microplate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany).

Neuronal cell isolation and fluorescent activated cell sorting (FACS) analysis

This was carried out as described by (42) and (43). Briefly, brain tissue was allowed to thaw on a cold glass plate, after which two drops of PBS-T (Sigma-Aldrich) was added, after which the tissue was minced (100 times orthogonally) using a pre-chilled razor. The minced tissue was transferred into microcentrifuge tubes using the razor, and 650 µl of cold PBS-T was added. The suspension was passed through a 19G needle 12 times. The mixture was centrifuged at 110 xg for 2 minutes, after which the supernatant was discarded. 1 ml

of cold Accutase enzyme (Life Technologies, CA, USA) was added slowly to the pellet and placed on a rotary shaker for 20 minutes at 200 rpm at room temperature. The tissue was centrifuged and washed in cold PBS-T. The tissue was then passed through 4 rounds of trituration in 600µl cold PBS-T with 12 times passes once in 21G and 23G and twice in 25G needles. After each pass, the cloudy supernatant was collected and placed in a separate tube. All supernatant was collected and passed through a 40 µm mesh cell strainer (Corning, Sigma Aldrich, South Africa). Cells were incubated with Hoechst 33342 (1mM) and Propidium iodide (PI) (250 micrograms/ml) for 20 minutes (Invitrogen, V35118), which provides simultaneous detection of early and late apoptotic cells (44–46). Labeled cells were analyzed on a BD FACSCanto II (BD Bioscience, Franklin Lakes, NJ, USA), Hoechst 33342, excitation/emission maxima ~350/461 nm, and PI, ~535/617 nm. Internal control was performed using the (CST) beads, and BD ™ Compbead compensation particles were used to compute for spectra overlap, according to the manufacturer's instructions (BD Bioscience, Franklin Lakes, NJ, USA).

Quantitative Polymerase Chain Reaction (RT-qPCR)

Brain tissue was homogenized in DNA/RNA Shield™ (1:6) using a Tissue Sonicator (BuenoBiotech, China). Then, total RNA was extracted using the Zymogen extraction kit. The concentration of the extracted RNA was determined using the NanoDrop™ Spectrophotometer (Thermofisher Scientific, USA). The RNA purity value of 1.7–2.1 from optical density 260/280 nm was used for cDNA synthesis. Reverse transcription was carried out using the iScript™ cDNA synthesis kit (Biorad). In addition to the cDNA template, the PCR reaction mixture contained 20 µM of each primer (Table 1), SYBR green (Biorad), and nuclease-free water to a total volume of 10 µl, and was run on a Roche LightCycler 96 system. The reaction condition was set as follows: denaturation at 95°C, 10 seconds; primer annealing at 61°C (Nrf2), 61°C (Keap1), 60°C (NQO1), 61°C (HO-1), and 60°C (GAPDH) for 20 seconds; elongation at 72°C, 20 seconds. Gene expression was normalized against GAPDH (reference gene) using the $2^{-\Delta\Delta}$CT method (47,48).

Table 1. List of primers

Gene	Forward	Reverse
Nrf2	CAGCATGATGGACTTGGAATTG	GCAAGCGACTCATGGTCATC
Keap1	AACTCGGCAGAATGTTACTACCC	CTACGAAAGTCCAGGTCTCTGTCTC
NQO1	CATTCTGAAAGGCTGGTTTGA	CTAGCTTTGATCTGGTTGTCG
HO-1	AGAGGCTAAGACCGCCTTCC	ATTTTCCTCGGGGCGTCTCT
GAPDH	TGTGAACGGATTTGGCCGTA	ATGAAGGGGTCGTTGATGGC

Statistical Analysis

All data represent means ± SEM. Fear extinction test was analyzed using a two-way repeated-measures ANOVA followed by Dunnett's multiple comparisons test. Other post-lesion analyses were conducted using two-way ANOVA followed by Tukey's post hoc analyses. Data were analyzed using GraphPad Prism version 7 (San Diego, CA, USA). $P<0.05$ was considered significant.

2.3 **Results**

Footshocks increase freezing levels in the SEFL paradigm

As shown in Table 2, four groups of animals viz control, shock, Aβ(1-42), and shock+Aβ(1-42) were analyzed for freezing behavior after exposure to footshocks. Two-way ANOVA showed a significant interaction between shock administration across days of exposure on freezing levels ($F (6, 56) = 56.48$, $p<0.0001$). There was also a time effect ($F (2, 56) = 192$, $P<0.0001$) and a shock effect ($F (3, 28) = 142.9$, $p<0.0001$) on freezing levels. Post hoc analyses revealed a significant increase in freezing levels on day 1 in the animals that received footshocks when compared to the animals that did not receive any footshock (shock; shock+Aβ(1-42) vs. control; Aβ(1-42), $p<0.0001$) (Table 2A). This effect was reversed in Context B before introducing the single footshock on day 2 (Table 2B). On (day 3), twenty-four hours after a single footshock was administered on day 2 in context B, freezing levels significantly increased in the animals that received footshocks compared to the ones that did not receive any footshock (shock; shock+Aβ(1-42) vs. control; Aβ(1-42), $p<0.0001$) (Table 2C).

Table. 2: Effect of pre-lesion footshocks on freezing levels

	Control (%freezing)	**Shock (%freezing)**	**Aβ(1-42) (%freezing)**	**Shock+Aβ(1-42) (%freezing)**
(a) Fear to trauma - day 1	9.5± 2.62	79.41± 6.01[a]	12.75± 2.77	77.76± 6.14[a]
(b) Fear generalisation - day 2	4.62± 1.53	9.5±1.27	5.62±1.43	7.38±1.86
(c) Fear to mild shock - day 3	2.52± 1.28	85.26± 6.97[a]	8.49± 2.62	93.1±2.21[a]

(a) Fear to trauma - day 1: after 15 (1mA, 1s) footshocks (b) Fear generalisation - day 2 (c)) Fear to mild shock - day 3: 24 hours after exposure to a single 1mA, 1s footshock. Data expressed as mean ± SEM. Two-way ANOVA followed by Tukey's post hoc test. Interaction, $F (6, 56) = 56.48$, $p<0.0001$; time effect, $F (2, 56) = 192$, $P<0.0001$; shock effect, $F (3, 28) = 142.9$; [a]$p<0.0001$, shock; shock+Aβ(1-42) vs. control; Aβ(1-42), $p<0.0001$, n=8/group.

Aβ(1-42) lesion impaired fear extinction

As shown in Figure 2, four groups of animals viz control, shock, Aβ(1-42), and shock+Aβ(1-42) were analyzed for fear extinction. Two-way repeated measure ANOVA showed a significant interaction between the effects of condition (footshocks and Aβ(1-42)-lesion) and extinction days (time) on freezing levels ($F(15, 135) = 4.154$, $p<0.0001$). Post hoc analyses revealed that the shock group displayed a gradual decline in freezing behavior up to day 5 compared to its corresponding pre-lesion freezing level ($p<0.001$). In contrast, Aβ(1-42) group showed a progressive increase in freezing when compared to their pre-lesion freezing level ($p<0.01$). However, shock+Aβ(1-42) showed sustained increased freezing levels for the 5 days compared to pre-lesion freezing levels ($p>0.05$).

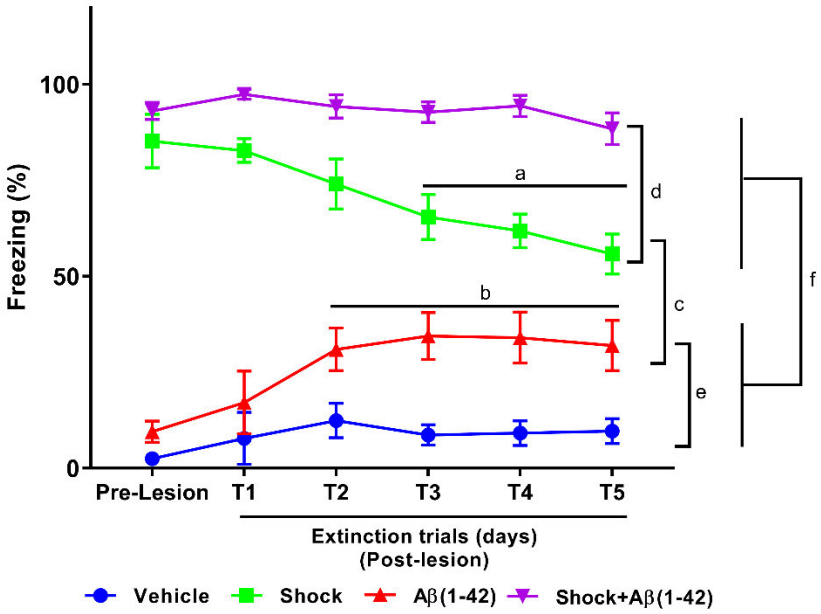

Figure 2: Effect of shock and Aβ(1-42) on percentage freezing time in fear extinction test. Interaction, $F(15, 135) = 4.154$. Post hoc analyses: $p<0.0001$. [a]$p<0.01$ shock vs. corresponding pre-lesion freezing, [b]$p<0.01$, Aβ(1-42) vs. corresponding pre-lesion freezing level. [c]$p<0.001$, Shock vs. Aβ(1-42). [d]$p<0.001$, shock+Aβ(1-42) vs. shock. [e]$p<0.001$, Control vs. shock; [f]$p<0.001$, shock+ Aβ(1-42); shock vs. Control; Aβ(1-42). Data represents means ± SEM. Two-way repeated measure ANOVA followed by Dunnett's multiple comparisons (within groups) and Tukey's test (between groups). n=7-8/group.

Footshocks increases anxiety-like behavior in Aβ(1-42) lesion

As shown in Figure 3, four groups of animals viz control, shock, Aβ(1-42), and shock+Aβ(1-42) were analyzed for anxiety-like behavior in the elevated plus maze test. There was a significant interaction between the effects of Aβ(1-42) lesion and footshocks on time spent in the closed arms ($F(1, 27) = 5.365$, $p=0.0284$). Post hoc analyses showed that the Aβ(1-42) group had increased time spent in the closed arms ($p<0.01$). This response was exacerbated in the shock+Aβ(1-42) group (Aβ(1-42) vs. shock+ Aβ(1-42), $p<0.01$, Figure 3a). Also, there was a significant interaction between the effects of Aβ(1-42) lesion and footshocks in the number of entries into the enclosed arms ($F(1, 27) = 12.71$, $p=0.0014$). However, post hoc analyses showed that

shock+Aβ(1-42) group showed a significantly decreased number of entries into the closed arms compared to control ($p<0.05$, Figure 3b). There was a significant interaction between the effects of Aβ(1-42) lesion and footshock on exploratory activity ($F(1, 27) = 4.747$, $p=0.0382$). Post hoc analyses further revealed that Aβ(1-42) group animals had reduced total exploration when compared to control ($p<0.05$), which was further reduced in the shock+Aβ(1-42) group (control vs. shock+ Aβ(1-42), $p<0.01$, Figure 3c).

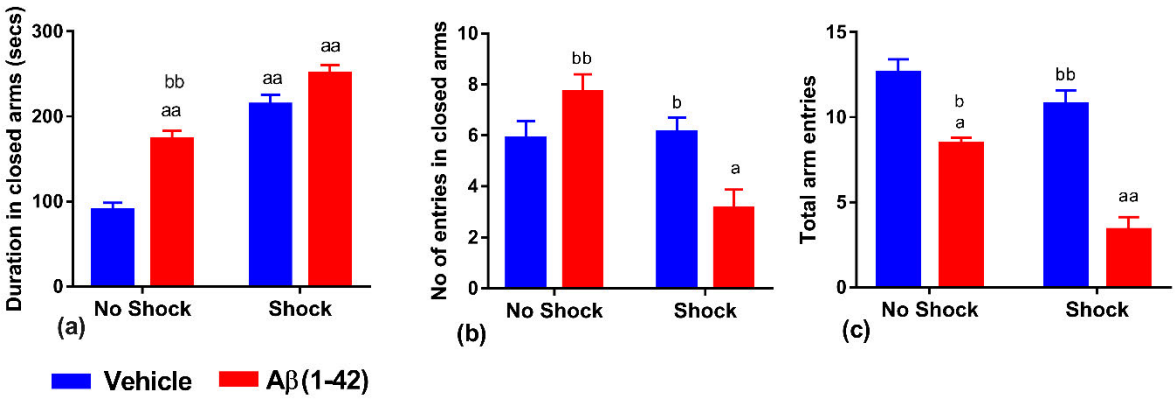

Figure 3: Effect of footshocks and Aβ(1-42) on anxiety-like behavior in elevated plus maze test

(a) duration in closed arms. Interaction, $F(1, 27) = 5.365$, $p=0.0284$; (b) number of entries in the closed arms. Interaction, $F(1, 27) = 12.71$, $p=0.0014$ (c) total arm entries. Interaction, $F(1, 27) = 4.747$, $p=0.0382$. Data represents means ± SEM. Two-way ANOVA followed by Tukey's post hoc test. $^{a}p<0.05$, $^{aa}p<0.01$ vs. control; $^{b}p<0.05$, $^{bb}p<0.01$ vs. shock+Aβ(1-42). n=7-8/group.

Neurochemical analyses

Footshocks exacerbate Aβ(1-42)-induced oxidative stress.

As shown in Table 3, four groups of animals viz control, shock, Aβ(1-42), and shock+Aβ(1-42) were analyzed for oxidative stress parameters. In the amygdala (Table 3a), there was a significant shock effect ($F(1, 12) = 20.4$, $p=0.0007$) and Aβ(1-42) lesion effect ($F(1, 12) = 45.46$; $p=0.0001$) on the concentration of MDA. Further post hoc analyses showed that all the groups had a significant increase in MDA concentration compared to control ($p<0.05$). However, there was a further increase in MDA concentration in the shock+Aβ(1-42) group compared to the group that received shock alone ($p<0.05$). In addition, there was a significant shock and Aβ(1-42) lesion effects on the concentration of SOD (shock effect, $F(1, 12) = 72.8$; $p<0.0001$ and Aβ(1-42) lesion effect $F(1, 12) = 35.15$; $p<0.0001$) and CAT (shock effect ($F(1, 12) = 11.66$; $p=0.0051$ Aβ(1-42) lesion effect ($F(1, 12) = 9.892$; $p=0.0084$). Post hoc analyses showed a reduction in SOD concentration in the amygdala of all groups when compared to control ($p<0.05$). In addition, there was a further reduction in the shock+Aβ(1-42) group when compared to shock or Aβ(1-42) alone ($p<0.01$). Post hoc analyses also revealed a reduction in CAT concentrations in the amygdala of shock+Aβ(1-42) groups

when compared to the control ($p<0.01$). Similarly, there was a significant interaction between the effects of footshocks and Aβ(1-42) lesion on the concentration of GSH in the amygdala of the animals ($F(1, 12) = 6.821$; $p=0.0227$). There was also a shock effect ($F(1, 12) = 26.18$, $p=0.0003$) and Aβ(1-42) lesion ($F(1, 12) = 26.68$, $p=0.0002$). Post hoc analyses showed a reduction in GSH concentration in the amygdala of all groups when compared to control ($p<0.001$).

Furthermore, as shown in Table 3b, the concentration of oxidative stress parameters in the hippocampus follows a similar trend as in the amygdala. Two-way ANOVA revealed a significant shock effect ($F(1, 12) = 30.65$, $p=0.0001$) and Aβ(1-42) lesion effects ($F(1, 12) = 84.86$, $p<0.0001$) on the concentration of MDA. Post hoc analyses showed a reduction in MDA concentration in the hippocampus of all groups when compared to control ($p<0.05$). In addition, there was a further reduction in the shock+Aβ(1-42) group when compared to shock or Aβ(1-42) alone ($p<0.01$).

There was a significant interaction between the effects of footshocks and Aβ(1-42) lesion on the concentration of SOD in the hippocampus of the animals ($F(1, 12) = 10.91$; $p=0.0063$). Post hoc analyses showed a reduction in SOD in the hippocampus of all groups when compared to control ($p<0.0001$). In addition, there was a further reduction in the shock+Aβ(1-42) group when compared to shock or Aβ(1-42) alone ($p<0.05$). There was also a significant interaction between the effects of footshocks and Aβ(1-42) lesion on the concentration of CAT ($F(1, 12) = 4.852$; $p=0.0479$) and GSH ($F(1, 12) = 8.316$; $p=0.0137$) in the hippocampus of the animals. Post hoc analyses showed a reduction in CAT ($p<0.001$) and GSH ($p<0.05$) in the hippocampus of all groups when compared to control. In addition, there was a further reduction in CAT concentration in the shock+Aβ(1-42) group when compared to Aβ(1-42) alone ($p<0.05$).

Table 3. Effect of footshocks and Aβ(1-42) lesion on the concentration of oxidative stress parameters

(a) Amygdala				
	MDA (U/mg protein)	**SOD (ng/ml)**	**CAT (U/mg protein)**	**GSH (μM/mg protein)**
Control	0.049 ± 0.0045	0.42 ± 0.022	13 ± 1.9	1.2 ± 0.14
Shock	$0.064 \pm 0.0063^{a,bb}$	$0.3 \pm 0.013^{aa,bb}$	8.1 ± 0.66	0.61 ± 0.035^{aaa}
Aβ(1-42)	0.094 ± 0.0073^{aa}	$0.34 \pm 0.014^{a\ bbbb}$	8.4 ± 0.25	0.61 ± 0.027^{aaa}
Shock+Aβ(1-42)	0.16 ± 0.012^{aaaa}	0.17 ± 0.017^{aaaa}	5.5 ± 1^{aa}	0.41 ± 0.064^{aaaa}
(b) Hippocampus				
Control	0.06 ± 0.01	0.5 ± 0.018	16 ± 1.1	1 ± 0.14

Shock	$0.11 \pm 0.01^{a,bbbb}$	$0.33 \pm 0.017^{aaaa,bbb}$	7.9 ± 1^{aaa}	0.61 ± 0.044^{aa}
Aβ(1-42)	$0.15 \pm 0.012^{aaa,bb}$	$0.26 \pm 0.01^{aaaa,b}$	$9 \pm 1.1^{aaa,b}$	0.51 ± 0.045^{a}
Shock+Aβ(1-42)	0.22 ± 0.01^{aaaa}	0.19 ± 0.014^{aaaa}	4.7 ± 0.25^{aaaa}	0.56 ± 0.058^{aa}

(a) Amygdala and (b) Hippocampus. Data represents means ± SEM. Two-way ANOVA followed by Tukey's post hoc test. n=4/group. $^{a}p<0.05$, $^{aa}p<0.01$, $^{aaa}p<0.001$, $^{aaaa}p<0.0001$ vs. control; $^{b}p<0.05$, $^{bb}p<0.01$, $^{bbb}p<0.001$, $^{bbbb}p<0.0001$ vs. shock+Aβ(1-42).

Footshocks increased neuronal apoptosis in the amygdala and hippocampus of Aβ(1-42) lesioned animals

As shown in Table 4, two-way ANOVA revealed significant shock and Aβ(1-42) effects in the amygdala ($F(1, 14) = 94.75$; $p<0.0001$, $F(1, 14) = 52.43$; $p<0.0001$) and hippocampus ($F(1, 14) = 29.84$; $p<0.0001$, $F(1, 14) = 111.6$; $p<0.0001$) respectively. Furthermore, post hoc analyses showed a significant increase in the percentage of total apoptotic cells in the amygdala in all groups compared to the control ($p<0.001$). In addition, there was a further increase in the percentage of total apoptotic cells in the amygdala of the shock+Aβ(1-42) group when compared to shock or Aβ(1-42) alone ($p<0.01$) (Table 4a). Post hoc analyses also revealed a significant increase in the percentage of total apoptotic cells in the hippocampus in all groups compared to the control ($p<0.01$). In addition, there was a further increase in the percentage of total apoptotic cells in the hippocampus of the shock+Aβ(1-42) group when compared to shock or Aβ(1-42) alone ($p<0.05$) (Table 4b).

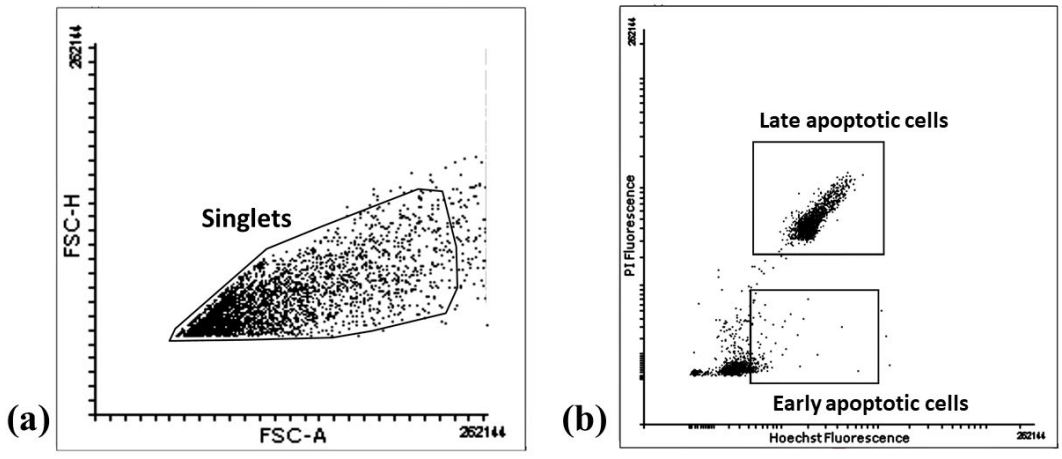

Figure 4. Gating strategy for the quantification of apoptotic cells

(a) illustrates the use of unstained cell samples of forward scatter height (FSC-H) and the forward scatter area (FSC-A) properties to identify the singlet neural cells population in a control sample. (b) percentage of early apoptotic (propidium iodide (PI)-/Hoechst +) cells and late apoptotic (PI+/Hoechst +) cells.

Table 4. Percentage of the total (early+late) apoptotic cells after exposure to footshocks and Aβ(1-42) lesion

% Apoptotic cells	Control	Shock	Aβ(1-42)	Shock+Aβ(1-42)
(a) Amygdala	14.45±1.025	$36.53\pm1.607^{aaaa,bb}$	$31.43\pm2.963^{aaa,bbb}$	49.22 ± 2.514^{aaaa}
(b) Hippocampus	17.88±2.227	$34.18\pm2.083^{aa,bbbb}$	$47.74\pm1.914^{aaaa,b,c}$	60.49 ± 3.286^{aaaa}

(a) amygdala and (b) hippocampus. Data represents means ± SEM. Two-way ANOVA followed by Tukey's post hoc test. n=3-5/group. $^{aa}p < 0.01$, $^{aaa}p < 0.001$, $^{aaaa}p < 0.0001$, vs. control; $^{b}p<0.05$, $^{bb}p<0.01$, $^{bbb}p<0.001$, $^{bbbb}p<0.0001$ vs. shock+Aβ(1-42), $^{c}p < 0.05$ vs. shock.

