EFFECT OF AMBIENT TEMPERATURE ON METHYLENEDIOXYMETHAMPHETAMINE (MDMA)-INDUCED CHANGES IN BODY TEMPERATURE, NEUROBEHAVIOUR AND NEUROTOXICITY IN RODENTS

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DECLARATION

I, Alhassan Abdul Wahab declare that the work in this dissertation entitled "**Effect of Ambient Temperature on Methylenedioxymethamphetamine (MDMA)-induced Changes in Body Temperature, Neurotoxicity and Neurobehaviour in Rodents**" has been carried out by me in the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria under the supervision of Professor M.A. Mabrouk, Professor J.O. Ayo and Dr. A.U. Zezi. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

_____ _____

Alhassan Abdul Wahab **Date**

CERTIFICATION

This dissertation entitled "**Effect of Methylenedioxymethamphetamine (MDMA, ECSTASY) on Body Temperature, Neurobehaviour and Neurotoxicity in Rodents**" meets the regulations governing the award of Doctorate in Philosophy in Human Physiology of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to the Almighty Allah, my late father, my wife Laila, my sons, Yasir and Alhassan.

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ABSTRACT

Studies on the effect of ambient temperature on methylenedioxymethamphetamine (MDMA)-induced changes in body temperature, neurobehaviour and neurotoxicity was carried out in mice and rats. The study was carried out in four (4) different categories and designed to determine variations in ambient temperature on MDMA-induced changes on body temperature, neurobehaviour and neurotoxicity. Category 1, made up of twenty male C57BL/6J mice were grouped into four groups of 5 each (n=5). Group 1a (control: normal saline and Temperature of 21°C), group IIa (Temperature of 21°C and MDMA (4 x 20 mg/kg). Group Ib (control: normal saline and Temperature of 27°C), group 11b (Temperature of 27°C and MDMA (4 x 20 mg/kg). Core body temperature was measured each hour after each vehicle or MDMA administration. Category 2, mice were sacrificed 48 hrs after the last administration of MDMA and coronary striatal sections were used for neurotoxicity analysis with glial fibrilliary acidic protein (GFAP) and CD 11b as markers of astroglia and microglia. Category 3, assessment of short-term spatial memory using Y-maze in rats and divided into four groups of 10 each (n=10). Groups 1a and 1b served as control and were given normal saline and kept at an environmental temperatures of 21°C and 27°C respectively. Groups IIa and 11b were administered MDMA (10 mg/kg) and kept at an environmental temperatures of 21°C and 27°C respectively. Category 4, assessment of non-spatial working memory using novel object recognition task in rats grouped into four groups of 10 each (n=10). Groups 1a and 1b served as control and were given normal saline and kept at an environmental temperatures of 21°C and 27°C respectively. Groups IIa and 11b were administered MDMA (10 mg/kg) and kept at an environmental temperatures of 21°C and 27°C respectively. All administrations were i.p. 24 hrs apart over four consecutive days. The results showed that body temperature of

MDMA-treated mice was significantly higher ($p<0.05$) than mice treated with vehicle at 21°C. Similarly, body temperature of MDMA-treated mice was significantly higher ($p<0.05$) than mice treated with vehicle at the 27°C. The body temperature of MDMA-treated mice at 27°C was significantly higher ($p<0.05$) than mice treated at 21°C. Mice exposed to 21°C and 27°C, MDMA-treated mice showed a higher CD 11b immunoreactivity (microgliosis) was observed as compared with vehicle- treated mice. Mice treated at 27°C showed a higher CD 11b immunoreactivity compared with mice treated at 21°C. Exposure to 21°C and 27°C, MDMA induced a significantly higher GFAP ($p<0.05$) immunoreactivity (Astrogliosis) compared with vehicle treated mice. MDMA-treated mice at 27°C showed a significantly higher ($p<0.05$) GFAP immunoreactivity compared with mice exposed at 21°C. At 21°C and 27°C there was a significant ($p<0.001$) impairemant in both spatial and non-spatial memory in MDMA-treated rats as compared to the vehicle treated. Impairment in spatial and non-spatial memory in MDMA-treated rats at 27°C was significantly higher than in MDMA-treated rats at 21°C ($p<0.001$). In conclusion, MDMA induced hyperthermia, neurotoxicity and impairement in both spatial and non-spatial memory depending on variations in ambient temperature.

TABLE OF CONTENTS

Cover page - - - - - - - - - i

Title page - - - - - - - - - ii

Declaration - - - - - - - - - iii

Certification - - - - - - - - - iv

Dedication - - - - - - - - - v

Acknowledgement - - - - - - - - vi

Abstract - - - - - - - - - vii

Table of contents - - - - - - - - viii

List of tables - - - - - - - - - xii

List of figures - - - - - - - - - xiii

List of plates - - - - - - - - - xiv

List of appendices - - - - - - - - xvi

List of abbreviations - - - - - - - - xvii

Chapter One: Introduction

1.0 Introduction - - - - - - - - 1

1.1 Statement of Research Problem - - - - - 1

1.2 Justification - - - - - - - - 4

1.3 General Aim - - - - - - - - 4

1.4 Specific Objectives - - - - - - - 5

Chapter Two: Literature Review

2.1 History of MDMA - - - - - - - 6

2.2 Chemical structure and mechanisms of action of MDMA - - 9

2.3 Effects in human users - - - - - - 11

2.3.1 Desirable - - - - - - - 11

2.3.2 Acute adverse effect - - - - - - 12

2.3.3 Long term adverse effect - - - - - 13

2.4 Mechanisms involved MDMA-induced temperature changes - 14

2.4.1 Hypothalamic-pituitary-thyroid axis - - - - 14

2.4.2 Role of monoamine neurotransmitters - - - 15

2.4.3 Sympathetic Nervous System - - - - 17

2.4.4 Uncoupling proteins - - - - - 18

2.4.5 Role of atrial nituretic peptide - - - - 19

2.4.6 Blood Brain Barrier - - - - - - 20

2.4.7 Role of Pro-Inflammatory Cytokines - - - 20

2.5 Toxicity of MDMA - - - - - - - 21

2.5.1 In Humans - - - - - - - 21

2.5.2 In Rats - - - - - - - 22

2.5.3 In Mice - - - - - - - 22

2.6 Mechanism involved in MDMA toxicity - - - - 23

2.6.1 Oxidative stress - - - - - - 23

2.6.2 Excitotoxicity - - - - - - 24

2.6.3 Mitochondrial Dysfunction - - - - - 24

2.6.4 Formation of toxic MDMA metabolites - - - 26

2.6.5 Neuroinflammation - - - - - - 29

2.7 Glial cells - - - - - - - - 30

2.7.1 Microglial cells - - - - - - 32

2.7.2 Astroglia - - - - - - - 34

2.8 Cognition - - - - - - - - 39

Chapter Three: Materials and Methods

3.1 Materials - - - - - - - - 41

3.1.1 Drugs and chemicals - - - - - - 41

3.1.2 Instruments and devices - - - - - 41

3.1.3 Animals - - - - - - - 42

3.2 Experimental site - - - - - - - 42

3.3 Methodology - - - - - - - 43

3.3.1 Drug preparation - - - - - - 43

3.3.2 Temperature measurements - - - - - 43

3.3.3 Immunohistochemistry for markers of astroglia and microglia 43

3.3.4 Image Analysis - - - - - - 43

3.3.5 Assessment of short-term spatial memory using Y-maze - 44

3.3.6 Assessment of non-spatial working memory using NOR - 46

3.4 Data analysis - - - - - - - - 47

Chapter Four: Results

4.1 Role of ambient temperature variation on MDMA-induced hyperthermia 48

4.2 Inflammatory response in the striatum - - - - - 49

4.2.1 CD11b Immunoreactivity in the Striatum - - - 49

4.2.2 GFAP Immunoreactivity in the Striatum - - - 53

4.3 Effect of MDMA on cognition - - - - - 57

4.3.1 Effect of MDMA on spatial memory - - - - 57

4.3.2 Effect of MDMA on non- spatial memory - - - 60

Chapter Five: Discussion

5.1 Effect of external temperature on MDMA-induced hyperthermia - 63

5.2 Effect of external temperature on MDMA-induced neurotoxicity - 66

5.3 Effect of external temperature variation on MDMA-induced cognitive changes - - - - - - - - - 69

Chapter Six: Summary, Conclusion and Recommendations

6.1 Summary - - - - - - - - 77

6.2 Conclusions - - - - - - - - 77

6.3 Recommendations - - - - - - - 78

References - - - - - - - - - 79

Appendices - - - - - - - - - 105

LIST OF TABLES

Table 2.1: Milestones from the history of Methylenedioxymethamphetamine (MDMA) - - - - - - - 8

LIST OF FIGURES

Figure 1.1: MDMA tablets - - - - - - - 2

Figure 2.1: Chemical structure of MDMA - - - - - 10

Figure 2.2: MDMA induced mitochondrial complex I inhibition and free radical formation - - - - - - 26

Figure 2.3: Pathways of 3,4-methylenedioxymethamphetamine (MDMA) metabolism in rats and in humans. Isoenzymes of cytochrome P450 (CYP) involved in the N-demethylation and demethylenation metabolic reactions in rats are highlighted in blue whereas those corresponding to enzymes in humans are shown in red - - - - - - - 28

Figure 2.4: Balance of Inflammation. (Abbreviations: Helper T-cell Type I (TH1), Tumor necrosis factor (TNF), Interleukin-1 (IL-1), Matrix Metalloprotease (MMP), Helper T-cell Type II (TH2), Transforming Growth Factor (TGF), Brain Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF), Neurotropic Factor 3 (NT3), and Glial-Derived Neurotrophic Factor (GDNF) - 31

Figure 2.5: Glutamate transporters in astrocytes are responsible for synaptic glutamate homeostasis - - - - - - 38

Figure 4.1: Effect of repeated administration of MDMA (4 x 20 mg/kg, i.p.) on MDMA induced hyperthermia at 21^0C and 27^0C - - 48

Figure 4.2: Effect of repeated administration of MDMA (20mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker at 21°C and 27°C Histogram from the striatum immunostained for CD11b - - - - - - - - 52

Figure 4.3: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker at 21°C and 27°C. Histogram from the striatum immunostained for GFAP. Values are reported as a percentage of GFAP-positive cells with respect to vehicle-treated mice and are expressed as mean ± SEM - - - - - - - - 56

Figure 4.4: Effect of MDMA (10 mg/kg) and normal saline on Spatial memory at 21°C - - - - - - - - 57

Figure 4.5: Effect of MDMA (10 mg/kg) and normal saline on spatial memory at 27°C - - - - - - - - 58

Figure 4.6: Effect of MDMA (10 mg/kg) on Spatial memory at 21°C and 27°C 59

Figure 4.7: Discrimination ratio of novel object recognition task of rats treated with MDMA (10 mg/kg) and vehicle at 21°C - - - 60

Figure 4.8: Discrimination ratio of novel object recognition task of rats treated with MDMA (10 mg/kg) and vehicle at 27°C - - - 61

Figure 4.9: Discrimination ratio of novel object recognition task of rats treated with MDMA (10 mg/kg) and vehicle at 21°C and 27°C - 62

LIST OF PLATES

Plate 4.1: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker at 21°C (x 20) - - - - - - - 50

Plate 4.2: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker at 27°C (x 20) - - - - - - - 51

Plate 4.3: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker at 21°C (x 20) - - - - - - - 54

Plate 4.4: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker at 27°C (x 20) - - - - - - - 55

LIST OF APPENDICES

Appendix 1: International drug abuse research society (IDARS) - - 105

Appendix 2: 11th International conference of the society of neuroscientist of Africa - - - - - - - - 106

Appendix 3: Immuno-histochemistry for GFAP - - - - 108

Appendix 4: Immuno-histochemistry for CD11b - - - - 110

Appendix 5: Y-maze - - - - - - - 112

Appendix 6: Novel Object Recognition Apparatus - - - 113

Appendix 7: Table: Y-maze Percentage Preference at 21°C - - 114

Appendix 8: Table: Y-maze Percentage Preference at 27°C - - 115

Appendix 9: Table: Novel Object Recognition at 21°C (MDMA) - 116

Appendix 10: Table: Novel Object Recognition at 21°C (Saline) - - 117

Appendix 11: Table: Novel Object Recognition at 27°C (MDMA) - 118

Appendix 12: Table: Novel Object Recognition at 27°C (Saline) - - 119

Appendix 13: Effect of on MDMA- induced hyperthermia at 21°C - - 120

Appendix 14: Effect of MDMA - induced hyperthermia at 27°C - - 121

LIST OF ABBREVIATIONS

5-HIAAC	-	5-hydroxyl indole acetic acid
5-HT	-	5-hydroxytryptamine
ALS	-	Amyotrophic lateral sclerosis
AMPH	-	Amphetamine
ANP	-	Atrial natriuretic peptide
AR	-	Adrenergic receptors
ATP	-	Adenosine triphosphate
BBB	-	Blood brain barrier
CNS	-	Central nervous system
COMT	-	Catechol-O-methyl transferase
COX-2	-	Cyclooxygenase 2
DA	-	Dopamine
DHBA	-	Dihydroxybenzoic acid
EMCDDA	-	European Monitoring Centre for Drugs and Drug Addiction
ER	-	Endoplasmatic reticulum
ERK	-	Extracellular signal-regulated kinase
ETC	-	Electron transport chain
ETC	-	Electron transport chain
FFA	-	Free fatty acid
GABA	-	Gamma-amino butyric acid
GFAP	-	Glial fibrillary acidic protein
GLT	-	Glutamate transporters

GPCRs	-	G-protein-coupled-receptors
HHA	-	Dihydroxyamphetamine
HHMA	-	3,4–dihydroxymethamphetamine
HMA	-	3-methoxy, 4-hydroxyamphetamine
I.C.V	-	Intracerebroventricular
i.p.	-	Intraperotoneally
IFN γ	-	Interferon γ
IgG	-	Immunoglobulin G
IL-1b	-	Interleukin-1b
iNOS	-	Inducible nitric oxide synthase
IP_3	-	Inositol triphosphate
LPS	-	Lipopolysaccharide
MAO	-	Monoamine oxidase
MDA	-	3,4 methylenedioxyamphetamine
MDMA	-	3,4 methylenedioxymethamphetamine
METH	-	Methamphetamine
MS	-	Multiple sclerosis
NE	-	Norepinephrine
NMDA	-	N-methyl-D-aspartic acid
NMDA	-	N-methyl-D aspartic acid
NO	-	Nitric oxide
NOR	-	Novel object recognition
NPR	-	Nitriuretic Peptide Receptor

NPR-A	-	Nitriuretic Peptide Receptor A
PET	-	Positron emission tomography
PGE2	-	Prostaglandin E2
PKG	-	Protein Kinase G
PNS	-	Peripheral nervous system
POAH	-	Preoptic-anterior hypothalamus
ROS	-	Reactive oxygen species
SERT	-	Serotonin transporter
SNc	-	Substantia nigra pars compacta
SULT	-	Sulfotransferase
TH	-	Tyrosine hydroxylase
TNF	-	α tumour necrosis factor α
TPH	-	Tryptophan hydroxylase
UCP	-	Uncoupling proteins
UDPGT	-	Glucuronosyl transferase
VEH	-	Vehicle
VMAT-2	-	Vesicular monoamine transporter 2

CHAPTER ONE

1.0 Introduction

1.1 Background of the Study

Addiction is a chronic, relapsing brain disease characterized by compulsive drug-seeking and use, despite harmful consequences to the individual and society (Koob and Volkow, 2010). The disease affects both the brain and behaviour. It is considered a brain disease because drugs causing addiction also change the structure and function of the brain (Alan 1997). The brain changes may be long-lasting, causing harmful behaviours seen in people who abuse drugs. People of all ages suffer the harmful consequences of drug abuse and addiction. The national institute on drug abuse in 2008 estimated 2.1 million Americans aged 12 and older had abused 3,4-Methylenedioxymethamphetamine (MDMA) at least once in their life. Over 32 million people or almost 10% of the adult population in the European Union used the drug in 2008, according to the annual report of the European Monitoring Center for Drugs and Drug Addiction (EMCDDA). Around 2 million drug users in Europe preferred amphetamine while ecstasy was used by 2.5 million people. Approximately 12 million have tried amphetamine and 10 million have tried ecstasy at least once in their lives. The use of ecstasy is overtaking the other amphetamines and getting the second place after cannabis, in both general population and school surveys approximately represent 22% of total drug abusers (EMCDDA 2008).

Figure 1.1: MDMA Tablets (www.drugabuse.org) (2013)

3,4-methylenedioxymethamphetamine (MDMA) is a commonly used recreational drug, often ingested at dance clubs. Hypothermia and hyperthermia are the two adverse effects of MDMA. Subjective thermal responses, such as 'feeling hot' or 'hot and cold flushes,' have also been frequently noted (Davison and Parrott, 1997; Parrott *et al*., 2006). However, the predominant severe adverse effect following MDMA by recreational users is life-threatening hyperthermia (Sprague *et. al.*, 2003). In hospital emergency department admissions, the MDMA-related casualties present with both low and high body temperatures (Green *et al.,* 2009). Halpern *et al.,* (2011) described the medical profiles of MDMA-related admissions to hospital emergency departments in Israel. They noted that: 'The most common manifestations were restlessness, agitation, disorientation, shaking, high blood pressure, headache and loss of consciousness.

Methylenedioxymethamphetamine is a ring-substituted amphetamine that is widely used among youths in many countries, especially in the West (Green *et al.*, 2003) and also throughout the regions of Asia and in many African countries (Krolikowski and Koyfman, 2014). The use of amphetamines has reached epidemic proportions around the world (Koesters and Rogersn, 2002). In Africa, methamphetamine has become a major drug of abuse (Kapp, 2008) .The United Nations estimates that 280,000–780,000 South Africans used amphetamines in 2011 (World Drug Report, 2011). The South African Medical Research Council estimates that 7% of the population in Cape Town is using methamphetamine (Morris and Parry, 2006). The first report of psychoactive property of the drug in human users was published in 1978 (Anderson *et al.,* 1978). Users of MDMA exhibit feelings of depressed mood, loss of energy, muscle cramping, fatigue, nausea, and in severe cases, compromised mental status. Such symptoms may begin several hours

after ecstasy consumption and can persist for days. These after-effects of ecstasy use are referred to as either the "mid-week blues' (Parrott, 2002) or as the 'ecstasy hangover" (Traub *et al*., 2002). Methylenedioxymethamphetamine is now used extensively at dance clubs, and parties, and it is this new use that has given rise to a substantial increase in the number of reports of toxic reactions and deaths (Schwartz and Miller, 1997). Hyperthermia is one of the causes of death due to MDMA. In rats, the hyperthermic response is similar to that of mice. In humans, MDMA induces effects, including hyperthermia, hepatotoxicity, cardiotoxicity, hypertension, hyponatraemia, mydriasis, thrombocytopenia and other physiological and behavioural modifications (Parrott 2001; Green *et al.,* 2003; Schifano *et al.,* 2003; Freedman *et al*., 2005; Easton and Marsden 2006).

1.2 Statement of Research Problem

The use of MDMA has escalated at an alarming proportion in the last few years around the globe despite the fact that dozens of young people die as a result of its abuse every year and its associated impairment in cognition, neurotoxicity and hyperthermia. There is no pharmacologically specific treatment. Furthermore, the associations between hyperthermia and many of the pathological changes induced by MDMA is not clearly understood. Moreover, the use of these drugs has led to many youth ending up in psychiatric hospitals and a lot of burden on the socio-economic infrastructure. In Nigeria, there are only few or no reported cases with a tendency for a further research.

1.3 Justification for the Study

Whilst substantial evidence exists demonstrating the hyperthermic and toxic effects of MDMA, especially on the brain, the mechanisms underlining them is still debatable.

