The Identification of the Dopamine Transporter (DAT) and the Effects of Dopamine on Human Sperm Parameters

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Declaration

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Abstract

Introduction: Catecholamines such as dopamine are found throughout the mammalian reproductive system, where they are hypothesized to play some role in fertilization. A dopaminergic phenotype has been discovered in the male spermatozoan of many mammalian species. Dopamine regulates a wide range of functions including capacitation, and the induction of sperm motility and the acrosome reaction through mechanisms involving dopamine receptor (D2) activation. A dose-dependent response to dopamine has been observed in sperm, whereby low concentrations were shown to be protective and high concentrations were observed to negatively impact sperm functions. This biphasic effect can only be explained by the existence of a transporter protein; however, the Dopamine Transporter (DAT) is yet to be well explored on human sperm membranes.

Objectives: To identify DAT on human sperm membranes and determine the effects of dopamine, at high, medium, and low concentrations, on human sperm parameters.

Methods: Utilizing an indirect immunofluorescence assay, spermatozoa were stained with a primary anti-DAT antibody against the fluorescent secondary Alexa Fluor® 488. Fluorescence was visualized on a confocal microscope. Western blot analyses were employed as a second assay to show the presence of DAT on human sperm membranes with rat brain tissue used as a positive control. The membranes were exposed to anti-DAT primary antibody against a Horseradish Peroxidase (HRP)-linked secondary antibody. Furthermore, the effect of dopamine on sperm parameters, at different dopamine concentrations were tested for three time periods (0, 1, 3 and 6 hours). Sperm were exposed to high (1 mM), medium (10 µM) and low (100 nM) dopamine concentrations at 37°C for 1, 3 and 6 hours, thereafter functional sperm parameters were analysed. Using CASA and the SCA® software system, total motility, progressive motility, and sperm kinematics were analysed. Sperm viability and the acrosome reaction were additionally assessed.

Results: The immunofluorescence assay indicates a strong immunoreaction in the positive controls vs. the negative controls and it could conclude that DAT is positively expressed on human sperm membranes. Western blot analyses further confirmed the existence of DAT in human sperm membranes when compared to the positive brain controls. Protein bands of 62 kDa and 48 kDa appear in the Western Blot lanes loaded with sperm samples, thereby positively correlating with bands found in the brain tissue. High concentrations of dopamine were found to negatively affect motility parameters as early as 60 minutes (43.05% ± 12.72 vs. 18.75% ± 8.75). Sperm kinematics were also negatively affected by the addition of high concentrations of dopamine when compared to the controls. Sperm viability was significantly

decreased by high dopamine concentration after 3 hours of incubation (51.86% ± 7.220 vs. 34.36% ± 13.03). The high concentration of dopamine was found to elicit a premature acrosome reaction upon addition of treatment (26.88% ± 3.199 vs. 31.91% ± 2.85).

Conclusion: It can be concluded that DAT is found on human sperm membranes and that dopamine regulates sperm functions through mechanisms involving the dopamine D2 receptor and DAT. These findings suggest a possible regulatory role for DAT inhibitory drugs such as Ritalin on sperm parameters.

Opsomming

Inleiding: Katekolamiene soos dopamien kom dwarsdeur die voortplantingstelsel van soogdiere voor, waar hulle veronderstel is om 'n rol in bevrugting te speel. 'n Dopaminerge fenotipe is in die spermatozoë van baie soogdierspesies ontdek. Dopamien reguleer 'n wye verskeidenheid funksies, insluitend kapasitering, en die induksie van spermmotiliteit en die akrosoomreaksie deur meganismes wat dopamienreseptoraktivering (D2) insluit. 'n Dosisafhanklike reaksie op dopamien is waargeneem in sperma, waardeur lae konsentrasies beskermend is en hoë konsentrasies spermfunksies negatief beïnvloed. Hierdie bifasiese effek kan slegs verklaar word deur die bestaan van 'n vervoerderproteïen; die Dopamien-vervoeder (DAT) moet egter nog goed ondersoek word op menslike spermmembrane.

Doelstellings: Om DAT op menslike spermmembrane te identifiseer en die effek van dopamien teen hoë, medium en lae konsentrasies op menslike spermparameters te bepaal.

Metodes: Met behulp van 'n indirekte immunofluoressensie toets is spermatozoa gekleur met 'n primêre anti-DAT-teenliggaam teen die fluoresserende sekondêre Alexa Fluor® 488. Fluoressensie is in 'n konfokale mikroskoop gevisualiseer. Western Blot ontledings is gebruik as 'n tweede toets om die teenwoordigheid van DAT op menslike spermmembrane met rotbreinweefsel as 'n positiewe kontrole te toon. Die membrane is blootgestel aan anti-DAT primêre teenliggaam teen 'n Horseradish Peroxidase (HRP)-gekoppelde sekondêre teenliggaam. Verder is die effek van dopamien op spermparameters by verskillende dopamienkonsentrasies vir drie tydperke (0, 1, 3 en 6 uur) getoets. Sperma is gedurende 1, 3 en 6 uur aan hoë (1 mM), medium (10 µM) en lae (100 nM) dopamienkonsentrasies by 37 ° C blootgestel, waarna funksionele spermparameters geanaliseer is. Met behulp van CASA en die SCA® sagtewarestelsel is totale beweeglikheid, progressiewe beweeglikheid en spermakinematika geanaliseer. Daarbenewens is die lewensvatbaarheid van die saad en die akrosoomreaksie beoordeel.

Resultate: Die immunofluoressensie toets dui op 'n sterk immuunreaksie in die positiewe kontroles teenoor die negatiewe kontroles, en dit kan aflei dat DAT positief tot uitdrukking kom op menslike spermmembrane. Western Blot ontledings het die bestaan van DAT in menslike spermmembrane bevestig in vergelyking met die positiewe breinkontroles. Proteïenbande van 62 kDa en 48 kDa verskyn in die Western Blot-bane gelaai met spermmonsters, wat sodoende positief korreleer met bande wat in die breinweefsel voorkom. Daar is gevind dat hoë konsentrasies dopamien beweeglikheidsparameters negatief beïnvloed al in 60 minute (43,05% ± 12,72 teenoor 18,75% ± 8,75). Sperma-kinematika is ook negatief beïnvloed deur

die toevoeging van hoë konsentrasies dopamien in vergelyking met die kontroles. Sperma-lewensvatbaarheid is aansienlik verminder deur hoë dopamienkonsentrasie na 3 uur inkubasie (51,86% ± 7,220 versus 34,36% ± 13,03). Daar is gevind dat die hoë konsentrasie dopamien 'n voortydige akrosoomreaksie uitlok by toevoeging van die behandeling (26,88% ± 3,199 versus 31,91% ± 2,85).

Gevolgtrekking: Daar kan tot die gevolgtrekking gekom word dat DAT op menslike spermmembrane voorkom en dat dopamien spermfunksies reguleer deur meganismes wat die dopamien D2-reseptor en DAT insluit. Hierdie bevindings dui op 'n moontlike regulerende rol vir DAT-remmende middels soos Ritalin op spermparameters.

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Dedication

I would like to dedicate this thesis to my late grandmother:

Maudlin Kathleen Africa.

Ma, I wish you were here to see me finish this, but I know you are watching over me and that I have made you proud.

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List of Abbreviations

%	Percent
°C	Degree Celsius
α	Alpha
β	Beta
µg/mL	Microgram per millitre
µL	Microlitre
µM	Micromolar
µm/s	Micrometres per second
AADC	Aromatic Amino Acid Decarboxylase
AC	Adenyl Cyclase
ADHD	Attention-Deficit Hyperactivity Disorder
AGS	Accessory Gland Secretions
AKAP-3	A-Kinase Anchoring Protein 3
ALH	Amplitude of Lateral Head Displacement
AMPH	Amphetamine
ANOVA	Analysis of Variance
APS	Ammonium Persulfate
APUD	Amine Precursor Uptake and Decarboxylation
ASP^+	4-[4-(*Dimethylamino*)styryl]-N-methylpyridinium iodide
ATP	Adenosine Triphosphate
bDAT	Bovine Dopamine Transporter
BCF	Beat Cross Frequency
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CAF	Central Analytical Facility

CAMKII	Ca^{2+}-calmodulin dependent kinase
cAMP	Cyclic Adenosine Monophosphate
CASA	Computer-Aided Sperm Analysis
CB1	Cannabinoid Receptor 1
Cl^-	Chloride ion
CNS	Central Nervous System
COMT	Catechol-O-Methyltransferase
CO_2	Carbon Dioxide
CuP	Copper Phenanthroline
D1	Dopamine Receptor 1
D2	Dopamine Receptor 2
D3	Dopamine Receptor 3
D4	Dopamine Receptor 4
D5	Dopamine Receptor 5
DAG	Diacylglycerol
DAT	Dopamine Transporter
DBH	Dopamine Beta-Hydroxylase
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DOPAL	3,4-dihydroxyphenylacetaldehyde
DOPAC	3,4-dihyroxyphenylacetic acid
DRD2	Dopamine Receptor 2
ECL	Enhanced Clarity™ Chemiluminescence
EDTA	Ethylene Diamine Tetra-acetic Acid