Footshocks downregulate Nrf2, NQO1, and HO-1 expression in the amygdala and hippocampus of Aβ (1–42)-lesioned animals

In the amygdala, two-way ANOVA revealed a significant interaction between the effects of footshocks and Aβ(1-42) lesion in the mRNA expression of Nrf2 ($F(1, 12) = 7.527$; $p=0.0178$) and Keap1 ($F(1, 12) = 5.308$; $p=0.0399$). In addition, there was a significant shock and Aβ(1-42) effect in the mRNA expression of NQO1 ($F(1, 12) = 16$; $p=0.0018$, $F(1, 12) = 33.64$; $p<0.0001$) and HO-1 ($F(1, 12) = 5.116$; $p=0.0431$, $F(1, 12) = 14.48$; $p=0.0025$) respectively. Post hoc analyses showed that there was a significant reduction in the levels of Nrf2 and NQO1 in the Aβ(1-42) group when compared to the control group ($p<0.01$), which was further reduced in the shock+Aβ(1-42) group (shock vs. shock+Aβ(1-42), $p<0.05$) (Figure 5a,c). Also, the mRNA expression of HO-1 was significantly reduced in the Aβ(1-42) group when compared to the control group ($p<0.05$), and this was further reduced in the shock+Aβ(1-42) group (shock+Aβ(1-42) vs. control, $p<0.01$) (Figure 5d). In contrast, there was a significant increase in the levels Keap1 in Aβ(1-42) group when compared to the control group ($p<0.05$), which was further increased in the shock+Aβ(1-42) group (shock+Aβ(1-42) vs. control, $p<0.01$) (Figure 5b). In the hippocampus, there was a significant interaction between the effects of footshock and Aβ(1-42) lesion in the mRNA expression levels of Nrf2 ($F(1, 12) = 12.55$; $p=0.0041$), NQO1 ($F(1, 12) = 7.633$; $p=0.0172$) and HO-1 ($F(1, 12) = 9.046$; $p=0.0109$). Also, there was a significant shock ($F(1, 12) = 44.41$; $p<0.0001$) and Aβ(1-42) ($F(1, 12) = 67.45$; $p<0.0001$) effects in the mRNA expression level of Keap1. Post hoc analyses revealed a significant reduction in the mRNA expression levels of Nrf2, NQO1, and HO-1 in Aβ(1-42) group compared to control ($p<0.05$). There were further reductions in the shock+Aβ(1-42) group: Nrf2 (shock vs. shock+Aβ(1-42), $p<0.05$) (Figure 5e) and NQO1 (shock+Aβ(1-42) vs. control, $p<0.05$) (Figure 5g). However, no further reduction was seen in the

mRNA expression levels HO-1 in the shock+Aβ(1-42) group, although still significantly reduced compared to control (shock+Aβ(1-42) vs. control, $p<0.01$) (Figure 5h). Also, expression levels of Keap1 significantly increased in the Aβ(1-42) group when compared to control ($p<0.05$), which was further increased in the shock+Aβ(1-42) group (shock vs. shock+Aβ(1-42), $p<0.01$) (Figure 5f).

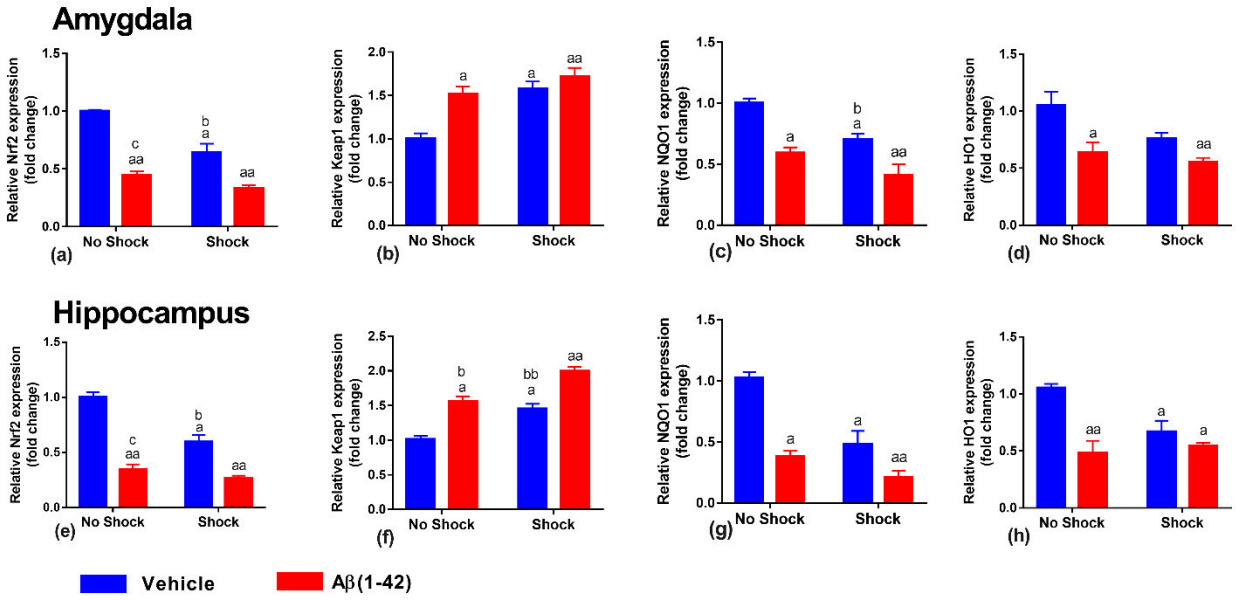

Figure 5. Effect of footshocks on mRNA expression of Nrf2, Keap1, NQO1, and HO-1 in the amygdala and hippocampus of Aβ(1-42)-lesioned animals. (a-d) Amygdala: Nrf2, Keap1, NQO1, and HO-1 (e-f) Hippocampus: Nrf2, Keap1, NQO1, and HO-1. Data represent mean ± SEM. Two-way ANOVA followed by Tukey's post hoc test. n=4/group. $^{a}p<0.05$, $^{aa}p<0.01$ vs. Control, $^{b}p<0.05$, $^{bb}p<0.01$ vs. shock+Aβ(1-42), $^{c}p<0.01$ vs. shock.

2.4 **Discussion**

This study examined the interactive effects of footshock stress and Aβ(1-42) lesion on oxidative status and neuronal apoptosis in rats. We found an increased freezing level in animals that received footshocks that lasted up to 12 days (post-shock). This is in line with previous findings (26,27). We also tested for behavioral changes within 5 to 14 days post-lesion, based on our previous observation that the effect on Aβ(1-42) was evident between day 3 and 14 post-lesion (31,32). Similarly, Karthick and co-workers have reported evident Aβ(1-42) effects at 15 days post-lesion (49).

Furthermore, we observed that footshocks led to elevated freezing behavior and impaired fear memory extinction in animals lesioned with Aβ(1-42), indicating a defective memory consolidation. This is in line with a previous report showing that AD animals exhibit severely impaired memory consolidation, as indicated by an increased percentage of freezing (50). This could result from dysregulation of fear pathways

in the limbic system, as enhanced accumulation of Aβ in the amygdala has been associated with enhanced fear responses and cognitive deficits (51). Also, we assessed anxiety-like behaviors in the EPM test. Animals that received footshocks exhibited increased anxiety-like behavior. Our findings agree with the report of Perusini and co-workers who also showed that SEFL produced enhanced anxiety-like responses (52). Similarly, Aβ(1-42) lesioning increased anxiety-like behavior, which was exacerbated in animals that received footshocks in addition to Aβ(1-42) lesioning. These observations is in line with previous findings (51,53,54). This result, in a way, supports the sustained freezing levels seen in the fear extinction test. Thus, the possible neurotoxic effect of Aβ(1–42) may be responsible for the anxiogenic responses observed in this study.

To further investigate the underlying mechanisms associated with the observed anxiety-like and impaired fear memory behaviors, we determined the level of oxidative stress in the amygdala and hippocampus of the animals. Aβ(1–42) caused an increase in MDA (a marker of lipid peroxidation) and a corresponding decrease in antioxidants (viz. SOD, CAT, and GSH) in the amygdala and hippocampus, suggesting the establishment of oxidative stress. These results are consistent with previous findings (24,55). The induced oxidative stress was exaggerated in the amygdala and hippocampus of animals that received footshocks in addition to Aβ(1–42) lesion. SOD, which plays a protective role in neurodegeneration, is the first line of defense against superoxides. Although SOD has significant reactive oxygen species (ROS) scavenging properties, its activities also lead to hydrogen peroxide production, which is equally toxic to cells (56). It is only beneficial when this is accompanied by CAT and GSH activities, which further scavenges the hydrogen peroxide from superoxide dismutation (57,58). Therefore, it is possible that oxidative stress in the amygdala and hippocampus was involved in disrupting cell communication, which may be responsible for exaggerated anxiety-like behavior and enhanced fear expression (59,60).

The response to cellular oxidative stress is coordinated by an intricate network of antioxidant molecules and genes, including Nrf2, Keap1, NQO1, and HO-1 (61–63). The Nrf2-Keap1 signaling pathway plays a vital role in the cellular defense against oxidative stress (64,65). Nrf2 regulation is thought to be primarily driven by Keap1, which targets Nrf2 for proteasomal degradation (64,65). In our study, Aβ(1-42) decreased Nrf2 expression and increased Keap1 expression. This finding is in line with previous reports (24,55). Similarly, footshocks stress reduced Nrf2 expression. This is also in line with previous findings (25). However, when footshocks preceded Aβ(1-42) lesion, these alterations were further exaggerated. The increased expression of Keap1 suggests a reduction in the translocation of Nrf2 into the nucleus, thereby causing the downregulation of antioxidant genes. Indeed, we observed a decrease in the expression of antioxidant genes NQO1 and HO-1 following Aβ(1-42) lesion. In addition, decreased Nrf2 expression is associated with reduced antioxidant enzymes expression e.g., SOD and CAT (66). Besides, neurotoxins such as Aβ(1-42) activate ROS. ROS can, in turn, stimulate the Nrf2-Keap1 pathway, which can be influenced by stress (23,67,68).

The suppression of Nrf2 signaling pathway results in cellular apoptosis (64,66,69,70). For instance, Aβ(1-42) and footshock induces oxidative stress by increasing ROS generation (71,72). The increased oxidative

stress, in turn, activates Nrf2-Keap1 pathway to upregulate antioxidants e.g., NQO1 and HO-1 (73). However, sustained oxidative stress due to Aβ(1-42) neurotoxicity and footshocks suppress the Nrf2-Keap1 pathway causing downregulation of antioxidant molecules viz. NQO1, HO-1, SOD, CAT, and GSH (24,74). Besides, it has been shown that increased oxidative stress causes damage to biological molecules, eventually leading to apoptosis (75–78). Specifically, suppression of Nrf2 leads to increased apoptosis in the amygdala and hippocampus as well as inducing anxiety-like behaviors in rats (79). This study found that oxidative stress was accompanied by increased apoptosis in the amygdala and hippocampus. In fact, we showed that Aβ(1-42) increased the number of apoptotic cells that were further elevated in the presence of footshocks, suggesting that footshock stress may exacerbate Aβ AD-like pathology. These results are in line with previous reports that showed that stress and Aβ induce neuronal apoptosis (68,71,72,80). Similarly, stress exposure-induced oxidative stress facilitates apoptosis in the amygdala, which leads to increased anxiety-like behavior (22,81,82).

It, therefore, seems that footshocks, as well as Aβ(1-42) lesion, induced oxidative stress by suppressing the Nrf2-Keap1 pathways and the associated genes and that the interaction of footshocks and Aβ(1-42) lesion led to exaggerated apoptosis in the amygdala and hippocampus, which is evident by increased anxiety-like responses and impaired fear extinction memory.

In conclusion, footshock stress can precipitate molecular changes in the amygdala and hippocampus that can be exacerbated in AD-like pathology.

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Declarations of interest: none

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Prologue

Learning and memory changes are largely implicated in the pathologies of post-traumatic stress disorder (PTSD) and Alzheimer's disease (AD). Chapter 3 examines the effect of PTSD-like induction on learning and memory outcomes in Aβ(1-42) lesion rat model of AD, and how it is influenced by glutamate excitotoxicity and stress dysregulation in the hippocampus and amygdala. This chapter is prepared in a manuscript format and has been published in Neurobiology of Learning and Memory journal, Impact factor: 2.877 https://doi.org/10.1016/j.nlm.2022.107611 See Appendix VII.

3 CHAPTER 3

Trauma-like exposure alters neuronal apoptosis, Bin1, Fkbp5 and NR2B expression in an amyloid-beta (1-42) rat model of Alzheimer's disease

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Abstract

Post-traumatic stress disorder (PTSD) is a risk factor in the development and progression of Alzheimer's disease (AD), with unclear underlying mechanisms. Recently, we provided data showing the effect of

trauma-like stress on *Bin1* and *Fkbp5* expression in the prefrontal cortex of $A\beta_{(1-42)}$ lesioned animals. This present work sought to expand the study by examining the involvement of the amygdala and hippocampus, in addition to highlighting the role of NR2B in the co-occurrence of trauma-like stress and an Aβ AD-like pathology.

Trauma-like condition was induced by exposing the animals to footshocks. $A\beta_{(1-42)}$ was injected into the hippocampus of the animals to induce AD-like pathology. Cognitive functions were assessed in the Morris water maze (MWM) and novel object recognition tests, after which amygdala and hippocampus tissues were harvested for neurochemical analyses.

We found that the combination of footshocks and $A\beta_{(1-42)}$ lesion caused a decrease in the number of crossings in the target quadrant of the Morris water maze test, indicating memory deficits. Footshocks caused a further downregulation of *Bin1* in the amygdala of $A\beta_{(1-42)}$-lesioned rats. Prior exposure to footshocks downregulated NR2B in the hippocampus of $A\beta_{(1-42)}$-lesioned rats. In addition, a combination of footshocks and $A\beta_{(1-42)}$ lesion sustained the upregulation of *Fkbp5* in the hippocampus and amygdala. A combination of footshocks and $A\beta_{(1-42)}$ lesion increased neuronal apoptosis in the hippocampus and amygdala.

In conclusion, exposure to a trauma-like condition may influence AD-like pathology, leading to exaggerated behavioural and molecular changes in the amygdala and hippocampus.

Keywords**:** Post-traumatic stress disorder; Alzheimer's disease; Apoptosis; Amyloid-beta, Cognition

3.1 Introduction

Post-traumatic stress disorder (PTSD) is a psychological disorder that results from experiencing a life-threatening trauma (Careaga et al., 2016). Alzheimer's disease (AD) is the most common form of dementia, affecting about 44 million people globally (Q. Li et al., 2017). The disease is characterized by extracellular deposition of amyloid-beta (Aβ), intracellular neurofibrillary tangles, neuronal loss, synaptic deficits, and learning and memory impairments (Hong et al., 2016; Sanabria-Castro et al., 2017). Sporadic AD, which accounts for the majority (60 – 80 %) of AD cases, contrasts with familial AD in that the former develops from multifactorial conditions while the latter is due to genetic mutations. (Gatz et al., 2006; Koukouli et al., 2016). It has been shown that people with PTSD have twice the risk of developing dementia, mostly AD

(Stilling et al., 2014; Yaffe et al., 2010). However, the underlying mechanism of the interaction is less understood.

Although neurodegeneration in the hippocampus is prominent in AD conditions (Facchinetti et al., 2018; Scuderi et al., 2014), several other brain areas are affected, including the amygdala (T. W. Lin et al., 2015). There is also crosstalk between the amygdala and hippocampus on regulating the effect of stress on cognition (Roozendaal, 2002). Also, Aβ deposits are found in the amygdala in AD conditions, which have shared circuitry to the hippocampus (Karthick et al., 2019; T. W. Lin et al., 2015). Therefore, the evaluation of the changes in the amygdala following intrahippocampal Aβ-lesion could be very useful to investigate region vulnerability in AD linked to Aβ deposition from one targeted brain structure to connected areas (Koukouli et al., 2016). Several studies have also shown that neurodegeneration occurs earlier in the amygdala than in the hippocampus, thereby suggesting amygdala function assessment as an important tool in diagnosing cognitive impairment in AD (T.-W. Lin et al., 2015; T. W. Lin et al., 2015).

Bridging integrator 1 (*Bin1*) has been identified as the second most prevalent genetic risk factor in sporadic AD (De Rossi et al., 2020; Glennon et al., 2013). *Bin1* is involved in the synaptic release of neurotransmitters and has also been shown to play a role in Amyloid Precursor Protein (APP) processing into Aβ and AD pathogenesis (Andrew et al., 2019; Miyagawa et al., 2016; Schneider et al., 2008). Our recent study showed that $A\beta_{(1\text{-}42)}$ injection or footshock stress caused downregulation of *Bin1* in the prefrontal cortex of rats (Faborode et al., 2021b). Despite the focus on *Bin1* in AD pathology, the involvement of *Bin1* in memory functions and whether Aβ alters *Bin1* expression remains unclear (Andrew et al., 2019; De Rossi et al., 2020; Zhu et al., 2021). Also, the involvement of *Bin1* in trauma-induced disease is less understood.

Trauma-like stress has been shown to elevate brain levels of Aβ (Carroll et al., 2011; Justice et al., 2015). A critical component in stress regulation in PTSD is the FK506 binding protein 51 (*Fkbp5*) (Yehuda, 2009; Young et al., 2018). *Fkbp5* regulates stress via glucocorticoid receptors (GRs), and its upregulation in the hippocampus has been associated with memory deficits (Aminyavari et al., 2019). Coincidentally, increased *Fkbp5* expression has been linked to AD progression (Aminyavari et al., 2019; Blair et al., 2013). It has been reported that Aβ aggregation can disrupt stress regulation through interaction with *Fkbp5* and GRs, and that stress-induced *Fkbp5* upregulation can alter neuronal functions (Rein, 2016). Similarly, we recently showed

that traumatic stress led to an upregulation of *Fkbp5* in the prefrontal cortex of rats, which correlated strongly with neuronal apoptosis and memory decline (Faborode et al., 2021b). However, the role of traumatic stress on the amygdala and hippocampal expression of *Fkbp5* in an AD condition is less understood.

Furthermore, there is evidence that increased expression of *Fkbp5* promotes the regulation of glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors-mediated altered neurotransmission and cognitive dysfunction (Blair et al., 2019). Similarly, excessive glutamate N-methyl-D-aspartate receptor (NMDAR) activation has been associated with sporadic AD and synaptic dysfunction (Liu et al., 2019; Morgan, 2011). Specifically, Aβ toxicity causes an upregulation of NMDA receptor subunit 2B (NR2B) (Karthick et al., 2019). Equally, trauma-like stress has been shown to increase NR2B expression (Yamamoto et al., 2008). Therefore, trauma-like stress in the presence of an AD condition may exacerbate NR2B expression, leading to severe pathological responses (Minnella et al., 2018). Hence, a continuous focus on the role of NR2B in traumatic stress and AD pathology is necessary.

Usually, mature neurons in the CNS are less susceptible to apoptosis (Kole et al., 2013), because they are well-adapted to handling stresses in their lifetime (Chi et al., 2018). Cell death usually occurs when these stresses accumulate beyond the homeostatic ability of the cells (Chi et al., 2018). This phenomenon leading to neuronal death is evident in neurodegeneration and trauma-induced conditions (Chi et al., 2018; Rami & Kögel, 2008). The role of trauma-like stress on neuronal cell death in the presence of an Aβ AD-like pathology remain elusive.

Given the involvement of *Bin1* and *Fkbp5* in the prefrontal cortex of PTSD and AD rats, this study aimed to further examine the effect of trauma-like exposure on the expression of *Bin1* and *Fkbp5* in the amygdala and hippocampus of an $A\beta_{(1-42)}$ rat model of AD pathology. We also determined the relationship of *Bin1* and *Fkbp5* expression with NR2B expression and neuronal apoptosis-mediated memory impairment in a bid to elucidate the common underlying molecular mechanisms of PTSD and AD.

3.2 Materials and methods

Animals

All procedures involving animals were approved by the animal research ethics committee of the University of KwaZulu-Natal (AREC/005/019D). Ten-week-old male Sprague Dawley rats were used for this study. Animals were housed under standard conditions (Temperature, 21 ± 2 °C; humidity, 55 – 60 %; lights on at 07:00 and off at 19:00 hours) in the Biomedical Resource Unit (BRU), University of KwaZulu-Natal. Food and water were freely available. Animals were grouped as follows: (1) Control: no footshock, vehicle injection (sham surgery); (2) Shock: received footshocks, vehicle injection (sham surgery); (3) $A\beta_{(1-42)}$: no footshocks, amyloid-beta (1-42) injection; (4) Shock+$A\beta_{(1-42)}$: received footshocks, amyloid-beta (1-42) injection.

Experimental design

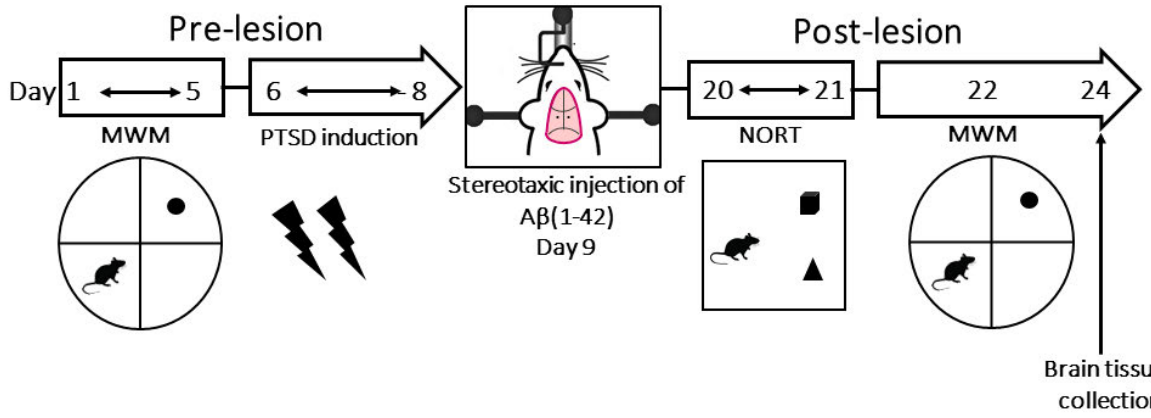


Figure 1: Experimental timeline. On days 1 to 3, rats were trained in the Morris water maze, rested on day 4, and a probe test was conducted on day 5. On days 6 to 8, rats received footshocks or no shock. Twenty-four hours later, they received a bilateral intrahippocampal infusion of $A\beta_{(1-42)}$ or vehicle solution. On days 20 and 21, rats were tested in the Novel object recognition test (NORT), followed by the Morris water maze test (MWM) on day 22. On day 24, brain tissue samples were collected for analysis.

Post-traumatic stress disorder (PTSD) animal model

A post-traumatic stress disorder-like condition was induced using the stress-enhanced fear-learning protocol as described previously (Rajbhandari et al., 2018). Briefly, on the first day of PTSD-like induction, the rats were placed in a conditioning chamber (Context A), where they received 15 (1 mA, 1 second) footshocks over 90 minutes. The following day, the rats were put in a new conditioning chamber (Context B), different

in colour, odour, and shape. After 3 minutes of habituation, rats received a single shock. On the third day of the induction, rats were returned to Context B, did not receive any footshock.

Stereotaxic surgery

Alzheimer's disease-like pathology was induced using an intrahippocampal lesion with Aβ $_{(1-42)}$ peptide, as described previously by Shallie and Mabandla (Shallie & Mabandla, 2020). Briefly, rats were deeply anaesthetized with a cocktail of anaesthetic drugs (ketamine, 100 mg/kg and xylazine, 10 mg/kg; intraperitoneally). After complete anaesthesia was established, rats were carefully positioned in the stereotaxic apparatus, and 5µl of 1 µg/µl solution of Aβ $_{(1-42)}$ peptide was infused into the hippocampus on both sides using a 25G Hamilton syringe at the rate of 1µl /min, at the coordinates: anteroposterior (AP) = −4.8 mm; mediolateral (ML) = ±3.4 mm; dorsoventral (DV) = −3.0 mm relative to Bregma, according to a rat brain atlas (Paxinos & Watson, 2007). After infusion, the needle was left in place for 2 minutes for proper diffusion of the solution. An equal volume (5µl) of phosphate-buffered saline (PBS) was infused into the hippocampus of the control rats. After removing the needle, the incision was sutured, cleaned with an antiseptic solution, and the rats were placed on a heating pad to recover before being returned to their cages.

Behavioural testing

Following shock and Aβ $_{(1-42)}$ lesioning, rats were tested for memory functions using the novel object recognition and Morris water maze tests.

Novel object recognition test (NORT)

The NORT apparatus comprised an opaque box of 55 × 55 × 35 cm. On Day 20 of the experiment, the rats were exposed to the box containing two identical objects and allowed to explore for 5 minutes. Twenty-four hours after, the rats were put in the same box containing the same objects. One hour later, the rats were returned to the box with one of the objects replaced by a new one with a different shape, colour, odour, and size. The rats were allowed to explore the box for 5 minutes. An observer blind to the experiment measured the time spent on each object. The discrimination index was determined as follows:

$$\frac{(\text{time spent on novel object} - \text{time spent on familiar object})}{(\text{time spent on novel object} + \text{time spent on familiar object})}$$ (Antunes & Biala, 2012).

Morris water maze (MWM) test

The MWM test was used to assess spatial memory in the rats (Morris, 1984; Qiang et al., 2017; Vorhees & Williams, 2006). The MWM apparatus consisted of a circular pool made of plastic (diameter, 140cm; height, 60cm). The pool was filled with water to a depth of 30 cm, and the temperature was maintained at 25 ± 1°C. An escape platform was placed 1cm below the water surface. An imaginary line was drawn to divide the pool into 4 quadrants viz North (N), South (S), West (W), and East (E). The test was divided into 2 phases: training trials and probe tests. The training consisted of 4 trials per day for 3 days.