Evidence for the occurrence of induced neurotoxic damage in human users remains equivocal. For example, some believe the hyperthermia is dose dependent without interference from ambient temperature. The drug is still under-investigated despite its growing popularity among the population. Most of the investigations did not take into consideration variations in ambient temperature where recreational users are subjected to. The results of the current study may be of value in educating the community and the general population on some of the consequences of recreational use of MDMA and the role of ambient temperature and its toxic effects.

1.4 General Aim of the Research

The aim of the present study was to investigate the effects of variation in ambient temperature in relation to body temperature and neurological changes induced by MDMA administration in rodents.

1.5 Specific Objectives

The specific objectives were to evaluate:

a) The role of ambient temperature variation on MDMA induced body temperature changes in mice.
b) Activation of striatal microglia through CD 11b as a marker for microgliosis in mice
c) Activation of striatal astroglia through GFAP as a marker for astroglyosis in mice
d) Assessment of spatial memory using Y-maze in relation to ambient temperature in Wistar rats.
e) Assessment of non- spatial memory using the Novel Object Recognition (NOR) test in relation to ambient temperature in Wistar rats.

CHAPTER TWO

2.0 Literature Review

2.1 History of MDMA

3,4-Methylenedioxymethamphetamine (MDMA) was first synthesized and patented by the German pharmaceutical company Merck in Darmstadt around 1912, merely as a precursor in a new chemical pathway which was patented in order to avoid an infringement of existing patent for the synthesis of the clotting agent hydrastinine (Freudenmann *et al*., 2006). The first formal animal study on MDMA was done by Hardman *et al*., (1973). In 1953-54 (US army studies) consisted of a number of LD_{50} determinations on five laboratory animal species, including the mouse. MDMA was first used by humans in the late 1960s, where it showed its properties of inducing feelings of well-being and increased communication (Watson and Beck, 1991). There was a phase of therapeutic enthusiasm for this drug as adjunct to psychotherapy due to its ability to enhance feelings of openness and trust and cause a sense of deep harmony in the self and in relationships with other persons (Grinspoon and Bakalar, 1986). The drug was introduced in clinical psychotherapeutic practice on the West Coast of the United States in the beginning of 1976 (Shulgin, 1990). There were reports of cases of toxicity and deaths from exposure to large doses of MDMA in United States and Europe (Dowling *et al*., 1987) and several researchers reported long-term neurotoxic effects of MDMA in laboratory animals (Ricaurte *et al*., 1985; Schmidt *et al*., 1986; Stone *et al*., 1986). Until the mid 1980s, drug use was restricted predominantly to people taking the drug when alone in a small party (Peroutka *et al*., 1988). Whereas, since the late 1980s, MDMA has been used as a street drug (street names include “Ecstasy”, “XTC”, “Adam”, “Essence”,

“Clarity”). The Food and Drug Administration placed the compound on Schedule I control substance on July1, 1985. MDMA is now used extensively at dance clubs at parties called “raves” and it is this new use that has probably given rise to a substantial increase in the number of reports of toxic reactions and deaths (Schwartz and Miller, 1997). Despite increasing reports of the potential neurotoxicity, as well as deaths, related to MDMA use, the popularity of this drug has increased tremendously over the years making it of high concern for mental health professionals.

Table 2.1: Milestones from the history of MDMA/Ecstasy (Freudenmann *et al*., 2006)

Year	*Event*
1912	First synthesis MDMA by Köllisch at Merck (Darmstadt, Germany), secured by German patent 2743350
1927	First pharmacological tests with MDMA by Oberlin at Merck
1952	Basic toxicological test with MDMA by van Schoor at Merck
1953/4	First formal animal study in five species using MDMA and seven other psychotropic drugs (University of Michigan); secret, US army-sponsored study, unpublished until 1973
1959	Re-synthesis of MDMA by Pruhstorfer at Merck
1960	First regular scientific paper on MDMA (in Polish) describing an MDMA synthesis
1970	First detection of MDMA in tablets seized in the streets of Chicago
1978	First MDMA studies in humans by Shulgin and coworkers reporting on chemistry, dosage, kinetics and psychotropic effects
1984	MDMA's street name 'ecstasy' was coined in California
1985-8	MDMA became a Schedule I controlled substance in the United States and Banned in most others soon thereafter

2.2 Chemical Structure and Mechanisms of Action of MDMA

As its chemical name implies, MDMA bears the intrinsic structure of amphetamine (AMPH) with an *N*-methyl group and a methylenedioxy-ring substitution on the third and fourth carbon of the phenyl ring (Figure 2.1). It is thought that this variation in structure is responsible for dichotomous effects on brain neurochemistry exerted by these two substances. More specifically, AMPH and its derivative, methamphetamine (METH) has potent and long lasting effects on the dopamine (DA) neuromodulatory system while, in the long-term, MDMA affects mainly the serotonergic system. Additionally, MDMA more subtly affects the dopamine, norepinephrine (NE), gamma-aminobutyric acid (GABA), glutamate, and other systems as well (Green *et al*., 2003).

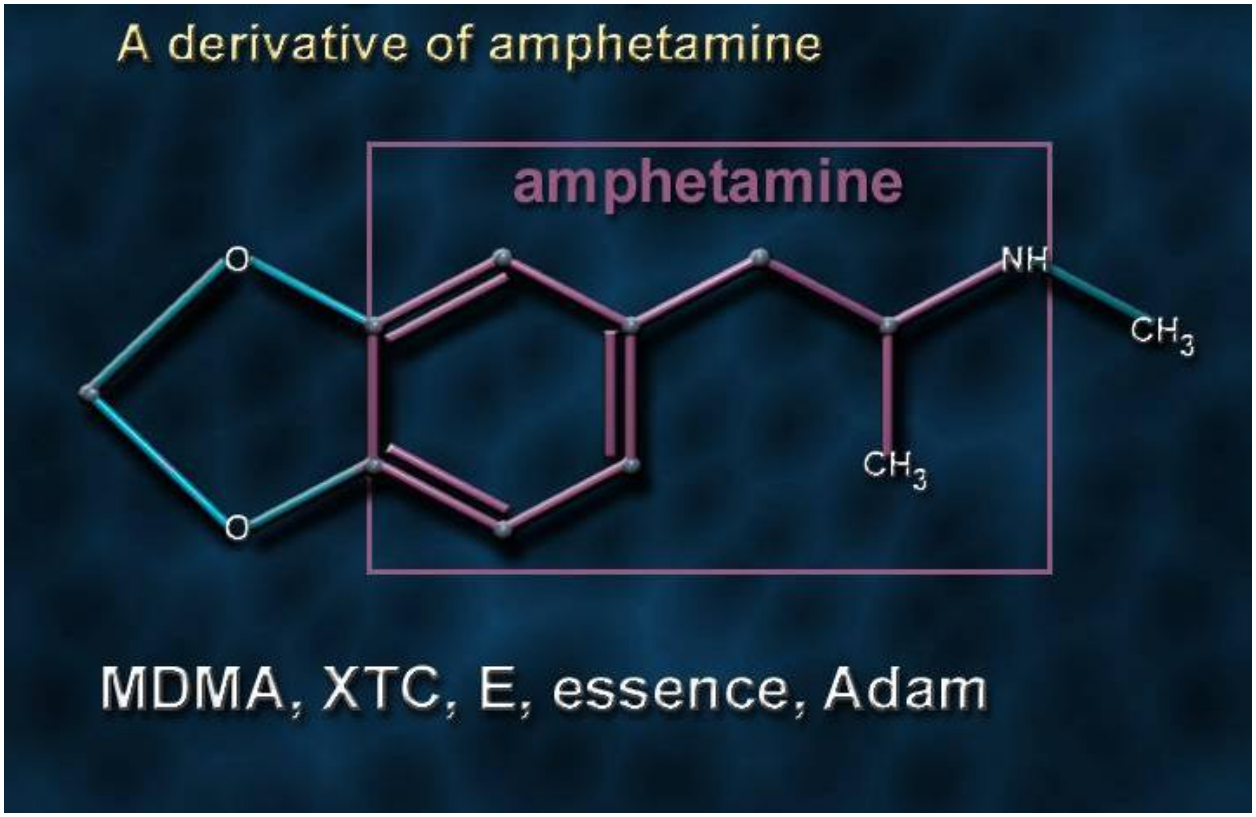


Figure 2.1: Chemical structure of MDMA (Green *et al*., 2003)

Acutely, MDMA causes a rapid efflux of serotonin (5-hydroxytryptamine; 5-HT), NE, and DA from respective monoaminergic terminals. In terms of serotonergic release, this effect is mediated by the drug's interaction with both the serotonin transporter (SERT) as well as with the intracellular vesicular monoamine transporter 2 (VMAT-2), both proteins being involved in the selective transport of 5-HT across phospholipid bilayers. More specifically, MDMA is a substrate for both SERT and VMAT-2, allowing it to enter the terminal and subsequently, vesicles bearing 5-HT, respectively (Rudnick and Wall, 1992). Once inside the vesicles, the slightly alkaline nature of MDMA causes dissipation of the proton gradient between the vesicle and the cytosol necessary for proper functioning of VMAT-2, and in this respect, it inhibits VMAT-2- mediated influx and proper storage of 5-HT in the terminal (Sulzer and Rayport, 1990). Coupled with its ability to cause functional reversal of both VMAT-2 and SERT, MDMA allows 5- HT to passively efflux from terminal vesicles and subsequently, from the neuron itself, ultimately leading to a global increase in extracellular 5-HT throughout brain regions bearing raphe afferents (Rudnick and Wall, 1992). This effect is further potentiated by MDMA-induced inhibition of 5-HT reuptake, as consequence of competition for SERT-binding by both 5- HT and MDMA.

2.3 Effects in Human Users

2.3.1 Desirable

The positively rated effects of MDMA consumption include euphoria, arousal, enhanced mood, increased sociability, and heightened perceptions (Jerrold, 2013). The desirable effects of MDMA, which lead to the use of ecstasy, are generally psychological. They include feelings such as euphoria, reduction of negative thoughts, increased energy,

happiness, friendliness, calmness, relaxation and heightened perception of sound, colour and touch (Baylen and Rosenberg, 2006; Davison and Parrott, 1997; Green *et al.*, 2003).

2.3.2 Acute adverse effect

There are also many adverse effects associated with ecstasy use. Acute adverse effects associated with MDMA use include motor and muscular problems, such as hyperactivity, muscle aches and tension, and bruxism and jaw clenching, as well as others such as elevated blood pressure and heart rate, nausea, chills, sweating, confusion and hyperthermia (Green *et al*, 2003; Lyles and Cadet, 2003; McCann *et al*, 1996). Although the epidemiological evidence indicates the incidence of major adverse effects are low (Byard *et al*, 1998), the events are unpredictable and can lead to death or morbidity (Gowing *et al*, 2002; Williamson *et al*, 1997). Hyperthermia is one of these major effects, which can lead to death due to cardiac arrhythmias, acute renal failure, rhabdomyolysis and disseminated intravascular coagulation (Lyles and Cadet, 2003; Screaton *et al*, 1992).

The clinical picture of an 'ecstasy overdose' victim is very similar to that described as 'serotonin syndrome'. The serotonin syndrome, believed to be caused by an excess of Synaptic serotonin (5-hydroxytryptamine; 5-HT), is usually associated with Inappropriate administration of therapeutic drugs which affect the 5HT system (Sun- Edelstein *et al*, 2008). The associated symptoms include hyperactivity, confusion, agitation, jaw clenching, hyperreflexia, hyperthermia, tachycardia, shivering, ocular oscillations, tremor as well as others (Parrott, 2002). Some of these symptoms such as hyperactivity, confusion and jaw clenching are considered normal effects of ecstasy by users, but some users develop the complications mentioned above. Unfortunately, we are still unaware of

why this occurs in some situations and not others, and why it is not a more predictable dose dependent phenomenon as animal studies would suggest.

2.3.3 Long term adverse effect

There are also longer term problems associated with the use of MDMA. Users report adverse feelings such as lethargy, moodiness, irritability, insomnia, paranoia and depression in the days after use of MDMA (Davison and Parrott, 1997; Green *et al.*, 2003).

It has also been suggested that MDMA use could lead to the development of depression, as long term depletion of serotonin in the brain has been shown in both humans and animals (Malberg and Seiden, 1998; McCann *et al*, 1998; Wang *et al*, 2004). For example, nuclear imaging studies have shown consistently that ecstasy users have reduced serotonin transporter (5HTT) levels, a possible indicator for loss of 5HT neurons (McCann *et al*, 1998; 2005; Reneman *et al*, 2002a; 2002b; 2002c). However, several studies show evidence that depression may actually precede MDMA use in many users (Guillot and Greenway, 2006; Lieb *et al.*, 2002; Soar *et al.*, 2001). There is evidence for other long term cognitive problems associated with ecstasy use, although high rates of poly drug use makes it difficult to interpret some results. Several studies have attempted to account for this in different ways. Halpern *et al*. (2004) found a group of MDMA users and non-users with low exposure to other types of drugs including alcohol and tobacco.

They were able to show that heavy MDMA users displayed deficits in mental processing speed and impulsivity compared to non-users. Other groups have compared ecstasy users to cannabis users, to control for the high cannabis use in the drug using population.

Quednow *et al.* (2006a; 2006b) found that MDMA users also showed higher impulsivity and lower decision making performance compared to cannabis users and non-drug users (Quednow *et al*, 2006b), as well as memory deficits compared to the same control groups (Quednow *et al.*, 2006a).

2.4 Mechanisms Involved MDMA-Induced Temperature Changes

Hypothermia and hyperthermia are the two adverse effects of MDMA. However, the predominant severe adverse effect following MDMA by recreational users is life-threatening hyperthermia (Sprague *et. al.*, 2003). In hospital emergency Department admissions, the MDMA-related casualties present with both low and high body temperatures (Green *et al.*, 2009). In chronic terms, the bioenergetic stress model notes that the adverse psychobiological effects of MDMA are heightened by various co-stimulatory factors, including heat stress. (Parrot 2012) MDMA increases core body temperature and thermal stress in humans (Parrot, 2012).

2.4.1 Hypothalamic-Pituitary-Thyroid Axis

According to Sprague *et al.* (2003, 2007) thermogenesis induced by MDMA involved the hypothalamic-pituitary-thyroid axis and the sympathetic nervous system. The hypothalamus is a portion of the brain with a variety of functions. It has the greatest concentration of nuclei at which set points are encoded and controlled, and so can be considered as the key brain region for the control of thermostasis. The hypothalamus is responsible for the regulation of body temperature which is essential because most of the metabolic processes and enzyme activities that are necessary for life are strongly temperature-dependent. The normal body temperature set-point is primarily determined by the activity of neurons in the medial preoptic and anterior hypothalamic nuclei as well

as by neurons in the adjoining medial septal nuclei. Collectively, this region is often termed the preoptic-anterior hypothalamus (POAH). Three types of neurons are involved in temperature set-point; warm-sensitive, cold-sensitive and temperature- insensitive neurons. MDMA disrupts the activation of warm-sensitive neurons and inhibit the dissipation of heat.

Thyroid-stimulating hormone secreted by about 5 percent of the basophilic cells in the pituitary called thyrotrophs and regulates thyroid function. Thyroid hormone is the primary endocrine regulator of metabolism and thermogenesis. MDMA-induced thermogenesis is also thyroid hormone-dependent (Sprague *et al.*, 2003, 2007). According to Martin *et al*., (2007), clinically, hyperthyroidism potentiates the hyperthermic effects of ecstasy. When administered to rats, MDMA acutely increases plasma levels of thyroid hormone T4 and induces a similarly acute and robust elevation in core temperature. Surgical removal of either the pituitary or thyroid glands abolished the hyperthermic response andproduced a significant hypothermia, in addition to blocking subsequent serotonergic neurotoxicity (Sprague *et. al*., 2003). Hypothyroidism has also been associated with anup-regulation of α_1 AR (Dicker *et al*., 1992) and β_3 AR (Rubio *et al*., 1995b), and a down-regulation of β_1 and β_2 receptors (Rubio *et al*., 1995a).

2.4.2 Role of Monoamine Neurotransmitters

The effect of MDMA on body temperature is complex because the drug has actions on all three major monoamine neurotransmitters 5-hydroxytryptamine (5-HT), dopamine and noradrenaline, both by amine release and by direct receptor activation (Docherty and Green, 2010). MDMA produces an acute and massive release of 5-HT from serotonergic nerve endings (Colado and Green, 1994; Mechan *et al.*, 2001) and dopamine (Koch and

Galloway, 1997; Sabol and Seiden, 1998; Colado *et al.,* 1999). It is assumed that MDMA-induced hyperthermia is a consequence of 5-HT release and subsequent stimulation of the 5-HT receptors involved in thermoregulation (Shankaran and Gudelsky, 1999). MDMA was first reported to cause release of 5-HT from synaptosomes (Nichols *et al*., 1982). MDMA has high affinity for the serotonin transporter (SERT), (Brian, 2008). SERT is a twelve-transmembrane-domain neural protein distributed along the body of the serotonergic axon and is responsible for the uptake of 5-HT from the extracellular space (Feldman *et al.*, 1997; Tao-Teng and Zhou, 1999). MDMA is most widely known for inducing long-term changes in several markers of serotonin system integrity and robust depletion in 5-HT (Green *et al.,* 2003; Thompson *et al.;* 2004). MDMA reduce the responsiveness of 5-HTIA receptor subtype involved in both thermoregulation and the serotonin syndrome (Darmani & Ahmad, 1999). It has long been known that acutely increasing 5-HT synthesis and release in the brain by administration of L-tyrptophan and a monoamine oxidase inhibitor result in hyperthermia (Grahame-Smith, 1971a; Green and Grahame-Smith, 1976). Acute 5-HT release is not directly responsible for hyperthermia, but 5-HT receptors are involved in modulating the hyperthermic response. In addition, the increased 5-HT function resulting from the MDMA-induced 5-HT release stimulates 5-HT2 receptors thereby further enhancing dopamine release (Nash, 1990; Gudelsky *et al.*, 1994; Schmidt *et al.*, 1990). MDMA has been shown to produce lipid peroxidation and to reduce antioxidant content in 5-HT nerve terminals, events that usually accompany free radical formation (Shankara *et al*., 2001).

MDMA has a direct dopamine- releasing effect via both calcium-dependent and independent mechanisms at the level of the nerve ending (Nash and Brodkin, 1991; Crespi *et al*., 1997; Koch and Galloway, 1997). Dopamine is the neurotransmitter that is central to both the hyperthermic and hypothermic effects of MDMA. MDMA-mediated dopamine release and subsequent activation of hypothalamic D1 receptors has been shown to play an essential role in this hyperthermic response (Mechan *et al.*, 2002). Activation of the hypothalamic axis following MDMA treatment is also confirmed by increased c-fos expression in the supraoptic and median preoptic nucleus of the hypothalamus (Stephenson *et al.*, 1999). However, there is now considerable evidence to suggest that 5-HT plays little or no role in the acute hyperthermic response. Pretreatment with a variety of selective and non-selective 5-HT receptor antagonists was found to have no effect on MDMA-induced hyperthermia (Mechan *et al*., 2002).