EGTA	Ethylene Glycol Tetra-acetic Acid
ERK	Extracellular-Signal Regulated Protein Kinase
FBS	Foetal Bovine Serum
FITC-PSA	Fluorescein Isothiocyanate Pisum Sativum Agglutinin
FMHS	Faculty of Medicine and Health Sciences
GABA	Gamma Amino Butyric Acid
GPCR	G protein-coupled receptors
hDAT	Human Dopamine Transpoter
H_2O	Water
H_2O_2	Hydrogen Peroxide
HCO_3	Bicarbonate
HCl	Hydrochloride
HPG	Hypothalamic-Pituitary-Gonadal
HREC	Health Research Ethics Committee
HRP	Horseradish Peroxidase
HVA	Homovanillic Acid
Hz	Hertz
ICF	Informed Consent Form
IF	Immunofluorescence
IgG	Immunoglobulin G
IP_3	Inositol triphosphate
IVF	*In Vitro* Fertilization
K^+	Potassium ion
kB	Kilo Base
kDa	Kilo Dalton
Km	Michaelis constant

LB	Lysis Buffer
L-DOPA	L-3,4-dihydroxyphenylalanine
LIN	Linearity Index
LSD	Lysergic Acid diethylamide
M	Molar
M/mL	Million per millilitre
mg	Milligram
mL	Millilitre
mM	Millimolar
MAPK	Mitogen Activated Protein Kinase
MAO	Monoamine Oxidase
MB-COMT	Membrane Bound Catechol-O-Methyltransferase
METH	Methamphetamine
Na^+	Sodium ion
NaCl	Sodium Chloride
Na_3VO_4	Sodium Orthovanadate
NE	Norepinephrine
NET	Norepinephrine Transporter
nM	Nanomolar
ng/mL	Nanogram per millilitre
NTT	Neurotransmitter Transporters
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PD	Parkinson’s Disease
pH	Potential of Hydrogen

PI	Protease Inhibitor
PKA	Protein Kinase A
PKC	Protein Kinase C
PMSF	Phenyl Methyl Sulfonyl Fluoride
PNMT	Phenylethanolamine-N-Methyltransferase
PTM	Post-Translational Modifications
rDAT	Rat Dopamine Transporter
rcf	Relative Centrifugal Force
rpm	Rotors Per Minute
ROS	Reactive Oxygen Species
RT	Room Temperature
sAC	Soluble Adenyl Cyclase
SCA	Sperm Class Analyser®
SB	Sample Buffer
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SERT	Serotonin Transporter
SLC	Solute Carrier
SLC6A3	Solute Carrier 6 member 3
SNc	Substantia nigra pars compacta
SNP	Single Nucleotide Polymorphism
STR	Straight-line Index
SURRG	Stellenbosch University Reproductive Research Group
TAAR1	Trace Amine-Associated Receptor 1
TBS	Tris-Buffered Saline

TEMED	Tetrametylethylenediamine
TM	Transmembrane
tmAC	Transmembrane Adenyl Cyclase
TH	Tyrosine Hydroxylase
UTR	Untranslated Region
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VMAT	Vesicular Monoamine Transporter
VNTR	Variable Number of Tandem Repeats
VSL	Straight-line Velocity
VTA	Ventral Tegmental Area
WB	Western Blot
WHO	World Health Organisation
WOB	Oscillation Index

Chapter 1

Introduction

1.1 Introduction and Rationale

Dopamine is a known neuromodulator within the brain, however its effects outside the central nervous system (CNS) are not well studied. Dopamine has been postulated to influence reproductive behaviours in mammals, but its direct effect on fertility is not clear. Catecholamines such as dopamine and norepinephrine have been found in high concentrations within the reproductive tract of mammals such as rats, cows, boars, and humans. In females, differential expression of dopamine has been observed throughout the oviduct and a dopaminergic phenotype has been established in males. The recent identification of the dopamine receptor 2 (D2), on sperm membranes has prompted investigations into the effects of agonists and antagonists of this receptor on sperm functions. Dopamine, the principle D2 receptor agonist, was found to modulate several sperm functional parameters (Otth *et al.*, 2007; de Angelis *et al.*, 2016) including capacitation, motility, and the acrosome reaction. The mechanisms through which dopamine brings about changes in these sperm parameters is thought to be through dopamine receptor activation. A dose-dependent effect has subsequently been observed in boar spermatozoa, whereby low concentrations was shown to be protective and high concentrations of dopamine negatively impacted on sperm parameters (Ramírez *et al.*, 2009). This effect could be explained by the presence of monoamine transporters, that function to remove catecholamines when an excess is detected. Recently Urra and colleagues discovered the presence of monoamine transporters in equine sperm, namely norepinephrine transporter (NET), serotonin transporter (SERT) and dopamine transporter (DAT) (Urra *et al.*, 2014). Equine spermatozoa were found to express functional DAT's and exposure to dopamine was shown to negatively impact sperm parameters as previously described by Ramírez and colleagues (Urra *et al.*, 2014). The biphasic effect of dopamine on sperm functions was demonstrated in various mammalian species including humans, however DAT is yet to be well explored on human sperm membranes. Considering the negative effects exerted by high doses of dopamine on sperm parameters, it is crucial to establish the presence of this transporter on human sperm membranes in order for the mechanism of dopamine handling by the reproductive system to be further illuminated.

1.2 Problem Statement

The dopamine pathway is involved in the modulation of sperm parameters however, the effects of dopamine on human sperm parameters are yet to be established and the presence of DAT on human sperm membranes needs to be further investigated.

1.3 Research Question

Are DATs present on human sperm membranes and what are the effects of different concentrations of dopamine on human sperm parameters?

1.4 Aims and Objectives

This study was divided into two parts based on the independent aims.

Aim 1

To purely identify and localize the DAT protein in human spermatozoa.

Objectives

i) Utilize Immunofluorescence (IF) to visually identify DAT protein on human sperm membranes.
ii) Use western blotting (WB) techniques to further confirm the identification of the DAT protein in human spermatozoa.

Aim 2

To determine the effects of various concentrations of dopamine on human sperm parameters.

Objective

Incubate spermatozoa with low (100 nM), medium (10 µM) and high (1 mM) concentrations of dopamine for 1, 3 and 6 hours. Investigate the effect of the dopamine concentrations on various functional parameters including, total motility, progressive motility, sperm kinematics, viability, and acrosome reaction.

1.5 Thesis Layout

This thesis comprises of an extensive literature review (Chapter 2) which explores the current knowledge regarding dopamine transporters, receptors, as well as the effect of dopamine on male reproductive parameters. Following this Chapter 3 will detail the materials and methods employed during the study. Chapter 4 will report the results of this study and Chapter 5 will discuss and interrogate these results. Finally, Chapter 6 will comprise of the conclusion of the study.

Chapter 2

Literature Review

2.1 Introduction

Fertilization of gametes is essential for the continuation of any species (Litscher and Wassarman, 2006). It is a complex process controlled by a great number of components. The main goal is for the spermatozoa to reach the ovum to begin the fertilization process, however there are many factors which influence this and may hinder the spermatozoan from reaching its goal.

Infertility is defined as the inability to conceive after 12 months of regular unprotected sexual intercourse (Gnoth *et al.*, 2005; WHO, 2010; Du Plessis, 2016; Starc *et al.*, 2019). Male factor infertility is said to account for 50% of all infertility cases and approximately 10-15% of men suffer from idiopathic infertility (Kothandaraman *et al.*, 2016). In recent years, a significant downward trend in male fertility was observed (Kumar and Singh, 2015; Levine *et al.*, 2017). A wide range of factors may contribute to male infertility, such as stress, genetics, environmental factors, and improper sperm functioning.

Dopamine is an important catecholamine, which functions as a neuromodulator and participates in many biological functions within the body- such as motor control, cognitive function, and reproductive behaviours (Daly and Salloway, 1994; Klein *et al.*, 2019). Outside of the central nervous system (CNS) dopamine is also located in peripheral tissues, where it functions as a local paracrine messenger, vasodilator as well as a modulator of food intake by regulating glucose homeostasis (Missale *et al.*, 1998; Rubí and Maechler, 2010).

Catecholamines, such as dopamine and norepinephrine have additionally been found in high concentrations within the reproductive tracts of mammals. This discovery led to the investigation into whether a catecholaminergic phenotype existed in the reproductive systems of mammals (Otth *et al.*, 2007; de Angelis *et al.*, 2016). Dopamine receptors were subsequently shown to be expressed throughout the male reproductive tract and dopamine was shown to modulate sperm functions in a dose-dependent effect (Otth *et al.*, 2007; Ramírez *et al.*, 2009). Low concentrations were shown to render sperm protection while high concentrations impacted negatively on sperm functions such as motility and acrosomal integrity. This biphasic effect could only be attributed to the presence of a transporter protein (Ramírez *et al.*, 2009; Urra *et al.*, 2014).

Dopamine receptors have been located on mammalian spermatozoa, including humans, and recently a functional dopamine transporter protein (DAT) has been found in equine spermatozoa (Ramírez *et al.*, 2009; Urra *et al.*, 2014). These authors also demonstrate that

DAT may be located on human sperm membranes and speculate that dopamine may modulate sperm parameters by mechanisms involving DAT (Ramírez *et al.*, 2009; Urra *et al.*, 2014).