On Day 1 of the experiment, the rat was trained in the MWM. The rat was gently lowered into one of the quadrants with its nose facing the wall of the pool and allowed to swim freely until it located the escape platform. After locating the platform, the rat was allowed to stay there for 15 seconds. The time taken to locate the escape platform (escape latency) was noted using a stopwatch. If the rat did not locate the platform within 120 seconds, it was guided to the platform and allowed to stay for 15 seconds. After each trial, the rat was dried with a towel and returned to its home cage. The subsequent trial was started from another quadrant. The training continued on Days 2 and 3. Following the 3-day training, the rat was allowed to rest for one day (day 4) before the probe trial on Day 5. On the day of the probe trial, the escape platform was removed from the pool. The rat was placed gently in the quadrant opposite the initial position of the escape platform. The number of crossings and the amount of time spent in the target quadrant were noted by an observer blind to the experiment. On Day 22, the probe trial was repeated.

After the behavioural tests, the rats were decapitated using a guillotine. The hippocampus and amygdala were dissected, weighed, transferred into Eppendorf tubes, snap-frozen in liquid nitrogen, and stored in a biofreezer at -80°C until further analyses. For the tissue processing, the whole brain area (e.g amygdala) was initially minced, then the recommended mass required for the tissue processing was weighed out. The remainder of the minced sample was stored away at -80°C.

Estimation of N-methyl D-aspartate (NMDA) receptor subunit 2B (NR2B) protein expression

The level of NR2B protein was measured using western blot. Brain tissues were homogenized in RIPA buffer (Invitrogen, USA). Protein concentration was determined using RC DC™ Protein Assay kit as per manufacturer's instructions (Bio-Rad, USA). Samples were diluted in 2X Laemmli sample buffer (Bio-Rad, USA) and heated at 95°C for 5 minutes. Heated samples were loaded into the wells of a pre-cast 4-20%

polyacrylamide gel (Bio-Rad, USA) and electrophoresed at 100 V for 60 minutes. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a wet transfer protocol and were run at 100 V for 90 minutes (Bio-Rad, USA). Membranes were blocked using the Odyssey blocking buffer (LI-COR, USA) for 2 hours. Membranes were then washed thoroughly 3 times with PBS-T (Sigma-Aldrich), after which they were incubated with NR2B primary mouse antibody (1:500; Invitrogen, USA) diluted in Odyssey blocking buffer (LI-COR, USA) at 4°C overnight. Membranes were thereafter washed in PBS-T (Sigma-Aldrich) and incubated in goat anti-mouse secondary antibody diluted in Odyssey blocking buffer (1:15 000) for 60 minutes in the dark at room temperature. Membranes were then washed and visualized in the Odyssey CLx Scanner. (LI-COR, USA). Band fluorescence was normalized using β-actin (Invitrogen, USA) as the housekeeping protein.

Neuronal cell isolation and flow cytometry

Neuronal cell isolation was carried out as previously described (Martin et al., 2017). Briefly, brain tissues were minced in 2 drops of PBS-T (Sigma-Aldrich) on a cold glass plate for 2 minutes with a razor. The minced tissue was homogenized in 650 µl PBS-T, and the homogenate was centrifuged at 110 × g for 2 minutes. The suspension was passed through a 19G needle 12 times, centrifuged, and the supernatant discarded. A 1 ml volume of Accutase cell dissociation solution (Life Technologies, CA, USA) was added, and the suspension was placed on a rocker for 20 minutes at 200 rpm. The tissue was centrifuged and washed in PBS-T. The suspension was passed through a 21G needle, followed by a 23G needle, and finally two times in a 25G needle - 12 times each after adding 650 µl of PBS-T. After each trituration, the clear supernatant was collected in a tube, and the residue was allowed to settle before adding another 650 µl of PBS-T for the next trituration. All the supernatants from the collection tubes were collected at the end of the triturations and passed through a 40 µm mesh strainer. The cell suspension was stored at 4°C until staining. Cells were incubated with 2.5 µL of PE Annexin V (BD Biosciences, USA, 560930) and 2.5 µL of FLICA™ (Invitrogen, USA, Vybrant FAM Caspase-3 and -7, V35118) reagent for 60 minutes at room temperature in the dark, according to the manufacturer's recommendation. After incubation, the cell suspension was centrifuged and washed. Fixing and permeabilization were carried out according to the manufacturer's protocol (Fix and Perm, Life Technologies), after which cells were incubated with 5 µg/ml of NeuN-

AlexaFluor 647 (Novusbio, USA, NBP1-92693AF647) for 30 minutes at room temperature according to the manufacturer's protocol. Labelled cells were analyzed on a BD FACSCanto II flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

Gating strategy for the quantification of apoptotic cells:

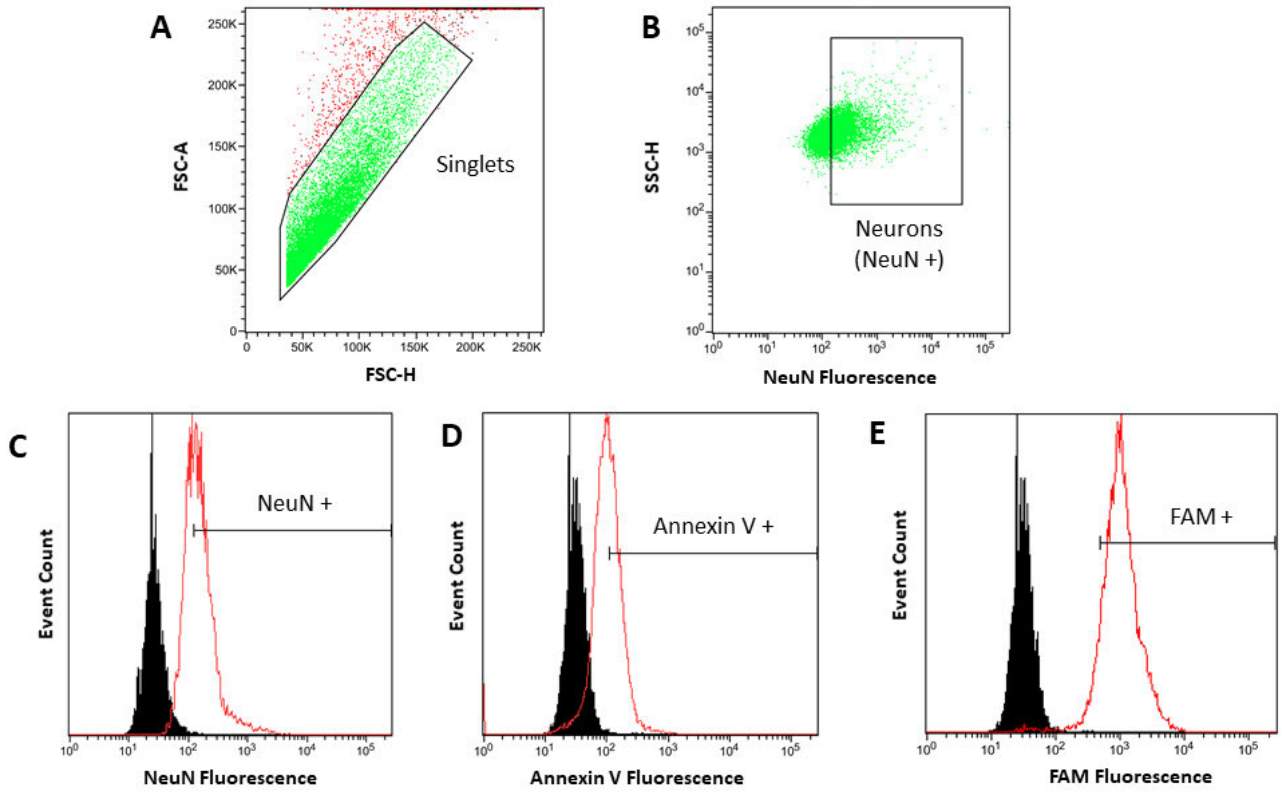


Figure 2: Gating strategy used to detect the percentage of Annexin V positive and caspase 3/7 activation in a neuron population (A) forward scatter to discriminate doublets using unstained cells. (B) NeuN-labelled cells to show neurons (C-E) unstained control to set negative and positive events for NeuN, Annexin V, and FAM Caspase 3/7.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Harvested brain tissues (amygdala and hippocampus) were lysed in RNA shield ™ and RNA extraction was carried out per kit manufacturer's protocol (Zymo Research, USA). The extracted RNA purity was determined using NanoDrop™ (Life Scientific, USA). Reverse transcription into cDNA was done using an iScript™ cDNA kit. A total reaction volume of 10 µl was used, consisting of cDNA template, forward and reverse primer, SYBR green master mix, and RNAse-free water. PCR was run with the following conditions on a light cycler (Roche, Germany): denaturation at 95 °C, 10 seconds; primer annealing at 51°C (*Bin1*),

51°C (*Fkbp5*), and 51°C (GAPDH) for 30 seconds; elongation at 72 °C, 20 seconds. GAPDH was used as the reference gene. Fold change was calculated using the ddCT method (Livak & Schmittgen, 2001).

Table 1. List of primers

Gene	Forward	Reverse
Bin1	AAGGCGGATGAAACGAAGGA	GGCTTTCTCAAGCAGCGAG
Fkbp5	ACTGACTCGCCTGACACAAG	GAGCGAGGTATCTGCCTGTC
Gapdh	TGTGAACGGATTTGGCCGTA	ATGAAGGGGTCGTTGATGGC

Statistical Analysis

Data were tested for normality using Shapiro-Wilk's test, and where data met requirements, parametric tests were used. Pre-lesion MWM training data were analyzed using two-way repeated-measures (RM) ANOVA followed by Tukey's multiple comparison test. Differences before and after exposure to footshocks and $A\beta_{(1-42)}$ lesioning in the MWM probe test were analyzed using three-way repeated measure ANOVA, followed by Tukey's multiple comparisons. Other behavioural and neurochemical data were analyzed using two-way ANOVA followed by Tukey's post hoc test. All data are presented as mean ± SEM. $P < 0.05$ was considered significant. Data were analyzed using GraphPad Prism version 7 (San Diego, USA).

3.3 Results

The effect of trauma-like exposure and $A\beta_{(1-42}$ lesion on learning and memory in rats

This study set out to determine the common underlying mechanisms in PTSD and AD using rat models. A trauma-like condition was induced using footshocks, while AD-like pathology was induced by lesioning the hippocampus with $A\beta_{(1-42)}$ peptide. We started our analyses by testing whether trauma-like exposure would influence the effect of $A\beta_{(1-42}$ lesion on learning and memory outcomes. The rats were randomly divided into 4 groups, namely control, shock, $A\beta_{(1-42)}$, and shock+$A\beta_{(1-42)}$ and trained in the Morris water maze test prior exposure to footshock and $A\beta_{(1-42)}$ lesioning. The rats learned progressively from Day 1 to Day 3 as

indicated by decreased latency to reach the escape platform in the MWM (Two-way RM ANOVA: $F_{(2, 56)}$ = 68.84, P < 0.0001; Tukey's post hoc: Day 1 vs. Day 2, P < 0.0001; Day 1 vs. Day 3, P < 0.0001; Figure 3A). There are no significant differences between the groups during the training trials in the MWM test before exposure to footshocks and $A\beta_{(1-42)}$ lesioning, suggesting that all the rats learned at a similar rate (Figure 3A). There are no significant differences in the swim speeds between the groups during the training trials before exposure to footshocks and $A\beta_{(1-42)}$ lesioning, suggesting no motor deficits in the rats used for the study (Table 2).

After the training, the escape platform was removed, and the rats were tested for memory retrieval before and after exposure to footshocks and $A\beta_{(1-42)}$ lesioning (Figure 1). All the groups, except control, spent less time searching the quadrant where the platform was initially located during the probe trial of the MWM after exposure to footshocks and $A\beta_{(1-42)}$ lesioning, when compared to their respective probe trials before exposure to footshocks and $A\beta_{(1-42)}$ lesioning, indicating that footshocks and $A\beta_{(1-42)}$ lesioning impaired memory recall (Three-way RM ANOVA: time effect ($F_{(1, 28)}$ = 36.15, P < 0.0001), lesion effect ($F_{(1, 28)}$ = 10.02, P = 0.0037), shock effect ($F_{(1, 28)}$ = 4.356, P = 0.0461), time × lesion effect ($F_{(1, 28)}$ = 14.21, P=0.0008), time × shock effect ($F_{(1, 28)}$ = 7.431, P = 0.0109; Tukey's post hoc: shock, P=0.0370; $A\beta_{(1-42)}$, P= 0.0028; shock+ $A\beta_{(1-42)}$, P = 0.0003, Figure 3B). Further analyses of the probe trial after footshocks and $A\beta_{(1-42)}$ lesioning revealed that all the experimental groups spent less time in the target quadrant when compared to control (Tukey's post hoc: shock vs. control, P = 0.0500; $A\beta_{(1-42)}$ vs. control, P = 0.0023; shock+ $A\beta_{(1-42)}$ vs. control, P < 0.0001, Figure 3B).

We also determined whether the footshocks and $A\beta_{(1-42)}$ lesioning influenced the number of crossings in the target quadrant where the escape platform was initially located in the MWM. We observed that only the shock+$A\beta_{(1-42)}$ group had a significantly lesser number of crossing during the probe trial after exposure to footshocks and $A\beta_{(1-42)}$ lesioning, when compared to the probe trial before exposure to footshocks and $A\beta_{(1-42)}$ lesioning, suggesting a combined effect of shock and $A\beta_{(1-42)}$ lesion on the impairment of long-term memory retrieval (Three-way RM ANOVA: ($F_{(1, 28)}$ = 13.76, P = 0.0009), shock effect ($F_{(1, 28)}$ = 10.79, P = 0.0027), time × lesion effect ($F_{(1, 28)}$ = 10.71, P = 0.0028; Tukey's post hoc: shock+$A\beta_{(1-42)}$, P = 0.0034, Figure 3C). Following exposure to footshocks and $A\beta_{(1-42)}$ lesioning, the rats were also assessed for short-

term recognition memory using the Novel object recognition test (NORT). $A\beta_{(1-42)}$ lesion, but not footshock exposure impaired short-term recognition memory, as indicated by a reduced discrimination indices (Two-way ANOVA: lesion effect ($F_{(1, 27)} = 12.53$, $P = 0.0015$); Tukey's post hoc: $A\beta_{(1-42)}$ vs. control ($P = 0.0421$), shock+$A\beta_{(1-42)}$ vs. control ($P = 0.0259$); Figure 3D). This data suggests that shock did not influence the effect of $A\beta_{(1-42)}$ lesion on short-term memory.

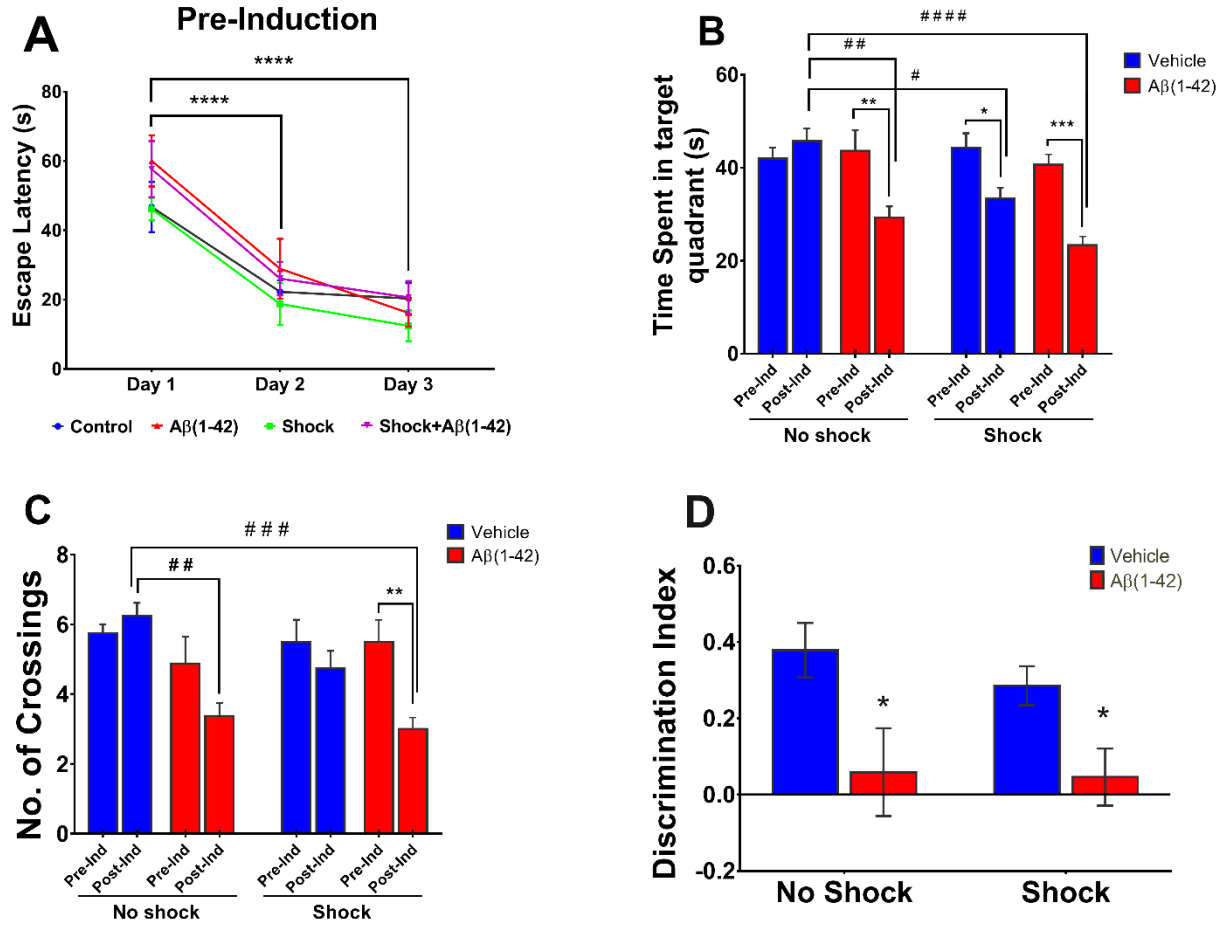


Figure 3: (A) Escape latency during training trials in the MWM test before exposure to footshocks and $A\beta_{(1-42)}$ lesioning (inductions). Day 1 vs. Day 2, ****P < 0.0001; Day 1 vs. Day 3, ****P < 0.0001; Two-way RM ANOVA, Tukey's post hoc test (B) Time spent in target quadrant during probe trial in MWM test. Pre- vs. post-induction: *P < 0.05, **P < 0.01, ***P < 0.001; Post-induction: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ vs. control, Three-way RM ANOVA, Tukey's post hoc test (C) Number of crossings in the target quadrant in the MWM test. Pre- vs. post-induction: **P < 0.01, Post-induction: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control, Three-way RM ANOVA, Tukey's post hoc test (D) Discrimination indices in the NORT. *P < 0.05 vs. control; Two-way RM ANOVA, Tukey's post hoc test. (n = 8/group). Pre-Ind=Pre-induction; Post-Ind=post-induction.

Table 2. Average swim speed of the rats during training trials in the Morris water maze before exposure to footshocks and Aβ $_{(1-42)}$ lesioning

Swim speed (cm/s)	Control	Shock	Aβ$_{(1-42)}$	Shock+Aβ$_{(1-42)}$
Day 1	14.91±1.87	17.91±1.19	16.60±0.92	15.78±2.07
Day 2	15.74±2.17	15.82±2.51	16.00±2.22	15.11±2.40
Day 3	16.99±1.57	13.72±1.65	16.80±2.46	13.82±2.01

Speed to reach the escape platform in the target quadrant in the Morris water maze during the training trials before exposure to footshocks and Aβ $_{(1-42)}$ lesioning. Data represents the average swimming speed (cm/sec ± SEM).

The effect of trauma-like exposure and Aβ$_{(1-42)}$ lesion on mRNA expression levels of *Fkbp5 and Bin1* in rats.

To investigate the mechanisms underlying the observed behavioural changes, we started by examining the mRNA expression levels of *Fkbp5* and *Bin1* in the amygdala and hippocampus of the rats. While *Fkbp5* is associated with stress regulation in trauma-induced conditions (Young et al., 2018), *Bin1* is implicated in AD's pathology (De Rossi et al., 2020).

In the amygdala, exposure to footshocks, as well as Aβ$_{(1-42)}$ lesion caused an upregulation of *Fkbp5*. Furthermore, a combination of the two conditions sustained the upregulation of *Fkbp5* (Two-way ANOVA: shock effect ($F_{(1, 12)} = 8.756$, $P = 0.0119$), lesion effect ($F_{(1, 12)} = 25.24$, $P = 0.0003$), shock × lesion effects ($F_{(1, 12)} = 8.473$, $P = 0.0131$); Tukey's post hoc: shock, P=0.0064 vs. control; Aβ$_{(1-42)}$ vs. control, P=0.0006; shock+Aβ$_{(1-42)}$ vs. control, P = 0.0005; Figure 4A).

Similarly, in the hippocampus, we observed that exposure to footshocks and Aβ$_{(1-42)}$ lesion separately caused and upregulation of *Fkbp5*, which was sustained when the two conditions co-occurred (Two-way ANOVA: shock effect ($F_{(1, 12)} = 6.59$, $P = 0.0247$), shock × lesion effect ($F_{(1, 12)} = 8.111$, $P = 0.0147$); Tukey's post hoc: shock vs. control, P = 0.0111; Aβ$_{(1-42)}$ vs. control, P=0.0218; shock+Aβ$_{(1-42)}$ vs. control, P = 0.0308; Figure 4B).

In the amygdala, it was observed that footshocks and $A\beta_{(1-42)}$ lesion separately led to a downregulation of *Bin1*. However, when the two conditions co-occurred, we observed a further downregulation of *Bin1*, suggesting an additive effect of footshocks and $A\beta_{(1-42)}$ lesion (Two-way ANOVA: shock effect ($F_{(1, 12)}$ = 57.97, $P < 0.0001$), lesion effect ($F_{(1, 12)}$ = 22.9, P = 0.0004); Tukey's post hoc: shock vs. control, P = 0.0007; $A\beta_{(1-42)}$ vs. control, P=0.0192; shock+$A\beta_{(1-42)}$ vs. control, $P < 0.0001$, shock+$A\beta_{(1-42)}$ vs. shock, P = 0.0306, shock+$A\beta_{(1-42)}$ vs. $A\beta_{(1-42)}$, P = 0.0010; Figure 4C).

In the hippocampus, footshocks and $A\beta_{(1-42)}$ lesion independently led to a downregulation of *Bin1*. However, a combination of footshocks and $A\beta_{(1-42)}$ lesion tends to sustain *Bin1* downregulation (Two-way ANOVA: lesion effect ($F_{(1, 12)}$ = 9.266, P = 0.0102), shock × lesion effect ($F_{(1, 12)}$ = 18.53, P = 0.0010); Tukey's post hoc: shock vs. control, P = 0.0116, $A\beta_{(1-42)}$ vs. control, P = 0.0011, shock+$A\beta_{(1-42)}$ vs. control, P=0.0550; Figure 4D).