2.4.3 Sympathetic Nervous System

Users of MDMA have elevated plasma catecholamine levels, which may be due to noradrenergic hyperactivity and cardiovascular complications (Stuerenburg *et al*., 2002). MDMA also inhibits monoamine oxidase (MAO) (Leonardi and Azmitia, 1994) to block metabolism of noradrenaline. After MDMA administration, there is 35-fold increase in plasma norepinephrine levels (Sprague *et al.*, 2007), which stimulates peripheral α_1, β_2 and β_3 adrenergic receptors (AR) (Himms-Hagen *et al.*, 1978; Kuusela *et al.*, 1997; Zhao *et al.*, 1997). Activation of α_1AR mediates peripheral vasoconstriction. MDMA activates central α_2AR and peripheral α_1AR to produce cutaneous vasoconstriction to restrict heat loss, and β_3AR in brown adipose tissue to increase heat generation (Pedersen and Blessing, 2001). Sprague *et al.* (2004) demonstrated the involvement of α_1, and β_3 AR in

the acute thermogenic effect of MDMA. According to Pedersen and Blessing (2001) and McDaid and Docherty (2001) cutaneous vasoconstriction contributes to the increase in core temperature seen after treatment with MDMA, showing that the vascular effects of MDMA may involve predominantely α_1 AR. Peripheral effects of MDMA at α_1 AR could explain a major component of its temperature actions, namely cutaneous vasoconstriction. The monoaminergic systems are interconnected and can influence each other, therefore, it has been suggested that under the conditions of increased extracellular levels of the three monoamines produced by MDMA, concomitant activation of the pre-synaptic α_2 AR results in a component of the hyperthermic response. Although vasoconstriction is mediated predominantly by, α_1 AR, α_2AR particularly, α_2AR also contribute to systemic vasoconstriction (Duka *et al*., 2000).

2.4.4 Uncoupling Proteins

Uncoupling proteins are mitochondrial carrier proteins that catalyse a regulated proton leak across the inner mitochondrial membrane, diverting free energy from ATP synthesis by the mitochondrial ATP synthase to the production of heat. There are five known types: UCP1 also known as thermogenin, UCP2, UCP3, UCP4 and UCP5 (Sprague, 2003, 2007) noted that the thermogenesis, induced by MDMA in animals involve uncoupling proteins. MDMA induces thermogenesis in a mitochondrial uncoupling protein 3-dependent manner (Hrometz *et al.,* 2011). There is evidence that this hyperthermia is mediated in part by the lipolytic release of free fatty acids that subsequently activate UCP3 in the mitochondria. Three other lines of evidence suggest that UCPs are involved in the thermogenic response to MDMA. First, clinical presentations of severe hyperthermia, induced by MDMA include rhabdomyolysis, wherein skeletal muscle cells

lose viability, lyse, and release myoglobin, which can lead to renal failure (Walubo and Seger, 1999). Second, MDMA regulates the levels of UCP-3 mRNA in skeletal muscle (Sprague *et al.*, 2007). Third, MDMA has also been shown to increase proton leak in rat striatum, a specific functional correlate of UCP activity (Burrows *et al.*, 2000). Peripherally, the increased body temperature seen with MDMA is due in part to a combination of peripheral vasoconstriction which prevents heat dissipation (Pedersen *et al.*, 2001)) and activation of mitochondrial uncoupling proteins (UCP) that generates heat in skeletal muscle and adipose tissues (Mills *et al*., 2003; Mills *et al*., 2004; Kelly *et al*., 2011). Free fatty acids (FFA) activate mitochondrial UCP3 and induce proton leak across the inner mitochondrial membrane. This process, referred to as mitochondrial uncoupling, dissociates the mitochondrial proton gradient from ATP synthesis, and releases the free energy as heat (Lowell *et al.*, 2000).

2.4.5 Role of Atrial Nituretic Peptide

According to Hrometz *et al.* (2011) atrial natriuretic peptide (ANP), a strong lipolytic mediator contributes to the induction and maintenance of MDMA-induced thermogenesis. Atrial natriuretic peptide (ANP), acting on the natriuretic peptide receptor (NPR) type A (NPR-A), stimulates lipolysis and FFA production through activation of protein kinase G (PKG). Plasma insulin levels, also increases following MDMA administration (Banks *et al*., 2009). Insulin stimulates uptake of FFAs into skeletal muscles by recruiting FFA transporters to the plasma membrane (Luiken *et al.*, 2002). ANP is released from cardiac myocytes in response to high temperature and stretch of the atria and/or ventricles. As a sympathomimetic, MDMA increases heart rate and blood pressure (Broadley, 2010), which likely accounts for MDMA induced ANP release. As

the release of FFA are essential in the MDMA-induced thermogenic model (Sprague, *et al.,* 2007), release of ANP would contribute to this increase in FFA release. Intra-cerebro-ventricular administration of ANP induces an increase in body temperature (Pataki *et al.*, 1999). Sengenes *et al.* (2002) however, reported that ANP-induced lipolysis occurs predominantly in adipose tissue in humans and primates and to a lesser extent in other mammals such as rats, mice, rabbits and dogs.

2.4.6 Blood Brain Barrier

The blood-brain barrier (BBB) protects the neural tissue from variations in blood composition and toxins. Astrocytes forming BBB are highly specialized to allow precise control over the substances that enter or leave the brain. Methylenedioxy-methamphetamine is known to induce alterations in neurochemical metabolism in the central nervous system CNS). It disrupts the BBB permeability to proteins and induces the formation of oedema and cellular stress, leading to cell injury. BBB breakdown is often associated with increased extravasation of plasma proteins and high levels of immunoglobulin G (IgG) in the brain. Sharma and Ali (2008) found increased albumin immunoreactivity, indicating breakdown of the BBB, and upregulation of glial fibrillary acidic protein (GFAP), suggesting activation of astrocytes. According to Ramirez *et al.* (2009), MDMA disrupts blood–brain barrier function by induction of oxidative stress in endothelial cells.

2.4.7 Role of Pro-Inflammatory Cytokines

Recent studies suggest that microglial activation and the subsequent release of the pyrogen interleukin-1b also contribute to MDMA-induced hyperthermia (Abraham *et al.*, 2003, Orio *et al.,* 2009). Interleukin-1b (IL-1b) is a pro-inflammatory cytokine that is

mainly produced by activated microglia following diverse forms of neurodegeneration and CNS inflammation (Pearson *et al.*, 1999; Touzani *et al.*, 1999; Chauvet *et al.*, 2001; Rothwell, 2003). Pro- inflammatory cytokines exacerbate and sustain the hyperthermia caused by MDMA (Salem *et al.*, 2011). MDMA induces changes in the upstream regulation of IL-1b signaling in the hypothalamus. IL-1b is expressed in microglial cells shortly after MDMA administration. Hypothalamic neurons are the main target cells for IL-1b. In the hypothalamus, the drug induces a pronounced and transient increase in IL-1b levels. Interleukin- 1b is constitutively expressed at very low levels in normal rodent brain (Quan *et al.*, 1996), but it is rapidly induced in response to acute experimental brain insults (Rothwell, 2003).

2.5 Toxicity of MDMA

2.5.1 In Humans

Methylenedioxymethamphetamine has various harmful health effects in humans. The neurotoxic dose of MDMA in non-human primates approaches the dose of MDMA typically taken by recreational MDMA users (www.drugtext.org/library/articles/ricaurte.htm).

The acute effects after taking MDMA are hyponatraemia (headaque, confusion or altered mental state, seizures), hyperthermia is one of the causes for death due to MDMA. Approximately 15 young persons die every year from acute MDMA toxic effects. The reason may be that MDMA is usually taken recreationally in dance clubs or in rave parties in hot, crowded rooms (both conditions leads to larger elevation of MDMA induced body temperature in animals) therefore clinically such conditions could increase the possibility of subsequent cerebral neurotoxic effect. Several case reports have been reported fatal hyperthermia after ingestion of ecstasy. The patient collapsed with the

seizures they tended to have a very fast heart rate and very low blood pressure (Green *et al*., 2004). Chronic studies by Mc Cann *et al*. (1994) found selective reductions in cerebrospinal fluid of 5 – hydroxyindoleacetic acid (5-HIAA) in MDMA users compared to control that never used it. Positron emission tomography (PET) studies showed evidence of decreased 5HT transporter sites correlated with the degree of MDMA exposure. It has also been found that, there is altered neuroendocrine function in MDMA users which can be correlated to alterations of hypothalamic 5HT function, suggestive of MDMA-induced 5-HT neurotoxicity. Neuropsychiatric testing methods found that MDMA users, compared to controls, had deficits in verbal and visual memory (Morgan 1999).

2.5.2 In Rats

MDMA administration in rats is known to release 5-HT in striatum and medial prefrontal cortex dose dependently which may lead to marked decrease in 5-HT concentration (Green *et al*., 2003). Treatement with MDMA also decreases 5-HT transporter level. A significant reduction in tryptophan hydroxylase (TPH), a rate limiting enzyme required for 5-HT synthesis is observed in the hippocampus, striatum and frontal cortex of hyperthermic animal's resulting in decreased cerebral tissue concentrations of 5-HT and 5-HIAA after MDMA administration. The release of dopamine by MDMA occurs through entering in dopamine nerve terminals and is modulated by 5-HT2A/2C receptors. MDMA administration to rats has generally been reported to produce a marked hyperthermic response. MDMA also induces increase in glial fibrillary acidic protein (GFAP) expression in the hippocampus (Green *et al*., 2003).

2.5.3 In Mice

Administration of MDMA in mice causes a small decrease in 5-HT and 5-HIAA in cortex, hippocampus and on striatum. Whereas it causes a rapid release of dopamine in striatum and reduces striatal content of both dopamine and its metabolites (Thomas *et al*., (2004) showed that MDMA induces significant microgliosis in striatum and substantial nigra compacta (SNc). MDMA induced hyperthermia though much more variable has been seen in mice.

2.6 Mechanism Involved in MDMA Toxicity

Studies have been carried out to understand mechanism involved in MDMA induced neurotoxicity still there is no much success. It is very well known that free radicals are involved in MDMA induced neurotoxicity but it is not yet known the source for free radicals which may be from release of excess of dopamine, from mitochondrial complex I inhibition, or from formation of toxic MDMA metabolites.

2.6.1 Oxidative stress

Several studies using animal models supported the involvement of oxidative stress in MDMA neurotoxicity. The role of oxidative stress is further supported by the findings that neurotoxic effects of MDMA can be attenuated by free radical scavengers and anti-oxidants. The reactive oxygen and nitrogen species involved in oxidative stress are suppose to be formed from release of excess of dopamine in cytosol or from formation of neurotoxic MDMA metabolites (Puerta *et al*., 2010). MDMA induces rapid and powerful release of dopamine in mice which is metabolised by MAO-B and leads to formation of DA quinones as well as hydrogen peroxide. Attenuation of DA release by lesioning DA neurons or blocking DA transporter has been shown to protect against the long term

toxicity of MDMA. In addition, oxidation of 5-HT, MDMA itself, and thioether metabolites of MDMA has also been implicated in MDMA neurotoxicity. MDMA has been shown to increase hydroxyl radical formation in rats; consequently these highly reactive free radicals can lead to the generation of lipid peroxidation and oxidize proteins in the nerve terminals (Quinton *et al*., 2006). Camarero *et al*., (2002) reported that administration of MDMA led to rise in the formation of 2,3 dihydroxybenzoic acid (2,3 DHBA) and malonyldialdehyde, a lipid peroxidation product, in mice striatum. In addition to reactive oxygen species, reactive nitrogen species now appear to play a major role in mediating MDMA-induced neurotoxicity. Neuronal nitric oxide synthase (NOS) activation seems to be involved in MDMA neurotoxicity which generates NO. This generated NO and peroxide radical from DA metabolism combines together to form peroxinitrite (ONOO-) which promotes autooxidation of DA to DA quinone and also has been found to inhibit DAT (Chipana *et al*., 2006).

2.6.2 Excitotoxicity

Excitotoxicity includes succession of several events, excessive glutamate release, activation of glutamate receptors and increase in intracellular calcium levels which leads to generation of free radicals and nitric oxide (Bruno *et al*., 1993; Yamamato *et al*., 2010). In fact glutamate and other excitatory amino acids have been linked to several neurodegenerative disorders suggesting a possible role of glutamate in Methamphetamine and MDMA-induced terminal degeneration (Lipton *et al*., 1994; Quinton *et al*., 2006). Colado *et al*., (2001) showed that there is no involvement or release of glutamate and calcium with MDMA in mice.

2.6.3 Mitochondrial Dysfunction

In addition to the increased oxidative stress, more recent evidence suggests an important role of the mitochondrial electron transport chain (ETC) in mediating the toxic effects of substituted amphetamines. The first evidence of inhibition of ETC by MDMA was given by Burrows et *al*. (2000) where they observed significant inhibition of cytochrome oxidase activity in the substantia nigra, the nucleus accumbens, and the striatum. In line with this study Puerta *et al*. (2010) reported that inhibition of complex I of the mitochondrial electron transport chain is one of the earlier events that take place in MDMA-induced neurotoxicity in mice. Aconitase, a krebs cylcle enzyme sensitive enough to reflect in situ reactive oxygen species (ROS) generation in mitochondria was significantly decreased after MDMA treatment. Methylenedioxymethamphetamine toxicity may derive from peroxinitrite that is formed by the diffusion related reaction of O_2^- with NO.

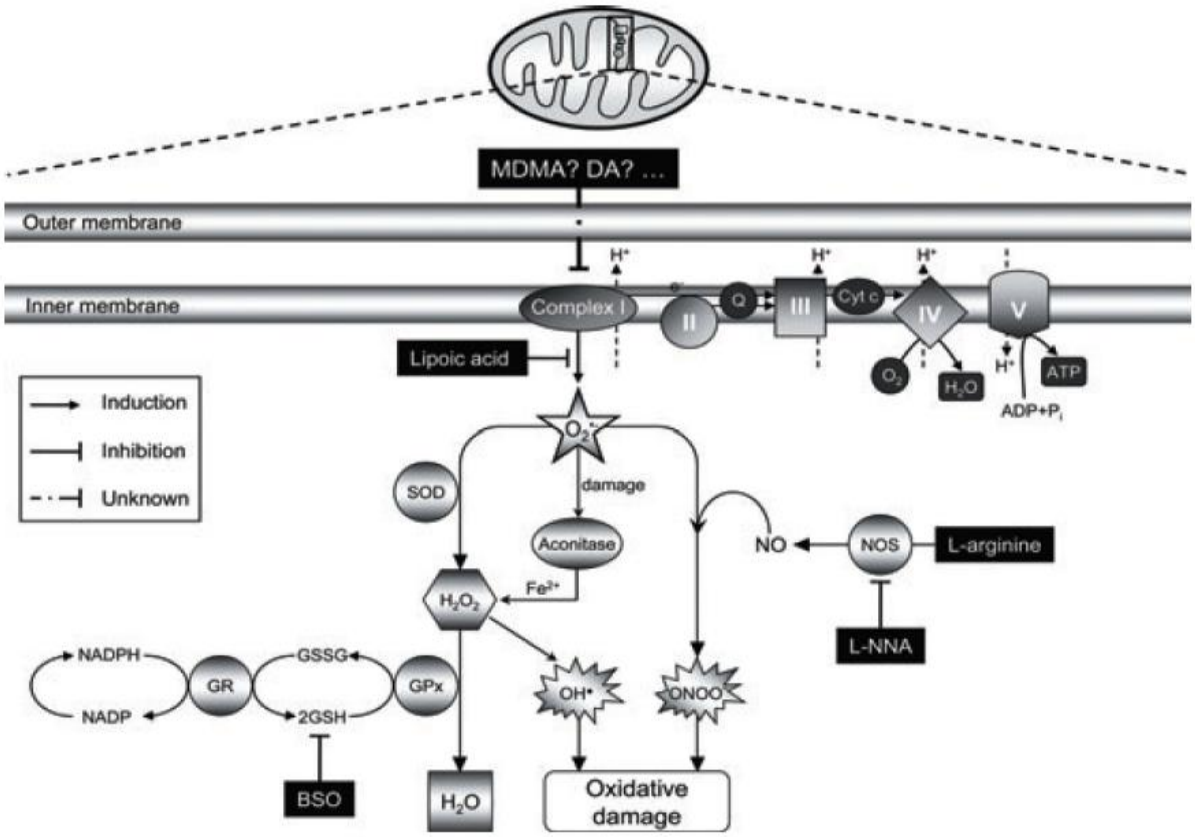


Figure 2.2: MDMA induced mitochondrial complex I inhibition and free radical formation (Puerta *et al*., 2010).

2.6.4 Formation of toxic MDMA metabolites

Yuan *et al*. (2010) reported that dopamine is not essential for the development of methamphetamine-induced neurotoxicity giving more stress on involvement of amphetamine metabolites in amphetamine toxicity. Several studies already reported that it's not MDMA but the metabolite of MDMA that induces different neurotoxic effects. With regard to MDMA toxicity Escobedo *et al*. (2005) showed that intrastriatal administration of MDMA at a dose much higher than the peripherally administered neurotoxic dose does not induce neurotoxicity. So as to produce neurotoxic effects, MDMA has to get metabolised peripherally and then the metabolite of MDMA induces different neurotoxic effects. The parent compound MDMA is N-demethylated to form 3,4 -methylenedioxyamphetamine (MDA) and O-demethylenated to form 3,4-dihydroxymethamphetamine (HHMA) which is further O-methylated to 4-hydroxy-3-methoxymethamphetamine (HMMA). In rats, N-demethylation to MDA is one of the main metabolic pathways, whereas in humans O-demethylenation to HHMA predominates. 3,4-Dihydroxyamphetamine (HHA) and HHMA are the precursors of neurotoxic species (De La Torre*et al*., 2004). The N- demethylation product 3,4-methylenedioxyamphetamine (MDA) which undergoes oxidation by GSH to form 5-(GSyl)-MeDA and might be the main neurotoxic metabolite involved in rats. MDMA-induced neurotoxicity is mainly dopamine - mediated as MDMA causes the release of dopamine, which leads to the generation of reactive oxygen species as a result of dopamine oxidation. In other animal species, including humans, hepatic metabolism is a key factor involved in the production of MDMA toxicity to 5-HT-containing neurons (De La Torre *et al*., 2004).

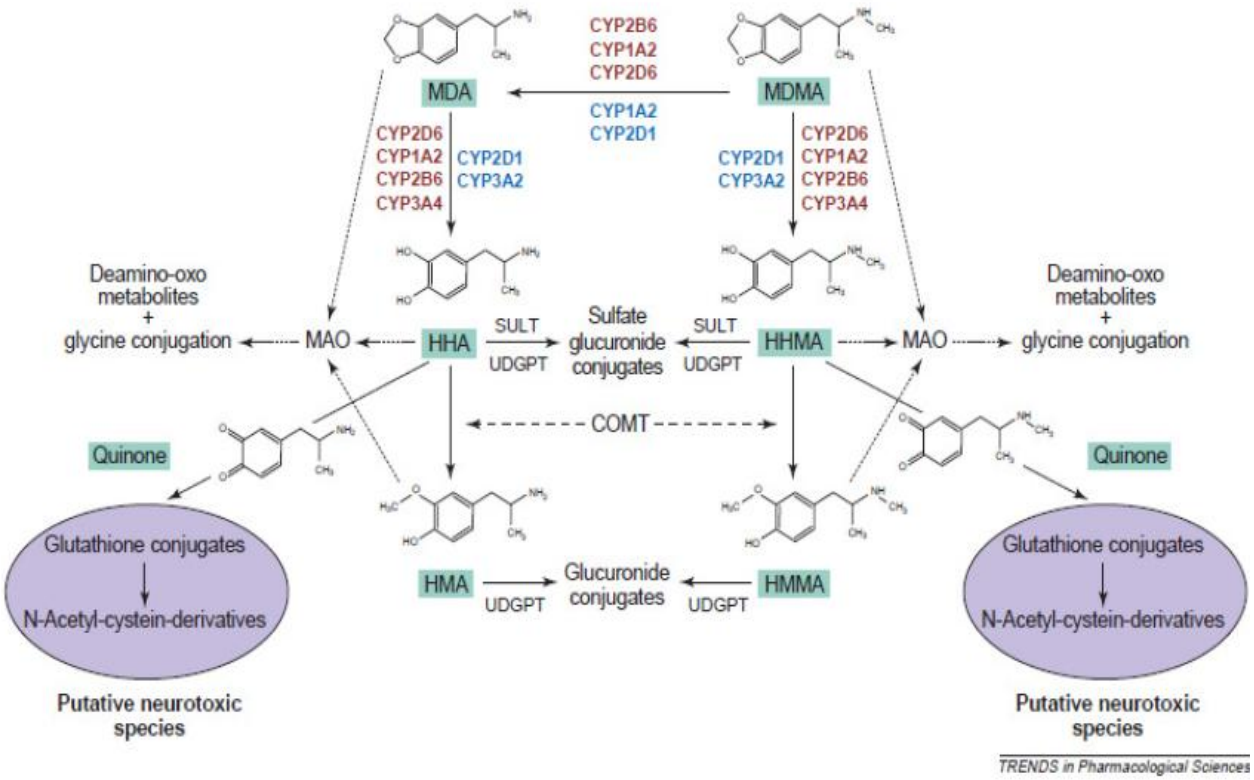


Figure 2.3: Pathways of 3,4-methylenedioxymethamphetamine (MDMA) metabolism in rats and in humans. Isoenzymes of cytochrome P450 (CYP) involved in the N-demethylation and demethylenation metabolic reactions in rats are highlighted in blue whereas those corresponding to enzymes in humans are shown in red (De la Torre *et al*., 2004).