This literature review will, therefore, define what dopamine is and how it functions; as well as outline the structure and functional relationships that exist between dopamine and its receptors. An in-depth analysis of DAT and how its structure relates to its functionality will be explored; and the effect of dopamine on the male reproductive system will be examined, specifically sperm parameters.

2.2 Dopamine

Catecholamines are chemicals released by the brain which transfer signals and act as hormones that regulate various physiological functions in the CNS (Laverty, 1978; Gnegy, 2012; Paravati and Warrington, 2019). Dopamine, also known as 3,4-dihydroxyphenethylamine, is the most prominent catecholamine neurotransmitter in the mammalian brain (Klein *et al.*, 2019). Dopamine participates in numerous biological processes including the control of cognition, emotion, food intake and endocrine regulation (Missale *et al.*, 1998). Dopamine is further characterised as a monoamine neurotransmitter, which are neuromodulators containing one amino group connected to an aromatic ring by a two-carbon chain (Yousuf and Kerr, 2016). Dopamine is produced in several areas of the brain, including the substantia nigra, the ventral tegmental area (VTA) and the hypothalamus, as seen in figure 1. The substantia nigra, a pigmented streak within the brain forms part of the basal ganglia and consists of three sub-regions, the pars compacta, the pars reticulata and the pars lateralis (Murray *et al.*, 2019). The substantia nigra contains a cluster of neurons that are involved in the regulation of motor movements (Kaufman and Milstein, 2013). The substantia nigra pars compacta (SNc) is the dopamine-containing region where neurons which produce this neurotransmitter project to the striatum and release the dopamine to various postsynaptic targets in this region of the brain. The striatum is the site of control for motor behaviour; the ability of the dopamine system to modulate neuronal interactions within this area is crucial to the normal functioning of the basal ganglia system (Albanese, Altavista and Rossi, 1986; Chinta and Andersen, 2005; Grace, 2008; Scarr *et al.*, 2013; Murray *et al.*, 2019). Two neural pathways, the mesolimbic and mesocortical pathways, originate in the VTA and are respectively involved in- reward and pleasure, and cognition and emotion (Wise, 2004; Scarr *et al.*, 2013; McHugh and Buckley, 2015).The hypothalamus is the site of origin of dopaminergic neurons which control the tuberoinfundibular pathway, a pathway which controls hormone secretion and regulation from or by the pituitary gland (Albanese, Altavista and Rossi, 1986; Scarr *et al.*, 2013; McHugh and Buckley, 2015).

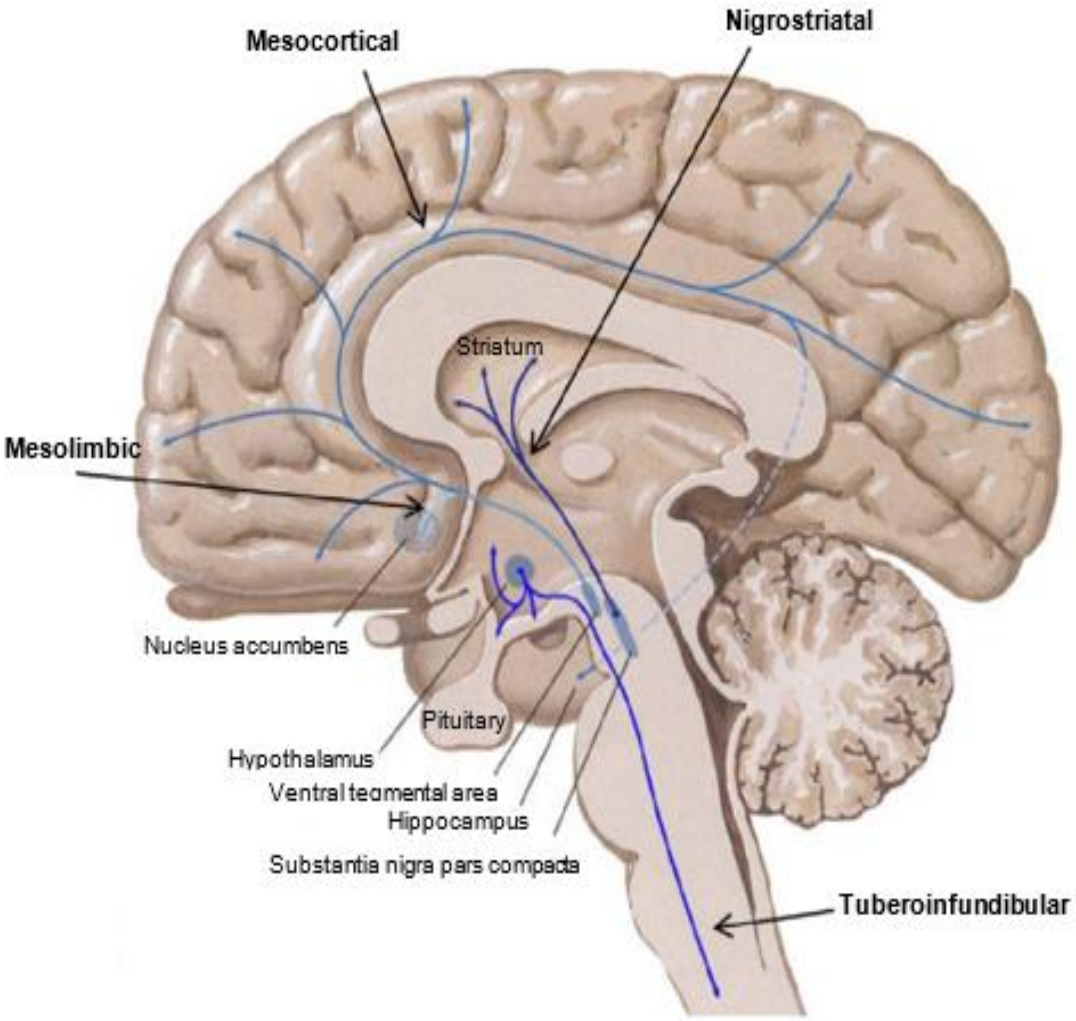


Figure 1: Dopaminergic pathways in the human brain. *(Adapted from Scarr et al., 2013; Felton, O'Banion and Maida, 2015).*

The loss of innervation of the dopamine neurons by degeneration or any mechanism which impedes the pathway to any extent, could lead to severe cognitive deficits and movement disorders (Chinta and Andersen, 2005; Grace, 2008). It is for this reason that the dopaminergic system has become a focal point in research for the past 30 years. The dysregulation of the dopamine pathway has since been implicated in several pathological conditions such as Parkinson's disease (PD), schizophrenia, hyperprolactinemia, drug addiction and attention-deficit hyperactivity disorder (ADHD) (Sotnikova *et al.*, 2006; Best, Nijhout and Reed, 2009; Rondou, Haegeman and Van Craenenbroeck, 2010).

2.2.1 The synthesis and actions of dopamine

The synthesis of catecholamines such as dopamine, epinephrine and norepinephrine are regulated by the rate-limiting enzyme Tyrosine Hydroxylase (TH) (Daubner, Le and Wang, 2011; Juárez Olguín *et al.*, 2016). Dopamine synthesis as seen in Figure 2, starts with the

amino acid Phenylalanine which is converted to Tyrosine through the phenylalanine hydroxylase enzyme. TH then converts Tyrosine to levodopa or L-3,4-dihydroxyphenylalanine (L-DOPA), a step which is subsequently followed by the conversion of L-DOPA to Dopamine via, aromatic amino acid decarboxylase (AADC), the L-DOPA decarboxylase enzyme (Kaufman and Milstein, 2013).