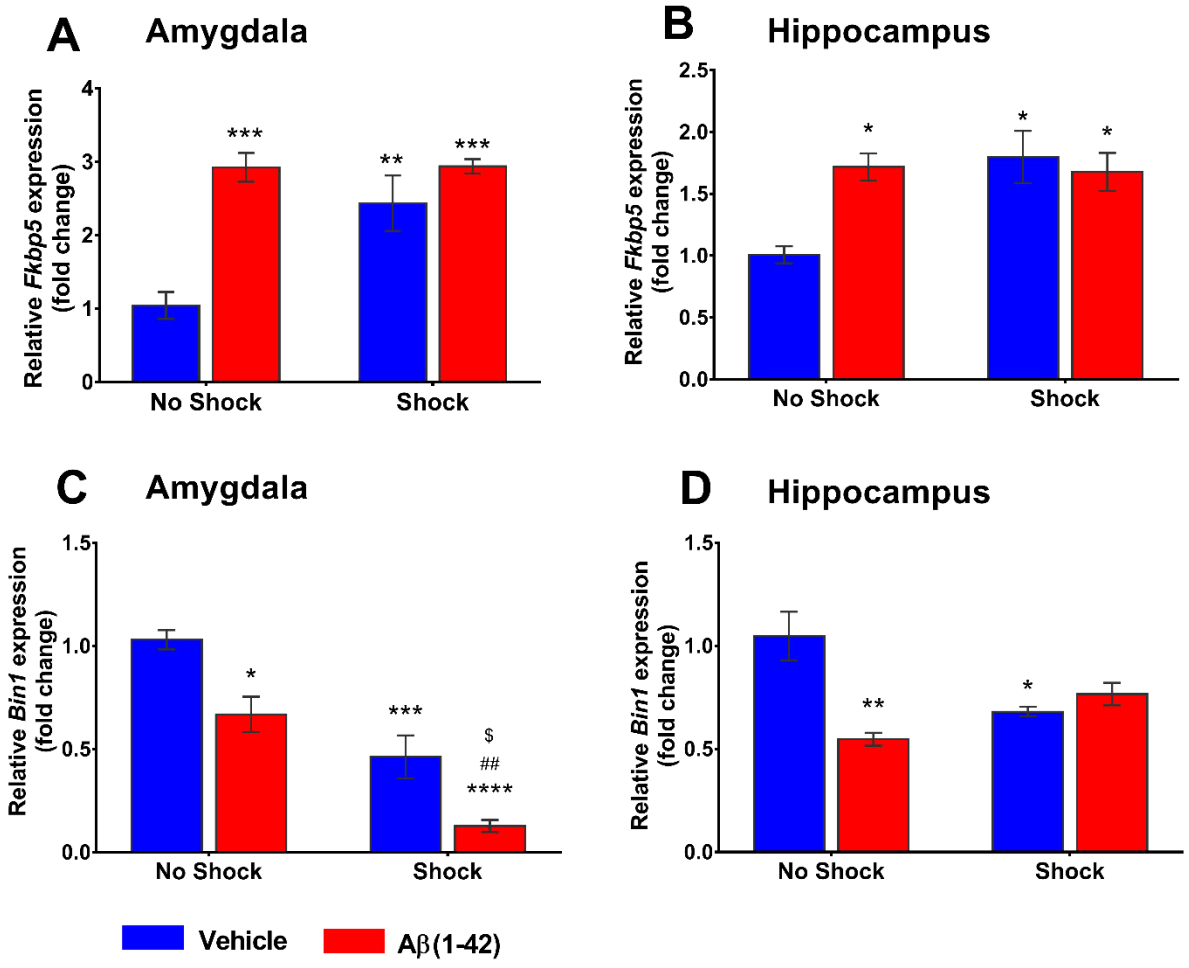


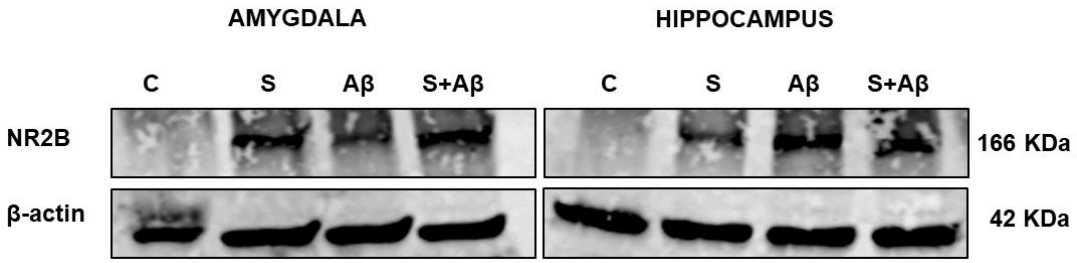
Figure 4: Effect of trauma-like exposure and $A\beta_{(1-42)}$ lesioning on the mRNA expression of (A) *Fkbp5* in the amygdala (B) *Fkbp5* in the hippocampus (C) *Bin1* in the amygdala (D) *Bin1* in the hippocampus. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. control; $^{\$}$P < 0.05 vs. shock; $^{\#\#}$P < 0.01 vs. $A\beta_{(1-42)}$ Two-way ANOVA followed by Tukey's post hoc test. Results expressed as mean ± SEM. (n = 4/group).

The effect of trauma-like exposure and $A\beta_{(1-42)}$ lesion on NR2B protein expression in rats.

The dysregulation of glutamate NR2B protein expression has been implicated in traumatic stress and AD-like pathology (Karthick et al., 2019; Yamamoto et al., 2008). We further investigated whether trauma-like exposure would influence the effect of $A\beta_{(1-42)}$ lesion on NR2B in the amygdala and hippocampus.

In the amygdala, $A\beta_{(1-42)}$ lesion increased NR2B expression. Similarly, footshocks increased NR2B expression. The co-occurrence of footshocks and $A\beta_{(1-42)}$ lesion caused a further increase in NR2B expression (Two-way ANOVA: shock effect ($F_{(1, 8)} = 96.92$, $P < 0.0001$), lesion effect ($F_{(1, 8)} = 24.11$, $P = 0.0012$); Tukey's post hoc: shock vs. control, $P = 0.0004$, $A\beta_{(1-42)}$ vs. control, $P = 0.0245$, shock+ $A\beta_{(1-42)}$ vs. control, $P < 0.0001$, shock+$A\beta_{(1-42)}$ vs. shock, $P = 0.0481$, shock+$A\beta_{(1-42)}$ vs. $A\beta_{(1-42)}$, $P = 0.0007$; Figure 5A).

In the hippocampus, $A\beta_{(1-42)}$ lesion greatly increased NR2B expression, while footshocks showed a slight increase, although not significant. Interestingly, when footshocks preceded $A\beta_{(1-42)}$ lesion, there was a reversal in the increased expression of NR2B, suggesting that footshocks influenced the effect of $A\beta_{(1-42)}$ lesion on NR2B expression in the hippocampus (Two-way ANOVA: lesion effect ($F_{(1, 8)} = 24.78$, $P = 0.0011$), shock × lesion effect ($F_{(1, 8)} = 11.06$, $P = 0.0104$); Tukey's post hoc: $A\beta_{(1-42)}$ vs. control, $P = 0.0017$, $A\beta_{(1-42)}$ vs. shock, $P = 0.0163$; Figure 5B).



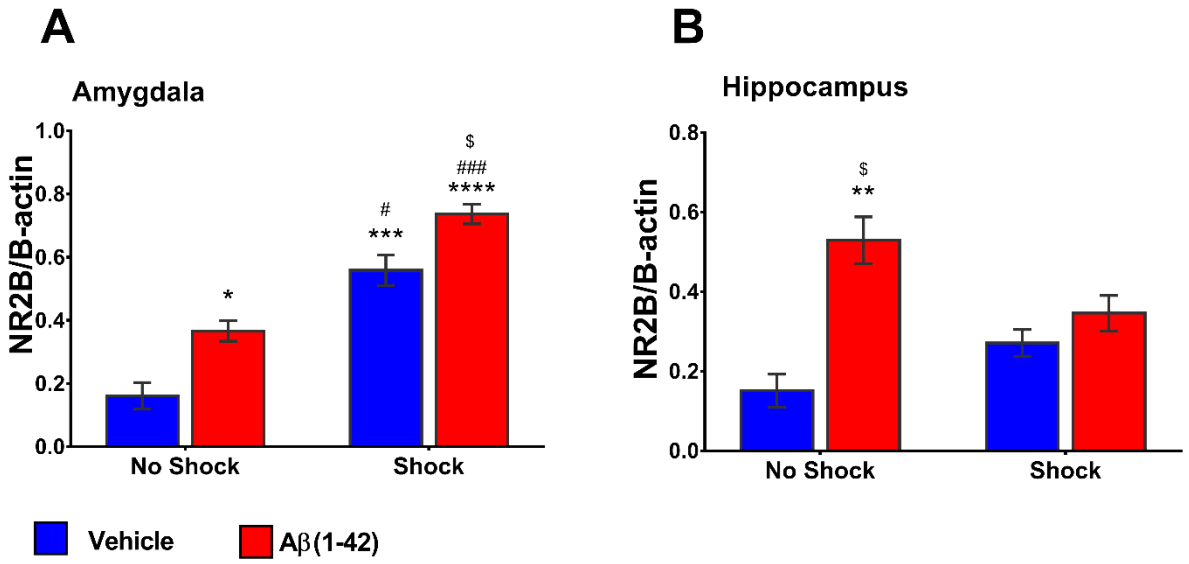


Figure 5: Effect of trauma-like exposure and $A\beta_{(1-42)}$-lesion on the NR2B protein expression in the (A) Amygdala (B) Hippocampus. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. control; $^{\#}$P < 0.05, $^{\#\#\#}$P < 0.001 vs. $A\beta_{(1-42)}$; $^{\$}$P < 0.05 vs. shock. Two-way ANOVA followed by Tukey's post hoc test. Results expressed as mean ± SEM. C = Control; S = Shock; Aβ = $A\beta_{(1-42)}$; S+Aβ = Shock+$A\beta_{(1-42)}$. (n = 3/group).

The effect of trauma-like exposure on neuronal apoptosis in the amygdala and hippocampus of $A\beta_{(1-42)}$-lesioned rats

Cell death can occur because of multiple stresses beyond the homeostatic capabilities of the cell in response to stresses (Chi et al., 2018). We further tested whether the combination of trauma-like stress and $A\beta_{(1-42)}$-lesion would exaggerate cellular apoptosis in the amygdala and hippocampus.

In the amygdala, it was observed that footshocks increased neuronal apoptosis. Similarly, $A\beta_{(1-42)}$-lesion led to an increase in apoptotic neurons. Furthermore, a combination of the two conditions merely sustained the increased neuronal apoptosis. (Two-way ANOVA: shock effect ($F_{(1, 14)}$ = 14.33, P = 0.0020), lesion effect $F_{(1, 14)}$ = 11.16, P = 0.0049), shock × lesion effect ($F_{(1, 14)}$ = 7.862, P = 0.0141); Tukey's Post hoc: shock vs. control, P = 0.0009, $A\beta_{(1-42)}$ vs. control, P= 0.0056, shock+$A\beta_{(1-42)}$ vs. control, P = 0.0004; Table 3a).

In the hippocampus, footshocks and $A\beta_{(1-42)}$ lesion independently increased neuronal apoptosis. However, the combination of the two conditions caused a further increase in neuronal apoptosis (Two-way ANOVA: shock effect ($F_{(1, 14)}$ = 45.96, P = 0.0001), lesion effect ($F_{(1, 14)}$ = 116.5, P < 0.0001), shock × lesion effect (F

$_{(1, 14)}$ = 7.184, P = 0.0179); Tukey's post hoc: shock vs. control, P = 0.0330, Aβ$_{(1-42)}$ vs. control, P = 0.0005, shock+ Aβ$_{(1-42)}$ vs. control, P < 0.0001), shock+Aβ$_{(1-42)}$ vs. shock, P < 0.0001, shock+Aβ$_{(1-42)}$ vs. Aβ$_{(1-42)}$, P = 0.0001; Table 3b).

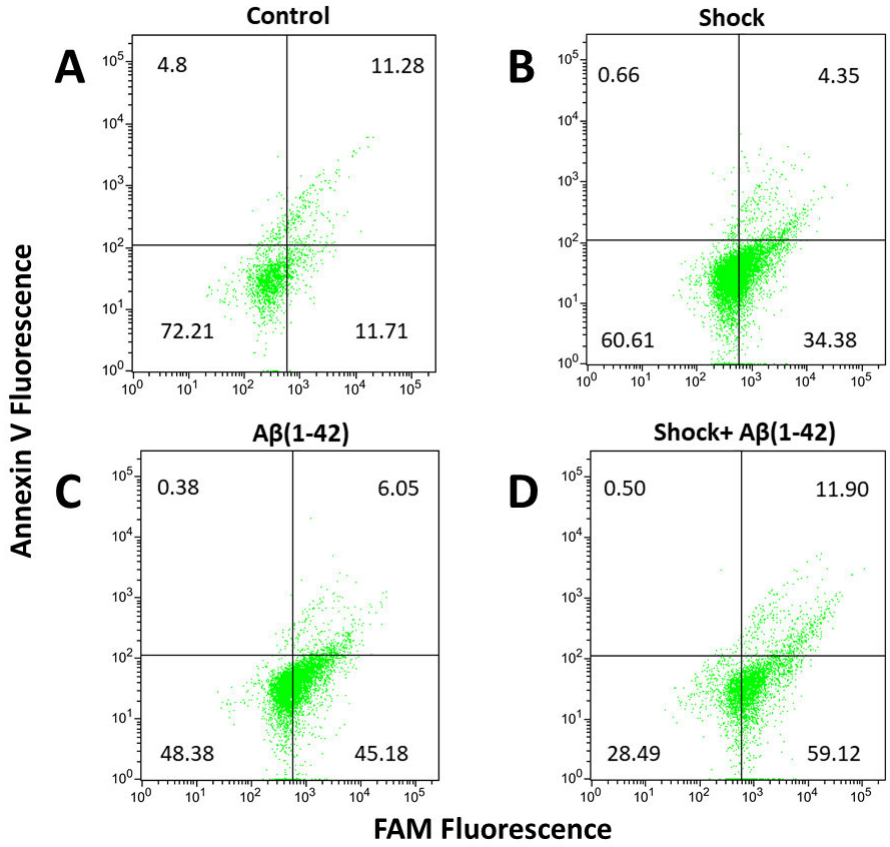


Figure 6: Annexin V staining and Caspase 3 and 7 activation used to measure apoptosis by flow cytometry. Representative dot plots of experimental groups: (A) Control (B) Shock (C) Aβ$_{(1-42)}$ (D) Shock+Aβ$_{(1-42)}$, showing percentage neurons stained with Annexin V and FAM caspase 3 and 7. Early apoptotic cells positive for FAM caspase 3 and 7 can be seen in the bottom right quadrant, and late apoptotic cells positive for annexin V and FAM caspase 3 and 7 in the top right quadrant. Normal cells are negative for both stains. Cells were analyzed on a BD FACSCanto II flow cytometer.

Table 3. Percentage of the total (early + late) apoptotic neuronal cells after exposure to footshocks and Aβ$_{(1-42)}$ lesion

% Total apoptotic cells	Control	Shock	Aβ$_{(1-42)}$	Shock+Aβ$_{(1-42)}$
(a) Amygdala	20.57±5.58	55.31±3.67***	52.96±5.05**	58.14±5.57***

(b) Hippocampus	21.50±2.96	33.87±3.22*	45.99±2.80***	74.53 ±2.41****,###,$$$$

(a) Amygdala (b) Hippocampus. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. control; ###P < 0.001 vs. $A\beta_{(1-42)}$; $$$$P < 0.0001 vs. Shock. Two-way ANOVA followed by Tukey's post hoc test. Results expressed as mean ± SEM. (n = 3-5/group).

Memory decline correlates with *Bin1*, *Fkbp5*, and NR2B expression

A Pearson correlation was performed to test the relationship between the expression of *Bin1*, *Fkbp5*, NR2B and time spent in the target quadrant of the MWM. We observed a positive correlation between time spent in the target quadrant and *Bin1* expression in the amygdala (r = 0.7381, P = 0.0011, Figure 7A) and hippocampus (r = 0.5354, P = 0.0326, Figure 7B). There was a negative correlation between time spent in the target quadrant and *Fkbp5* expression in the amygdala (r = -0.5297, P = 0.0348, Figure 7C) and hippocampus (r = -0.4984, P = 0.0494, Figure 7D). There was also a negative correlation between time spent in the target quadrant and NR2B expression in the amygdala (r = -0.7998, P = 0.0018, Figure 7E) and hippocampus (r = -0.6362, P=0.0261, Figure 7F).

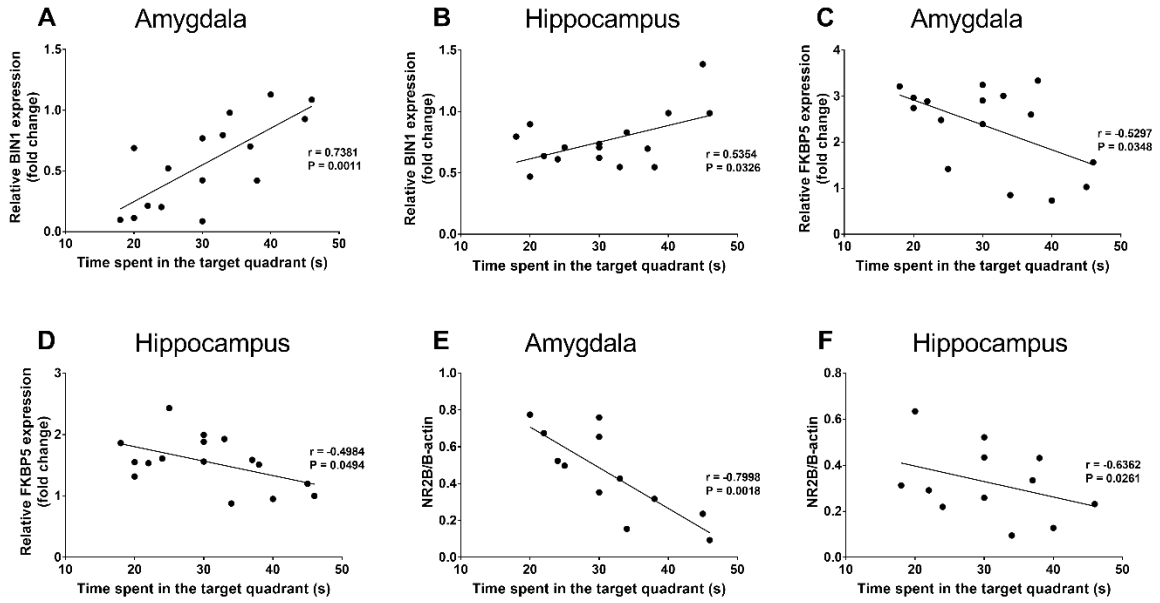


Figure 7: XY scatter plots of time spent in target quadrant of the Morris water maze and (A) *Bin1* expression in the amygdala, (B) *Bin1* expression in the hippocampus, (C) *Fkbp5* expression in the amygdala, (D) *Fkbp5*

expression in the hippocampus, (E) NR2B expression in the amygdala, (F) NR2B expression in the hippocampus of rats treated with footshocks and $A\beta_{(1-42)}$ lesion. Data are from rats in all groups.

Expression of *Bin1* and *Fkbp5* correlates with NR2B expression

A Pearson correlation was performed to test the relationship between *Bin1*, *Fkbp5* and NR2B expression in the amygdala and hippocampus of rats. There was a strong negative correlation between *Bin1* expression and NR2B expression in the amygdala (r = -0.9068, P = 0.00005, Figure 8A) and hippocampus (r = -0.7247, P = 0.0076, Figure 8B). In addition, there was a strong positive correlation between *Fkbp5* expression and NR2B expression in the amygdala (r = 0.6396, P = 0.0251, Figure 8C) and hippocampus (r = 0.6622, P = 0.0190, Figure 8D).

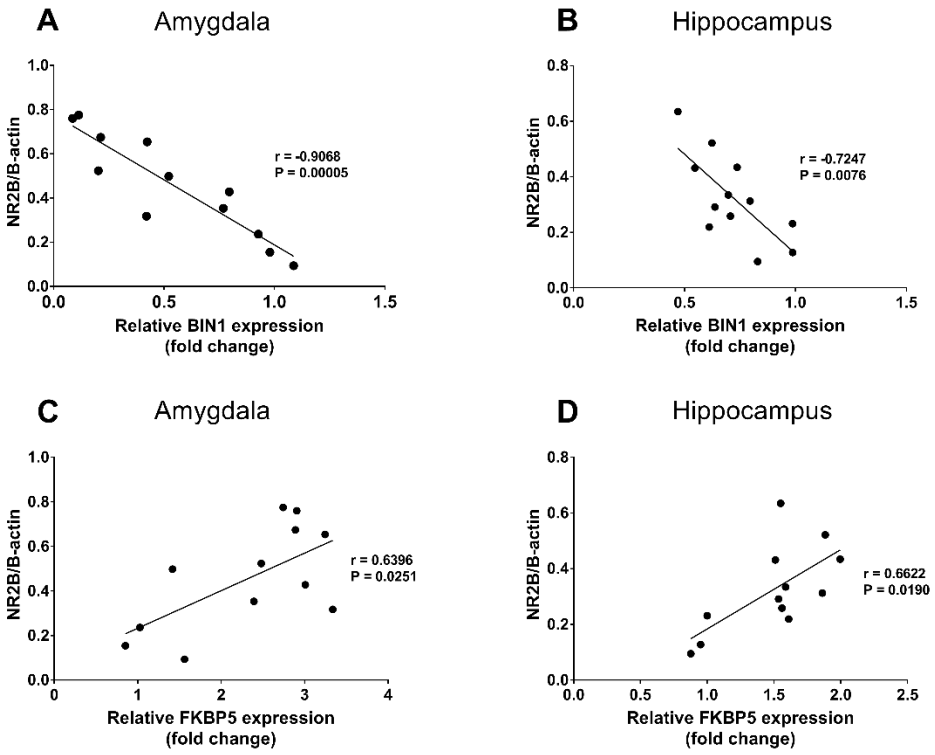


Figure 8: XY scatter plots of relative expression of *Bin1* and NR2B in the (A) amygdala (B) hippocampus; relative expression of *Fkbp5* and NR2B in the (C) amygdala (D) hippocampus of rats treated with footshocks and $A\beta_{(1-42)}$ lesion.

Memory decline is associated with neuronal apoptosis

There was a strong negative correlation between time spent in the target quadrant and percentage of apoptotic cells in the amygdala (r = -0.6584, P = 0.0030, Figure 9A) and hippocampus (r = -0.7465, P = 0.0004, Figure 9B)

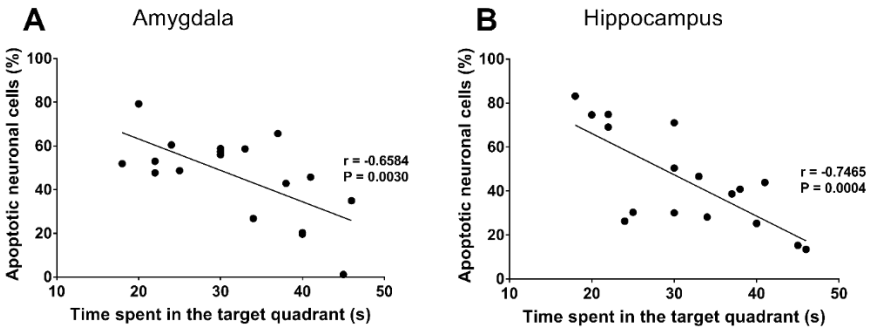


Figure 9: XY scatter plots of time spent in target quadrant of the Morris water maze and percentage apoptosis in the (A) amygdala (B) hippocampus of rats treated with footshocks and $A\beta_{(1-42)}$ lesion

3.4 Discussion

Post-traumatic stress disorder (PTSD) has been shown to be a risk factor in the development of Alzheimer's disease (AD), but the underlying molecular mechanisms remains unclear. Here, we examined the effect of trauma-like stress in an $A\beta_{(1-42)}$ lesion rat model of AD.

In this study, footshocks reduced time spent in the target quadrant of the MWM, as well as $A\beta_{(1-42)}$ lesion. This result is consistent with previous reports showing that footshocks or $A\beta_{(1-42)}$ injection led to spatial memory impairment in rats (Almaguer-Melian et al., 2012; Ding et al., 2015; Shallie et al., 2020; L. Zhang et al., 2015). Further assessment of memory functions in the MWM, by determining the number of times the rats crossed the quadrant where the escape platform was initially located, revealed a reduced number of crossings in rats with prior exposure to footshocks before $A\beta_{(1-42)}$ injection. This suggests that the interactive effects of footshocks and $A\beta_{(1-42)}$ injection could induce spatial memory deficits. Similarly, we have previously shown that a trauma-like exposure can influence $A\beta_{(1-42)}$-induced working memory deficits in rats (Faborode et al., 2021b). Taken together, these findings suggest that traumatic stress may worsen different types of memory in an AD condition.