2.6.5 Neuroinflammation

Neuroinflammation is found to be one of the factors involved in MDMA induced neurotoxicity (Zhang *et al*., 2006). Inflammatory response in CNS has been associated with many chronic neurodegerative conditions including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS). Whether neuroinflammation is a cause or a consequence of neurologic disease remains unclear. It is observed that infection, trauma, stroke, toxins and other stimuli may cause an acute neuroinflammatory response which leads to activation of the resident immune cells (microglia) resulting in a phagocytic phenotype and the release of inflammatory mediators such as cytokines and chemokines. This acute neuroinflammatory response may trigger oxidative stress which is short lived and unlikely to be detrimental to long-term neuronal survival. In contrast chronic neuroinflammation includes not only longstanding activation of microglia and subsequent sustained release of inflammatory mediators, but also results in increased oxidative stress. Rather than serving a protective role as does acute neuroinflammation, chronic neuroinflammation is most often detrimental and damaging to nervous tissue. Thus, whether neuroinflammation has beneficial or harmful outcomes in the brain may depend critically on the duration of the inflammatory response. Neuropathological and neuroradiological studies indicate that neuroinflammatory responses may begin prior to significant loss of neuronal populations in the progression of neurodegerative diseases (Frank-Cannon *et al*., 2009).

2.7 Glial Cells

Neuroglial cells of the central nervous system (CNS) include the astrocytes, oligodendrocytes, and microglia, whereas glia in the peripheral nervous system (PNS) is composed of Schwann cells. It is now well established that glial cells represent intimate partners to neurons throughout their lifespan. For example, during neurogenesis and early development, glial cell provide a scaffold for the proper migration of neurons and growth cones, a process mediated via the synthesis and secretion of a variety of growth factors and extracellular matrix components. Glial cells also provide guidance cues for neuronal proliferation and electrical differentiation of neurons. In the adult, glial cells maintain neuronal homeostasis, synaptic plasticity, and repair (Achner *et al*., 1999).

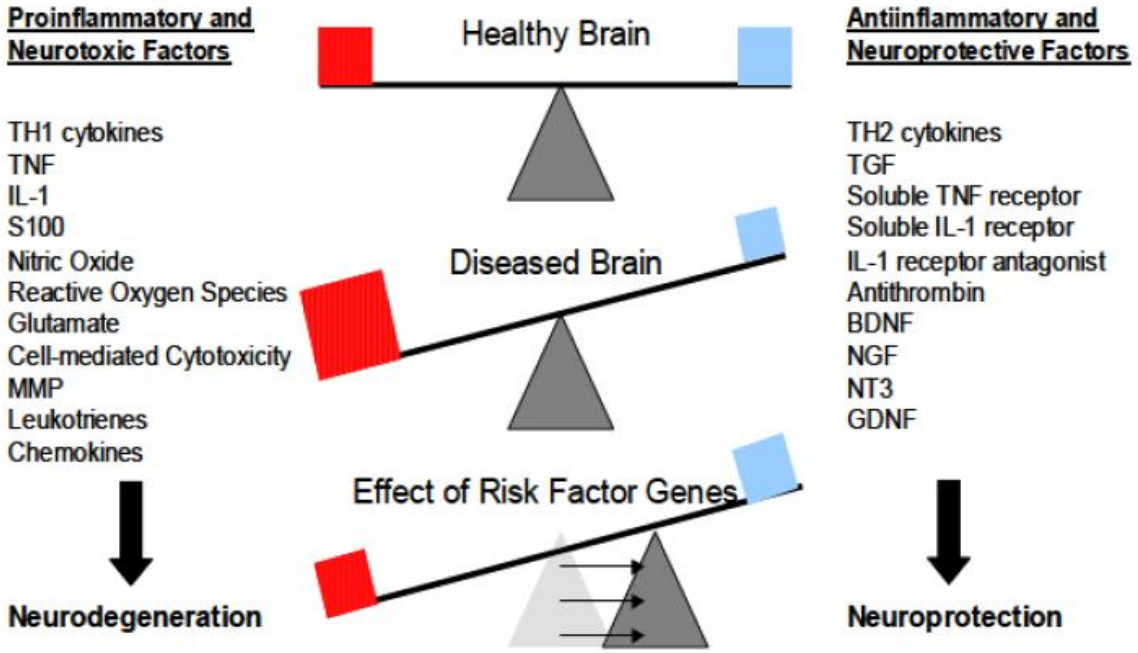


Figure 2.4: Balance of Inflammation. (Abbreviations: Helper T-cell Type I (TH1), Tumor necrosis factor (TNF), Interleukin-1 (IL-1), Matrix Metalloprotease (MMP), Helper T-cell Type II (TH2), Transforming Growth Factor (TGF), Brain Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF), Neurotropic Factor 3 (NT3), and Glial-Derived Neurotrophic Factor (GDNF). (Achner *et al*., 1999)

In the healthy brain glia often respond to stress and insults by transiently upregulating inflammatory processes. These processes are kept in check by other endogenous anti-inflammatory and neuroprotective responses that return the brain to homeostasis. In neurodegenerative disorders, however, pro-inflammatory processes predominate and contribute to the neuronal damage observed. Several examples of proinflammatory and neurodegenerative mediators are given as well as examples of anti-inflammatory and neuroprotective factors (Fig. 2.4).

2.7.1 Microglial cells

Microglia reside in the CNS, comprise approximately 12% of the brain (depending on brain region, health, or pathology), and serve as the brain's immune defense. Microglial cells are the resident macrophages in the central nervous system analogous to the role of macrophages and lymphocytes in the peripheryone (Kettenmann *et al.*, 2011). Role of microglia is to act as the brain's immune defense against disease and injury. Resting ramified, microglia cell bodies are spaced throughout the CNS to avoid cell body overlap, but have been shown to be present with variable density in different brain regions. In the developing brain and in areas of remodeling, microglia are responsible for the phagocytosis of cellular debris resulting from apoptosis and normal cell death. Microglia have been implicated as the "brain's electricians, in which the release of neurotrophic factors and anti-inflammatory cytokines from microglia has been shown to promote synaptic plasticity. In fact, the majority of microglial functions are beneficial and necessary for a healthy CNS, as activated microglia are critical for CNS wound healing. In addition, microglias have also been shown to release anti-inflammatory and trophic molecules to enhance the survival of surrounding neurons. In contrast microglia is a

predominant source of proinflammatory factors [TNF-(tumour necrosis factor), PGE_2 (prostaglandin E_2) and IFN- (interferon)] and oxidative stress which are toxic to neurons. Although microglia is necessary for normal function, microglia when activated by an extensive list of pro-inflammatory stimuli, such as lipopolysaccharide (LPS), and even neuron damage, can result in disastrous neurotoxic consequences. Any pathologic event in the brain leads to the activation of microglia, and they can communicate with macroglial cells and are considered the most susceptible sensors of brain pathology (Kettenmann *et al.*, 2011; 2013). Microglia also has been implicated to play both causative and exacerbating roles in neurodegerative diseases. Microglial and astroglial activation plays an important role in neurotoxicity andrepresent an early step in MDMA-induced neurotoxicity (Thomas *et al*., 2004; Zhang *et al*., 2006). Microglia, the immunocompetent cells of the central nervous system, react to chemical and structural changes in their environment with morphological and biochemical changes, switching from a silent ramified state to an active phagocytosing macrophage-like phenotype (Stoll and Jander 1999). Most neurological disorders involve activation and possibly, dysregulation of microglia. Microglia have the ability to proliferate, phagocytose apoptotic cells and migrate long distances toward their final destinations throughout all CNS regions, where they acquire a mature ramified morphological phenotype (Marín-Teva *et al.*, 2011). This morphological change is also accompanied by changes in signaling and gene expression that can result in changes in surface receptor expression, the release of pro- or anti-inflammatory factors, recruitment molecules, and ROS, among others. In the normal brain microglia were considered "resting," but it has recently become evident that they constantly scan the brain environment and contact synapses.

Activated microglia can remove damaged cells as well as dysfunctional synapses, a process termed "synaptic stripping" (Kettenmann *et al.*, 2013). Microglial dysfunction results in behavioral deficits, indicating that microglia are essential for proper brain function. This defines a new role for microglia beyond being a mere pathologic sensor (Kettenmann *et al.*, 2013).

2.7.2 Astroglia

Neurotoxicity induced by MDMA is cumulative and is related to the dose and frequency of its use (McKenna and Peroutka, 1990; Kalant, 2001). Neurotoxicity in heavy ecstasy users has been revealed by neuroimaging studies showing reduced SERT binding and increased 5-HT_{2A} receptor binding in several cortical and/or subcortical areas (Jerrold 2013). Neuroglial cells of the central nervous system (CNS) include the astrocytes, oligodendrocytes and microglia. It is well understood that drugs of abuse lead to plastic changes in synapses and that these long-term modifications have the potential to underlie adaptive changes of the brain. The changes lead to substance abuse and glial cells have been found to play some roles that are associated with synapses. In response to noxious stimuli to the CNS, astrocytes undergo a process of proliferation and morphological change (hypertrophy of cell bodies, thickening and elongation of astrocytic processes) and increase in the expression of glial fibrillary acidic protein. This process, which is termed astrogliosis, is associated with enhanced release of growth factors and neurotrophins that support neuronal growth, but might also lead to the formation of neuronal scars (Liberto and Albercht, 2004). Besides their important role in normal brain physiology, astrocytes are involved in pathological processes and in the etiology of neurological diseases such as Alzheimer disease and Parkinson’s disease (Halliday and

Stevens, 2011; Parpura *et al*., 2012) where the expression of the astrocyte-specific protein, glial Fibrillery acidic protein (GFAP) is known to increase (Mille, 2005). Exposure to drugs of abuse leads to reactive astrocytosis and altered GFAP expression (Sung and Zhan, 2001; Bowers and Kalivas, 2003; Narita *et al.*, 2006b).

In the brain, astrocytes fulfill a range of important and essential functions (Parpura *et al.*, 2012; Sofroniew and Vinters, 2010). These include the extracellular ion homeostasis, metabolic supply to neurons, the maintenance of the blood–brain barrier (BBB) as well as the modulation of synaptic transmission and synaptic plasticity (Parpura *et al.*, 2012; Sofroniew and Vinters, 2010). Astrocytes have a strategically important location in the brain, being in close contact to both neurons and to endothelial cells of brain capillaries.

They cover almost completely the brain capillaries (Mathiisen *et al*., 2010). Astrocytes are one of the two primary types of macroglia. They comprise nearly 35% of the total CNS cell population and like microglia are found in all regions of the CNS. Histologically, astrocytes can be visualized by immunolabeling with antisera specific for glial fibrillary acidic protein (GFAP), S100b or the astrocyte specific glutamate transporters, GLT1 and GLAST (Carson *et al*., 2006). In the healthy, uninjured CNS, astrocytes perform numerous functions absolutely essential for neuronal function. In case of injury, astrocytes can phagocytose injured cells after which they replace them and form a glial scar. They are slow to react to injury, but stay activated during the late recovery stage (Gehrmann *et al*., 1995). Astrocytes produce several growth factors and regulate the induction and maintenance of neurite outgrowth, axonal guidance and synapse formation (Gee and Keller, 2005). Their most amazing function is probably their role in the tripartite synapse, which consist of the presynaptic neuron, the postsynaptic

neuron and the astrocyte itself. Astrocytes act as a physical link between the pre- and postsynaptic neurons on one side and blood capillaries on the other, which is important for maintaining the external environment for optimal functioning of the neurons. Astrocyte interactions with the cerebrovasculature endothelium play a key role in the induction and maintenance of the tight junction's characteristic of the intact BBB and can influence BBB permeability when needed (Carson *et al*., 2006). Astrocytes detect synaptic activity by binding of neurotransmitters to receptors on the astrocyticmembrane. By secreting vasoactive substances, astrocytes can regulate the blood flow in reaction to varying levels of neuronal activity, since an active brain region requires more oxygen and energy (Parri and Crunelli, 2003; Benarroch, 2005). A large body of evidence indicates that astrocytes are involved in the control of glutamate homeostasis and susceptibility of the brain to excitotoxic injury. Glutamate transporters are expressed in many different types of brain cells, but astrocytes are primarily responsible for glutamate uptake. After uptake of glutamate into astrocytes, the enzyme glutamine synthetase converts glutamate into glutamine, which is then transported intoneurons where it is converted back into glutamate (Boison *et al*., 2010). Besides terminating the action of glutamate, astrocytes are also responsible for the clearance of aminobutyric acid (GABA) and glycine, which both are inhibitory neurotransmitters. However, maintaining synapses and uptake of neurotransmitter and thereby terminating the presynaptic signals are not the only functions of the astrocyte in the tripartite synapse. Astrocytes have G-protein coupled receptors that bind neurotransmitters, ATP or adenosine. This enables them to monitor the activity of neurons directly (Fields and Stevens-Graham 2002). Activation of these G-protein coupled receptor results in an intracellular [Ca^{2+}] rise in the astrocyte causing the

release of chemical transmitters, including several neurotransmitters, ATP and D-serine (Fields and Stevens-Graham 2002; Halassa *et al*., 2006). The secreted chemical transmitters are also called gliotransmitters and they give feedback to neurons, influencing neuronal excitability and synaptic strength. Different responses might be elicited by secreting different types of gliotransmitters, depending on the characteristics of the [Ca^{2+}] response evoked. One of these gliotransmitters is ATP, which can bind the presynaptic neuron thereby modulating synaptic transmission. ATP also binds receptors on neighbouringastrocytes, inducing inositol triphosphate (IP3) formation that subsequently triggers the release of Ca^{2+} stored in the endoplasmatic reticulum (ER). This way, astrocytes are capable of communicating with each other. Astrocytes can be a significant source of extracellular glutamate, which can be released by a variety of mechanisms. It has been demonstrated that Ca^{2+} elevations in astrocytes induce excitotoxic release of glutamate from these cells. It was shown that astrocytederived glutamate targets synaptic N-methyl-D-aspartic acid (NMDA) receptors providing a rationale explanation for the astrocyte-based control of neurotoxicity (Boison *et al*., 2010).

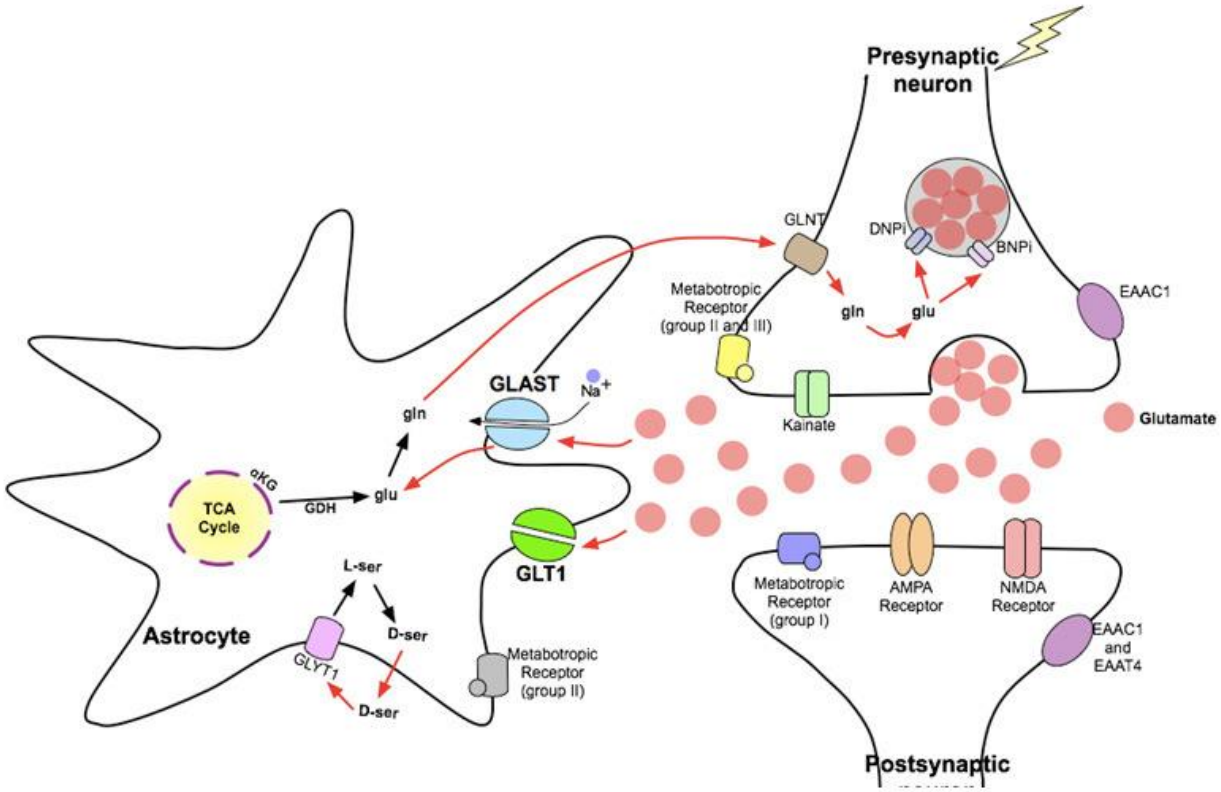


Figure 2.5: Glutamate transporters in astrocytes are responsible for synaptic glutamate homeostasis (Liu *et al*., 2008).

2.8 Cognition

Cognition is a global term that describes all the mental activities that are engaged in our thoughts (Carlson, 1981; Claxton, 2002). MDMA has been frequently associated with cognitive impairment. Several studies have shown that abstinent ecstasy users display cognitive deficits, such as verbal, spatial and working memory deficits (Cole and Sumnall, 2003; Parrott, 2001). MDMA has been found to have effect on the developing rodent brain (Dzietko *et al.*, 2010). The children of mothers who take Ecstasy/MDMA during pregnancy have developmental problems (Parrott, 2013). MDMA is associated with changes in learning, memory, attention, executive function, mood, and decision making (Montegomery and Fisk, 2008). MDMA exposure has been found to have adverse effects on the developing brain and behavior (Mathew *et al.*, 2008). The primary effect of this drug is to alter perception, cognition, or mood (Check, 2004).