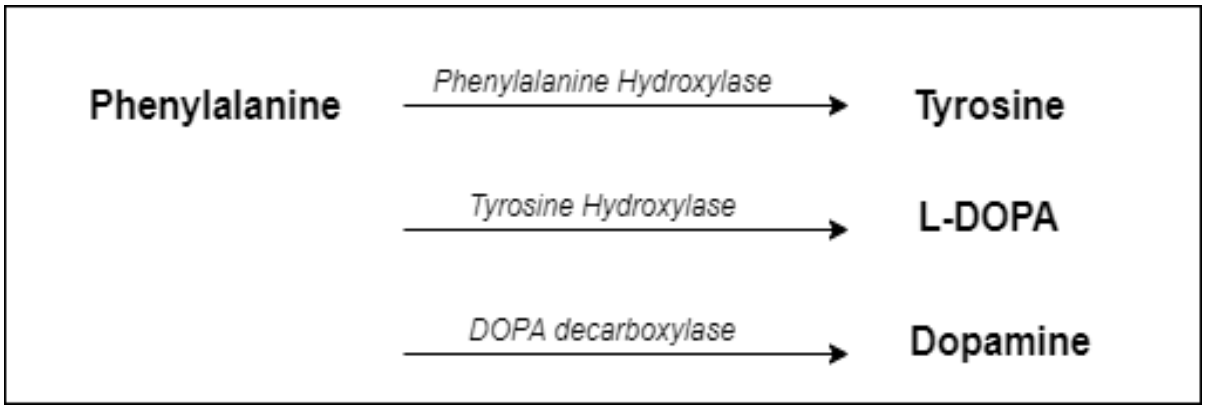


Figure 2: Synthesis of Dopamine *(Adapted from Kaufman and Milstein, 2013).*

After dopamine is synthesized, it is packaged into vesicles by vesicular monoamine transporters (VMATs), specifically VMAT2, which are integrated membrane proteins found on synaptic vesicles of presynaptic neurons as seen in figure 3 (Miller *et al.*, 1999; Best, Nijhout and Reed, 2009; Juárez Olguín *et al.*, 2016). These vesicles are stored in presynaptic neurons and await a stimulus for them to be transmitted across the synapse, whereafter the release of dopamine occurs through exocytosis (Agnati *et al.*, 1995; Juárez Olguín *et al.*, 2016). Once dopamine is released into the extracellular space (synaptic cleft) it can bind to metabotropic receptors located on postsynaptic targets or presynaptic auto receptors (Kandel *et al.*, 2000). After dopamine has bound to its extracellular targets and elicited its effect, the neurotransmitter quickly becomes unbound (Floresco *et al.*, 2003). The ‘free’ dopamine is now removed from the extracellular space and sequestered back into presynaptic cells to be recycled by a process mediated by the Dopamine Transporter (DAT) (Miller *et al.*, 1999; Schmitt, Rothman and Reith, 2013; McHugh and Buckley, 2015; Klein *et al.*, 2019). As previously mentioned, dopamine functions as both a hormone and a neurotransmitter (Danborg, Simonsen and Gotzsche, 2017). Consequently, dopamine acts as a neuromodulator, rather than a catecholamine that simply excites or inhibits targets, therefore signalling cascades are triggered by the modulation of synaptic transmission (Rondou, Haegeman and Van Craenenbroeck, 2010).

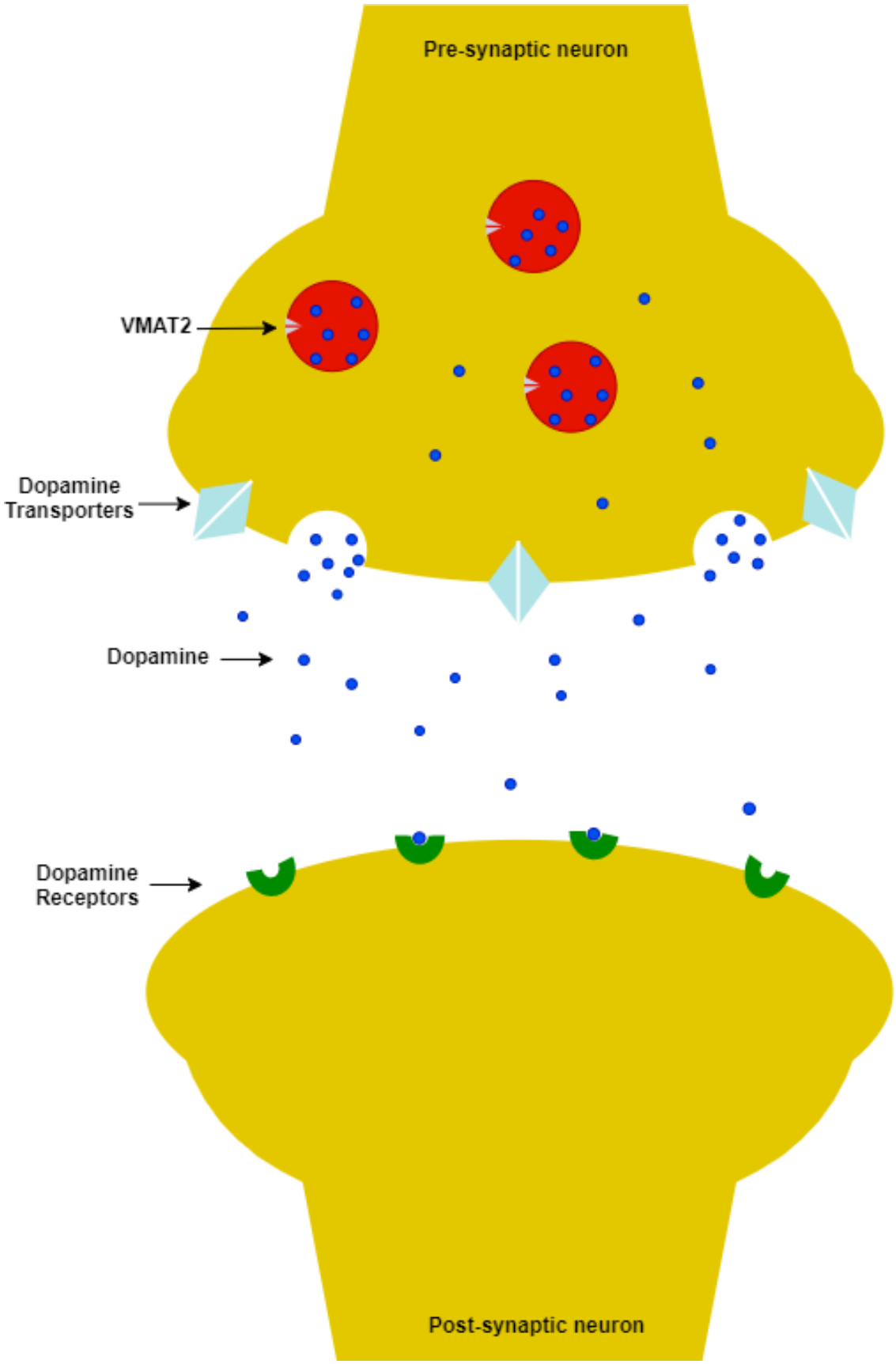


Figure 3: Release of dopamine at the synapse. *(Author's own illustration)*

Although this catecholamine has primarily been found in the brain, it has also been located in peripheral tissues where it performs several functions including the modulation of cardiovascular function, hormone secretion and gastrointestinal motility (Missale *et al.*, 1998). Since dopamine does not cross the blood-brain barrier and dopaminergic signalling within the CNS is functionally distinct from peripheral pathways, where would the dopamine found in these peripheral tissues arise from? Dopamine that has been found in peripheral tissues such as the kidney, liver, and heart, can originate from three different sources namely neuronal fibres, adrenal medulla, and neuroendocrine cells. Dopamine synthesis is therefore, both dependent and independent of neuronal elements (Rubí and Maechler, 2010). These neuroendocrine cells, called amine precursor uptake and decarboxylation (APUD) cells can produce and store biologically active neurotransmitters, by converting amino acid precursors (Norris and Carr, 2013). APUD cells have been found in the kidney, both the endocrine and exocrine pancreas and retinal cells (Wahbe *et al.*, 1982; Mappouras, Stiakakis and Fragoulis, 1990; Mezey *et al.*, 1996; Rubie *et al.*, 2001; Kubrusly *et al.*, 2008).

Vascular endothelial cells can produce dopamine, dopamine lowers blood pressure by stimulating cardiac contractility, which subsequently induces vasodilation in coronary vascular beds (Amenta *et al.*, 2002). Dopamine also acts as a natriuretic hormone by increasing the excretion of sodium in the urine thereby modulating the sodium balance and maintaining blood pressure (Rubí and Maechler, 2010). The dysregulation of dopamine-dependent mechanisms has therefore been implicated in the pathogenesis of hypertension (Zeng *et al.*, 2007). Dopamine indirectly modulates food intake and plays a role in the regulation of glucose homeostasis as well as body weight (Rubí and Maechler, 2010; Timper and Brüning, 2017). Once the hypothalamus releases dopamine, it tonically inhibits the secretion of prolactin from the pituitary and prolactin is required for the proliferation of β-cells of the pancreas and subsequently insulin secretion (Brelje, Parsons and Sorenson, 1994; Hügl and Merger, 2007; Auffret *et al.*, 2013). Additionally, the pancreatic β-cells also exhibit a dopaminergic phenotype, allowing dopamine, through the effects of metabotropic dopamine receptor D2, to actively inhibit insulin secretion (Rubi *et al.*, 2005). It is also assumed that the dopamine secreted into the small intestine, originates from the exocrine pancreas, and reduces gastrointestinal motility (Rubí and Maechler, 2010).

2.3 Dopamine Receptors

Dopamine is known to exert its modulatory effects through the binding and activation of metabotropic receptors (Seeman, 2010; Klein *et al.*, 2019). The earliest evidence of the existence of dopamine receptors came in 1972 when it was revealed that dopamine was able to stimulate the second messenger protein adenylyl cyclase (AC). In 1976 Alexander Rudolf Cools was the first person to propose the existence of multiple dopamine receptors (Cools and

Van Rossum, 1976). Earlier terms used to identify different types of dopamine receptors were alpha (α)- and beta (β)-dopaminergic receptors (Kebabian, 1978), although these terms are no longer in use since they were most easily confused with the α- and β-adrenoceptors (Kebabian and Calne, 1979). In 1979 Kebabian and Calne found that the actions of dopamine were mediated by two dopamine receptors - D1 and D2. In mammals, five subtypes of dopamine receptors have been identified, namely D1, D2, D3, D4 and D5. In humans, dopamine expresses a high binding affinity for these receptors, as well as for an additional receptor - Trace Amine-Associated Receptor 1 (TAAR1) (Seeman, 2010; Beaulieu, Espinoza and Gainetdinov, 2015; Bhatia and Saadabadi, 2019). The five subtypes of dopamine receptors can be divided into two families, namely D1-like and D2-like, based on their interaction with AC (explained later in the text) (Kebabian and Calne, 1979). The two families also differ in their binding profile and physiological effects (Weng, Li and Hsu, 2017). D1-like receptors are composed of the D1 and D5 receptors, whereas D2-like receptors are composed of the D2, D3 and D4 receptors (Mishra, Singh and Shukla, 2018). Dopamine receptors are found throughout the CNS and the relative density of the receptors are D1 > D2 > D3 > D5 > D4 (Missale *et al.*, 1998; Mishra, Singh and Shukla, 2018).