In addition, we observed that $A\beta_{(1-42)}$ lesion, but not footshocks impaired short-term recognition memory. This result agrees with a previous report which showed that footshocks did not impair short-term memory (Almaguer-Melian et al., 2012). Even when footshocks preceded $A\beta_{(1-42)}$ lesion, the combined effect on recognition memory remained similar to $A\beta_{(1-42)}$ lesion alone, suggesting that trauma-like stress did not influence $A\beta_{(1-42)}$-induced short-term memory deficits. This might be because trauma-like stress activates fear-related memory processes that predominantly involve the amygdala, not the dorsal hippocampus, essential for object recognition memory (Barbosa et al., 2013; Tuscher et al., 2018). Interestingly, our previous study found that a combination of footshock stress and $A\beta_{(1-42)}$ lesion exacerbated impaired fear memory (Faborode et al., 2021a).

ApoE ε4 is the single most important genetic factor associated with an increased risk of developing AD (Fenoglio et al., 2018; Harold et al., 2009). However, *ApoE ε2* is associated with a reduced risk of developing AD but with an increased risk of developing more severe memories in a PTSD condition (Johnson et al., 2019). Bridging integrator 1 (*Bin1*) has been identified as the second most prevalent genetic risk factor in sporadic AD (De Rossi et al., 2020; Glennon et al., 2013). In this study, we showed that *Bin1* expression was reduced in the hippocampus and amygdala after $A\beta_{(1-42)}$ lesion. Similarly, there was a reduction in *Bin1* expression in the amygdala and hippocampus after exposure to footshocks. This is the first report to show the effect of footshock stress on *Bin1* expression in the amygdala and hippocampus of rats. In the hippocampus, the effects of footshock stress and $A\beta_{(1-42)}$ lesion on *Bin1* downregulation was similar, even though the combination of both conditions merely sustained the level of *Bin1* downregulation. However, we observed a further decrease in *Bin1* expression in the amygdala of Aβ-lesioned rats with prior exposure to footshocks, indicating an additive effect of trauma-like stress and $A\beta_{(1-42)}$ lesion. The amygdala is known to respond largely to negative stimuli such as trauma (Tottenham & Sheridan, 2010), and this could exacerbate the effect of Aβ in the amygdala, thereby causing a further reduction in *Bin1* expression. This suggests *Bin1* may be involved in the enhancing effect of a trauma-like exposure in an AD-like condition. Since deficient *Bin1* expression favors Aβ production and consequent memory deficits, it is, therefore, possible that reduced *Bin1* expression following footshocks and $A\beta_{(1-42)}$ lesion may be involved in memory deficits seen in our rats (De Rossi et al., 2020; McAvoy et al., 2019).

Stress regulation involves the activation of glucocorticoid receptors (GRs) (de Kloet et al., 1998). In addition to GRs, *Fkbp5* is also involved in stress regulation by reducing the binding affinity of glucocorticoids to GRs (reduced GR sensitivity), thereby preventing glucocorticoid receptor translocation into the nucleus of the cell (Binder, 2009). The cells of the hippocampus and amygdala have a high expression of GRs, which are involved in regulating responses to stress and trauma exposure (Greenberg et al., 2014; Videlock et al., 2008). Here, we observed an increase in the expression of *Fkbp5* in the amygdala and hippocampus following footshocks. We also observed a similar effect of $A\beta_{(1-42)}$ lesion on increased *Fkbp5* expression in the amygdala and hippocampus of the rats. Therefore, the combined effect of footshocks and $A\beta_{(1-42)}$ lesion merely sustained the increased *Fkbp5* expression. The sustained upregulation of *Fkbp5* in the hippocampus could be involved in the memory deficits observed in this study because overexpression of *Fkbp5* in the hippocampus has been associated with spatial memory deficits (Blair et al., 2019). Although increased stress response in the amygdala is not crucially involved in memory retrieval (J. J. Kim et al., 2001; Roozendaal et al., 1996), however, it may contribute to the impairment of hippocampal-dependent memory retrieval (Roozendaal et al., 2003). These results, therefore, suggest that footshocks stress influences Aβ-induced *Fkbp5* expression in the amygdala and hippocampus, which can be associated with cognitive impairment (Aminyavari et al., 2019; Blair et al., 2019). Furthermore, our recent study also showed that footshock stress increased *Fkbp5* expression in the prefrontal cortex of rats that were injected with $A\beta_{(1-42)}$ (Faborode et al., 2021b). These data strongly suggest that *Fkbp5* dysregulation in critical brain areas (e.g. hippocampus, amygdala and prefrontal cortex) may be a common pathogenic mechanism underlying PTSD and AD.

Moreover, synaptic dysfunction in AD has been strongly correlated with cognitive decline (DeKosky & Scheff, 1990; Mucke & Selkoe, 2012), and there is evidence that increased levels of oligomeric Aβ alters glutamatergic synaptic transmission and induce cognitive deficits via overactivation of glutamate NR2B receptors (Karthick et al., 2019; Sheng et al., 2020). In this study, we found that $A\beta_{(1-42)}$ lesion increased NR2B protein expression in the hippocampus of the rats. This is consistent with a previous study by Karthick and co-workers, who showed that intrahippocampal injection of $A\beta_{(1-42)}$ increased NR2B expression in the hippocampus (Karthick et al., 2019). However, when footshocks preceded $A\beta_{(1-42)}$ lesion, we observed a reduction in the expression of NR2B. A possible explanation might be the loss of neurons in the

hippocampus. The hippocampus is highly susceptible to stress (McEwen, 2007; Semmler et al., 2005). Stress from footshocks in addition to $A\beta_{(1-42)}$ lesion might induce more severe effects in the hippocampus leading to increased apoptosis (J. Kim & Yoon, 1998; Sapolsky, 1996), thereby reducing the population of viable neurons expressing the NR2B protein. Indeed, we found a corresponding exaggerated neuronal apoptosis in the hippocampus (Table 3). The upregulation of NR2B could contribute to the observed memory deficits seen in this study (Hong et al., 2016; Mucke & Selkoe, 2012; Y. Zhang et al., 2016). However, the shock group had increased NR2B expression in the amygdala. This suggests that the amygdala shows a greater response to traumatic stress (Liberzon et al., 1999). Furthermore, we found that a combination of footshocks and $A\beta_{(1-42)}$ lesion caused a further increase in NR2B expression in the amygdala. This suggests that the presence of Aβ was able to exacerbate footshock-induced NR2B upregulation in the amygdala. This, in a way, could have also contributed to the memory deficits seen in the rats, as it has been previously shown that dysregulation of NMDARs in the amygdala influences the effect of stress on spatial learning and memory (Rafia et al., 2020; Roozendaal et al., 2003).

Previous studies have shown that presynaptic and postsynaptic glutamate receptors form protein complexes with *Bin1* to mediate synaptic functions, and reduced *Bin1* expression has been shown to induce glutamate receptor-mediated synaptic dysfunction (De Rossi et al., 2020; Schürmann et al., 2020). Even though we have not shown any evidence of a direct causal effect of reduced *Bin1* expression on NR2B overactivation, we found that the downregulation of *Bin1* correlates with increased NR2B expression in the amygdala and hippocampus. Similarly, *Fkbp5* induce memory deficits via alterations in the complexes formed with glutamate AMPA receptors (Blair et al., 2019). *Fkbp5* mediates stress regulation by reducing GR sensitivity (Binder, 2009), and there is evidence that GRs regulate glutamate NR2B functions (de Kloet et al., 1998; Huang et al., 2010). In our study, we found that an upregulation of *Fkbp5* correlates with an increase in NR2B expression, and upregulation of *Fkbp5* correlated with memory decline, in the amygdala and hippocampus. Increased *Fkbp5* due to stress dysregulation leads to increased glucocorticoid production (Criado-Marrero et al., 2018), which favours Aβ accumulation (Justice et al., 2015). Aβ accumulation can, in turn, induce NR2B-mediated excitotoxicity leading to synaptic dysfunction and hippocampal-dependent memory decline (Gao et al., 2014; Y. Zhang et al., 2016). In addition, the effect of stress on hippocampal-

dependent spatial memory impairment also requires inputs from the amygdala (Roozendaal et al., 2003; Tsoory et al., 2007), and memory dysfunction has been associated with abnormal synaptic function in PTSD (Chen et al., 2020).

Furthermore, there is a direct relationship between memory deficits and neuronal apoptosis in AD (Chi et al., 2018). In this study, exposure to footshocks increased neuronal apoptosis in the amygdala and hippocampus of the rats (Table 3, Figure 6). This result is consistent with a previous study showing that footshocks increased apoptosis in the amygdala and hippocampus of rats (Gao et al., 2014; Huang et al., 2010; Wang et al., 2013). This result suggests that trauma-like stress can induce cellular neuronal in the amygdala and hippocampus of rats. Similarly, $A\beta_{(1-42)}$ lesion increased neuronal apoptosis in the amygdala and hippocampus (Table 3, Figure 6). This is in line with previous reports (Ding et al., 2015; Han et al., 2017). In the amygdala, the combined effect of footshocks and $A\beta_{(1-42)}$ lesion was similar to the single effect of footshocks or $A\beta_{(1-42)}$ lesion. The level of neuronal apoptosis after shock exposure was relatively higher in the amygdala than in the hippocampus, suggesting that the amygdala shows a heightened response to traumatic events (Liberzon et al., 1999). Interestingly, $A\beta_{(1-42)}$ lesion had a relatively similar effect in the amygdala and hippocampus even though the injection site was only in the hippocampus. This result indicates that as AD progresses, other associated brain areas are susceptible to the effect of Aβ pathology (Faborode et al., 2021b; Karthick et al., 2019; Thal et al., 2002). However, when footshock stress preceded $A\beta_{(1-42)}$ lesion, there was greater apoptosis in the hippocampus. The hippocampus is vulnerable to stress (McEwen, 2007; Semmler et al., 2005). It is possible that footshock stress and other stresses due to the direct effect of Aβ injection in the hippocampus added up, thereby enhancing apoptosis in the hippocampus. In addition, we also found that increased neuronal apoptosis correlated with memory decline. It, therefore, seems that cellular death in these brain areas could contribute to the behavioural deficits associated with the pathology of PTSD and AD (J. Li et al., 2010; Wen et al., 2016; Zare et al., 2015). This is because multiple stresses resulting from neurodegeneration or trauma can induce neuronal cell death (Chi et al., 2018; Rami & Kögel, 2008)

Conclusion

Footshock stress and $A\beta_{(1-42)}$ lesion have a similar effect on *Fkbp5* expression in the amygdala and hippocampus, *Bin1* expression in the hippocampus, and neuronal apoptosis in the amygdala. However, an

additive effect of footshock stress and $A\beta_{(1-42)}$ lesion on impairment of spatial memory, *Bin1* and NR2B expression in the amygdala, NR2B expression and neuronal apoptosis in the hippocampus. These results suggest that AD risk in those diagnosed with PTSD may be partly driven by selective mechanisms sensitive to the compound effects of stress and Aβ pathology. More studies are needed to elucidate the involvement of other pathways underlying the risk of AD in PTSD.

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Prologue

In addition to the hippocampus and amygdala, the prefrontal cortex (PFC) is implicated in the pathophysiology of post-traumatic stress disorder (PTSD) and Alzheimer's disease (AD). This chapter investigated associated changes in the PFC following PTSD-like induction in Aβ(1-42) lesion rat model of AD. This manuscript has been published in the journal Behavioural Brain Research journal, Impact factor: 3.332. https://doi.org/10.1016/j.bbr.2021.113679. See appendix VII

4 CHAPTER 4

Inescapable footshocks induce molecular changes in the prefrontal cortex of rats in an amyloid-beta-42 model of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) affects several brain areas, including the prefrontal cortex (PFC) involved in execution, working memory, and fear extinction. Despite these critical roles, the PFC is understudied in AD pathology. People with post-traumatic stress disorder (PTSD) have twice the risk of developing AD, and the underlying mechanisms linking these two diseases are less understood. Here, we investigated the effect of footshock stress on behavioural vis-a-vis molecular changes in the PFC of an amyloid-beta (Aβ)-42 lesion rat model of AD.

Trauma-like conditions were induced by exposing the animals to several footshocks. AD-like condition was induced via intra-hippocampal injection of Aβ-42 peptide. Following Aβ-42 injections, animals were tested for behavioural changes using the Open Field Test (OFT) and Y-maze test. The PFC was later harvested for neurochemical analyses.

Our results showed an interactive effect of footshocks and Aβ-42 lesion on: reduced percentage alternation in the Y-maze test, suggesting memory impairment; reduced number of line crosses and time spent in the centre square of the OFT, indicating anxiogenic responses. Similarly, there was an interactive effect of footshocks and Aβ-42 lesion on: increased FK506 binding protein 51 (FKBP5) expression, which can be associated with stress-induced anxiogenic behaviours; and increased neuronal apoptosis in the PFC of the animals. In addition, footshocks, as well as Aβ-42 lesion, reduced superoxide dismutase levels and Bridging Integrator-1 (BIN1) expression in the PFC of the animals, which can be linked to the observed memory impairment.

In conclusion, our findings indicate that footshocks exaggerate PFC-associated behavioural and molecular changes induced by an AD-like pathology.

Keywords**:** Alzheimer's disease; Post-traumatic stress disorder; Anxiety; Memory; Amyloid-beta; Prefrontal cortex.

4.1 Introduction

Alzheimer's disease (AD) is the most common form of neurodegenerative disease that affects patients' cognitive abilities. The disease is characterised by intracellular neurofibrillary tangles and extracellular amyloid plaques [1,2]. On the other hand, post-traumatic stress disorder (PTSD) is an affective disorder that develops after exposure to an intense trauma [3]. Reports have shown that people who have PTSD are two times at risk of developing AD [4,5]. To the best of our knowledge, few studies have attempted to elucidate the underlying mechanisms that link PTSD to AD. For instance, an earlier study investigated the interaction between acute oligomer amyloid-beta (Aβ)-40 injection and stress. They found that increased glucocorticoid receptor/mineralocorticoid receptor ratio, expression of corticotrophin-releasing factor 1 (CRF-1) receptor, increased Aβ accumulation, oxidative stress, and apoptotic signalling in the hippocampus of mice was associated with severely impaired spatial learning and memory, following a combination of footshocks and Aβ-40 injection. Therefore, they concluded that stress might play an essential role in the early stages of AD [6]. Another work by Justice and co-workers found that exposure to PTSD-like trauma elevated CSF Aβ levels in mice which accelerated AD pathogenesis [7]. They also found that expression of human familial AD mutations in amyloid precursor protein and presenilin 1 led to sensitivity to trauma-induced PTSD-like behavioural and endocrine changes. In addition, they showed that PTSD-like induction and Aβ elevation depends on CRF-1 receptor signalling, highlighting a potential mechanism by which Aβ influences PTSD-like phenotypes [7]. In addition, Agís-Balboa and co-workers found that young mice lacking the Formin2 (Fmn2) gene exhibited PTSD-like phenotypes and developed an accelerated age-associated memory decline, suggesting that the downregulation of Fmn2 in a PTSD mouse model accelerated AD-related pathology [8]. In addition, a recent study using human datasets suggested immune response as a common pathway for PTSD and AD, implicating MS4A6A, a gene encoding cellular membrane proteins involved in signal transduction and regulation of cell activation [9,10]. Despite these reports, the link between PTSD and AD remains unestablished [11,12].

The direct infusion of oligomeric Aβ-42 into the hippocampus of rats is widely used as a model of AD amyloidopathy since the hippocampus is one of the brain areas most affected by neurodegeneration in AD conditions [13,14]. However, several other brain areas are affected, including the PFC [15]. Besides, Aβ deposits are also found in the cortex in AD conditions, which have shared circuitry to the hippocampus [16–18]. The evaluation of the changes in the PFC following intrahippocampal Aβ-

lesion could be very useful to investigate region vulnerability in AD linked to Aβ deposition from one targeted brain structure to connected areas [19]. Also, it avoids additional effects due to direct insult to the PFC tissue via injection [13]. The prefrontal cortex (PFC) is an essential part of the brain involved in several cognitive functions, including working memory and decision making [20,21]. Disruption in the activities of the PFC is implicated in AD and has been associated with Aβ pathology [15,22]. Besides, cognitive deficits involving PFC activities may precede impairments in the functions of other brain areas in an AD condition [23]. In addition, while the PFC is vulnerable to neurodegeneration, it is relatively less studied in AD [24]. Preclinical studies have also shown that Aβ increase in the PFC is associated with a dysregulation in synaptic transmission and cognitive functions [25,26]. Besides, the pathophysiology of AD terminates in apoptosis-mediated neurodegeneration in addition to oxidative stress and Aβ pathologies [27,28]. There is also evidence that Aβ forming plaques induce progressive loss of neurons [29]. Investigation of the vulnerability of PFC in AD as a result of Aβ deposition alongside disruption of neuronal activities is less examined [19].

Bridging Integrator-1 (BIN1) (also called Amphiphysin 2) is the second most important risk factor in sporadic AD and its downregulation has been linked to learning and memory deficits [30,31]. However, its role in mediating PFC functions in AD pathology is also unclear. Equally, the PFC is involved in the regulation of stress and anxiety disorders [21,32]. Stress regulation in the PFC requires a complex interaction between several factors, including FK506 binding protein 51 (FKBP5) and glucocorticoid receptors (GRs). The FKBP5 is upregulated following stress exposure by inhibiting the activities of GRs [33,34]. However, the relationship between stress and FKBP5 activity in the PFC in AD is less understood. The interaction of the PFC with other subcortical structures such as the hippocampus has been suggested as a common molecular target for investigating AD's link with stress-induced diseases [26]. Furthermore, the involvement of PFC in working memory and anxiety regulation in AD pathology suggests it is a critical brain region involved in trauma-induced disease and AD. This study, therefore, aimed to examine the effect of footshock stress on behavioural and molecular changes in the PFC of rats in an Aβ-42 model of AD.

4.2 Materials and methods

Animals

Adult male Sprague Dawley rats (10 weeks old) were used in this study. The rats were randomly divided into four groups of 10 animals each. Animals were kept in the animal facility of the University of KwaZulu-Natal. Animals were housed 4 per cage under standard conditions (lights on 07:00 and off at 19:00 hours; humidity, 55–60%). Animals were provided with standard rat chow and water *ad libitum*. Animals were grouped as follows: (a) *Control*: received no footshocks and a sham

surgery; (b) *Shock*: received footshocks and sham surgery; (c) *Aβ-42*: received no footshock and Aβ-42 lesion; (d) *Shock+Aβ-42*: received footshocks and Aβ-42 lesion. The experimental protocol used in this study was approved by the animal ethics of the University of KwaZulu-Natal, with ethics number AREC/005/019D.

Experimental design:

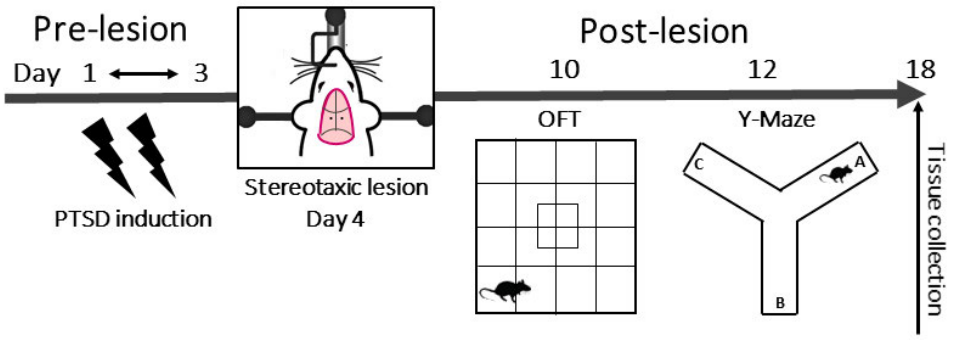

Figure 1: Experimental timeline. On Days 1 to 3, rats received footshocks or nothing. Twenty-four (24) hours later, they received a bilateral intra-hippocampal infusion of Aβ-42 or vehicle solution (Day 4). On Day 10, rats were tested for anxiety and exploratory activities using the open field test. On Day 12, working memory was assessed on the Y-maze test. Finally, on Day 18, brain tissue samples were collected.

Post-traumatic stress disorder rat model

To induce a traumatic stress-like condition, we used the stress-enhanced fear learning (SEFL) protocol as previously described by [35]. This animal model presents a crucial characteristic of PTSD, including fear learning resulting from exposure to a stressor. The experiment lasted for three days. On day 1 of the experiment, the rat was put in a conditioning chamber with a series of floor grids connected to an electric source (Context A). The rat was administered 15 footshocks (1mA, 1 second) distributed over 90 minutes. Freezing was measured after the footshocks. Freezing is defined by the absence of movements except the ones involved in respiration. The inter-stimulus interval was 6 minutes. After the footshocks, the rat was returned to its home cage. In between rats, the chamber was cleaned with an alcohol solution to remove odour cues. On day 2, the rat was placed in a new chamber (Context B) that was different in shape, colour, and odour to differentiate it from the first chamber (Context A). The rat was allowed to acclimatise for 3 minutes. Freezing was recorded in this period. After acclimatisation, the rat was administered one footshock, after which the rat was returned to its home cage. On day 3 of the experiment, the rat was returned to the chamber used on day 2 (Context

B), and freezing was measured for 5 minutes. Sham-treated rats were also placed in the chambers for the same duration and days without footshocks.

Alzheimer's disease-like model

The intra-hippocampal injection of Aβ-42 is a widely accepted AD model, as it reproduces critical aspects of the disease, including neuronal death and memory impairment [13,14]. To induce an AD-like condition, the rats were infused with a solution of Aβ-42 peptide (Invitrogen, USA) into the hippocampus as described by [36]. Before the surgery, the rat received an intraperitoneal injection of a cocktail of anaesthesia (ketamine, 100 mg/kg, and xylazine, 10 mg/kg). The surgery procedure only started when the animal had been deeply anaesthetised, confirmed by a toe pinch. The rat skull was shaved to expose the skin on the scalp. The rat was gently placed in the stereotaxic frame and firmly fixed with the aid of two ear bars. The skin was incised to expose the skull, two holes were gently drilled on the surface of the skull according to the following coordinates: from bregma: anteroposterior (AP) = −4.8 mm; mediolateral (ML) = ±3.4 mm; dorsoventral (DV) = −3.0 mm) [37]. A 25G Hamilton syringe was inserted into the CA1 area of the hippocampus from the drilled holes, and 5 µl (1 µg/µl) of the Aβ-42 solution was injected at the rate of 1 µl /minute. After injection, the needle was left in place for another 2 minutes to allow for proper diffusion of the injected solution. Following this, the needle was withdrawn, the skull cleaned with a sterile swab, the skin sutured and cleaned with an antiseptic. The rat was placed under the heating pad to recover. Temgesic (0.05 mg/kg) was administered subcutaneously to the rats as a postoperative analgesic. Sham treated rats were infused with the same volume of the vehicle solution (phosphate-buffered saline, PBS).

Behavioural testing

Animals were examined for exploration, anxiety-like behaviour, and memory functions using the Open Field apparatus and Y-maze tests.

Open Field Test (OFT)

The open field is a widely used test for accessing exploratory and anxiety-like behaviour in rodents [38]. The apparatus was made from a wooden box of the dimension: 45 ×100 ×100 cm (H × L × W). The floor was divided into 16 equidistant squares and a centre square. The rat is placed into the centre square and allowed to explore the box for 5 minutes. A camera is placed above the box to record the behaviour of the rat. The parameters measured include time in the centre square, number of line crossings, grooming, rearing and defaecation frequency [39].

Y-maze test

The Y-maze was used to assess short-term working memory [40]. The apparatus comprises three enclosed arms (50 ×10 cm) labelled A, B, and C. The arms were inclined at 120 degrees to each other.

Spontaneous alternation assesses spatial working memory. It is based on the tendency for rats to explore unvisited areas [40]. Briefly, the rat was placed at the distal part of the arm "A" facing the centre and allowed to explore the maze for 5 minutes. The animal was left undisturbed during the test session. The succession of arms entered, and the number of entries was noted within the 5 minutes. A video camera was placed above the apparatus to record the experiment. The apparatus was cleaned with alcohol solution between rats to remove odour cues. An entry was defined as all four limbs entering an arm. Alternation was defined as when the rat entered all three arms consecutively. Percentage alternation was calculated as follows: $\% \, Alternation = \frac{\text{Number of Alternations}}{(\text{Total number of arm entries} - 2)} \times 100$ [41].

Sample collection

Following behavioural testing, rats were euthanised using a guillotine. The brain was quickly removed, sections of the prefrontal cortex were obtained by a coronal cut stereotaxically (+4 mm from the bregma) [37], snap-frozen in liquid nitrogen, and stored at -80°C for later analyses.