Declarative memory, prospective memory, and higher cognitive skills are often impaired (Parrot, 2013). Repeated or high-dose MDMA use has been associated with tolerance, depressive symptomatology, and persisting cognitive deficits, particularly in memory tests (Meyer 2013, Jerrold 2013). The hippocampus, and cerebral cortex found to suffer 5-HT damage due to Ecstasy use are strongly associated with memory function (Parrott, 2000). MDMA users produce impaired delayed recall and in encoding information into long-term memory (McCardle *et al*., 2004) and retrieving learnt information from long-term memory (Fox *et al*., 2001). One area of the brain affected by MDMA exposure is the forebrain, including the frontal cortex and this brain region is thought to be important in executive functioning (Heffernan *et al*., 2001) which involves controlling many higher cognitive functions such as monitoring and updating working memory, inhibition, task-

shifting, planning, concept formation and cognitive flexibility (Roesch-Ely *et al*., 2005; Dafters, 2006).

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

3.1.1 Drugs and Chemicals

The following drugs and chemicals were used for the experiment:

i. Methylenedioxymethamphetamine (MDMA) from Sigma–Aldrich, Milan, Italy.

ii. Momoclonal mouse anti-GFAP (1:400) sigma- Aldrich

iii. Momoclonal rat anti CD11b (1:10000) Serotec, United Kingdom.

iv. Ethanol Sigma–Aldrich, Milan, Italy.

v. Sodium azide Sigma–Aldrich, Milan, Italy.

vi. Hydrogen peroxide Sigma–Aldrich, Milan, Italy.

vii. Chlorhydrate Sigma–Aldrich, Milan, Italy.

viii. Formaldehyde Sigma–Aldrich, Milan, Italy.

3.1.2 Instruments and Devices

i. The following instruments and devices were used for the experiment.

ii. Portable digital rectal thermometer (CHY 502A)

iii. Syringe and needle

iv. PixeLink camera Software (National Institute of Health) Software

v. Vibratome

vi. Scion image software (National Institute of Health)

vii. Atlas of Paxinos and Watson (1998)

viii. Y-maze Apparatus (3.3.5)

ix. Novel Object Recognition Apparatus (3.3.6)

3.1.3 Animals

Twenty (20) male C57BL/6J mice, 12 Weeks old and eighty (80) male Wistar rats, 3 months old (Charles River, Milan, Italy) were used for the study. They were given food and water *ad libitum.* The animals were allowed to acclimate to the environment for 1 week after arrival before being handled daily for 3 min by the experimenter over three consecutive days, then allowed to acclimate to the room over an additional 3 days.

3.2 Experimental Site

The study was carried out in Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria. Zaria is located between latitudes 11° and 3°N, and between 7° and 42° E, at an altitude of 670 m above the sea level and 664 km away from the sea, in the Northern Guinea Savannah zone. The average rainfall in Zaria is approximately 1000 mm, mainly during the month of March to October. The maximum ambient temperature range in Zaria is 27-35°C with a dry and wet season (Marthins, 2006).

3.3 Methodology

3.3.1 Drug preparation

MDMA was dissolved in saline and the amount calibrated and injected intraperotoneally (i.p.)

3.3.2 Temperature measurements

Twenty (20) mice were grouped into four (4) groups of five each (5) and received repeated administration of vehicle or MDMA (4 x 20 mg/kg). All administrations were done intraperitoneally (i.p.). at 2 hour intervals. Groups 1a and 1b made up of 5 mice each served as the control and were given normal saline and kept at an environmental temperature of 21°C and 27°C. Group 2a and 2b made up of 5 mice each were kept at an environmental temperature of 21°C and 27°C respectively and administered MDMA (4x 20 mg/kg), (Thomas *et al.,* 2004). Core body temperature was measured using a portable digital rectal thermometer (CHY 502A) with an accuracy of 0.1°C each hour after each vehicle or MDMA administration. Mice were sacrificed 48 hrs after the last administration of MDMA for immunohistochemistry processing (Granado *et al.,* 2008).

3.3.3 Immunohistochemistry for markers of astroglia and microglia

3.3.4 Immunohistochemistry

Animals were anaesthetized with chloral hydrate (450 mg/kg, i.p.) and perfused with 4% paraformaldehyde in phosphate buffer 0.1 M (pH 7.4). Brains were isolated and kept in fixing solution for 2 hrs and later in PBS plus sodium azide. Coronal sections (50 μm thick) of mice brain were cut on a vibratome and immunostained for glial fibrillary acidic protein (GFAP) and CD11b in order to analyze astroglial and microglial activation respectively. Immunohistochemistry was carried out in free-floating sections with

standard avidin–biotin (ABC; Vector Laboratories, UK) immunohistochemical protocols. Sections were treated with hydrogen peroxide to block endogenous peroxidases and then incubated overnight with the following specific primary antisera: monoclonal mouse anti-GFAP (1:400; Sigma–Aldrich) and monoclonal rat anti-CD11b (1:1000; Serotec, UK). 3,3 – diaminobenzidine (DAB) was used as a chromogen for visualization . After getting colour, these sections were mounted on chromealum-gelatine coated slides dried and dehydrated with ethanol respectively.

3.3.5 Image Analysis

Images were captured under constant light conditions using a PixeLink PL-A686 camera at 20 x magnification for the striatum. For each animal, three sections from striatum (A = 1.10, 0.74, 0.38 mm from bregma), were analyzed for each protein evaluated in the study. Quantification of GFAP immunoreactivity in the striatum, one dorsolateral and one ventromedial portion were analyzed from both the left and right parts using PixeLink image analysis software. Analysis of CD11b immunoreactivity in the striatum was done with the analysis software SCION Image (Scion Corporation, Frederick, MD, USA). Within each frame, the area occupied by gray values above the threshold was automatically calculated. In order to obtain averages for GFAP and CD11b, values were normalized with respect to vehicle.

3.3.5 Assessment of short-term spatial memory using Y-maze

The spatial memory task (Vermeeren *et al*., 1995) assesses short-term memory for spatial information. Forty Wistar rats were used and grouped into four groups of ten (10) each. Group 1a and 1b made up of ten (10) Wistar rats each, served as the control and were given normal saline and kept at an environmental temperature of $21^{o}C$ and $27^{o}C$

respectively. Groups 2a and 2b made up of ten (10) rats each were kept at an environmental temperature of 21°C and 27°C respectively and administered MDMA (10 mg/kg). All administrations were i.p. 24 hrs apart over four consecutive days.

Evaluation of spontaneous alternation behaviour in a Y-maze is commonly employed to investigate short-term spatial memory in rodents (Maurice *et al*., 1994; Yamada *et al*., 1996). The apparatus was made of black PVC, consisting in three equal arms (length 50 cm, width 20cm, height 35cm), named A, B and C. Arms converged onto a central triangular area and the floor of the maze was covered in sawdust, which was changed in between rat tests. Rats were placed in the central area and left free to explore the whole apparatus for a single 8 minute trial during which their performance was videotaped. A rat was considered inside a specific arm when it had all its four paws inside that arm. Percentage of spontaneous alternation was calculated on the basis of the sequence of arm entries as reported by Yamada *et al*. (1996). An alternation was defined as entries into all three arms on consecutive occasions (i.e., ABC, ACB, CAB, etc.). The number of maximum alternations is the total number of arm entries minus two and the percentage of alternation was calculated as (actual alternations/maximum alternations) x 100. For example, if the rat performed ABCACBACCAB, the number of arm entries would be 11, and the successive alternations: ABC, BCA, ACB, CBA, BAC, CAB. Therefore, the percentage of alternation would be given as:

$[6/(11)]x100 = 66.7.$

3.3.6 Assessment of non-spatial working memory using NOR

The novel object recognition (aka novel object preference) task capitalizes on the findings of Berlyne (1950), where it was found that rats prefer to explore objects that they have not previously encountered over objects that are familiar. Measurement of novel object recognition is widely used for evaluating non-spatial working memory in rodents (Ennaceur and Delacour, 1988) not involving the use of primary reinforcement (e.g., food, shock), and comparable to similar procedures employed in human and non-human primate subjects (Ennaceur, 2010). Preferences to explore the various objects are noted, and a tendency to explore the novel object over the familiar sample is interpreted as evidence of memory for the training exposure (Ennaceur and Delacour, 1988; Gaskin *et al*., 2010). Object recognition experiments were performed in a black wooden box (length 60 cm, width 40 cm, height 30 cm) with the floor covered in sawdust. Objects to be discriminated were made of plastic, deferring in shape and colour. Objects had no genuine significance and had not been previously associated to rewarding or aversive stimuli. Two days before the test, rats were allowed to explore the box twice for 5 minute, in order to acclimatize. Forty Wistar rats were used and grouped into four groups of ten (10) each. Group 1a and 1b made up of ten (10) Wistar rats each served as the control and were given normal saline and kept at an environmental temperature of 21°C and 27°C respectively. Groups 2a and 2b made up of ten (10) rats each were kept at an environmental temperature of 21°C and 27°C respectively and administered MDMA (10mg/kg). All administrations were i.p. 24 hrs apart over four consecutive days.

On the testing day each rat was placed in the box for two 4 min sessions and left to explore objects freely. During the first session (S1), two copies of the same object were

present, whereas in the second session (S2), rats were exposed to a copy of the objects presented previously in S1 plus a novel object. S1 and S2 were separated by a 15 min interval. The 15-min interval was chosen because this delay was found to be sensitive to MDMA treatment (Morley *et al*., 2001). Exploration was defined as the rats sniffing, gnawing or touching the object with the nose, whereas sitting and/or turning around the object were not considered as exploratory behaviours. To avoid the presence of olfactory cues, objects were thoroughly cleaned after each session. Moreover, the combination of objects (novel vs. old) and their respective position (right vs. left) were counter balanced to prevent biased preferences for particular objects or positions. The performance of the rats was videotaped and the following parameters were evaluated: (a) time spent by the rats in exploring the objects during either S1 or S2, and (b) novel object recognition. The latter was calculated as the percentage of time spent in exploring the new object with respect to the total amount of time spent in exploring the two objects during S2 (c) The discrimination-ratio is the duration of exploration of the novel object divided by the total exploration duration of both objects during the test phase. A discrimination ratio equal to 0.5 indicates chance behavior, with scores above 0.5 indicating preference for the novel object and therefore, memory of the familiar object.

3.4 Data Analysis

All values were expressed as mean ± SEM and statistically analyzed with two-way ANOVA followed by the Newman-Keuls, post hoc test using Graph Pad Prism version 4.0 and Student t-test using SPSS. Values of $p < 0.05$ were considered significant.

CHAPTER FOUR

4.0 Results

4.1 Role of Ambient Temperature Variation on MDMA-Induced Hyperthermia

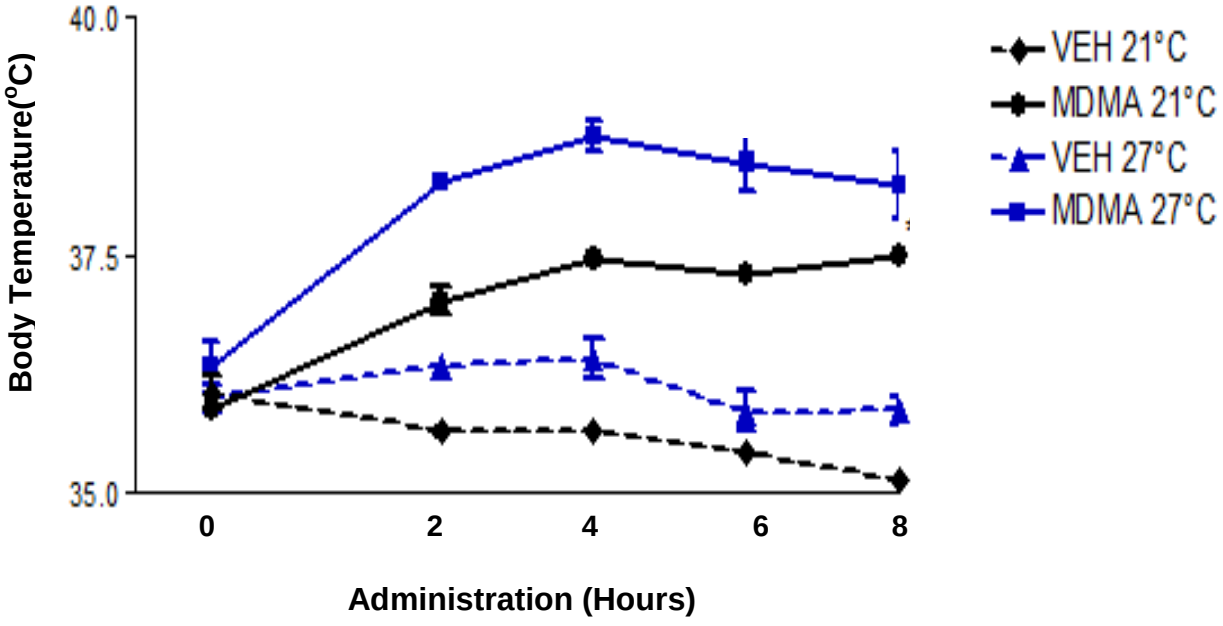


Figure 4.1: Effect of repeated administration of MDMA (4 x 20mg/kg, i.p.) on MDMA induced hyperthermia exposed to 21°C and 27°C

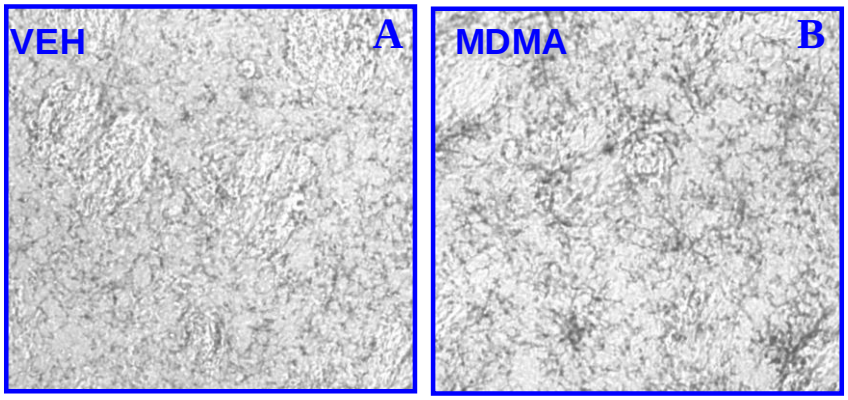
Body temperature was recorded in MDMA and vehicle-treated mice at 21°C and 27°C respectively (Fig 4.1). Body temperature of MDMA-treated mice was significantly higher ($p<0.05$) than mice treated with vehicle at the 21°C. Similarly, body temperature of MDMA-treated mice was significantly higher ($p<0.05$) than mice treated with vehicle at the 27°C. The body temperature of MDMA-treated mice at 27°C was significantly higher ($p<0.05$) than mice treated at 21°C.

4.2 Inflammatory Response in the Striatum

4.2.1 CD11b immunoreactivity in the striatum

Microglia activation of MDMA and vehicle-treated mice were analyzed through CD11b

At 21°C MDMA-treated mice showed an increase in CD11b immunoreactivity in the striatum compared with vehicle treated-mice (Plate 4.1). Similarly, at 27°C MDMA-treated mice showed a higher microglia reactivity than the vehicle treated-mice (Plate 4.2). Mice treated mice at 27°C showed a significantly ($p<0.05$) higher CD 11b immunoreactivity compared with mice treated at 21°C (Fig. 4.2).



21 °C

Temperature

Plate 4.1: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker at 21°C and 3,3-diaminobenzidine (DAB) as a chromogen for visualization (x 20)
At 21°C, MDMA-treated mice **(B)** showed an increase in CD 11b immunoreactivity in the striatum compared with vehicle-treated mice **(A)**

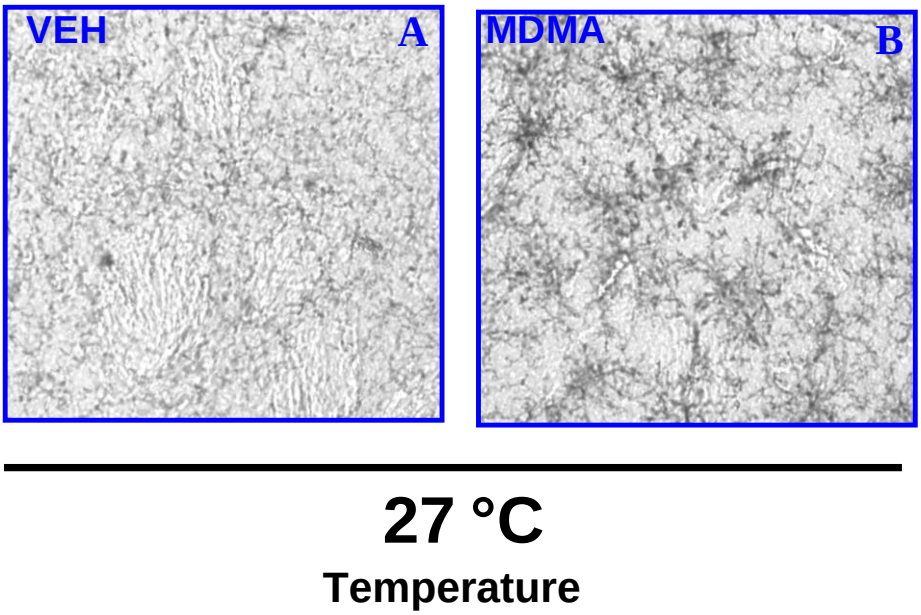


Plate 4.2: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker at 27°C and 3,3-diaminobenzidine (DAB) as a chromogen for visualization (x 20).
At 27°C, MDMA **(B)** induced a higher CD11b immunoreactivity in the striatum compared with vehicle treated mice **(A)**

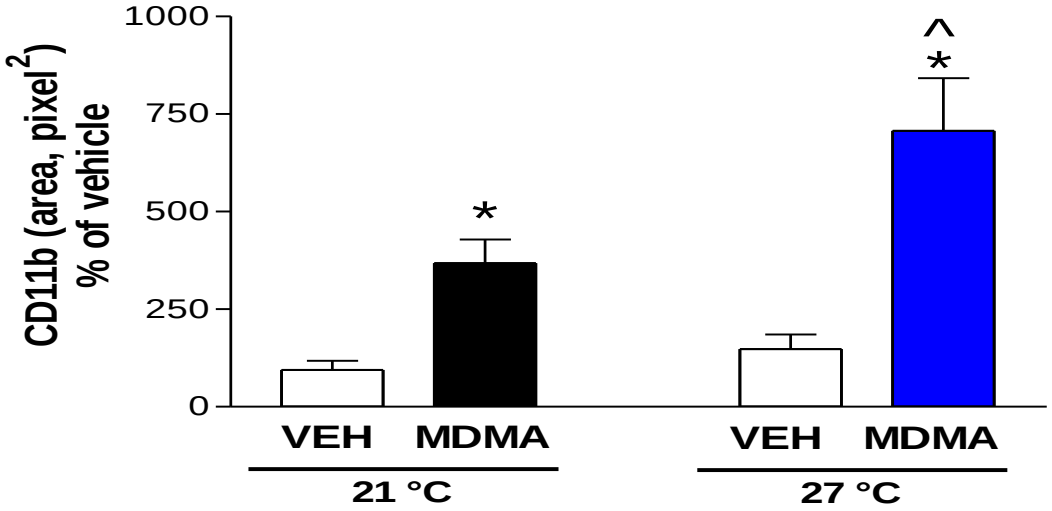


Figure 4.2: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker at 21°C and 27°C Histogram from the striatum immunostained for CD11b showing significantly higher CD 11b immunoreactivity at 27°C compared with mice treated at 21°C

*$p < 0.05$ compared with vehicle-treated mice,
^$p < 0.05$ compared with MDMA-treated mice at 21°C

4.2.2 GFAP Immunoreactivity in the striatum

Astroglia activation of MDMA-treated and vehicle-treated mice were analyzed through GFAP. At 21°C, MDMA induced a higher GFAP immunoreactivity in the striatum compared with vehicle treated mice (Plate 4.3). Similarly, at 27°C, MDMA-treated mice showed a higher GFAP positive cells compared with vehicle treated mice (Plate 4.4). More MDMA-treated mice at 21°C showed a significantly ($p<0.05$) higher GFAP immunoreactivity in the striatum compared with mice treated at 21°C (Fig. 4.3).