2.3.1 Dopamine Receptor structure

Dopamine receptors are seven transmembrane (TM) G protein-coupled receptors (GPCR). GPCRs form part of a huge superfamily of integral proteins which are membrane spanning. They are activated by extracellular ligands, and upon activation, pass signals to the cell interior via signal transduction pathways (Missale *et al.*, 1998; Bhatia and Saadabadi, 2019). Ligands, such as dopamine, may bind either to the extracellular N-terminus and loops or within the transmembrane helices (Trzaskowski *et al.*, 2012; Bhatia and Saadabadi, 2019). Structurally the extracellular N-terminus is connected to seven TM α-helices, which are made up of three extracellular and three intracellular loops, and a cytoplasmic C-terminus (Bhatia and Saadabadi, 2019). There are many similarities and differences between the D1-like and the D2-like receptors (Civelli, Bunzow and Grandy, 1993; Gingrich and Caron, 1993; O'Dowd, 1993; Jackson and Westlind-Danielsson, 1994). The D1 and D5 receptors share 80% homology in their TM domains, the D2 and D3 receptors share 75% homology in their TM domains and the D2 and D4 share 53% homology in their TM domains (Civelli, Bunzow and Grandy, 1993; Gingrich and Caron, 1993; O'Dowd, 1993; Jackson and Westlind-Danielsson, 1994). The D1 and D5 receptor subtypes each possess two N-linked glycosylation sites whereas the D2 receptor contains four such sites, the D3 has three, and the D4 possesses only one such site (Missale *et al.*, 1998). N-linked glycosylation is the attachment of an oligosaccharide or glycan to the nitrogen atom on the asparagine (N) residue of a protein - this

process is enzyme catalysed and can influence the protein folding or the stability of the glycoprotein it is coupled to (Imperiali and O'Connor, 1999; Ranganathan, Wongsai and Helena Nevalainen, 2006). In the D1-like receptor family the COOH terminal is about seven times longer than that of the D2-like receptors (Civelli, Bunzow and Grandy, 1993; Gingrich and Caron, 1993; O'Dowd, 1993; Jackson and Westlind-Danielsson, 1994). Both the D1-like and the D2-like receptors express two cysteine residues in extracellular loops 2nd and 3rd, which has been suggested to act as a stabilizer by forming a disulphide bridge (Fraser, 1989; Dohlman *et al.*, 1990).

2.3.2 Dopamine Receptor function and mechanism of action

Dopamine receptors regulate many functions in the human body, in particular motor and non-motor functions (Mishra, Singh and Shukla, 2018). The effects of these receptors occur via signal transduction pathways and are mediated by the type of dopamine receptor activated. The two families of dopamine receptors, although all GPCRs, have different mechanisms of action. Once activated by ligands, these receptors undergo a conformational change to an active state - the receptor then reorientates the side chains of amino acids within the TM domain, causing a conformational change for G protein recruitment to the intracellular side (Trzaskowski *et al.*, 2012; Weng, Li and Hsu, 2017). The primary distinction between the two dopamine receptor families is the positive and negative coupling to AC, where D1-like receptors increase the level of cyclic adenosine monophosphate (cAMP) by activating AC and D2-like receptors decrease the level of cAMP by inhibiting AC (Missale *et al.*, 1998). Once a ligand binds, guanosine nucleotide-binding proteins (G proteins) either activate or inhibit AC, and in doing so, affect the levels of second messengers, such as cAMP, Diacylglycerol (DAG) and Inositol triphosphate (IP_3), produced.

A pivotal requirement to discern between the individual receptor subtypes' functions is the identification of their agonists and antagonists (Missale *et al.*, 1998; Klein *et al.*, 2019). Both receptors in the D1-like family are linked to stimulatory G-proteins and express near identical pharmacological profiles, but generally antagonists present a higher affinity for the D1 than for the D5 receptor (Tiberi *et al.*, 1991). The D1 receptor is the most abundant dopamine receptor in the brain and specifically plays an integral role in the regulation of the reward system, memory, attention, and locomotive activity. (Daly and Salloway, 1994; Mishra, Singh and Shukla, 2018). Similarly, the D5 receptor regulates cognition, attention and decision making, although dopamine was found to have a 10-fold higher affinity for D5 receptors in comparison to D1 receptors (Daly and Salloway, 1994; Mishra, Singh and Shukla, 2018). All the D2-like receptors are linked to inhibitory G-proteins. D2 regulates learning and memory, as well as

sleep and reproductive behaviour (Mishra, Singh and Shukla, 2018). D2 receptors can be localized both pre- and post-synaptically and is of particular interest in pharmacological studies as it is the main receptor for most antipsychotic drugs (Seeman and Lee, 1975; Creese, Burt and Snyder, 1976; Meltzer, Matsubara and Lee, 1989).

Researchers have found that the D2 receptor exists in two isoforms: the long form (D2L) and the short form (D2S) (Daly and Salloway, 1994; Picetti *et al.*, 1997; Mishra, Singh and Shukla, 2018). The long form functions as a traditional postsynaptic receptor while the short form is localized pre-synaptically and acts as an auto receptor, which regulates the levels of dopamine in the synaptic cleft (Beaulieu and Gainetdinov, 2011). The long form differs from the short form with the insertion of 29 amino acids on the third intracellular loop, the loop at which the receptor couples to different G proteins (Picetti *et al.*, 1997). Although found to exhibit minor differences in affinities for certain G-proteins (Daly and Salloway, 1994), analysis of their pharmacological profiles shows that there are no compounds (agonists and antagonists) which discern any differences between the long and short forms of the D2 receptors (Missale *et al.*, 1998). D2 receptors were shown to undergo homodimerization where TM 4 was the main site of interaction (Guo, Shi and Javitch, 2003; Lee *et al.*, 2003). Evidently dopamine has been suggested to play a role in cell proliferation control and cell survival via mechanisms involving the D2 receptor activation, interestingly as activation of dopamine receptors exerts an inhibitory effect on cancer growth (Ishibashi *et al.*, 1994; Rubí and Maechler, 2010). This paradoxical effect has previously been observed by endogenous substances, endocannabinoids, by binding to cannabinoid CB1 receptors they suppress cancer cell proliferation (De Petrocellis *et al.*, 1998; Velasco *et al.*, 2007). Through the mechanism of receptor crosstalk, CB1 and D2 receptors have been shown to form functional complexes, where they act as heterodimers (Kearn *et al.*, 2005; Navarro *et al.*, 2010). The CB1 and D2 receptors are linked to stimulatory G proteins and inhibitory G proteins, respectively, thus, the consequence of this interaction is the shift of signalling in favour of CB1 receptor signalling, by mechanisms involving Extracellular Signalling Kinases (ERK 1/2) (Kearn *et al.*, 2005).

The D3 and D4 receptors seem to be pharmacologically similar to the D2 receptor and also function by inhibiting AC and thereby decreasing the levels of cAMP. However, D3 appears to affect AC very weakly (Daly and Salloway, 1994; Missale *et al.*, 1998). D3 receptors are generally found in areas of the brain which regulate impulse control, cognition, and locomotive activity, which may indicate its mediatory effect on drug-seeking behaviour (Le Foll *et al.*, 2014). Dopamine was found to have a 20-fold higher affinity for the D3 receptor than the D2 receptor (Sokoloff *et al.*, 1990). The D4 receptor functions are very similar to the other D2-like receptor subtypes- regulation of cognition, impulse control, sleep, and reproductive behaviours (Mishra, Singh and Shukla, 2018).

2.4 Regulation of Dopamine

The regulation of dopamine is controlled by several different factors. These include the quantity in which it is synthesized and released, the number of dopamine receptors (DR's) onto which dopamine can bind, and the termination of dopamine activity at the synapse via several proteins (McHugh and Buckley, 2015). These proteins include Monoamine Oxidase (MAO), Catechol-O-methyltransferase (COMT) and Aldehyde Dehydrogenase (ALDH) (Shih and Thompson, 1999; Kaufman and Milstein, 2013; Juárez Olguín *et al.*, 2016). MAO has been implicated in the pathogenesis of PD. It metabolizes free dopamine within the presynaptic neuron to produce highly toxic and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and 3,4-dihydroxyphenylacetaldehyde (DOPAL) (Doorn *et al.*, 2014). While ALDH is responsible for maintaining subcellular homeostasis by metabolizing endogenous and exogenous reactive compounds (Wang and He, 2018). It further metabolizes DOPAL to 3,4-dihyroxyphenylacetic acid (DOPAC) (Doorn *et al.*, 2014). Furthermore, DOPAC is then finally metabolized by COMT to Homovanillic Acid (HVA) (Eisenhofer, Kopin and Goldstein, 2004; Kaufman and Milstein, 2013). COMT is involved in the inactivation of dopamine by degradation mainly in the extracellular space. COMT is of particular interest as there has been much controversy regarding the orientation of membrane-bound COMT (MB-COMT) in the CNS (Schott *et al.*, 2010; Chen *et al.*, 2011), as well as how its activation pathway is mediated. One study performed by Schott *et al.,* in 2010, on cultured neocortical neurons, found that the MB-COMT was found to be orientated intracellularly. They then suggested that there may be a DAT1-independent postsynaptic uptake mechanism for dopamine, prior to its degradation via COMT. However, another study has concluded that the catalytic domain of MB-COMT is found in the extracellular space, which suggests that MB-COMT may metabolize dopamine synaptically and extrasynaptically on the surface of presynaptic and postsynaptic neurons (Chen *et al.*, 2011). This suggests that these enzymes not only play a major role in the recycling of the individual components of dopamine, but also metabolize dopamine and keep the concentration of dopamine lower than the rate at which it is being produced (Shih and Thompson, 1999).