Enzyme-linked immunosorbent assay (ELISA)

To determine the concentrations of superoxide dismutase (SOD) in the prefrontal cortex (PFC), we used a commercially available rat SOD kit (Elabscience Biotechnology, China), as per the manufacturer's instructions. First, 50 mg of PFC tissue was homogenised in phosphate-buffered saline (PBS) (Sigma-Aldrich) at a ratio of 1:9 using a tissue sonicator (BuenoBiotech, China). The homogenates were spun at 5000xg for 5 minutes at 4°C with a centrifuge (HERMLE Labortechnik GmbH, Germany) to obtain supernatant. Then, 50 µL of each sample and biotinylated detection antibody working solution was added to the pre-coated 96-well plate. The plate was then incubated at 37°C for 45 minutes. After incubation, the wells were washed, then 100 µL of Horseradish peroxidase (HRP) solution was added to the plate, which was incubated for 30 minutes at 37°C. The plate was thereafter decanted of the solution and washed five times. Following the washes, 90 µL of substrate reagent was added, and the plate was again incubated for 15 minutes at 37°C, protected from sunlight. Following the incubation, 50 µL of the stop solution was added to each well. The optical density was read at 450 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Germany).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Brain tissue (prefrontal cortex) were lysed using the RNA shield ™, after which RNA extraction was done using a commercially available RNA kit according to the manufacturer's protocol (Zymo Research, USA). The purity of the extracted RNA was determined using the NanoDrop™ (Life Scientific, USA). RNA was converted to complementary DNA (cDNA) by reverse-transcription using the iScript™ cDNA Synthesis Kit according to the manufacturer's protocol (Bio-Rad, USA). A 10 µl

PCR reaction volume containing cDNA template, forward and reverse primer, SYBR green master mix, and RNAse-free water was prepared and run in duplicates on a light cycler (Roche, Germany), according to the following reaction conditions: denaturation at 95°C, 10 seconds; primer annealing at 51°C (BIN1), 51°C (FKBP5), and 51°C (GAPDH) for 30 seconds; elongation at 72°C, 20 seconds. The following primers were used: BIN1 forward: 5'-AAGGCGGATGAAACGAAGGA-3'; reverse: 5'-GGCTTTCTCAAGCAGCGAG-3'; FKBP51 forward: 5'-ACTGACTCGCCTGACACAAG-3'; reverse: 5'-GAGCGAGGTATCTGCCTGTC-3', GAPDH forward: 5'-TGTGAACGGATTTGGCCGTA-3'; reverse: 5'-ATGAAGGGGTCGTTGATGGC-3'. GAPDH was used as the reference gene, and fold change was calculated using the delta-delta CT method [42,43].

Neuronal cell isolation and flow cytometry

Single-cell suspension from brain tissue (prefrontal cortex) was done as described by [44,45]. The tissue was placed on a glass plate and minced in 2 drops of PBS-T (Sigma-Aldrich) for 2 minutes. The minced tissue was then homogenised in 650 µl of PBS-T at 110xg for 2 mins. The resulting suspension was passed through a 19G needle 12 times. The suspension was centrifuged, and the supernatant was discarded. A volume of 1 ml of Accutase enzyme solution (Life Technologies, CA, USA) was added to the pellet and placed on a rocker for 20 minutes at 200 rpm. After digestion, the suspension was centrifuged and washed in PBS-T. The suspension went through four triturations in 650 µl of PBS-T: once through 21G needle, followed by a 23G needle, and twice in a 25G needle. Each trituration involved passing the solution through the needle 12 times, after which the suspension is left standing to settle. The supernatant was then collected before adding another 650 µl of PBS-T for the next trituration. The supernatant from each trituration was collected in a tube through a 40 µm mesh strainer (Corning, USA). After the four triturations, all supernatants were collected and stored at 4°C until antibody labelling.

The cell suspension was incubated with 2.5 µL of PE Annexin V (BD Biosciences, USA, 560930) followed by 2.5 µL of FLICA™ reagent (Invitrogen, USA, Vybrant FAM Caspase-3 and -7, V35118) for 60 minutes at room temperature away from light, according to the manufacturer's instructions. After that, the cells were fixed and permeabilised using the Fix and Perm ® Cell Permeabilization kit (Life Technologies), according to the manufacturer's instruction. After permeabilization, the cells were incubated with 5 µg/ml of NeuN-AlexaFluor 647 (Novusbio, USA, NBP1-92693AF647) for 30 minutes at room temperature. Labelled cells were analysed on the BD FACSCanto II flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

Gating strategy for the quantification of apoptotic cells:

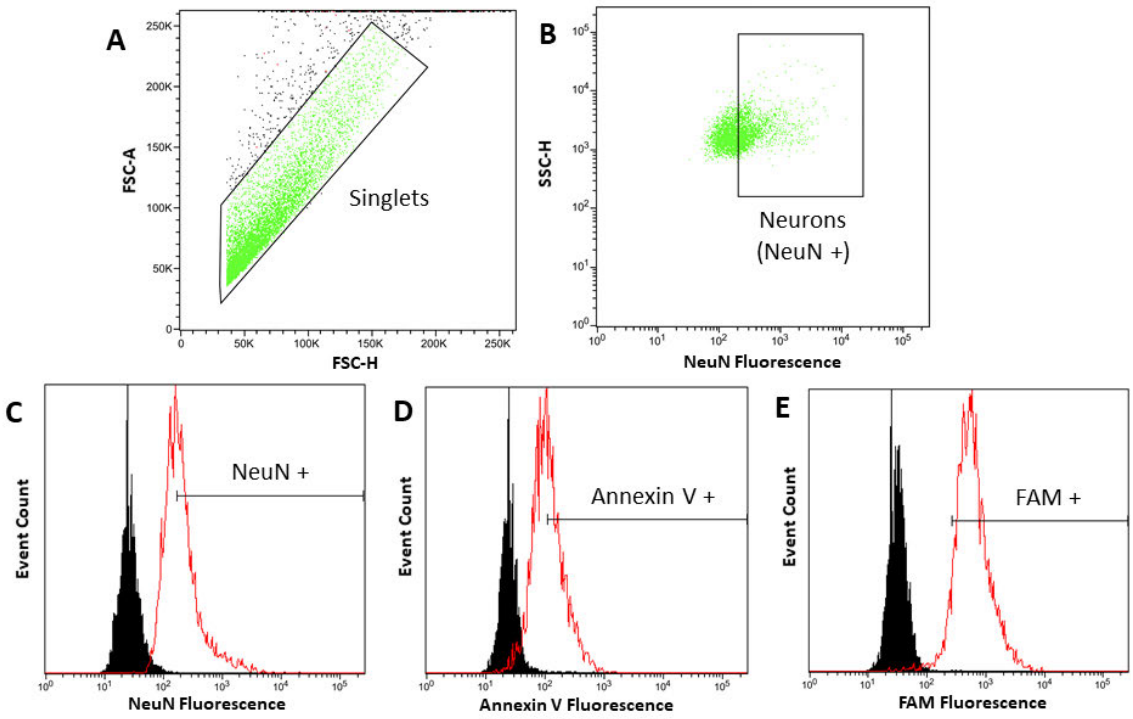

Figure 2: Gating strategy used to detect the percentage of Annexin V positive and caspase -3/-7 activation in a neuron population (A) forward scatter to discriminate doublets using unstained cells. (B) NeuN-labelled cells to show neurons (C-E) unstained control to set negative and positive events for NeuN, Annexin V, and FAM Caspase 3/7.

Statistical Analysis

Data were tested for normality using Shapiro-Wilk's test. Analyses were done using two-way ANOVA followed by Tukey's post hoc test. Pearson correlation coefficients were used to determine the relationship between observed parameters. All data represents as mean ± SEM. $P<0.05$ was considered significant. Data were analysed using GraphPad Prism version 7 (San Diego, USA).

4.3 Results

Footshocks increased freezing levels in rats

The stress-enhanced fear learning (SEFL) protocol was used to induce the trauma-like condition. Four groups of animals viz control, shock, Aβ-42, and shock+Aβ-42 were used. Two-way repeated measure (RM) showed a significant shock × time effect on freezing levels ($F (6, 72) = 338.4$, $P<0.0001$). There was also a significant shock ($F (3, 36) = 1145$, $P<0.0001$) and time ($F (2, 72) = 1017$, $P<0.0001$) effects. Post hoc analyses showed that rats that received footshocks (i.e., shock and shock+Aβ-42 groups) had increased freezing after several footshocks delivered on Day 1 in context A

(fear to trauma) when compared to rats that did not receive any footshock (shock vs control, P <0.0001; shock+Aβ-42 vs control, P <0.0001). This was reversed on Day 2 in context B before single footshock was delivered. On day 3 (fear to mild footshock treatment), rats that received single footshock (i.e., shock and shock+Aβ-42 groups) on Day 2 in context B had increased freezing when compared to rats that did not receive any footshock (shock vs control, P <0.0001; shock+Aβ-42 vs control, P <0.0001).

Footshocks impaired working memory in Aβ-42-lesioned rats

After Aβ-42 lesion, the four groups of rats viz control, shock, Aβ-42, and shock+Aβ-42 were tested in the Y-maze for working memory. Two-way ANOVA revealed a significant shock × lesion effect on percentage alternation (F (1, 36) = 4.954, P=0.0324). There was also a significant shock effect (F (1, 36) = 21.89, P<0.0001) and lesion effect (F (1, 36) = 35.86, P<0.0001). Post hoc analyses revealed a significant reduction in percentage alternation in all groups when compared to control (P<0.001) (Figure 3A). However, two-way ANOVA revealed a shock but not lesion effect on the number of arm entries (F (1, 36) = 7.291, P=0.0105). Post hoc analyses showed a significant reduction in arm entries in the shock groups when compared to control (P<0.05) (Figure 3B).

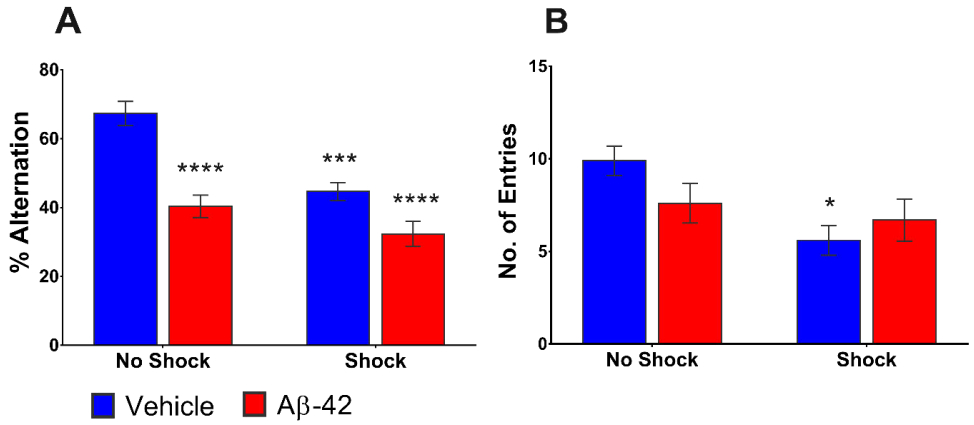

Figure 3: Effect of footshocks and Aβ-42 lesion on (A) percentage alternation in Y-maze [shock × lesion effect, F (1, 36) = 4.954, P=0.0324; shock effect, F (1, 36) = 21.89, P<0.0001; lesion effect, F (1, 36) = 35.86, P<0.0001], and on (B) number of arm entries in Y-maze [shock effect, F (1, 36) = 7.291, P=0.0105]. Results expressed as mean ± SEM, Two-way ANOVA, Tukey's post hoc test, *P<0.05, ***P<0.001, ****P<0.0001 vs. control group. n=10/group.

Footshocks induced anxiety-like behaviours in Aβ-42-lesioned rats

The rats were also tested for anxiety-like and exploratory behaviours in the Open Field Test (OFT). Two-way ANOVA showed a significant shock × lesion effect on time spent in the centre square ($F(1, 36) = 6.376$, $P=0.0161$). There was also a shock effect ($F(1, 36) = 16.17$, $P=0.0003$) and lesion effect ($F(1, 36) = 26.46$, $P<0.0001$). Post hoc analyses showed a significantly reduced time spent in the centre square in all the groups when compared to the control ($P<0.001$) (Table 1a). Similarly, there was a significant shock × lesion effect on exploratory behaviour as revealed by a reduction in the number of lines crosses in the OFT ($F(1, 36) = 4.629$, $P=0.0382$). There was also a shock effect ($F(1, 36) = 61.55$, $P<0.0001$) and lesion effect ($F(1, 36) = 15.85$, $P=0.0003$). Post hoc analyses also showed a significant reduction in the number of lines crossed in all groups compared to control ($P<0.001$). There was a further reduction in the shock+Aβ-42 when compared to Aβ-42 group ($P<0.01$) (Table 1b). In addition, two-way ANOVA revealed a significant shock effect on grooming time ($F(1, 36) = 9.363$, $P=0.0042$). Post hoc analyses showed a significant reduction in grooming time in the shock+Aβ-42 when compared to control ($P<0.05$) and Aβ-42 ($P<0.05$) groups (Table 1c). Two-way ANOVA also revealed a significant shock effect on defecation frequency ($F(1, 36) = 34$, $P<0.0001$). Post hoc analyses showed a significant increase in defecation frequency in the shock ($P<0.01$) and shock+Aβ-42 ($P<0.001$) groups when compared to control. There was also a significant increase in the shock+Aβ-42 group when compared to Aβ-42 ($P<0.001$) (Table 1e). However, there were no significant changes in the number of rearings (Table 1d).

Table 1. The effect of footshocks and Aβ-42 lesion on exploration and anxiety parameters in the Open Field Test

	Control	**Shock**	**Aβ-42**	**Shock+Aβ-42**
(a) Time in centre square (s)	6.1±0.73	2.6±0.52***	2±0.47****	1.2±0.33****
(b) Number of lines crossed	71.1±4.44	32.3±3.57****	47.3±3.89***	25.2±3.55****, ##
(c) Grooming time (s)	7.5±1.34	5.2±0.89	6.8±1.24	2.6±0.6*, #
(d) Number of rearing	7.1±1.44	6.4±1.33	7.9±0.92	6.5±1.27
(e) Defecation frequency	1±0.47	5.2±0.84**	1.5±0.60	6.2±1.02***, ###

(a) Time in centre square: shock × lesion effect, $F(1, 36) = 6.376$, $P=0.0161$; shock effect, $F(1, 36) = 16.17$, $P=0.0003$; lesion effect ($F(1, 36) = 26.46$, $P<0.0001$) (b) number of line crosses: shock × lesion effect: $F(1, 36) = 4.629$, $P=0.0382$; shock effect, $F(1, 36) = 61.55$, $P<0.0001$; lesion effect, $F(1, 36) = 15.85$, $P=0.0003$ (c) grooming time: shock effect, $F(1, 36) = 9.363$, $P=0.0042$ (d) number of rearing: no significant changes (e) defecation frequency: shock effect, $F(1, 36) = 34$, $P<0.0001$.

Results expressed as mean ± SEM, Two-way ANOVA, Tukey's post hoc test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. Aβ-42. n=10/group.

Effect of footshocks and Aβ-42 lesion on SOD levels in the prefrontal cortex of rats

Following footshocks and Aβ-42 lesion, we measured the level of SOD in the prefrontal cortex of the rats. As shown in Figure 4, two-way ANOVA revealed a significant shock effect (F (1, 12) = 13.1, P=0.0035) and lesion effect (F (1, 12) = 30.21, P=0.0001). Post hoc analyses revealed a significant reduction in all groups compared to control (P<0.05). There was also a significant reduction in the shock+Aβ-42 group compared to shock group (P<0.05).

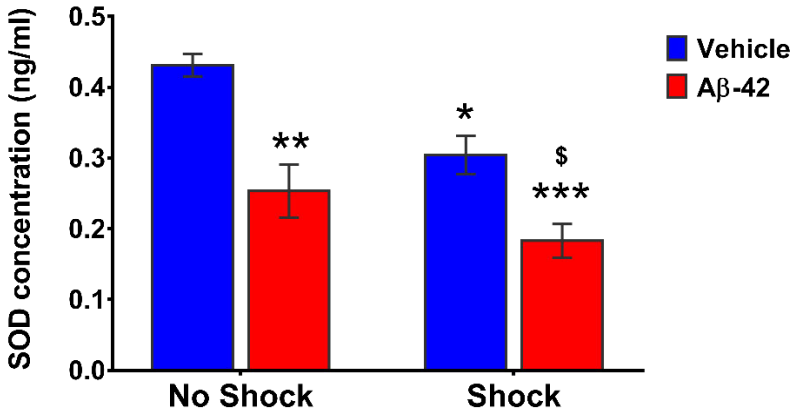

Figure 4: Effect of trauma-like exposure and Aβ-lesion on SOD levels in the prefrontal cortex of rats.

Shock effect, F (1, 12) = 13.1, P=0.0035; lesion effect, F (1, 12) = 30.21, P=0.0001. Results expressed as mean ± SEM, Two-way ANOVA, Tukey's post hoc test, *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\$}$P<0.05 vs. Shock+Aβ-42. n=4/group.

Footshocks increases FKBP5 mRNA expression in the prefrontal cortex of Aβ-42-lesioned animals

As shown in Figure 5A, two-way ANOVA revealed a significant shock × lesion effect on FKBP5 mRNA expression (F (1, 12) = 5.033, P=0.0445). There was a significant shock effect (F (1, 12) = 100.5, P<0.0001) and lesion effect (F (1, 12) = 48.77, P<0.0001). Post hoc analyses revealed a significant increase in FKBP5 mRNA expression in all groups compared to control (P<0.05). In addition, there was a significant increase in the shock+Aβ-42 when compared to the shock group (P<0.001) and Aβ-42 group (P<0.0001). Furthermore, we also determined the level of BIN1 mRNA expression, two-way ANOVA showed a significant shock effect (F (1, 12) = 15.65, P=0.0019) and

lesion effect (F (1, 12) = 21.65, P=0.0006). Post hoc analyses revealed a significant reduction in all groups compared to control (P<0.05) (Figure 5B).

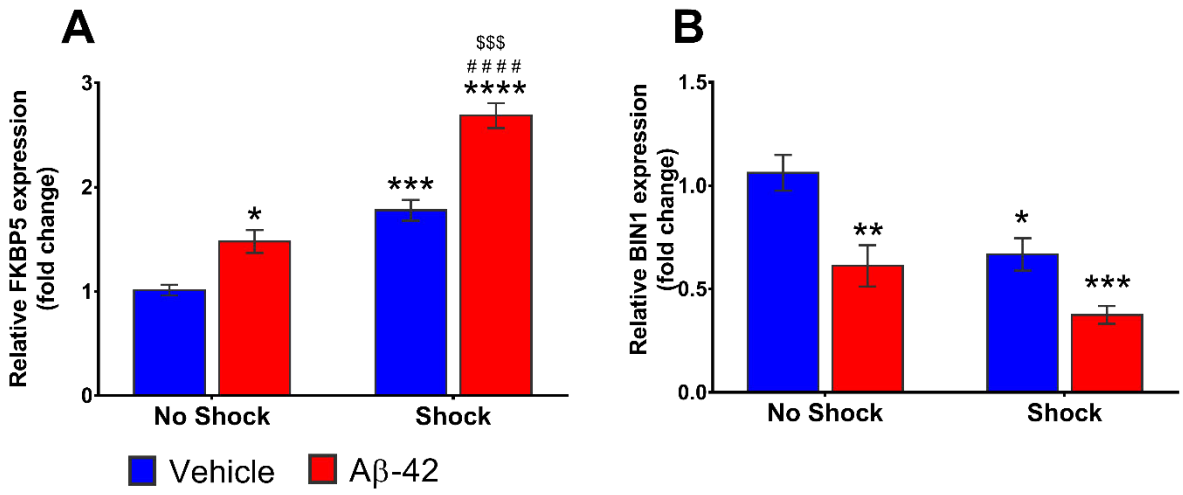

Figure 5: Effect of trauma-like exposure and Aβ-42 lesion on the mRNA expression of (A) FKBP5 [shock × lesion effect, F (1, 12) = 5.033, P=0.0445; shock effect, F (1, 12) = 100.5, P<0.0001; lesion effect, F (1, 12) = 48.77, P<0.0001], (B) BIN1 [shock effect, F (1, 12) = 15.65, P=0.0019; lesion effect (F (1, 12) = 21.65, P=0.0006]. Results expressed as mean ± SEM, Two-way ANOVA, Tukey's post hoc test, *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\$\$\$}$P<0.001 vs. shock, $^{\#\#\#\#}$P<0.0001 vs. Aβ-42. n=4/group.

Footshocks induced neuronal apoptosis in the prefrontal cortex of Aβ-42-lesioned rats

As shown in Figure 6, two-way ANOVA revealed a significant shock × lesion effect on apoptotic neurons in the prefrontal cortex (F (1, 14) = 5.581, P=0.0332). There was a significant shock effect (F (1, 14) = 14.69, P=0.0018) and lesion effect (F (1, 14) = 25.84, P=0.0002). Post hoc analyses revealed a significant increase in apoptosis expression in all groups compared to control (P<0.01).

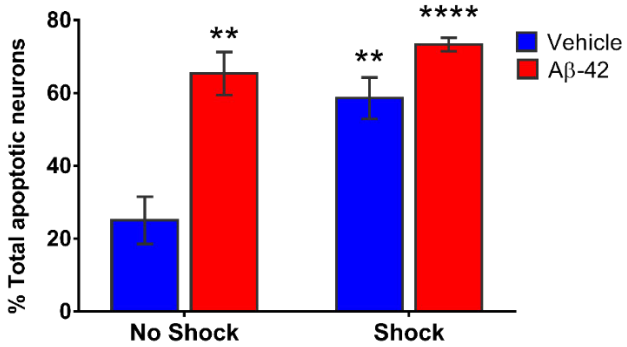

Figure 6: Effect of footshocks and Aβ-42 lesion on neuronal apoptosis in the prefrontal cortex.

Total (early+late) apoptosis: shock × lesion effect, F (1, 14) = 5.581, P=0.0332; shock effect, F (1, 14) = 14.69, P=0.0018; lesion effect, F (1, 14) = 25.84, P=0.0002. Results expressed as mean ± SEM, Two-way ANOVA followed by Tukey's post hoc test, **P<0.01, ****P<0.0001 vs. control; ###P<0.001, n=3/5 per group.

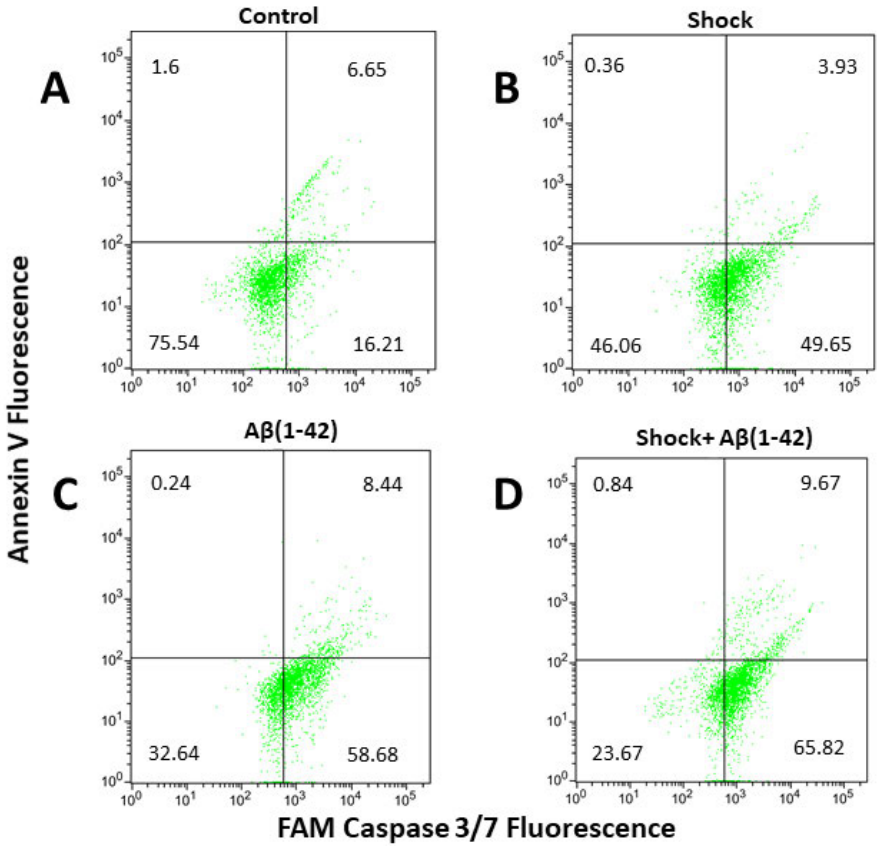

Figure 7: Annexin V staining and caspase 3 and 7 activation to measure neuronal apoptosis by flow cytometry. Representative dot plots of treatment groups: (A) Control (B) Shock (C) Aβ-42 (D) Shock+Aβ-42, showing percentage neurons stained with Annexin V and FAM caspase 3 and 7. Early apoptotic cells positive for FAM caspase 3 and 7 can be seen in the bottom right quadrant, and late apoptotic cells positive for annexin V and FAM caspase 3 and 7 in the top right quadrant. Normal cells are negative for both stains. Cells were analysed on a BD FACSCanto II flow cytometer.