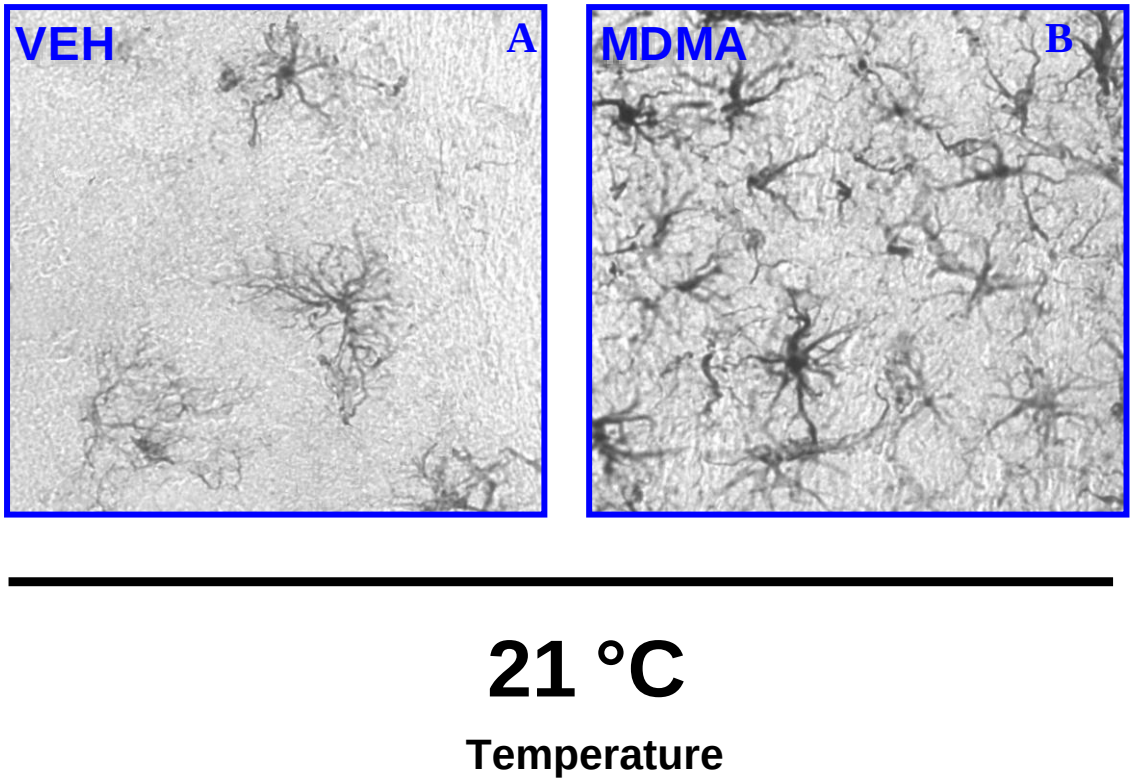


Plate 4.3: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker at 21°C and 3,3-diaminobenzidine (DAB) as a chromogen for visualization (x 20)
At 21°C, MDMA **(B)** induced a higher GFAP immunoreactivity in the striatum compared with vehicle treated mice **(A)**

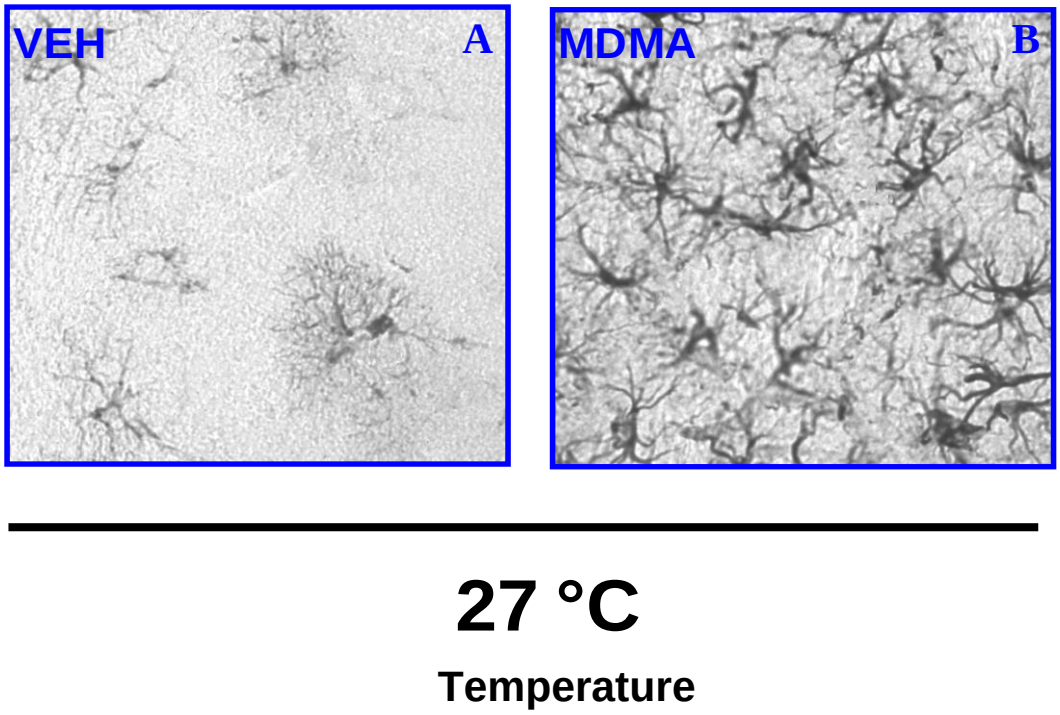


Plate 4.4: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker at 27°C and 3,3-diaminobenzidine (DAB) as a chromogen for visualization (x 20)
At 27°C, MDMA-treated mice **(B)** showed a higher GFAP positive cells compared with vehicle treated mice **(A)**

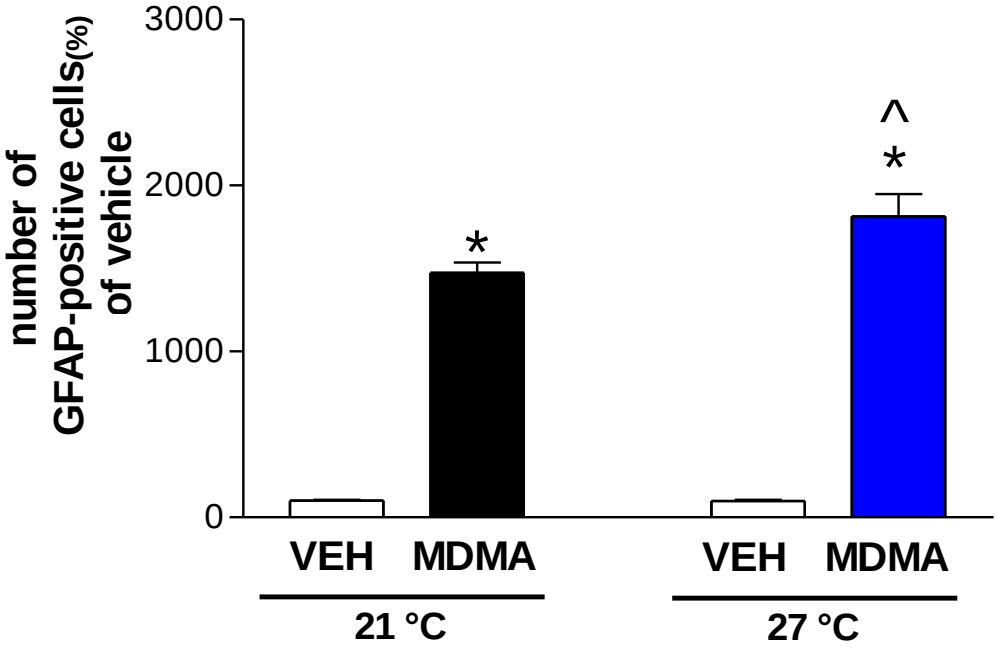


Figure 4.3: Effect of repeated administration of MDMA (20 mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker at 21°C and 27°C. Histogram from the striatum immunostained for GFAP showing significant immunoreactivity at 27°C as compared to 21°C

*$p < 0.05$ compared with vehicle-treated mice,
^$p < 0.05$ compared with MDMA-treated mice at 21°C

4.3 Effect of MDMA on Cognition

4.3.1 Effect of MDMA on spatial memory

At 21°C there was a significant impairemant in spatial memory in MDMA-treated rats as compared to the vehicle treated ($p<0.05$) (Fig. 4.4). Similarly, at 27°C there was a significant impairemant in spatial memory in MDMA-treated rats as compared to the vehicle treated ($p< 0.05$) (Fig. 4.5). At 27°C there was an impairement in sapatial memory in MDMA-treated rat as compared to MDMA treated rat at 21°C (Fig. 4.6)

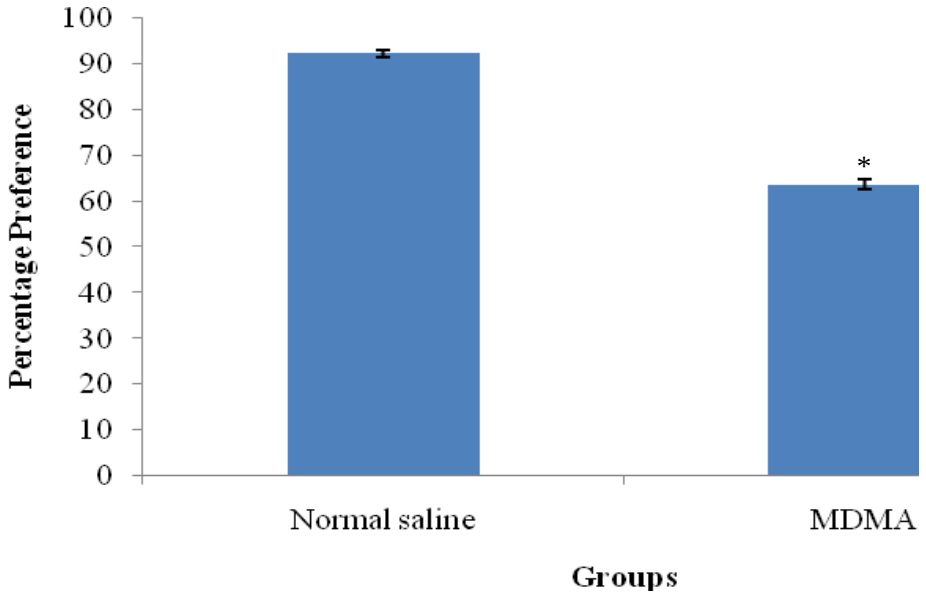


Figure 4.4: Effect of MDMA (10 mg/kg) and normal saline on spatial memory at 21°C There was a significant impairemant in spatial memory in MDMA-treated rats as compared to the vehicle treated

*$p < 0.05$ compared with normal saline-treated rats at 21°C

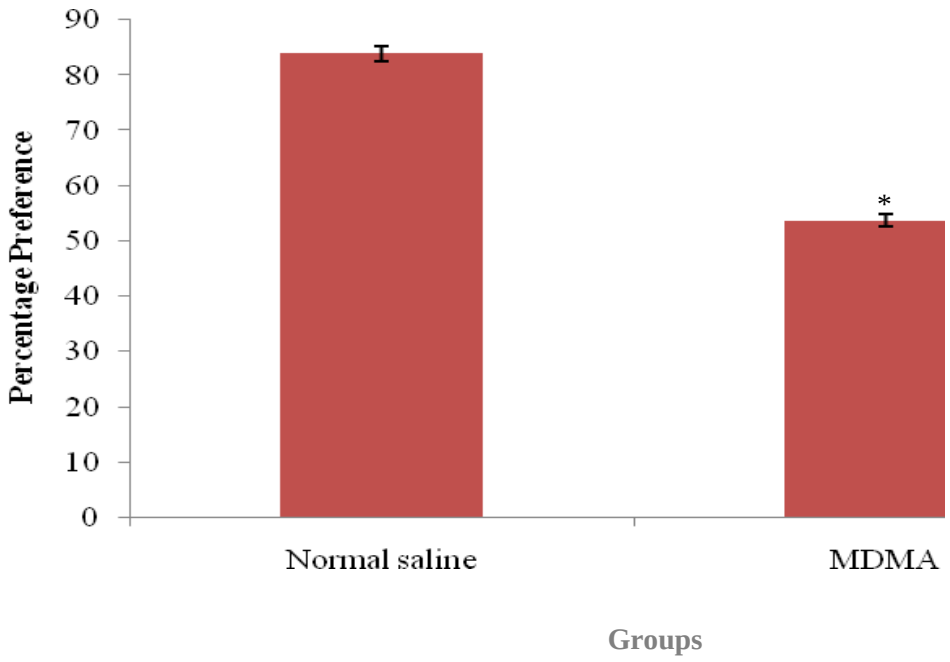


Figure 4.5: Effect of MDMA (10 mg/kg) and normal saline on spatial memory at 27°C There was a significant impairemant in spatial memory in MDMA-treated rats as compared to the vehicle treated

*p<0.05 compared with normal saline-treated rats 27°C MDMA-treated rats at 27°C

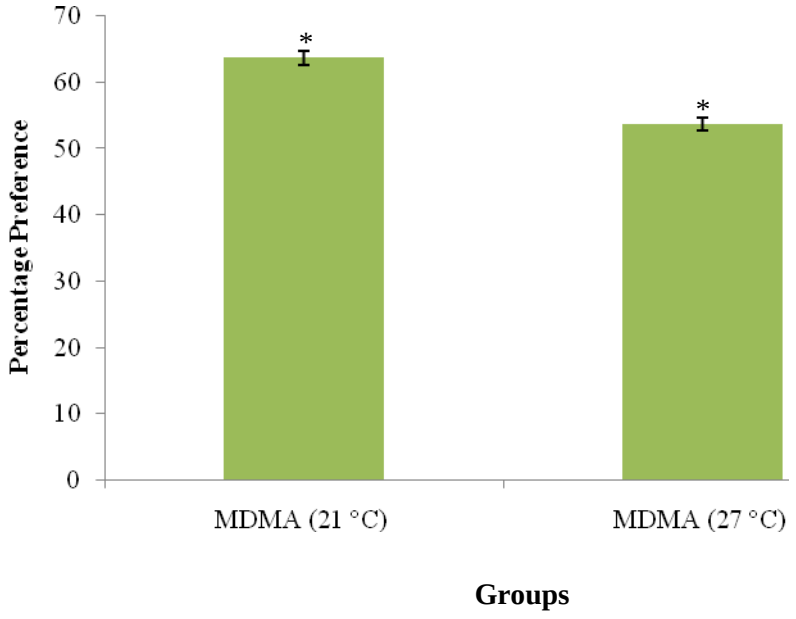


Figure 4.6: Effect of MDMA (10 mg/kg) on Spatial memory at 21°C and 27°C
At 27°C there was an impairement in sapatial memory in MDMA-treated rat as compared to MDMA treated rat at 21°C

*p<0.05 compared with MDMA-treated rats 21°C and 27°C

4.3.2 Effect of MDMA on non- spatial memory

At 21°C and 27°C vehicle treated rats showes higher significant ($p<0.001$) discrimination ratio as compared to MDMA treated rats respectively (Fig. 4.7, 4.8) MDMA treated rats at 21°C showed a significant ($p<0.001$) discrimination ratio than rats treated at 27°C (Fig 4.9).

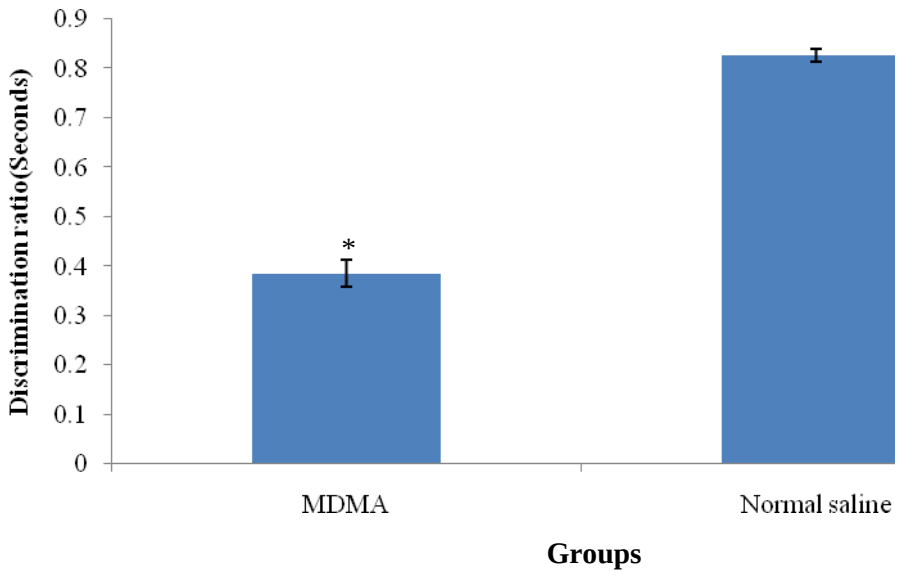


Figure 4.7: Discrimination ratio of novel object recognition task of rats treated with MDMA (10 mg/kg) and vehicle at 21°C

*$p<0.001$ compared with normal saline-treated rats 21°C

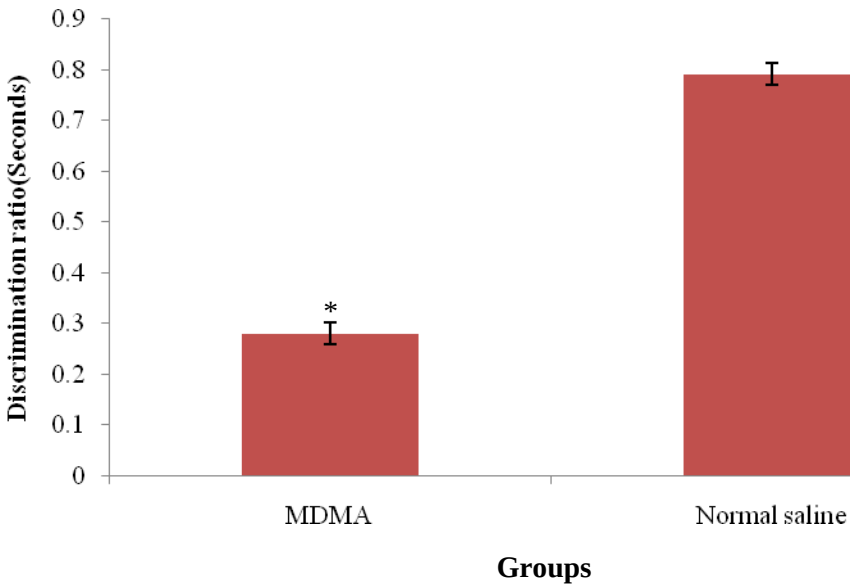


Figure 4.8: Discrimination ratio of novel object recognition task of rats treated with MDMA (10 mg/kg) and vehicle at 27°C