TAAR1 is a GPCR which regulates monoaminergic transmission and exhibits an inhibitory effect on dopamine transmission upon stimulation by an agonist (Miller, 2011; Asif-Malik, Hoener and Canales, 2017). TAAR1 and dopamine D2 auto receptors are known to form heterodimers and therefore modulate dopamine signalling by altering the transport capacity of DAT (Harmeier *et al.*, 2015). DAT is responsible for the reuptake of dopamine from the synaptic cleft into the presynaptic neuron when an excess of dopamine is detected in the synaptic cleft (Schmitt, Rothman and Reith, 2013; McHugh and Buckley, 2015; Klein *et al.*, 2019). DAT is therefore the target of addictive drugs such as cocaine, amphetamine (AMPH),

methamphetamine (METH) and Ritalin (Vaughan and Foster, 2013) as the inhibition of this transporter leads to an accumulation of dopamine at the synaptic cleft, making it more available for the downstream effects of DA receptor activation. It is however important to note that another protein, the Norepinephrine Transporter (NET), may also be partially responsible for some reuptake of dopamine (Horn, 1973; Raiteri *et al.*, 1977), which may have a higher affinity for dopamine in some areas of the brain than DAT itself (Giross *et al.*, 1994; Gu, Wall and Rudnick, 1994; Eshleman *et al.*, 1999).

2.5 Dopamine Transporter

As mentioned, DAT is responsible for the reuptake of dopamine from the synaptic cleft into the presynaptic neuron. Similar to the dopamine receptor, DAT is an integral protein, spanning the plasma membrane where it interacts with extracellular dopamine (Nirenberg *et al.*, 1996). It is a Na^+ and Cl^- dependent transporter which forms part of the solute carrier (SLC) family and is denoted as solute carrier 6 member 3 (SLC6A3). DAT forms part of a superfamily of transporter proteins called the neurotransmitter transporters (NTT), it displays amino acid homology with other neurotransmitter transporters, namely the norepinephrine transporter (NET) serotonin transporter (SERT) and gamma (γ)-amino butyric acid (GABA) transporter (Chen and Reith, 2000; Pramod *et al.*, 2013). It has the highest homology with NET but less so with SERT and the GABA transporter. DAT has been localized in areas of the brain with established dopaminergic circuitry e.g., mesocortical and mesolimbic pathways (Nirenberg *et al.*, 1996; Ciliax *et al.*, 1999). DAT levels in the brain may vary from normal - this could be attributed to a range of factors such as - degeneration of dopamine neurons, hyper-innervation of these neurons and drug-induced alterations of their neural networks (Miller and Madras, 2002). In the mid-1950's Julius Axelrod was the first to propose that reuptake was an important mechanism for the inactivation of neurotransmitters including dopamine. It would take another 30 years for the genes that encode these transporters, which are responsible for monoamine reuptake, to be identified (Torres, Gainetdinov and Caron, 2003).

2.5.1 DAT genetics

The human DAT gene (DAT1) has been localized on chromosome 5 (Giros and Mestikawy, 1992; Vandenbergh *et al.*, 1992). This gene spans over 64kB and consists of 15 coding exons separated by 14 non-coding introns (Figure 4) (Kawarai *et al.*, 1997). Polymorphisms within the DAT gene have been of particular interest through the decades, as heterogeneity in this gene may influence the functionality thereof and its expression levels within the brain and other organs (Miller and Madras, 2002). These polymorphisms are also thought to be the route of

many disorders related to dopamine. On the 3' Untranslated Region (UTR) of the DAT gene lies a variable number of tandem repeats (VNTR) which spans 40-base pairs (Vandenbergh *et al.*, 1992; Miller and Madras, 2002). This is the most extensively studied polymorphism within the DAT gene, alleles of this VNTR sequence range from 3 to 13 repeats (Bannon *et al.*, 2001). Previously differences in the VNTR of the gene have been shown to affect basal levels of transporter expression and functionality (Vandenbergh *et al.*, 1992; Miller and Madras, 2002). Interesting to note is that as the VNTR lies within the 3' UTR, a non-coding region, allelic variants of the gene should not result in any structural or functional differences within the protein. However, it was revealed that VNTR's can function as transcriptional and translational regulators (Nakamura, Koyama and Matsushima, 1998; Mill *et al.*, 2002). Investigations to link the number of tandem repeat units to various diseases has led to the revelation that an association may exist between VNTR polymorphisms; the 10-repeat length is associated with ADHD, whereas the 9-repeat length has been associated with alcohol and nicotine addictions (Cook *et al.*, 1995; Muramatsu and Higuchi, 1995; Miller and Madras, 2002). However, a systematic review and meta-analysis performed by Tunbridge *et al.,* in 2019 argues against any associations between the 3' UTR VNTR and various disease states. They conclude, from their findings, that the VNTR could not play a major role in influencing DAT function (Tunbridge *et al.*, 2019). Various single nucleotide polymorphisms (SNP) along with rare conservative amino acid substitutions have been identified within the coding region of the human DAT (hDAT) gene. However, these were all found to be silent subsequently leading to no change in functionality of the protein or its expression (Bannon *et al.*, 2001). Five SNPs were identified within the 5'UTR. Investigations have led to the discovery of possible associations between these polymorphisms and disease states, such as schizophrenia. However, authors support the undertaking of further research to confirm the associations between the polymorphisms within this region of the gene and the functional expression of this protein in disease states (Sacchetti *et al.*, 1999; Rubie *et al.*, 2001; Stöber *et al.*, 2006).

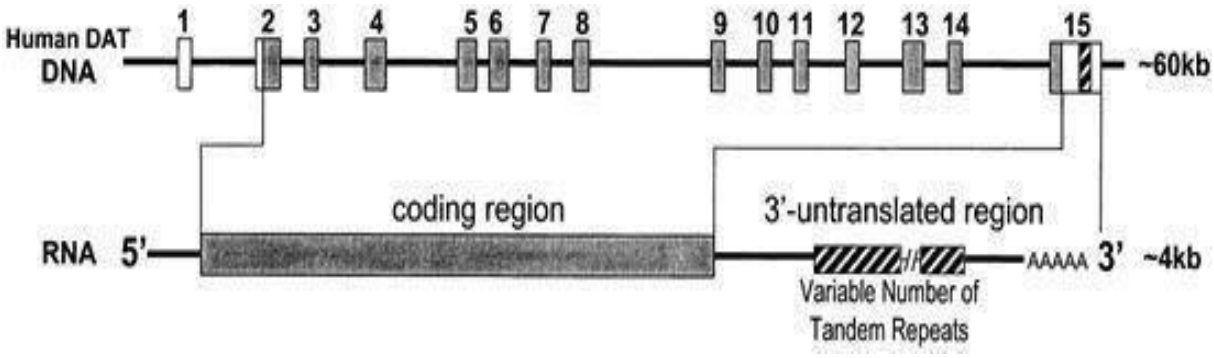


Figure 4: Schematic representation of the human dopamine transporter gene. Shaded exons in the DNA form the coding region, white exons contribute to the 5′ and 3′ non-coding regions. The 3′-untranslated region of the RNA is derived from a major portion of exon 15 and includes a variable number tandem repeat (VNTR) region (stripes) *(Miller and Madras, 2002).*

2.5.2 DAT structure

The identification of NTTs such as DAT was built on the elucidation of the high-resolution structure of a bacterial leucine transporter (LeuT) first identified in 2005 (Yamashita *et al.*, 2005). Although LeuT and NTTs only possess a 20-25% homology with each other the use of LeuT as a structural template for NTTs is fundamental in the understanding of their structure-function relationships (Kristensen *et al.*, 2011; Pramod *et al.*, 2013). NTTs have been shown to be highly homologous and therefore share a comparable general structure (Kristensen *et al.*, 2011). Photoaffinity labelling studies show that DAT is a glycoprotein with a molecular weight of between 60kDa and 80kDa, when in its native form in neuronal membranes (Sallee *et al.*, 1989; Milner, Béliveau and Jarvis, 1994; Chen and Reith, 2000; McHugh and Buckley, 2015; Foster and Vaughan, 2017). The protein, 620 amino acids in length, is composed of 12 TM helices, which are connected by alternating extracellular and intracellular loops (Figure 5). DAT contains six extracellular and five intracellular loops (Figure 5). TMs 1, 2 and 8 are conserved across DAT, NET and SERT (Madras, Miller and Fischman, 2005). The hDAT displays a 92% homogeneity with the rat dopamine transporter (rDAT) and an 84% homogeneity to the bovine DAT (bDAT) (Giros and Mestikawy, 1992).