Correlation between anxiety-like behaviour and neurochemical changes in the prefrontal cortex of rats treated with footshocks and Aβ-42 lesion

A Pearson correlation was performed to test whether the molecular changes in the PFC of the rats were associated with anxiety-like behaviour in the OFT post-treatment (footshocks and Aβ-42 lesion). As shown in Table 2a, there was a strong correlation between time spent in the centre square of the OFT and SOD level (r=0.5511, P=0.0269), FKBP5 mRNA expression (r=-0.5955, P=0.0149), and

BIN1 mRNA expression (r=0.7430, P=0.0009) in the PFC of the rats. There was also a strong correlation between the number of line crossings in the OFT and SOD level (r=0.6450, P=0.0069), FKBP5 mRNA expression (r=-0.7489, P=0.0008), and BIN1 mRNA expression (r=0.7309, P=0.0013) in the PFC of the rats (Table 2b). In addition, there was a strong correlation between grooming time in the OFT and SOD level (r=0.5985, P=0.0143), FKBP5 mRNA expression (r=-0.6257, P=0.0095), and BIN1 mRNA expression (r=0.7676, P=0.0005) in the PFC of the rats (Table 2c). There was also a strong correlation between defecation frequency in the OFT and SOD level (r=-0.5187, P=0.0395), FKBP5 mRNA expression (r=0.8328, P=0.00006), and BIN1 mRNA expression (r=-0.5126, P=0.0423) in the PFC of the rats (Table 2d).

Table 2. The association of anxiety-like behaviour with neurochemical changes in the prefrontal cortex of rats treated with footshocks and Aβ-42 lesion

		SOD concentration (ng/ml)	FKBP5 mRNA expression (fold change)	BIN1 mRNA expression (fold change)
a.	Time spent in the centre square of the OFT (s)	r=0.5511, P=0.0269	r=-0.5955, P=0.0149	r=0.7430, P=0.0009
b.	Number of line crossing in the OFT	r=0.6450, P=0.0069	r=-0.7489, P=0.0008	r=0.7309, P=0.0013
c.	Grooming time in the OFT (s)	r=0.5985, P=0.0143	r=-0.6257, P=0.0095	r=0.7676, P=0.0005
d.	Defecation frequency in the OFT	r=-0.5187, P=0.0395	r=0.8328, P=0.00006	r=-0.5126, P=0.0423

(a) time spent in the centre square of the OFT vs SOD level; FKBP5 mRNA expression; and BIN1 mRNA expression (b) number of line crossing in the OFT vs SOD level; FKBP5 mRNA expression; and BIN1 mRNA expression (c) grooming time in the OFT vs SOD level; FKBP5 mRNA expression; and BIN1 mRNA expression (d) defecation frequency in the OFT vs SOD level; FKBP5 mRNA expression; and BIN1 mRNA expression. r=Pearson correlation coefficient.

Correlation between memory decline and neurochemical changes in the prefrontal cortex of rats treated with footshocks and Aβ-42 lesion.

To test whether the molecular changes in the PFC of the animals was associated with memory decline in the Y-maze post-treatment (footshocks and Aβ-42 lesion), a Pearson correlation was performed. As shown in Figure 8A, there was a strong correlation between percentage alternation and SOD level in the PFC of the rats (r=0.5742, P=0.0200). There was also a strong correlation between percentage alternation and FKBP5 mRNA expression in the PFC of the rats (r=-0.5527, P=0.0264, Figure 8B). There was a strong correlation between percentage alternation and BIN1 mRNA expression in the PFC of the rats (r=0.7346, P=0.0012, Figure 8C).

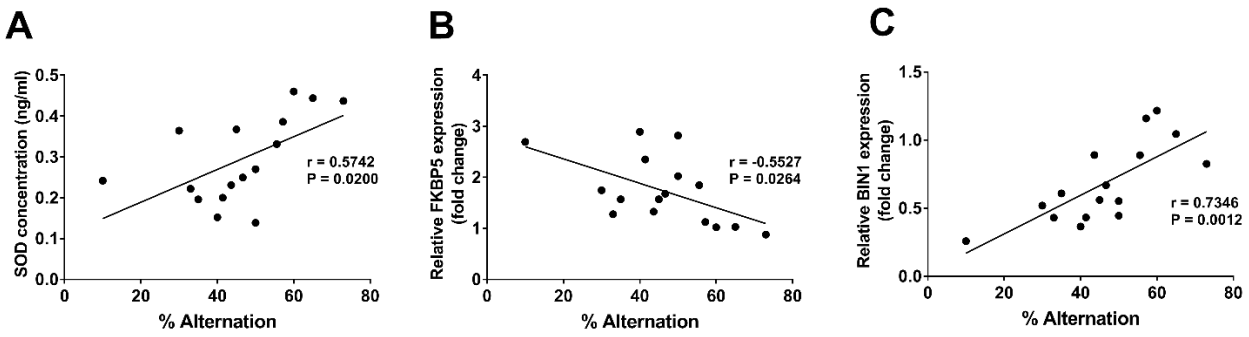

Figure 8: XY scatter plots of percentage alternation in the Y-maze and (A) SOD concentration (B) relative expressions of FKBP5 (C) relative expressions of BIN1 in the prefrontal cortex of rats treated with footshocks and Aβ-42 lesion.

Correlation between anxiety-like behaviour, memory decline, neurochemical changes and apoptosis in the prefrontal cortex of rats treated with footshocks and Aβ-42 lesion.

To test whether anxiety-like behaviour, memory decline, and neurochemical changes were associated with apoptosis in the PFC of rats post-treatment (footshocks and Aβ-42 lesion), a Pearson correlation was performed. As shown in Figure 9A, there was a strong correlation between time spent in the centre square of the OFT and the percentage of apoptotic cells in the PFC of the rats (r=-0.8230, P=0.00002). There was also a strong correlation between the number of line crossings in the OFT and the percentage of apoptotic cells in the PFC of the rats (r=-0.7446, P=0.0004, Figure 9B). There was a moderate correlation between grooming time in the OFT and the percentage of apoptotic cells in the PFC of the rats (r=-0.4921, P=0.0380, Figure 9C). There was a strong correlation between defecation frequency in the OFT percentage of apoptotic cells in the PFC of the rats (r=0.6246, P=0.0055, Figure 9D). In the Y-maze test, there was a strong correlation between percentage alternation and percentage of apoptotic cells in the PFC of the rats (r=-0.6347, P=0.0047, Figure 9E). In addition, there were strong correlations between the percentage of apoptotic cells and SOD levels (r=-0.7073, P=0.0032,

Figure 9F); FKBP5 mRNA expression (r=0.7486, P=0.0013, Figure 9G); BIN1 mRNA expression (r=-0.6871, P=0.0047, Figure 9H) in the PFC of the rats.

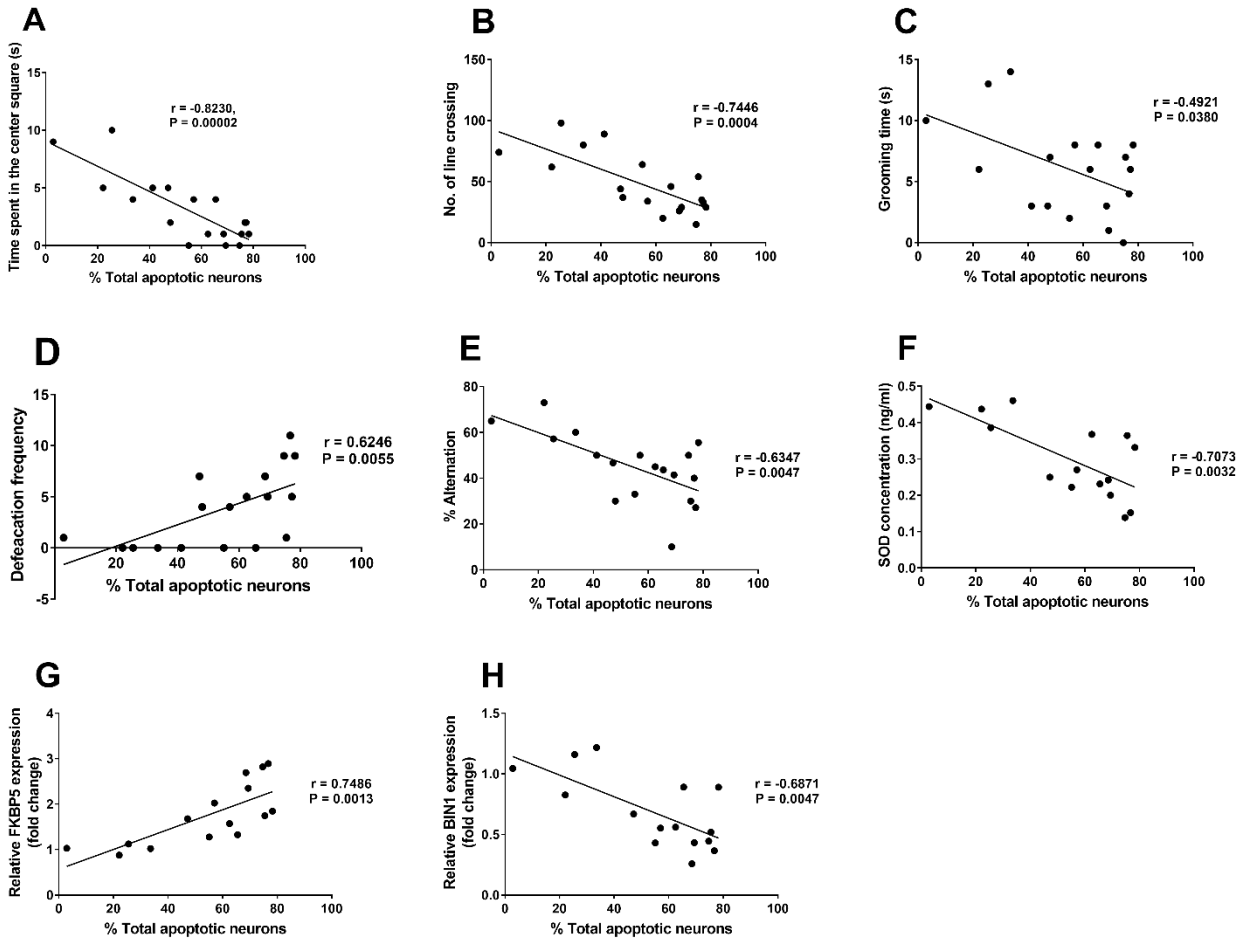

Figure 9: XY scatter plots of percentage apoptosis in the prefrontal cortex and (A) time spent in the centre square of the OFT (B) number of line crossing in the OFT (C) grooming time in the OFT (D) defecation frequency in the OFT and (E) percentage alternation in the Y-maze of rats treated with footshocks and Aβ-42 lesion (F) SOD concentration (G) FKBP5 mRNA expression (H) BIN1 mRNA expression.

4.4 Discussion

This study examined the effect of footshocks on behavioural and molecular changes in the prefrontal cortex of Aβ-42 rat model of Alzheimer's disease.

In this study, the animals that received footshocks showed increased freezing on Day 1 after the footshocks were administered. This increased freezing subsided when exposed to a new Context B on

Day 2 before introducing a single shock. However, when a single shock was administered as a reminder of the previous trauma-like experience, the freezing levels increased, as measured on Day 3. This indicates that footshocks induced fear responses in the animals that can be enhanced following a reminder. This confirms our PTSD-like induction [35]. This rat model of PTSD employs procedures that reduce any generalisation to the new Context B by differing transport to the experiment room, odour, shape and colour of the chamber. Therefore, unlike regular fear conditioning, the stress-enhanced fear learning (SEFL) paradigm is a non-associative process. It creates a new fear learning void of environmental cues directly associated with the traumatic experience [35]. Evidence suggests that increased FKBP5 expression in the PFC is associated with enhanced fear following trauma exposure [34]. Our results showed that footshocks influenced an increase in FKBP5 expression in the PFC. This is consistent with the report of Guidotti and co-workers, who showed that stressed animals had increased FKBP5 in the PFC [33]. FKBP5 is well expressed in the PFC [34], and its upregulation has been associated with enhanced stress and anxiety responses [46].

In this study, we injected Aβ-42 into the CA1 of the hippocampus to mimic AD-like pathology since the hippocampus is of the brain areas most affected by neurodegeneration in AD conditions [13,14]. The direct infusion of oligomeric Aβ-42 into the brain of rodents provides an excellent *in vivo* model of AD amyloidopathy [47]. Besides, the intra-hippocampal injection of Aβ-42 is a widely accepted model of AD [13,48]. Amyloid-beta deposition in the hippocampus and PFC is known to induce cognitive deficits [49].

Although the injection site was in the hippocampus, Aβ deposits have been found in the cerebral cortex of rats up to 15 days following intra-hippocampal injection of oligomeric Aβ-42, which was also associated with impaired working memory [16]. Besides, alterations in PFC-mediated behaviours and function can be induced by intra-hippocampal injection of Aβ [17,18]. Another *in vitro* study showed that in the presence of oligomeric Aβ-42, there was an inhibition of excitatory inputs from rat's hippocampus into the prefrontal cortex at both the population and single-cell levels [17]

Furthermore, it has been found that following intra-hippocampal injection of Aβ-42, its effects can also be seen in surrounding areas which have projections to the hippocampus. For instance, a 20-50% reduction in neuronal loss has been found in the forebrain of mice within 7-14 days following intra-hippocampal injection of Aβ-42 [50,51]. Similarly, molecular changes have been found in the PFC of animals 7 days following intra-hippocampal injection of oligomeric Aβ-42 [18].

Indeed, we found that Aβ-42 lesion increased the expression of FKBP5 in the PFC of the rats. This is in agreement with a previous report that found an increase in FKBP5 expression in the cortex of AD animals [52]. Besides, higher levels of FKBP5 have been associated with AD progression [53].

Moreover, we observed that footshocks caused a further increase in FKBP5 expression in the PFC of our Aβ-42-lesioned rats, suggesting an interaction between the effects of footshocks and Aβ-42 lesion. Perhaps, the Aβ-42 lesion in our study was sufficient to increase FKBP5 upregulation, which in turn worsen stress responses. Indeed, when our animals were further tested in the OFT, we observed that animals exposed to footshocks using SEFL spent less time exploring the box, suggesting anxiety-like behaviour [38]. This is consistent with a report which showed that footshocks increased anxiety-like responses in the OFT [54].

Similarly, Aβ-42 lesion reduced exploration time in the OFT. This is in agreement with the findings of Wong and co-workers, which showed that infusion of Aβ-42 reduced exploratory behaviour in rats [55]. Moreover, there was a further reduction in exploratory behaviour in rats exposed to footshocks in addition to Aβ-42 lesion. Defecation and grooming, which are also regarded as markers of anxiety, also increased and decreased, respectively, suggesting anxiety-like behaviour [56]. Again, Aβ deposits have been found in the cortex following intra-hippocampal injection of oligomeric Aβ-42 [16], and elevated levels of Aβ in the cortex has been associated with anxiety-like behaviours in rodents [55]. Thus, it is possible that Aβ-42-induced Aβ deposition in the PFC may have contributed to changes that led to the enhanced anxiety-like responses seen in the rats following footshocks. We also found a positive correlation between FKBP5 expression and anxiety-like behaviours. This relationship suggests that increased FKBP5 expression may somehow be associated with the anxiety-like responses observed in the animals. These findings, therefore, indicate that a prior trauma-like exposure could upregulate FKBP5 in the PFC of rats leading to an increased anxiety-like behaviour in the presence of an AD-like pathology.

Furthermore, we found that Aβ-42 lesion downregulated BIN1 expression in the PFC of the rats. To our knowledge, this is the first report showing an indirect effect of Aβ-42 on BIN1 expression in the PFC *in vivo*. However, it has been shown that reduced expression of BIN1 in cortical neurons of mice leads to Aβ production [57]. Besides, Aβ-42 has been shown to inhibit excitatory synaptic transmission in PFC neurons [24]. This, therefore, suggests that the reduction of BIN1 expression is involved in the neurotoxic effect of Aβ-42 in the prefrontal cortex of rats. In addition, there is clinical evidence showing a reduced BIN1 expression in the PFC of AD patients [58].

Similarly, we observed that footshocks also downregulated BIN1 expression in the PFC of the rats. To the best of our knowledge, this is the first preclinical report showing a reduced BIN1 expression in the PFC of rats in response to footshock stress. Moreover, when footshocks preceded Aβ-42 lesion, there was a slight decrease in BIN1 mRNA expression compared to animals that received footshocks alone, although no interactive effect was found. BIN1 depletion has been linked to learning and memory deficits [30]. Our study also showed that Aβ-42 lesion reduced percentage alternation in the Y-maze test, indicating an impairment in working memory. This is consistent with the report of [59]. The use

of rat models to investigate memory impairment has been helpful to understand essential aspects of the development of AD because memory deficit is a key symptom of the disease [18].

As a measure of spatial working memory, spontaneous alternation can be assessed by allowing rats to explore all three arms of the Y-maze. This is based on an innate curiosity of rodents to explore previously unvisited areas [60]. A rat with an intact working memory will remember the arm of the maze that it has already visited and tend to visit a new arm. Therefore, it is considered an index of active retrograde working memory and a high percentage alternation is regarded as good working memory, as it implies that the rat has recalled which arms it has already visited [61,62]. Conversely, decreased percentage alternation indicates working memory deficits as the rats cannot remember the previously visited arm [61,62]. The ability to perform these tasks correctly relies on the intact prefrontal cortex [40,63,64].

Besides, the interplay between the hippocampus and the prefrontal cortex is involved in spatial working memory encoding, and Aβ-42 induces working memory impairment due to functional disruption of this network [18,65]. Therefore, it is possible that the intra-hippocampal Aβ-42 lesion in our study contributed to the decreased PFC activity, which resulted in impaired working memory seen in the Y-maze test [40,64]. In addition, we observed that the combination of footshocks and Aβ-42 lesion influenced a reduction in percentage alternation in the Y-maze test, suggesting that stress may enhance the Aβ-42-induced memory impairment. Using the Y-maze test to measure working memory, % alternation is primarily estimated from the number of correct alternations [40]. The decreased entries observed in the Y-maze after footshocks, in a way, supports the anxiogenic measures in the open field test. The open field test assesses emotional responses to stimuli. For instance, time spent in center square, number of line crossing, rearing, defaecation, grooming are measures of anxiety-like behaviour in the open field test [38,56,66,67]. In addition, studies that are screening anxiolytic treatment using OFT do not measure the effects of treatment on locomotion rather on the emotional responses to a stress-induced inhibition of exploration behaviour [38]. Therefore, the interaction of footshock stress and Aβ lesion on memory deficits in the Y-maze is unlikely due to motor deficits but other stress factors. For instance, increased stress elevates corticosteroid levels and FKBP5 expression [7,68]. An increase in corticosteroids favour Aβ accumulation [7], which may, in turn, exacerbate Aβ-mediated memory decline [18]. In addition, we found a positive correlation of BIN1 expression in the PFC with memory decline, indicating that treatment (footshocks and Aβ-42 lesion)-induced reduction in BIN1 expression is associated with working memory deficits. Although we have not shown a direct effect of BIN1 activity on neuronal apoptosis, we found that a decrease in BIN1 expression correlated with an increase in neuronal apoptosis (via caspase 3 and 7 activation) in the prefrontal cortex of rats treated with footshocks and Aβ-42 lesion.

Furthermore, we observed a decrease in SOD levels in the PFC of Aβ-42 lesioned animals. This result is in agreement with previous reports, which showed that Aβ toxicity is inversely proportional to SOD levels in neuronal cells [69,70]. In addition, footshock stress reduced SOD levels in the PFC. This is in contrast with previous findings that found no changes in SOD activity in the PFC of rats following footshock stress [71,72]. The difference might be due to the duration of exposure to the footshocks. We exposed the rats for 90 minutes, which is in contrast with the 20-minute exposure of earlier works [71,72]. Perhaps, the severity of footshocks was responsible for the decreased SOD levels in the PFC observed in our study. Although there was no interactive effect of footshocks and Aβ-42 on SOD levels in the PFC, we found that animals that received both footshocks and Aβ-42 had a further reduction in SOD than the rats that were exposed to shock only, suggesting a tendency for Aβ-42 to influence the decrease in SOD level following stress. Besides, evidence shows that prolonged stress decreases SOD levels and favours Aβ toxicity [73,74]. SOD, the first line of defence against superoxides, is an important antioxidant, and its dysregulation has been linked to apoptosis and AD progression [75]. The superoxide radical is one of the most abundant reactive oxygen species within the cell, and it is involved in memory impairment, synaptic dysfunction, and neuronal apoptosis [76]. This suggests an important scavenging role for SOD [75]. Besides, Aβ has a direct inhibitory effect on SOD activity [77]. Indeed, we found an inverse relationship between SOD levels and neuronal apoptosis in the PFC of animals treated with footshocks and Aβ-42 lesion. Decreased SOD levels also correlated with anxiety-like behaviour and memory deficits.

In conclusion, our findings showed that footshock stress exaggerated molecular changes in the PFC of rats in an Aβ AD-like condition, which was associated with working memory deficits and increased anxiety-like behaviour. Therefore, this study suggests that prior traumatic stress may exacerbate AD pathology.

Declarations of interest: none

Ethics statement

All procedures were reviewed and approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (AREC/005/019D)

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5 CHAPTER 5: SYNTHESIS AND CONCLUSION

5.1 Synthesis

Alzheimer's disease is recognised as the most common form of dementia affecting over 50 million people globally (Shi et al., 2018). To date, there has not been an effective cure for AD (Huang et al., 2020). The lack of effective therapy may be due to inadequate knowledge regarding the molecular mechanism of the disease (Gulyaeva et al., 2017). Meanwhile, PTSD is a neuropsychiatric disorder that results from exposure to perceived life-threatening trauma. It has been shown that the risk of developing AD in people diagnosed with PTSD is doubled (Yaffe et al., 2010), and the underlying mechanisms linking these two diseases are currently unclear. It is, therefore, necessary to investigate the common underlying molecular mechanism of the diseases.

In our study, we investigated the effects of trauma-like exposure in an AD-like condition using rat models. We induced an AD-like condition by injecting oligomeric Aβ(1–42) peptides into the dorsal CA1 region of the hippocampus. We observed that the injection of the Aβ(1–42) peptides led to memory decline in the rats, which is characteristic of an AD condition (Faborode et al., 2022, 2021a; Shallie and Mabandla, 2020). On the other hand, we induced a PTSD-like condition by exposing the rats to footshocks, which led to exaggerated anxiogenic behaviour and impaired fear memory, which are characteristic responses in PTSD.

To determine underlying molecular mechanisms common to both conditions, i.e. PTSD-like and AD-like conditions, we tested for the level of oxidative stress known to promote Aβ toxicity in a vicious cycle. This interaction involves the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a molecule that regulates oxidative stress by promoting the production of antioxidant enzymes including NAD(P)H:quinone reductase (NQO1) and heme oxygenase-1 (HO-1) to combat the oxidant molecules (Faborode et al., 2021b). In our work, introducing a trauma-like condition led to abnormal regulation of the Nrf2-mediated antioxidant response in AD-like pathology by downregulation of antioxidant genes. The co-occurrence of trauma-like conditions and AD-like pathology also led to an increase in oxidative stress, which correlated with increased cell death in the hippocampus and amygdala of the rats. We believe that these molecular changes were involved in the observed impaired fear memory and enhanced anxiogenic behaviour in the rats.