*p<0.001 compared with normal saline-treated rats 27°C

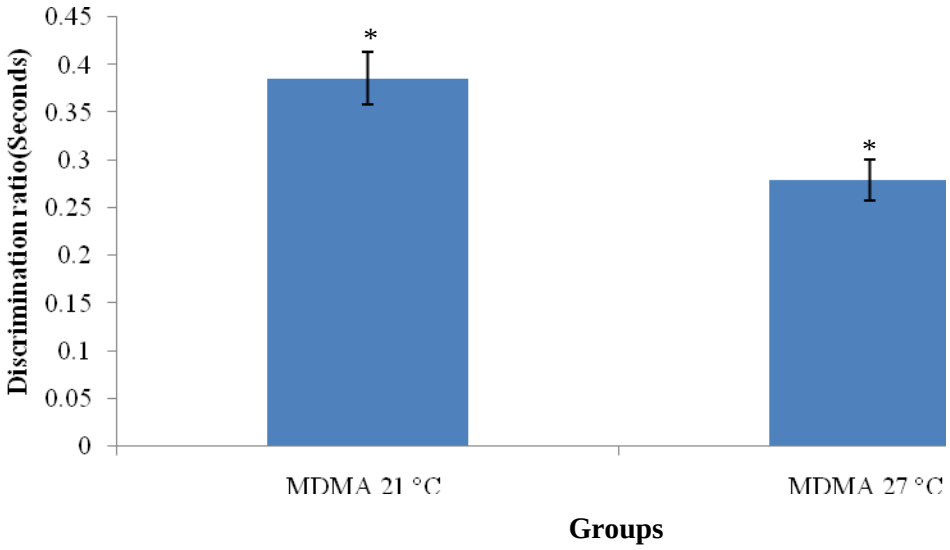


Figure 4.9: Discrimination ratio of novel object recognition task of rats treated with MDMA (10 mg/kg) and vehicle at 21°C and 27°C

*$p<0.05$ compared with MDMA-treated rats 21°C and 27°C

CHAPTER FIVE

5.0 Discussion

5.1 Effect of ambient temperature on MDMA-induced hyperthermia

Methylenedioxymethamphetamine dosing regimen in this experiment was designed to be clinically relevant for typical human recreational use patterns. The result showed MDMA-induced hyperthermia is temperature dependent relative to the dose. This finding is in contrast with that of Green *et al.* (2004), who reported that MDMA induces dose-dependent hyperthermia in experimental animals. However, the result is in line with Green *et al*. (2004) that the hyperthermia is influenced by ambient temperature and other housing conditions. These varied outcomes are actually in line with earlier research showing that the direction of core temperature response (hyperthermia or hypothermia) is dependent on the ambient temperature (Gordon *et al*., 1991). According to (Piper, 2006) the hyperthermic response to MDMA is both dose and age dependent. Reveron *et al*. (2005) determined that young-adult mice exhibited a significantly greater hyperthermia following MDMA. Both the dose administered and strain of mouse appears to influence the size and direction of response detected. Several groups have examined the response on temperature of female C57BL/6J mice after administration of MDMA (20 mg/kg s.c., 4 times, every 2 hr) and found that MDMA causes an elevation of body temperature (Johnson *et al*., 2000, 2002b; Miller and O'Callaghan, 1994). In contrast, the same laboratory (Johnson *et al*., 2002a) using male BALB/c mice and lower doses of MDMA (5 and 10 mg/kg s.c. every 2 hrs for 4 doses) observed a dose-dependent hypothermic response that was still evident 24 hr after administration of the higher dose studied. Carvalho *et al*. (2002) measured the subcutaneous temperature of male Charles River

mice and reported that a single administration of MDMA (5, 10, and 20 mg/kg i.p.) produced an increase in body temperature that reached its maximum (2°C) at approximately 30 min and remained elevated for more than 4 hrs. Using Swiss-Webster mice, O'Shea *et al*. (2001) reported that repeated administration of MDMA (3 times at 3 hr intervals i.p.) altered the body temperature biphasically in such a way that hypothermia was the predominant effect following MDMA at the dose of 10 mg/kg, while a higher dose (30 mg/kg) induced hyperthermia followed by hypothermia. In contrast, the same group using male NIH/Swiss mice and given a similar protocol of MDMA (20–25 mg/kg i.p., 3 times at 3 hrs intervals) observed a pronounced hyperthermic response immediately after each injection lasting over 2 hrs. The magnitude of hyperthermic response being more pronounced after the first and second injection (Colado *et al*., 2001). There are many published reports on the fact that administration of single or multiple doses of MDMA to rats results in a long-term depletion of 5-HT and 5-HIAA (Laverty and Logan, 1990; McKenna and Peroutka, 1990; Nash and Yamamoto, 1992; Colado *et al*., 1993; Farfel and Seiden, 1995; Malberg *et al*., 1996; O'Shea *et al*., 1998; Shankaran and Gudelsky, 1998; Wallace *et al*., 2001). One factor that has to be borne in mind in evaluating these reports is that different strains of rats have been used by different investigators and the strains have different sensitivities to both the acute (Malpass *et al*., 1999) and long-term neurotoxic effects of MDMA. Therefore, the dose required to induce neurotoxicity is strain-dependent. The most obvious example is the Dark Agouti strain, which requires a single dose (10–15 mg/kg) of MDMA to produce a clear 30 to 50% or greater loss in cerebral 5-HT content (Colado *et al*., 1995; O'Shea *et al*., 1998).

MDMA disrupts normal thermoregulatory integration so that the different thermal control mechanisms become dissociated. Increase body temperature has been found in both humans (Mallick and Bodenham, 1997) and rodents (Gordon *et al.*, 1991) treated with MDMA have been reported to produce hyperthermia, regardless of ambient temperature (Freedman *et al.*, 2005). Methamphetamine-induced hyperthermia has been shown to involve dopamine release (Bronstein and Hong, 1995), which implies that dopamine could also be involved in MDMA-induced hyperthermia, given the fact that MDMA and methamphetamine release both 5-HT and dopamine. Administration of MDMA to rats has generally been reported to produce a marked hyperthermic response (Nash *et al*., 1988; Schmidt *et al*., 1990a; Colado *et al*., 1993; Dafters, 1994; Broening *et al*., 1995; Che *et al*., 1995; Malberg *et al*., 1996; O'Shea *et al*., 1998). However, an acute decrease in temperature has also been reported in a few studies. Marston *et al*. (1999) reported a hypothermic response in Hooded Lister rats, and Malberg and Seiden (1998) demonstrated a hypothermic response in Holtzman rats. Gudelsky and Nash (1996) demonstrated a dose related increase in extracellular 5-HT concentrations in the striatum and medial prefrontal cortex following peripheral administration of MDMA.

There is growing evidence that serotonergic neurotoxicity to MDMA does not always parallel changes in core body temperature (McGregor *et al*., 2003; Meyer *et al*., 2004). Hhyperthermia due to MDMA is not yet clear, although it could involve changes in any of a number of systems that contribute to MDMA-related temperature dysregulation, including the serotonergic, dopaminergic, and cytokine systems (Green *et al*., 2004). These results are similar to those obtained with other amphetamines, such as methamphetamine and MDA (Miller and O'Callaghan, 1994; O'Callaghan and Miller,

1994), both of which also produce hyperthermia but without cognizance of external temperature variations. Gordon and Fogelson (1994) demonstrated an enhanced hyperthermic response to MDMA when the cage construction failed to assist body heat loss (an acrylic floor rather than a grid). Such data suggest that the conditions at dance parties, where people are grouped and there is loud music, high ambient temperatures, and sometimes lack of drinking water, could result in increased acute MDMA induced adverse effects in comparison to ingestion in quiet surroundings.

5.2 Effect of Ambient Temperature on MDMA-Induced Neurotoxicity

The results of the study showed that acute repeated administration of MDMA induced a neuroinflammatory (neurotoxicity) process in mice, characterized by microgliosis and astrogliosis in the striatum of mice. These findings have generally been interpreted to reflect a pruning of serotonergic fibers (sometimes referred to as a distal axotomy) in the affected areas (Green *et al*., 2003; Wang *et al*., 2004; 2005). Administrations of MDMA have been found to be toxic to the human brain (de Win *et al*., 2004; Grob, 2002; McCann *et al*., 2000; Turner and Parrott, 2000).

Numerous studies in laboratory animals have shown that MDMA can lead to persistent changes in the serotonergic system. Forebrain target areas such as the neocortex, hippocampus, and striatum exhibit reductions in a variety of different serotonergic markers, including tryptophan hydroxylase activity, serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations, 5-HT transporter (SERT) binding, and the density of 5-HT-immunoreactive fibers (Lyles and Cadet, 2003).

MDMA reduces brain levels of 5-HT, the 5-HT metabolite 5-HIAA, and SERT binding (Green *et al*. 2003). Immunohistochemical studies using antibodies against 5-HT or SERT have also found decreased immunoreactive fiber density in several forebrain areas of animals treated with MDMA (O'Hearn *et al*., 1988; Wilson *et al*., 1993; Xie *et al*., 2006).

Possible MDMA neurotoxicity in recreational users has been a controversial issue; however, clinical studies have found evidence for decreased SERT binding capacity, reduced 5-HIAA levels in cerebrospinal fluid, and blunted hormonal responses to serotonergic challenge drugs in MDMA users compared to non-users (Green *et al*., 2003). Administration of MDMA primarily activates serotonergic neurons, with secondary activation of noradrenergic and dopaminergic terminals (Rothman *et al*., 2001).

Schmued (2003) found that a single high dose of MDMA can cause degeneration of non-serotonergic neurons, raising the possibility that at least some MDMA-induced functional alterations result from damage to other neural systems. Oxidative stress, excitotoxicity and mitochondrial dysfunction appear to play a major role in the neurotoxicity produced by the substituted amphetamines (Maria *et al*., 2006).

An increase in body temperature has been suggested to be a contributing factor to MDMA induced toxicity (Mc Namara *et al*., 2006). This is in line with the present study. This result therefore, suggest that increase in body temperature may potentiatiate the neurotoxic effects by MDMA. Increase in neuroinflammatory effects appears to not be correlated with changes in this parameter. According to Vanattou-Saïfoudine *et al*.

(2010), MDMA induced neurotoxicity and hyperthermia are associated with dopamine D1 responses.

Neurotoxicity in heavy ecstasy users has been revealed by neuroimaging studies showing reduced SERT binding and increased 5-HT_{2A} receptor binding in several cortical and/or subcortical areas (Jerrold, 2013). Gudelsky and Nash (1996) demonstrated a dose related increase in extracellular 5-HT concentrations in the striatum and medial prefrontal cortex following peripheral administration of MDMA. The size of the acute hyperthermic response plays a major role in determining the severity of the subsequent neurotoxicity. The results show that an increase in ambient temperature leads to severity of the neurotoxicity at constant dose. Mechanism of toxicity has generally been assumed to be directly related to raised body temperature (Craig and Kupferberg, 1972), acute toxicity can occur without marked hyperthermia (Wolf and Bunce, 1973). Metabolism of MDMA leads to the formation of reactive intermediates and/or toxic oxidation products, which may be responsible for the toxicity exerted by MDMA (Capela *et al*., 2009; Song *et al*., 2010).

Astrocyte hypertrophy can occur as a result of neuronal injury and can lead to the enhanced expression of glial fibrillary acidic protein (GFAP). This marker of neuronal damage has been used in several studies in assessing MDMA-induced toxicity in mice. (Miller and O'Callaghan, 1995; Johnson *et al*., 2002a,b). The results agree with O'Callaghan and Miller (2002) who reported an increase in the production of GFAP in response to MDMA. Due to its depletion of 5-HT and GFAP expression, MDMA is frequently described as a "selective 5-HT neurotoxin" (Green *et al.,* 2003; Morgan *et al.*, 2000).

Finally, the neurotoxic effects of MDMA may not be limited to serotonergic neurons. Schmued (2003), reported that high doses of MDMA led to cellular damage in the rat forebrain (where no serotonergic soma are present) as indicated by staining with Fluoro-Jade B, a marker for neurodegeneration that has been validated with several known neurotoxins (Schmued and Hopkins, 2000). This pattern of findings suggests that some changes in behavioral function may not be the result of serotonergic deficits. Such a conclusion has potential clinical relevance for the treatment of ecstasy users who are suffering from mood or anxiety disorders that may be related to their substance use.

5.3 Effect of Ambient Temperature Variation on MDMA-Induced Cognitive Changes

The results indicated impairment of spatial memory as shown by the percentage preference. Similarly, there was impairment in non-spatial memory as indicated by the discrimination ratio. Therefore, MDMA impairs both spatial and non-spatial memory.

Investigators have identified a constellation of changes, which they refer to as the MDMA syndrome, that includes a reduction in cognitive function in the object-recognition test, increased anxiety-like behavior in the emergence test, and decreased social behavior in the social-interaction test (Gunman *et al*., 2002; McGregor *et al*., 2003a, 2003b; Morley *et al*., 2001, 2004). McGregor and colleagues found that MDMA lowers the discrimination ratio in the novel object-recognition test particularly when the drug is administered in a hot environment (McGregor *et al*., 2003; Morley *et al*., 2001) This is in line with the findings of this study. A reduction in the discrimination ratio is suggestive of a deficit in memory. Performance in tasks utilizing non-spatial stimuli have also shown impairments following binge MDMA administration. Morley *et al.* (2001) found MDMA treated rats were impaired on an object recognition task using a 15 minute

delay but not at 60 minute. Piper and Meyer (2004) administered a chronic regime of MDMA to adolescent rats. When tested in adulthood they were impaired on an object recognition task with a delay of 15 minutes. Therefore, Piper and Meyer (2004) argued that using an intermittent MDMA regime, closer to that found in human MDMA use, impaired non-spatial working memory. In an extension of this study, Piper *et al.,* (2005) used a different regime of MDMA and found MDMA treated animals showed impaired object recognition performance. The reduction in the discrimination ratio was caused by the effect of MDMA and exacerbated by increase in temperature. Rats exposed to MDMA and housed in varying ambient temperatures potentiated serotonin depletions and exhibited reductions in novel object recognition performance. Thus, the present results add to the growing literature showing learning or memory deficits in animals given MDMA under varying conditions of treatment regimen, and environmental conditions.

Cognitive impairments include deficits in spatial memory in an avoidance task (Stuchlik and Vales, 2006), impairments in spatial memory in the radial arm maze (Seamans *et al*., 1998; Floresco and Phillips, 2001), deficits in spatial working memory and the ability to learn an alternation rule in a T-maze (Zahrt *et al*., 1997) and a Y-maze (Kozlov *et al*., 2001). The results in this study showed imparement in spstial memory using the Y-maze. When rats are administered acute doses of MDMA, they produce significantly more reference memory errors than working memory errors in the partially baited radial arm maze (Kay *et al*., 2009). Forebrain structures that are essential for cognitive function like the hippocampus and frontal cortex are highly sensitive to MDMA (Green *et al*., 2003). The serotonin system in these regions undergoes dynamic (Chen *et al*., 1997; Galineau *et al*., 2005; Patel *et al*., 2005) and protracted development (Moll *et al*., 2000). A deficit in

learning and memory is a highly consistent finding of cross-sectional investigations with experienced adult ecstasy users (Morgan *et al*., 2000). Reports of structural and functional changes also exist in ecstasy users (Parrott, 2001). An example of structural changes is the lower densities of the serotonin transporter that are being found in the brains of heavy ecstasy users compared with non-users. These lowered densities are dose-related and thought to be of temporary nature (Reneman *et al*., 2006). Also, the development of chronic tolerance to MDMA could be an indication for structural changes (modified receptor sensitivity) or perhaps even structural damage (Parrott, 2005). That is, structural brain changes following repeated use may precipitate the need for higher doses in order to achieve desired drug effects. Ecstasy users describe their first time experience as the best, and experience with continued use the need to augment their normal dose in order to experience beneficial mood effects (Parrott, 2005).

Serotonin is a widespread neurotransmitter that is involved in diverse physiologic and cognitive processes. An example of a cognitive function in which 5-HT is implicated is memory performance. In several studies it has been shown that disruption of the normal 5-HT levels results in cognitive deficits (Meeter et al., 2006). Because MDMA acts generally on the serotonin (5-HT) system, and 5-HT has vasoconstrictor properties, it is possible that MDMA has a general effect on the cerebral blood flow (CBF) in the whole brain (Cohen *et al*., 1996; Frackowiak, 2004; Meyer *et al*., 2006).

It is less likely to attribute MDMA to its actions on the dopaminergic system. An obvious culprit is serotonin (5-HT), as it is known that this neurotransmitter is involved in memory processes. As a powerful, indirect 5-HT agonist MDMA disrupts the normal functioning of the serotonergic system. The disruption of 5-HT affects also affects other

systems involved in normal memory functioning i.e. glucocorticoid hormones (i.e. cortisol) (van Praag, 2004). Studies have shown that at low doses, and post-training, glucocorticoid hormones can enhance memory consolidation. At high doses however, glucocorticoglucoids can impair memory performance, although not permanently (Andreano and Cahill, 2006; Brunner *et al*., 2006). Increased levels of 5-HT have also been shown to increase corticosteroid release (van Praag, 2004). Lock *et al.*, (2006) showed that the levels of cortisol were significantly increased in ecstasy users after self-administration of MDMA, in a club (Lock *et al*., 2006). The increase in cortisol levels can cause the 5-HT1a receptor system to be more responsive (van Praag, 2004). These 5-HT1a receptors are implicated in memory performance, with more stimulation being related to poorer memory performance (Meneses and Hong, 1997; Yasuno *et al*., 2003). This increased availability of 5-HT in addition to the augmented responsiveness of the 5-HT1a receptors would explain the memory deficits caused by an acute dose of MDMA.

These findings indicate that MDMA's effect on spatial memory is not due to its action within the dopaminergic system. Though MDMA exerts its effects in the brain through a multitude of transmitter systems, it is generally assumed that MDMA induced memory deficits that are related to changes in serotonergic function (Bolla *et al*., 1998; McCann *et al*., 1994; Reneman *et al*., 2002; Verkes *et al*., 2001). In addition, support for this serotonin hypothesis also comes from animal studies that have shown impairment of spatial memory after treatment with MDMA (Broening *et al*., 2001; Sprague *et al*., 2003).

The behavioral after-effects of MDMA have been the recipient of substantial attention, especially over the previous five years. McGregor *et al.*, (2003a) at the University of Sydney have identified a cluster of functional deficits following MDMA administration

to rats that they refer to as the “MUMPS syndrome”. The MDMA syndrome includes a reduction in cognitive performance and an increase in anxiety-like and depression-like behavior and these alterations are observed many weeks after MDMA administration (Thompson *et al*., 2004). McGregor *et al.,* (2003b) have documented impairments in working memory in the object-recognition test and an anxiogenic pattern in the emergence test, elevated plus-maze, and social interaction assessments. A slight alteration in a spatial memory has also been documented following MDMA (Sprague *et al*., 2003). Heavy users reportedly suffer from cognitive deficits, depressed mood, and other behavioral disturbances (Parrott, 2001).

Slikker *et al*., (1989) examined a variety of behavioral endpoints following MDMA administration to rats and nonhuman primates. No changes were found in schedule-controlled behavior, nociception, and maze learning ability in rats, or spontaneous behaviors in rhesus monkeys. Two independent meta-analyses (cross-study statistical analyses) of memory in ecstasy users arrived at somewhat contradictory conclusions (Laws and Kokkalis 2007; Zakzanis *et al*., 2007). While both analyses detected an association between ecstasy use and impaired performance on at least some measures of memory, one analysis, that of Laws and Kokkalis (2007) reported that this association had a medium to large effect size and found no effect of ecstasy dose.