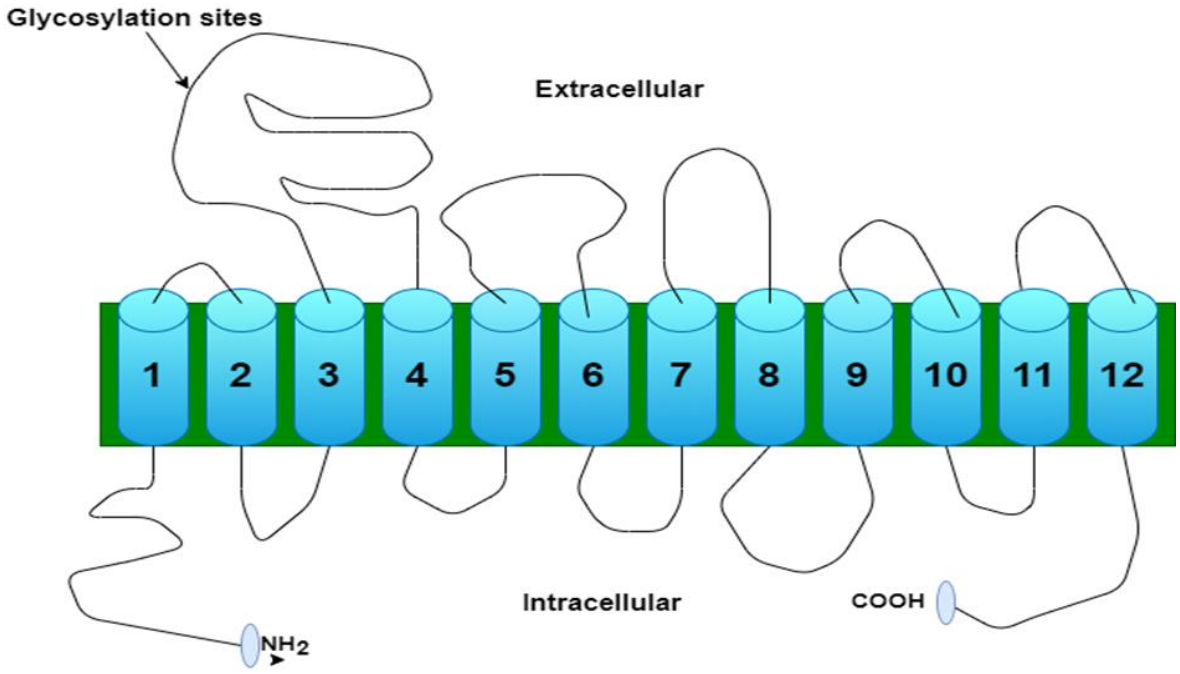


Figure 5*:* Simplified model of the Dopamine Transporter (DAT) protein structure. The 12 transmembrane (TM)-spanning hydrophobic domains are depicted as blue cylinders, linked by amino acid chains. The amino- and carboxyl terminals are located intracellularly, and N-linked glycosylation sites can be located within the large second extracellular loop. *(Adapted from Madras, Miller and Fischman (2005).*

Nearly all the TMs in DAT are α-helical except for unwound segments of TM1 and TM6 which form the core for the active site of the protein and separate the helices into functional segments (Kristensen *et al.*, 2011; Vaughan and Foster, 2013). Several α-helical structures are located within the intracellular and extracellular loops while TM helices 1, 3, 6, and 8 form the substrate permeation pathway or substrate binding site (S1) (Chen and Reith, 2000; Kristensen *et al.*, 2011; Vaughan and Foster, 2013). The movement of the substrate i.e., Dopamine is controlled by intracellular and extracellular gates situated directly above and below the active site, which also dictate the inwardly or outwardly facing conformations of the protein (Vaughan and Foster, 2013). Two Na^+ binding sites, Na1 and Na2, along with a Cl^- binding site were located near the S1 binding site, supporting the idea that DAT translocates its substrates along with Na^+ and Cl^- ions (Kristensen *et al.*, 2011).

In X-ray crystallography studies, LeuT was structurally shown to adopt a unique folding pattern. This structural hallmark was critical to understanding how the structure-function relationship works and it was dubbed the LeuT fold (Forrest and Rudnick, 2009; Kristensen *et al.*, 2011). The LeuT fold is described as an inverted structural repeat, denoted as the '5 + 5 inverted repeat'. DAT was shown to present with the same fold along with many other NTTs (Cheng and Bahar, 2015). The 5 + 5 architecture of the LeuT fold reveals that TMs 1-5 and TMs 6-10 forms two bundles of 5 helices aligned invertedly to one another (Figure.6). The TMs 11 and 12 lie on the periphery of the 5 + 5 core and are assumed to play a role in regulation but not in transportation of substrates (Forrest and Rudnick, 2009; Kristensen *et al.*, 2011; Pramod *et al.*, 2013). It was observed that the inverted repeat structure contributes to the functionality of the transporter, as it allows the protein to change conformation (Forrest and Rudnick, 2009; Kristensen *et al.*, 2011; Pramod *et al.*, 2013). The LeuT structure identified in 2005 dictated an extracellularly orientated protein whereby the substrate binding site was only accessible from this side of the membrane. However, a model of the LeuT, where the two the bundles of TMs helices (1-5 and 6-10) were simply swapped, was generated - this revealed a cytoplasmic access pathway to the central binding site and simultaneously the extracellular pathway to this site became occluded (Forrest and Rudnick, 2009).

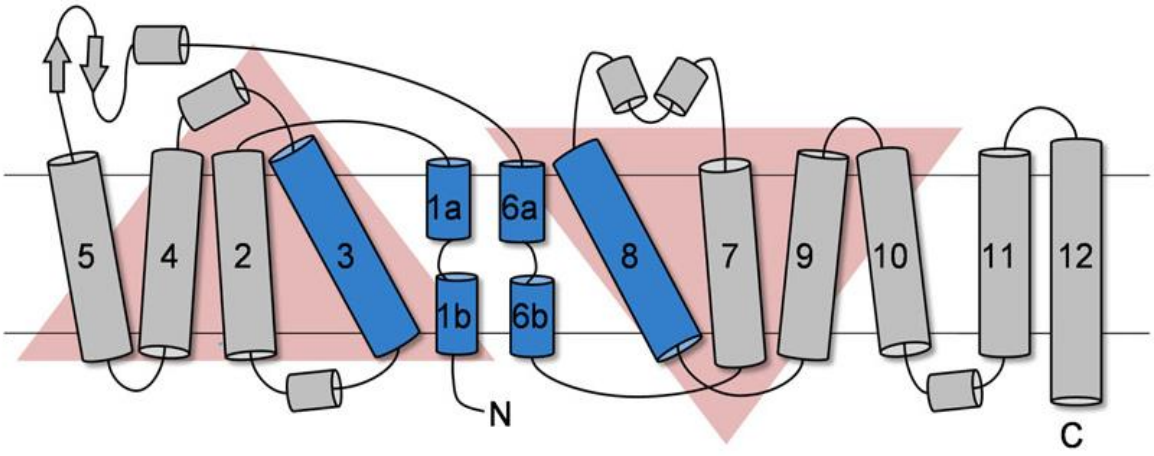


Figure 6: Schematic of the topology of LeuT with the inner ring (TM1, TM3, TM6, and TM8) that forms the substrate binding site shown in blue. The red triangles highlight the 5 + 5 inverted repeats, formed by TMs 1 to 5 and TMs 6 to 10, that are related by an apparent two-fold symmetry. *(Kristensen et al., 2011)*

Three N-linked glycosylation sites can be localized within the large second extracellular loop. The glycosylation sites can undergo phosphorylation and appear to be essential for the transport efficiency of DAT, as well as its stable membrane expression (Lin et al., 1999; Li et al., 2004; McHugh and Buckley, 2015). Important to note is that microheterogeneity exists within DAT, where differences have been observed in the content of the N-linked glycosylation sites (Lew et al., 1992; Patel, Uhl and Kuhar, 1993; Vaughan et al., 1996). DAT has been shown to be expressed in cells as either a mature or immature protein, the latter being either partially glycosylated or non-glycosylated (Daniels and Amara, 1999; Hastrup, Karlin and Javitch, 2001). Lew and colleagues have shown that DAT presents as different molecular sized proteins in the nucleus accumbens and the striatum of rat brains, with the striatum presenting a slightly lower molecular weight band (Lew et al., 1992). In addition to the microheterogeneity found within the different regions of a specific target organ (i.e., the brain), species-specific differences also exist. These species-specific variances could be attributed to the difference in the number of predicted N-linked glycosylation sites between rats and humans, for example (Patel, Uhl and Kuhar, 1993; Vaughan et al., 1996). A study by Nguyen and Amara demonstrated that N-linked oligosaccharides are required for the cell surface expression of monoamine transporter, NET, however they do not influence the affinity of substrates or inhibitors for transport by this protein (Nguyen and Amara, 1996). The question of whether glycosylation influences DAT function remained. In 2004 Li and colleagues demonstrated through site-directed mutagenesis that both glycosylated and non-glycosylated DAT (mature and immature) appear to be functional (reaches the surface membrane), albeit the non-glycosylated form functions at a slightly lower efficiency. The non-glycosylated DAT was suggested to be more susceptible to proteolytic degradation before

being able to reach the surface membrane (Li et al., 2004). N-linked glycosylation regulation of DAT might function to facilitate the formation of the homo-and hetero-oligomeric protein interactions, however non-glycosylated DAT monomers are still able to form homo-oligomers (Hastrup, Karlin and Javitch, 2001; Li et al., 2004).