Amyloid-beta (Aβ) promotes oxidative stress (Postu et al., 2019), which can lead to accelerated cellular damage and synaptic dysfunction. Bridging integrator 1 (BIN1) is a gene that regulates Aβ production. We found that BIN1 expression was reduced in the hippocampus and amygdala after Aβ(1–42) lesion and footshock exposure. We are the first to report an effect of Aβ(1–42) or footshock stress on BIN1 expression *in vivo*. Considering the nature of BIN1 in Aβ production, it is possible that

increased Aβ via exogenous injection of Aβ(1–42) overwhelmed the regulatory function of BIN1, thereby leading to increased endogenous Aβ production and consequent downregulation of BIN1. We also found a combined effect of footshocks and Aβ(1–42) lesion on BIN1 expression, suggesting that trauma-like exposure may exacerbate AD-like condition.

Furthermore, we found that footshocks enhanced Aβ(1–42)-induced glutamate excitotoxicity by increasing NR2B expression in the hippocampus of our rats. Overactivation of NR2B is known to induce neuronal apoptosis (Karthick et al., 2019). Indeed, we found that PTSD-like induction exaggerated neuronal apoptosis in the hippocampus, amygdala, and PFC of our Aβ-42-lesioned rats (Faborode et al., 2022, 2021a, 2021b). In addition, we measured the expression level of FKBP5, a gene involved in stress regulation. We found that footshock exposure exaggerated the expression of FKBP5, which can be associated with impaired synaptic functions and a consequent cognitive impairment (see Figure 5.1). Increased FKBP5 due to impaired stress regulation can lead to increased glucocorticoid production, which favours Aβ accumulation. Aβ accumulation can, in turn, induce NR2B-mediated excitotoxicity leading to synaptic dysfunction and memory decline.

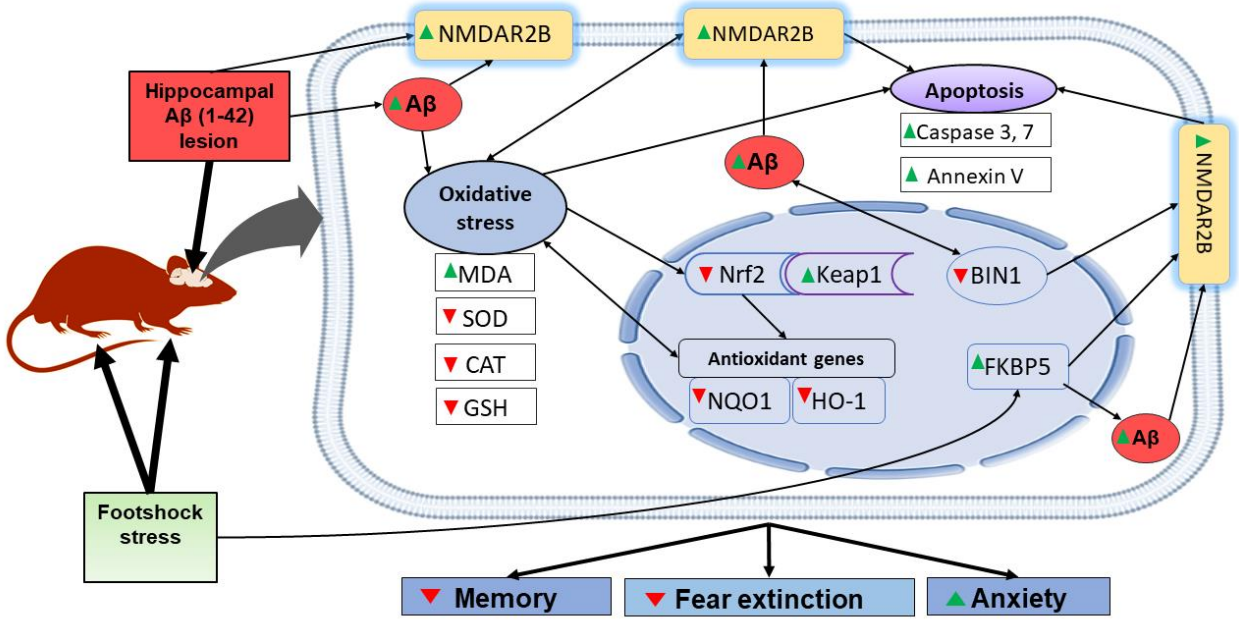

Figure 5.1. Effect of footshock stress on behavioral and molecular changes in the brain of Aβ(1-42) lesion rat model of Alzheimer's disease. Footshocks, in addition to Aβ(1-42) infusion, increase lipid peroxidation (MDA) and reduce antioxidants (SOD, CAT, GSH), resulting in oxidative stress. Sustained oxidative stress downregulates Nrf2 and upregulates Keap1 genes, which inhibits the upregulation of antioxidant genes (HO-1 and NQO1). Footshock stress upregulates FKBP5. FKBP5 upregulation favours Aβ accumulation leading to the overactivation of NMDAR2B. Overactivation of NMDAR2B, in turn, stimulates neuronal apoptosis via caspase 3/7 and membrane flipping (Annexin V). Aβ accumulation downregulates BIN1 expression, leading to a further increase in Aβ production.

An increase in apoptosis leads to memory decline, impaired fear extinction and increased anxiety. Aβ(1-42) (amyloid-beta 42); MDA (malondialdehyde); SOD (superoxide dismutase); CAT (catalase); GSH (glutathione); Nrf2 (nuclear factor erythroid 2-related factor 2); Keap1 (Kelch-like ECH-associated protein 1); HO-1 (Heme oxygenase 1); NQO1 (NAD (P) H: quinone oxidoreductase 1); NMDAR2B (N-methyl D-aspartate (NMDA) receptor subunit 2B, NR2B); BIN1 (Bridging integrator 1), FKBP5 (FK506 binding protein 51); Green triangle shapes represent an increase, and inverted red triangles represent a decrease.

Alzheimer's disease affects the hippocampus, but its effect is found in other brain regions, including the PFC. The PFC is known to regulate the activities of the amygdala and hippocampus in fear, anxiety, and memory. We found that footshocks caused a further increase in FKBP5 expression in the PFC of our Aβ-42-lesioned rats and these changes correlated with increased anxiety-like behaviour. We also found that footshocks or Aβ (1-42) lesion downregulated BIN1 expression in the PFC of the rats. This is a novel finding that has not been reported elsewhere. As previously reported that reduced BIN1 expression induces learning and memory deficits, we observed impaired working memory in the rats. In addition, we observed that footshocks enhanced cell death in the PFC of the Aβ (1-42) lesioned rats. Aβ deposition in the hippocampus and PFC is involved in cognitive deficits. Although the injection site was in the hippocampus, alterations in PFC-mediated behaviours and function can be induced by intra-hippocampal injection of Aβ. In all, changes in brain areas where the hippocampus projects (e.g., the amygdala and the prefrontal cortex) following intrahippocampal Aβ (1-42) injections have made our animal model robust and relevant to investigate novel therapeutical approaches for AD associated with PTSD. Our findings, in a way, support the clinical manifestation of AD beyond the main brain area (e.g., the hippocampus), which are affected by the disease.

5.2 Conclusions

Our study advances the understanding of the possible common underlying molecular changes associated with the heterogeneity and comorbidity of PTSD and AD. We were able to show that interaction of a PTSD-like and AD-like condition exaggerated oxidative stress by suppressing the Nrf2-Keap1 pathways and the associated genes in the brain of a rat. The interaction of PTSD-like and AD-like conditions also exacerbated the upregulation of FKBP5 and the downregulation of BIN1, which can be associated with NR2B overexpression. These changes led to exaggerated apoptosis in the brain, associated with increased anxiety-like behaviour, impaired fear extinction memory, spatial and working memory deficits.

Collectively, this knowledge suggests that in humans, traumatic stress may play an important role in the progression of Alzheimer's disease (AD) and that antioxidant, improved synaptic functions and anti-apoptotic strategy might be a potential therapeutic approach for stress-mediated disorders and, at the same time, preventing the development of AD later.

5.3 Recommendations for future studies

Our study provides an insight into the common molecular mechanisms involved in the effect of PTSD-like conditions in AD-like pathology. Albeit there is a need for further studies to:

- Examine plasma levels of BIN1 and FKBP5 as this will inform the development of blood-based AD biomarkers, which are of utmost importance in the early diagnosis of AD.
- To study the expression of additional genes using microarray or RNAseq will give more detailed information on the mixed interactions.
- Investigate the effect of PTSD-like induction in other models of AD and vice versa. For instance, this study only focused on the amyloid pathology of AD. Examining the effect of PTSD-like induction on tau pathology of AD would expand our knowledge on the co-morbidity of PTSD and AD.
- Inclusion of a different age bracket: The rats used in this study were at post-natal day (PND) 70 at the start of the experiments, considered young adults. We considered that normal ageing of the brain presents with neuronal loss, increased demyelination, a consequent cognitive decline, and neuropsychiatric disorders. Therefore, we sought our research subjects within the mid-adulthood age because these factors could introduce experimental biases.

Appendix I

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

20 June 2019

Mr Oluwaseun Samuel Faborode (218079802)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Mr Faborode,

Protocol reference number: AREC/005/019D
Project title: Alzheimer's disease Dementia risk in Post-traumatic Stress Disorder: Identification of Biomarkers and pathways involved using rat models

Full Approval – Research Application

With regard to your revised application received on 14 February 2019. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 12 April 2020.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

[redacted]

Dr Sanil D Singh, PhD
Chair: Animal Research Ethics Committee

/kr

cc Supervisor: Professor Musa V Mabandla

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Appendix II

SOUTH AFRICAN VETERINARY COUNCIL

ANNEXURE: CONDITIONS OF AUTHORISATION IN TERMS OF SECTION 23 (1) (c) OF THE VETERINARY AND PARA-VETERINARY PROFESSIONS ACT, ACT 19 OF 1982

MR OLUWASEUN SAMUEL FABORODE

This serves to confirm that **Mr Oluwaseun Samuel Faborode** with Passport No: **A09045822 (Nigeria)** is authorised, in terms of Section 23 (1)(c) of the Veterinary and Para-Veterinary Professions Act, Act 19 of 1982, subject to the following conditions: -

1. Authorisation is granted for the duration of the studies to enable you to perform the procedures as per the application with authorisation number **AR19/17733.**

2. The authorisation is limited to your studies and will lapse when you are no longer at the **Biomedical Resource Unit, University of Kwazulu-Natal.**

3. Your supervisor **submits a 6-monthly report** in respect of the procedures performed under the authorisation.

4. You render the services under supervision of a registered Veterinarian.

5. You are bound by the Veterinary and Para-Veterinary Professions Act 19 of 1982 and the Rules and Regulations promulgated in terms thereof.

6. You are authorised to perform the following procedures for research as follows: -

6.1 Rodents (rats):

- 6.1.1 Animal Handling;
- 6.1.2 Oral gavage;
- 6.1.3 Blood collection, tail prick;
- 6.1.4 Intra-peritoneal injections;
- 6.1.5 Subcutaneous injections;
- 6.1.6 Intramuscular injections; and
- 6.1.7 Stereotaxic surgery.

7. [redacted] the maintenance of a para-veterinary profession is due and

[redacted]

21/Nov/2019
Date

Appendix III

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

LMMS RESEARCH SYMPOSIUM 2020

CERTIFICATE OF PRESENTATION

Awarded to

Oluwaseun Faborode

University of KwaZulu-Natal

for presenting the oral:

Implication of amyloid-beta (1-42) lesioning on fear memory and anxiety in a rat model of posttraumatic stress disorder

At the

Annual Laboratory Medicine and Medical Sciences Research Symposium

2020 Which took place via Zoom, Durban, South Africa

On the 18th of September 2020

Yours sincerely,

[redacted]

Dr De Gama

Academic Leader Research SLMMS

Appendix IV

Neurochemistry International 150 (2021) 105170

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Exposure to footshock stress downregulates antioxidant genes and increases neuronal apoptosis in an Aβ(1–42) rat model of Alzheimer's disease

Oluwaseun Samuel Faborode *, Ernest Dalle, Musa Vuyisile Mabandla

Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Westville Campus, Durban 4000, South Africa

ARTICLE INFO

Keywords:
Alzheimer's disease
Post-traumatic stress disorder
Apoptosis
Fear extinction
Amyloid-beta
Oxidative stress

ABSTRACT

Post-traumatic stress disorder (PTSD) is a neuropsychiatric disorder that develops from exposure to trauma, mostly when normal psychological mechanisms fail. Studies have shown that people who have PTSD are susceptible to developing dementia, mostly Alzheimer's disease (AD), suggesting common underlying risk factors in the comorbidity. However, data elucidating links between these conditions is scarce. Here we show that footshock stress exacerbates AD-like pathology. To induce a trauma-like condition, the rats were exposed to multiple intense footshocks followed by a single reminder. This was followed by bilateral intrahippocampal lesions with amyloid-beta (Aβ) (1–42), to model AD-like pathology. We found that footshocks increased anxiety behavior and impaired fear memory extinction in Aβ(1–42) lesioned rats. We also found a reduced expression of nuclear factor erythroid 2-related factor 2 (Nrf2), NAD (P) H: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and an increased expression of Kelch-like ECH-associated protein 1 (Keap1) in the amygdala and hippocampus. Furthermore, oxidative stress level was sustained, which was associated with increased apoptosis in the amygdala and hippocampus.

Our finding suggests that AD-like pathology can induce oxidative changes in the amygdala and hippocampus, which can be exaggerated by footshock stress.

1. Introduction

Post-traumatic stress disorder (PTSD) is a fear-based psychological disorder that results from exposure to traumatic events (Careaga et al., 2016). It is characterized by the sufferer's inability to dissociate a previous adverse event from a perceived new threat. The ability to differentiate between threats occurring at different time points requires cognitive flexibility and extinction learning (Goswami et al., 2013). In humans, increased stress responses found in PTSD are associated with neuropsychiatric dysfunctions and increase vulnerability to other diseases, including Alzheimer's disease (AD) (Bao et al., 2008; Gold et al., 2005; Stuller et al., 2012). Coincidentally, studies have shown that people with PTSD have twice the risk of developing dementia, mostly AD (Burri et al., 2013; Stilling et al., 2014; Weiner et al., 2013; Yaffe et al., 2010). However, the underlying mechanisms are not well understood. It has also been shown that prolonged stress increased amyloid plaque formation and amyloid-beta (Aβ) release in AD animal models (Carroll et al., 2011; Devi et al., 2010; Rothman et al., 2012). Aβ peptides also influenced PTSD-like responses via the corticotropin-releasing factor 1 receptor activation, suggesting that PTSD-like trauma can exacerbate AD dementia, increasing PTSD responses (Justice et al., 2015). Also, the genetic mutation of Formin 2, a gene associated with synaptic transmission and memory function, was found to affect hippocampal plasticity, leading to fear memory impairment in AD animal models (Agís-Balboa et al., 2017; Almuqbil et al., 2013; Law et al., 2014; Peleg et al., 2010). Although stress has been implicated in dementia, the link between PTSD and AD remains unclear (Greenberg et al., 2014; Kuring et al, 2018, 2020). Recent studies have shown that oxidative stress plays a critical role in neuronal dysfunction and neurodegeneration (Dasuri et al., 2013). Similarly, oxidative stress is involved in stress-induced pathologies (Wilson et al., 2013). The nuclear factor erythroid 2-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) pathway regulates oxidative stress involved in AD pathology (Huang et al., 2020; Sun et al., 2020), as well as psychiatric disorders (Zhang et al., 2018). In this study, we tested if footshock stress could exacerbate fear and anxiety-like behaviors in Aβ(1–42) rat AD model. We also aimed to determine if footshocks stress would exaggerate the activities of Nrf2-Keap1 and its downstream antioxidant genes in the

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Available online 19 August 2021

Appendix V

CERTIFICATE OF ATTENDANCE

PSSA
Physiology Society of Southern Africa

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

This certificate confirms that

Oluwaseun Faborode

Attended

The 1st Virtual AAPS-PSSA Congress

held from the

12th to the 15th of September 2021

Appendix VI

SOCIETY OF NEUROSCIENTISTS OF AFRICA

Certificate of Attendance

This is to certify that

Mr. Oluwaseun Faborode

has attended the

15th SONA/4th GNS Hybrid Conference 2021
Accra, Ghana.

held on

27-30 October 2021

Attendee type: Poster Presenter

[redacted]
PROFESSOR AMADI O IHUNWO
Secretary-General, SONA

[redacted]
ROBERT PETER BINEY
Co-Chair, Local Organizing Committee

[redacted]
DR PATRICK AMOATENG
Chair, Local Organizing Committee
President, SONA 2021

Appendix VII

Behavioural Brain Research 419 (2022) 113679

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Inescapable footshocks induce molecular changes in the prefrontal cortex of rats in an amyloid-beta-42 model of Alzheimer's disease

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Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Westville Campus, Durban 4000, South Africa

ARTICLE INFO

Keywords:
Alzheimer's disease
Post-traumatic stress disorder
Anxiety
Memory
Amyloid-beta
Prefrontal cortex

ABSTRACT

Alzheimer's disease (AD) affects several brain areas, including the prefrontal cortex (PFC) involved in execution, working memory, and fear extinction. Despite these critical roles, the PFC is understudied in AD pathology. People with post-traumatic stress disorder (PTSD) have twice the risk of developing AD, and the underlying mechanisms linking these two diseases are less understood. Here, we investigated the effect of footshock stress on behavioural vis-a-vis molecular changes in the PFC of an amyloid-beta (Aβ)-42 lesion rat model of AD. Trauma-like conditions were induced by exposing the animals to several footshocks. AD-like condition was induced via intra-hippocampal injection of Aβ-42 peptide. Following Aβ-42 injections, animals were tested for behavioural changes using the Open Field Test (OFT) and Y-maze test. The PFC was later harvested for neurochemical analyses. Our results showed an interactive effect of footshocks and Aβ-42 lesion on: reduced percentage alternation in the Y-maze test, suggesting memory impairment; reduced number of line crosses and time spent in the centre square of the OFT, indicating anxiogenic responses. Similarly, there was an interactive effect of footshocks and Aβ-42 lesion on: increased FK506 binding protein 51 (FKBP5) expression, which can be associated with stress-induced anxiogenic behaviours; and increased neuronal apoptosis in the PFC of the animals. In addition, footshocks, as well as Aβ-42 lesion, reduced superoxide dismutase levels and Bridging Integrator-1 (BIN1) expression in the PFC of the animals, which can be linked to the observed memory impairment. In conclusion, our findings indicate that footshocks exaggerate PFC-associated behavioural and molecular changes induced by an AD-like pathology.

1. Introduction

Alzheimer's disease (AD) is the most common form of neurodegenerative disease that affects patients' cognitive abilities. The disease is characterised by intracellular neurofibrillary tangles and extracellular amyloid plaques [1,2]. On the other hand, post-traumatic stress disorder (PTSD) is an affective disorder that develops after exposure to an intense trauma [3]. Reports have shown that people who have PTSD are two times at risk of developing AD [4,5]. To the best of our knowledge, few studies have attempted to elucidate the underlying mechanisms that link PTSD to AD. For instance, an earlier study investigated the interaction between acute oligomer amyloid-beta (Aβ)-40 injection and stress. They found that increased glucocorticoid receptor/mineralocorticoid receptor ratio, expression of corticotrophin-releasing factor 1 (CRF-1) receptor, increased Aβ accumulation, oxidative stress, and apoptotic signalling in the hippocampus of mice was associated with severely impaired spatial learning and memory, following a combination of footshocks and Aβ-40 injection. Therefore, they concluded that stress might play an essential role in the early stages of AD [6]. Another work by Justice and co-workers found that exposure to PTSD-like trauma elevated CSF Aβ levels in mice which accelerated AD pathogenesis [7]. They also found that expression of human familial AD mutations in amyloid precursor protein and presenilin 1 led to sensitivity to trauma-induced PTSD-like behavioural and endocrine changes. In addition, they showed that PTSD-like induction and Aβ elevation depends on CRF-1 receptor signalling, highlighting a potential mechanism by which Aβ influences PTSD-like phenotypes [7]. In addition, Agís-Balboa and co-workers found that young mice lacking the Formin2 (Fmn2) gene exhibited PTSD-like phenotypes and developed an accelerated age-associated memory decline, suggesting that the down-regulation of Fmn2 in a PTSD mouse model accelerated AD-related pathology [8]. In addition, a recent study using human datasets suggested immune response as a common pathway for PTSD and AD, implicating MS4A6A, a gene encoding cellular membrane proteins

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Appendix VIII

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Trauma-like exposure alters neuronal apoptosis, Bin1, Fkbp5 and NR2B expression in an amyloid-beta (1–42) rat model of Alzheimer's disease

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ABSTRACT

Post-traumatic stress disorder (PTSD) is a risk factor in the development and progression of Alzheimer's disease (AD), with unclear underlying mechanisms. Recently, we provided data showing the effect of trauma-like stress on *Bin1* and *Fkbp5* expression in the prefrontal cortex of $A\beta_{(1-42)}$ lesioned animals. This present work sought to expand the study by examining the involvement of the amygdala and hippocampus, in addition to highlighting the role of NR2B in the co-occurrence of trauma-like stress and an Aβ AD-like pathology.

Trauma-like condition was induced by exposing the animals to footshocks. $A\beta_{(1-42)}$ was injected into the hippocampus of the animals to induce AD-like pathology. Cognitive functions were assessed in the Morris water maze (MWM) and novel object recognition tests, after which amygdala and hippocampus tissues were harvested for neurochemical analyses.

We found that the combination of footshocks and $A\beta_{(1-42)}$ lesion caused a decrease in the number of crossings in the target quadrant of the Morris water maze test, indicating memory deficits. Footshocks caused a further downregulation of *Bin1* in the amygdala of $A\beta_{(1-42)}$-lesioned rats. Prior exposure to footshocks downregulated NR2B in the hippocampus of $A\beta_{(1-42)}$-lesioned rats. In addition, a combination of footshocks and $A\beta_{(1-42)}$ lesion sustained the upregulation of *Fkbp5* in the hippocampus and amygdala. A combination of footshocks and $A\beta_{(1-42)}$ lesion increased neuronal apoptosis in the hippocampus and amygdala.

In conclusion, exposure to a trauma-like condition may influence AD-like pathology, leading to exaggerated behavioural and molecular changes in the amygdala and hippocampus.

1. Introduction

Post-traumatic stress disorder (PTSD) is a psychological disorder that results from experiencing a life-threatening trauma (Careaga, Girardi, & Suchecki, 2016). Alzheimer's disease (AD) is the most common form of dementia, affecting about 44 million people globally (Li, Liu, & Sun, 2017). The disease is characterized by extracellular deposition of amyloid-beta (Aβ), intracellular neurofibrillary tangles, neuronal loss, synaptic deficits, and learning and memory impairments (Hong et al., 2016; Sanabria-Castro, Alvarado-Echeverría, & Monge-Bonilla, 2017). Sporadic AD, which accounts for the majority (60–80 %) of AD cases, contrasts with familial AD in that the former develops from multifactorial conditions while the latter is due to genetic mutations. (Gatz et al., 2006; Koukouli, Rooy, & Maskos, 2016). It has been shown that people with PTSD have twice the risk of developing dementia, mostly AD (Stilling et al., 2014; Yaffe et al., 2010). However, the underlying mechanism of the interaction is less understood.

Although neurodegeneration in the hippocampus is prominent in AD conditions (Facchinetti, Bronzuoli, & Scuderi, 2018; Scuderi et al., 2014), several other brain areas are affected, including the amygdala (T.-W. Lin et al., 2015). There is also crosstalk between the amygdala and hippocampus on regulating the effect of stress on cognition (Roozendaal, 2002). Also, Aβ deposits are found in the amygdala in AD conditions, which have shared circuitry to the hippocampus (Karthick et al., 2019; T.-W. Lin et al., 2015). Therefore, the evaluation of the changes in the amygdala following intrahippocampal Aβ-lesion could be very useful to investigate region vulnerability in AD linked to Aβ deposition from one targeted brain structure to connected areas (Koukouli et al., 2016). Previous studies have also shown that neurodegeneration occurs earlier in the amygdala than in the hippocampus, thereby suggesting amygdala function assessment as an important tool in diagnosing cognitive impairment in AD ().T.-W. Lin et al., 2015; T.W. Lin et al., 2015

Bridging integrator 1 (*Bin1*) has been identified as the second most

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