One potential confounding factor within the research that examines the cognitive impairments found in MDMA users is that they tend to be polydrug users (Morefield *et al*., 2011 Mohamed *et al*., 2011: Yubero-Lahoz, 2011) and that, there is some debate as to whether it is the Ecstasy that produces the cognitive deficits or other drugs they have ingested. Croft *et al*. (2001) found cognitive impairments in MDMA users who also used

cannabis and also in MDMA free cannabis users. There were no significant differences between the two groups on the degree of cognitive impairment. Therefore, Croft *et al*. (2001) argued that cannabis use is an important confounding factor in the neurotoxic effect MDMA. Rodgers (2000) and Dafters *et al*. (2004) also found that MDMA and cannabis users and cannabis only users both showed significant memory impairments suggesting the deficits found in the Ecstasy users could be due to cannabis use rather than MDMA use.

However, other researchers that used controlled polydrug use have found supporting evidence that Ecstasy use is the contributing factor to the cognitive impairments found in Ecstasy users. Morgan (1999) found Ecstasy users were significantly impaired on tests assessing memory compared to Ecstasy free poly drug users and drug free controls. Other researchers have also found that the memory impairments found in Ecstasy users remained significant when other drug use, including cannabis, were taken into account (Heffernan *et al*., 2001; McCardle *et al*., 2004; Wareing *et al*., 2004). Gouzoulis-Mayfrank *et al*. (2000) and Dafters (2006) found participants who used Ecstasy and cannabis were significantly impaired on several cognitive measures but participants who used only cannabis did not show these impairments. Therefore, there is evidence that MDMA use and not polydrug use is associated with cognitive deficits.

Methylenedioxymethamphetamine users typically take one or more tablets during the evening or night (Farre *et al*., 2004) and this is often accompanied by insufficient rest. Sleep deprivation (stressor) interferes with allostasis, a process which serves to maintain a balance between several mediators (e.g., glucocorticoids, cytokines) in the normal functioning body (McEwen 2006).

It has been shown that very limited sleep restriction (i.e. 6 hours sleep/night) can cause an imbalance in these mediators (e.g. cortisol) and can consequently damage structures implicated in important functions e.g. memory (McEwen, 2006; Parrott, 2006). Performance on different types of memory tasks (verbal, spatial) deteriorated independent of treatment, as a function of sleep deprivation. Methylenedioxy-methamphetamine treatment produced a stable memory impairment that was consistent during the night and added to the memory impairment due to sleep deprivation. The fact that memory was impaired after administration of MDMA was in line with previous studies conducted during daytime. These acute studies showed impairment of verbal and spatial memory after administration of MDMA (Kuypers and Ramaekers, 2005; Kuypers and Ramaekers, 2006).

The existence of functional disturbances i.e. cognitive disturbances (memory, decision making, impulsivity), and psychiatric problems may be a strong indication for the existence of neuronal damage (Cole and Sumnall, 2003; Morgan *et al.*, 2002; Parrott, 2001). Recent studies have shown that impairment of verbal memory was even present in ecstasy users that had been abstinent for over 2 years compared with their performance on previous measures and/or compared with a control group (Thomasius *et al*., 2006; Ward *et al*., 2006).

There is also some concern as to whether memory deficits in Ecstasy users remain after they have stopped using the drug. Some studies have examined Ecstasy users that have abstained from drug use for short periods, usually one to two weeks, and found they still show impairments in cognitive tasks (Bolla *et al*., 1998; Gouzoulis-Mayfrank *et al*., 2000; Fox *et al*., 2001; Verkes *et al*., 2001; Zakzanis and Young, 2001; Fox *et al*., 2002;

Gouzoulis-Mayfrank *et al*., 2003; Dafters *et al*., 2004; Von Geusau *et al*., 2004; Montgomery *et al*., 2005). However these studies do not inform us about the long-term consequences of Ecstasy use. There has been evidence that Ecstasy users who have abstained for longer periods such as two to four months (Rodgers, 2000; Reneman *et al*., 2000; McCardle *et al*., 2004) and even up to six months (Wareing *et al*., 2000; Wareing *et al*., 2004) still show significant deficits on a number of cognitive tasks. In addition there have been reports of Ecstasy users showing cognitive impairments after abstaining from Ecstasy use for several years (Reneman *et al*., 2001; Morgan *et al*., 2002) suggesting with long-term drug cessation the deficits in memory function may not fully recover.

CHAPTER SIX

6.0 Summary, Conclusion and Recommendations

6.1 Summary

Methylenedioxymethamphetamine is a recreational drug commonly used by youths in parties and dance clubs where the temperature is high. The study was designed to investigate the effect of ambient temperature variations on MDMA-induced changes in body temperature, neurobehaviour and neurotoxicity in rodents. The results revealed hyperthermia, neurotoxicity (indicated by astrogliosis and microgliosis), impairement in both spatial and non-spatial memory. All the findings were exacerbated by an increase in external temperature.

6.2 Conclusion

The study showed body temperature of MDMA-treated mice exposed to 270C ambient temperature was significantly higher than MDMA-treated mice exposed to 21 0C. Mice treated at 270C had significantly higher microglia and astroglia activation than mice exposed at 210C. Impairment of spatial and non-spatial memory was significantly higher in MDMA-treated than control rats. These results suggest that any MDMA-induced hyperthermia and neurological deficits will be exacerbated by an increase in ambient temperature.

6.3 Recommendations

Based on the findings of this study, the following recommendations for further research were made:

1. To identify the amino acid, monoamine or neuropeptide systems that mediate the enduring behavioral consequences of MDMA could greatly enhance our understanding of the long-term risks of recreational ecstasy use.
2. To study the involvement of the 5-HT1a receptor in relation with the MDMA-induced memory impairment.
3. To include some factors related to the 'party lifestyle' of MDMA users i.e. sleep deprivation, and prolonged physical exertion, to increase the ecological validity of the experimental studies.
4. Medical interventions aimed at increasing the efficiency of whole-body cooling by targeting blood vessel constriction in the skin could be therapeutically relevant for counteracting the development of MDMA-induced hyperthermia.

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APPENDICES

Appendix 1

International Drug Abuse Research Society (IDARS)

International Drug Abuse Research Society (IDARS)

IDARS

George Koob
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San Diego, CA
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Newsletter Editor
Emmanuel Onaivi

Website:
www.idars.org

February 25, 2013

Dr. Abdul Wahab Alhassan
Department of Human Physiology and
University Health Clinic
Faculty of Medicine
Ahmadu Bello University
Zaria, Kaduna State, Nigeria

Dear Dr. Abdul Wahab,

We would like to invite you to attend the 4th International Drug Abuse Research Society (IDARS) meeting. The meeting will be held at the Hyatt Regency Hotel, Mexico City, Mexico on April 15-19, 2013. The theme of the conference is "Recent Frontiers and Advances in Drug Addiction" and main objective of this meeting is to bring together basic scientists and clinical investigators from international communities with an interest in CNS acting drugs, drugs of abuse and addiction. Therefore, we would like to invite you as a speaker of our meeting to give a lecture on "ROLE OF EXTERNAL TEMPERATURE ON MDMA-INDUCED NEUROINFLAMATION IN MICE. Hope you consider the invitation and will attend the meeting.

Enclosed you will find information which includes: a meeting registration form, a hotel accommodation/reservation form and a sample abstract. Abstract will be accepted as a Word document sent to bonnie.robinson@fda.hhs.gov, syed.ali@fda.hhs.gov along with the enclosed registration form filled out in full.

The deadline for receipt of abstract and registration form is March 15, 2013. Accommodations have been reserved at the Hyatt Regency Hotel. Since the meeting is limited to around 100 participants, and the hotel has a limited number of rooms reserved, we encourage you to submit your hotel reservation form along with your registration as soon as possible.

I hope you will be able to attend the meeting. If you need more information, don't hesitate to contact me by phone, fax or email. .

Sincerely,

Syed Ali

Syed Ali, Ph.D. and George Koob, Ph.D.
Co-Organizer, 4th IDARS meeting
National Center for Toxicological Research
Jefferson, AR 72079; Tel: 870-543-7123
syed.ali@fda.hhs.gov

Appendix 2

11th International Conference of the Society of Neuroscientist of Africa, Faculty of Sciences, Mohammed V-Agdal University. Rabat Morocco. June, 13-17 2013.

P03-26-The effect of prenatal stress on the vulnerability to addiction in rats
Nadia Said, Casablanca, Morocco

P03-27-A retrospective study on the impact of opioate abuse on the development of NEUROAIDS in intravenous drug users
George M. Wanderi, Nairobi, Kenya

Neurodegenerative Disease, Brain diseases and related syndroms

P04-1-Transcriptome analysis indicates early signs of neurodegeneration in Drosophila model of Parkinson's disease
Flora Stephano, Kiel, Germany

P04-2-Dopamine control of the globus pallidus and its impact on the subthalamic nucleus and the pars reticulate of substantia nigra
Mamad Omar, Rabat/ Bordeaux, Morocco/France

P04-3-Beta-arrestin signaling in patients with Parkinson›s disease suffering from depression.
Sofia Avissar, Negev, Israel

P04-4-Effects of postnatal stress on locomotor activity: Implications for Parkinson's disease
Thabisile Mpofana, Durban, South Africa

P04-5-Immunohistochemical Investigation of p53, Bax and NSE; the Link between Energy Metabolism and Cell Cycle Dysregulation in Degenerating Cells of the Cerebellar Cortex
Mike Ogundele, Ado Ekiti, Nigeria

P04-6-Homocysteine levels are associated with the Fat mass and obesity associated gene [FTO (intron 1 T>A) polymorphism] in MS patients
William Davis, Stellenbosh, South Africa

P04-7-Association of an iron-related genetic variant with functional iron deficiency and its effect on multiple sclerosis risk in the South African population
Kelebogile E. Moremi, Stellenbosh, South Africa

P04-8-Validating the Arabic versions of the rowland universal dementia assessment scale and 16-item informant questionnaire on cognitve decline in the elderly
Thien Kieu Thi Phung, Beirut, Lebanon

P04-9-Evidence for a decrease in dendrites of pyramidal cells in neonatal thalamic lesioned rat's prefrontal cortex: implication in Autism and Schizophrenia.
Ouhaz Zakaria, Marrakesh, Morocco

P04-10-Oxytocin and Schizophrenia
Ghita Zairi, Casablanca, Morocco

P04-11-Role of external temperature on MDMA-induced neuroinflamation in mice
Alhassan Abdul Wahab, Zaria, Nigeria

Appendix 3

Immuno-histochemistry for GFAP

(Italian version)

IMMUNOISTOCHIMICA per GFAP

- Lavare in PBS 3X10 minuti (1 ml)
- Blocco in H_2O_2 all'1% in PBS per 30 minuti (400 µl)
- Pulire bene i pennelli
- Lavare in PBS 1X10 minuti (1 ml)
- Lavare in PBS + Triton allo 0.5% 2X10 minuti (1 ml)
- Blocco in siero: NGS allo 1.5% in PBS + Triton allo 0.5% per 2h (400 µl)
- Anticorpo **mouse anti** GFAP 1:400 in PBS + Triton allo 0.5% + NGS allo 1.5%per 1 notte (400 µl)
- Lavare in PBS 3X10 minuti (1 ml) 2 da 5 1 da 10
- Anticorpo secondario **goat anti mouse** 1:400 in PBS + Triton allo 0.5% + NGS all' 1.5% per 1h (400 µl)
- Lavare in PBS 3X10 minuti (1 ml) → i primi 2 da 5 min, il terzo da 10 min
- ABC: 1:100 in PBS + Triton allo 0.5% + NGS all' 1.5% per 1h (400 µl)
- Lavare in PBS 3X10 minuti (1 ml)
- Incubare con una soluzione di tampone fosfato 0.1M contenente 5 mg di DAB + 4 mg NH_4Cl + 20 mg di glucosio in 10 ml di soluzione(5 ml di H_2O + 5 ml di TF 0.2M) per 5 minuti (500 µl)
- Aggiungere 50 µl di una soluzione contenente 2 mg di glucosio ossidasi in 25 ml di H_2O per 1h
- Lavare in PBS 3X10 minuti (1 ml)
- Montare su vetrini in gelatina e lasciare asciugare
- Disidratare con la scala crescente degli alcoli (70°x2'- 95°x2'-100x2'x2') per montare il coprioggetto

Immuno-histochimistry for GFAP

(English version)

- Rinse sections in PBS 3X10 minute (1 ml)
- Block in H_2O_2 all'l% in PBS per 30 minute (400 µl)
- Rinse brushes in clean PBS
- Rinse in PBS 1X10 miriute (1 ml)

. Rinse in PBS + Triton 0.5% 2X10 minute (ml)

- Block in: NGS 1.5% in PBS + Triton 0.5% per 2h (400 µl)
- Primary Antibody : mouse anti GFAP 1:400 in PBS + Triton allo 0.5% + NGS allo 1.5% per - notte (400 µl)
- Wash in PBS 3X10 minute (1 ml)
- 1 Night (400 µl)
- Secondary antibody goat anti mouse 1:400 in PBS + Triton 0.5% + NGS 1.5% per lh (400 µl)
- Rinse in PBS 3X10 minute (1 ml)
- ABC solution : 1:100 in PBS + Triton allo 0.5% ÷ NGS all' 1.5% per lh (400 µl)
- Rinse in PBS 3X10 minute (1 ml)
- Incubate with phosphate solution 0.1M containing 5 mg di DAB + 4 mg NH_4Cl + 20 mg of glucose in 10 ml of solution (5 ml di H_2O + 5 ml di TF 0.2M) per 5 minuti (500 µl)
- Aggregate with 50 µl solution containing 2 mg glucose oxidase in 25 ml di H_2O per lh
- Rinse in PBS 3X10 minute (1 ml)
- Mount sections on gelatin-coated slides and dry

De hydrate sections in the following grades of alcohol (70°x2'- 95°x2'-100x2'x2') and mount four visualization.

Appendix 4

Immuno-histochemistry for CD11b

(Italian version)

IMMUNOISTOCHIMICA per il CD11b

- Lavare in PBS 3X10 minuti (1 ml)
- Blocco in H_2O_2 all'1% in PBS per 15 minuti (400 µl)
- Pulire bene i pennelli
- Lavare in PBS 3X10 minuti (1 ml)
- Blocco in siero: NGS al 5% in PBS + Triton allo 0.2% per 1h (400 µl)
- Anticorpo rat anti mouse CD11b 1:1000 in PBS + Triton allo 0.2% NGS al 5% per 1 notte (RT,400 µl)
- Lavare in PBS 3X5 minuti (1 ml)
- Anticorpo secondario goat anti rat 1:200 in PBS + Triton allo 0.2% + NGS al 5% per 1h (400 µl)
- Lavare in PBS 3X5 minuti (1 ml)
- ABC: 1:100 in PBS + Triton allo 0.2% + NGS al 5% per 1h (400 µl)
- Lavare in PBS 3X5 minuti (1 ml)
- Incubare con una soluzione di tampone fosfato 0.1 molare contenente 5 mg di DAB + 4 mg NH_4Cl + 20 mg di glucosio in 10 ml di soluzione per 5 minuti (500 µl)
- Aggiungere 50 µl di una soluzione contenente 2 mg di glucosio ossidasi in 25 ml di H_2O per circa 1h
- Lavare in PBS 3X10 minuti (1 ml)
- Montare su vetrini in gelatina e lasciare asciugare
- Disidratare con la scala crescente degli alcoli per montare il coprioggetto

Immuno-histochimistry for CD11b

(English version)

- Rinse sections in PBS 3X10 minute (1 ml)
- Block in H_2O_2 all'l% in PBS per 15 minute (400 µl)
- Rinse brushes in clean PBS
- Rinse in PBS 3X10 miriute (1 ml)
- Block in: NGS 1.5% in PBS + Triton 0.5% per 2h (400 µl)
- Primary Antibody : rat anti –mouse CD11b 1:1000 in PBS + Triton 0.2% + NGS 1.5% .
- 1 Nigyt (400 µl)
- Wash in PBS 3X5 minute (1 ml)
- 1 Night (400 µl)
- Secondary antibody goat anti-rat 1:200 in PBS + Triton 0.2% + NGS 1.5% per lh (400 µl)
- Rinse in PBS 3X5 minute (1 ml)
- ABC solution : 1:100 in PBS + Triton 0.2% ÷ NGS ' 1.5% per lh (400 µl)
- Rinse in PBS 3X5 minute (1 ml)
- Incubate with phosphate solution 0.1M containing 5 mg di DAB + 4 mg NH_4Cl + 20 mg of glucose in 10 ml of solution (5 ml di H_2O + 5 ml di TF 0.2M) per 5 minuti (500 µl)
- Aggregate with 50 µl solution containing 2 mg glucose oxidase in 25 ml of H_2O per lh
- Rinse in PBS 3X10 minute (1 ml)
- Mount sections on gelatin-coated slides

De hydrate sections in the following grades of alcohol (70°x2'- 95°x2'-100x2'x2') and mount four visualization.

Appendix 5

Y-maze

www.stoelting.europe (2014)

Appendix 6

Novel Object Recognition Apparatus

www.stoelting.europe (2014)

Appendix 7

Table: Y-maze Percentage Preference at 21°C

S/No.	Saline (Percentage Preference)	MDMA (Percentage Preference)
1	95	60
2	90	68.6
3	91	64
4	90	69
5	95	62.9
6	92	61.9
7	90.3	62.5
8	90	65.5
9	95	62
10	95	60.5

Appendix 8

Table: Y-maze Percentage Preference at $27^{O}C$

S/No.	Saline (Percentage Preference)	MDMA (Percentage Preference)
1	80	49.6
2	83.33	53
3	80	50.5
4	85	56
5	75.9	54
6	80.4	60
7	85	55
8	87	51
9	88.2	57
10	88.2	51

Appendix 9

Table: Novel Object Recognition at 21°C (MDMA)

S/No.	Training (Both Objects)	Novel Object (Test)	Familiar Object (Test)
1	27	5	22
2	30	10	20
3	25	12	22
4	26	10	21
5	32	11	24
6	29	10	22
7	28	9	20
8	30	11	24
9	27	10	22
10	26	6	20

Appendix 10

Table: Novel Object Recognition at 21°C (Saline)

S/No.	Training (Both Objects)	Novel Object (Test)	Familiar Object (Test)
1	29	20	11
2	26	19	10
3	28	22	12
4	30	23	11
5	27	20	10
6	28	22	12
7	31	24	11
8	32	23	12
9	29	22	12
10	28	19	13

Appendix 11

Table: Novel Object Recognition at 27°C (MDMA)

S/No.	Training (Both Objects)	Novel Object (Test)	Familiar Object (Test)
1	22	9	13
2	39	5	29
3	31	6	13
4	32	8	22
5	33	9	20
6	37	10	24
7	29	8	20
8	34	9	22
9	31	7	20
10	30	6	22

Appendix 12

Table: Novel Object Recognition at 27°C (Saline)

S/No.	Training (Both Objects)	Novel Object (Test)	Familiar Object (Test)
1	33	21	8
2	30	18	6
3	23	19	3
4	35	25	6
5	31	28	2
6	38	30	7
7	30	20	8
8	29	21	7
9	31	23	6
10	32	23	7

Appendix 13

Effect of on MDMA- induced hyperthermia at 21°C

DRUGS	Basal Temp	1H MDMA	2H MDMA	3H MDMA	4H MDMA	Overall mean
Vehicle	35.52±0.232	35.32±0.1547	35.23±0.292	35.10±0.367	35.01±0.286	35.16±0.213
MDMA	35.45±0.218	36.52±0.368	37.32±0.319*	37.27±0.461*	37.4±0.212*	37.13±0.272*

Appendix 14

Effect of MDMA - induced hyperthermia at 27°C

DRUGS	Basal Temp	1H MDMA	2H MDMA	3H MDMA	4H MDMA	Overall mean
Vehicle	35.5±0.111	35.80±0.187	35.93±0.137	35.61±0.191	35.75±0.075	35.77±0.163
MDMA	36.05±0.105	37.82±0.087*	38.2±0.213*	37.93±0.133*	37.728±0.218*	37.79±0.180*