Also localized on the second extracellular loop is a disulphide bond, thought to play a role in the biosynthesis of the transporter (Chen *et al.*, 2007). Large N- and C- terminal domains are located in the cytosol and contain sites for post translational modifications and regulatory binding partners such as Syntaxin 1A (Syn1A) and D2 Dopamine Receptors (Vaughan and Foster, 2013). Structurally, DAT expresses multiple phosphorylation sites (serine, threonine, and tyrosine) in the cytoplasm (Vaughan and Foster, 2013).

2.5.3 DAT function and mechanism of action

As dopamine is known to be an essential modulatory neurotransmitter, DAT plays a crucial role in dopamine homeostasis (McHugh and Buckley, 2015). DAT is a monoamine transporter and is responsible for the clearance of dopamine in the synaptic cleft, thereby terminating the signal of this neurotransmitter. For decades, the mechanism behind the transport of solutes across membranes has been modelled after the alternating access mechanism. In this model, solutes can bind to sites on both sides of the membrane as it may undergo conformational changes to face inwardly or outwardly; this conformational change allows for alternating access to the substrate binding site. (Forrest *et al.*, 2008; Forrest and Rudnick, 2009). Figure 7 depicts the alternating access mechanism. The alternating access mechanism allows for many forms of transport: uniport, displayed by glucose transporters in cells; antiport, where solutes are transported across the membrane, but before it can reorientate itself, another solute binds and is transported in the opposite direction; symport, which involves the transport of solutes across the membrane in the same direction (Forrest and Rudnick, 2009). For a conformational change to occur, an intermediate state is likely to exist in transporters. In this case, the intermediate state is called the occluded state, where the substrate binding site is inaccessible from either side of the membrane, ensuring that the transporter does not become a channel through the membrane (Forrest and Rudnick, 2009).

DAT is a symporter, which couples the movement of dopamine across the plasma membrane to the movement of two Na^+ ions and one Cl^- ion against their concentration gradients. The ion-concentration gradient generated by the Na^+/K^+ ATPase pump is the driving force for DAT-mediated dopamine reuptake (Torres, Gainetdinov and Caron, 2003). Sodium and chloride ions bind to their sites within the transporter, whereafter dopamine binds to S1. Once dopamine

binds, the protein undergoes a conformational change, which allows the ions and dopamine to dissociate on the other side of the membrane (Forrest and Rudnick, 2009).

Substrate translocation is tightly controlled and follows a strict set of steps, whereby the transporter changes conformation and the substrates dissociate. The rocking bundle mechanism has been proposed to account for the transition of the outwardly facing conformation to the inwardly facing conformation (Forrest and Rudnick, 2009). TM's 1 and 6 form a four-helix bundle along with TM's 2 and 7 and the remaining TM's 3, 4, 5, 8, 9 and 10 form a scaffold around this bundle. The simple rocking of this bundle in relation to the scaffold results in the transition between two alternate orientations and would therefore open or close the pathway to S1 (Forrest *et al.*, 2008; Forrest and Rudnick, 2009; Pramod *et al.*, 2013). Occupation of S1 by the substrate leads to the outwardly facing occluded state whereby the substrate is trapped. The protein rearranges to the inwardly facing conformation whereby S1 is orientated towards the cytoplasm and the substrate dissociates, thus conformational changes are under the control of ligand binding (Forrest and Rudnick, 2009; Kristensen *et al.*, 2011).

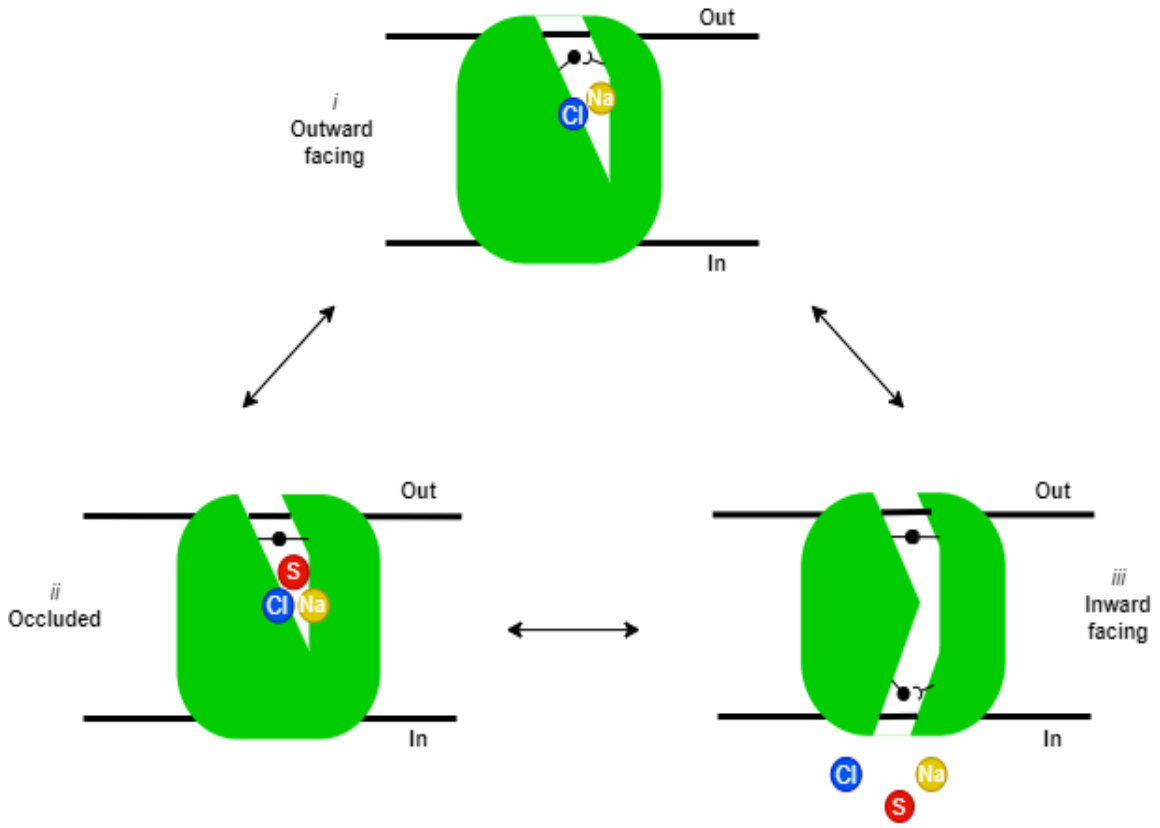


Figure 7: Illustration of alternating access and channel modes of transport. Substrate (S) and ions (Na and Cl) access the binding site of the outward facing transporter (i) via the open outer gate (black sticks); the outer gate closes yielding the occluded state (ii) which then transitions to the inward facing state (iii), releasing the substrate and ions to cytosol. The transporter then rectifies to the outward facing structure (i). *(Adapted from Pramod et al., 2013)*

Initially literature focused on DAT functioning as a monomer, however recent studies suggest that DAT may undergo oligomerization. This may impact upon protein functions by regulating protein translocation to the membrane affecting DAT transport efficiency (Pramod *et al.*, 2013; Cheng *et al.*, 2019). DAT has subsequently been shown to form dimers, trimers and even tetramers via cross-linking, radiation inactivation and co-immunoprecipitation studies (Zhen and Reith, 2018). Cross-linking experiments in mouse brain striatal membranes showed that DAT may exist as a dimer in the plasma membrane (Hastrup, Karlin and Javitch, 2001). Through radiation inactivation studies canine brain striatal membranes yielded protein sizes of 278±16kDa; this along with other studies is indicative that the neuronal native form of DAT is ~70kDa and is assembled in a tetramer (Berger *et al.*, 1994; Milner, Béliveau and Jarvis, 1994). It was found that DAT possesses two distinct symmetrical interfaces which interact with one another to form a dimer of a dimer, or simply a tetramer (Hastrup, Sen and Javitch, 2003). The assembly of the monomers of DAT into oligomers was found to play a critical role in the functioning and expression of the protein (Torres *et al.*, 2003). DAT oligomers were found to cooperate because one protomer can influence the properties of another protomer of DAT (Sitte, Schütz and Freissmuth, 2015). The functional role of DAT heterooligomers was investigated with cocaine analogues. When DAT contained different combinations of promoters with different affinities for inhibitors, only one protomer of an oligomer was found to be active at a time, and the binding affinity of a protomer in this organization may be influenced by the active protomer. Therefore, the active protomer influences the other protomers affinity for inhibitors (Zhen *et al.*, 2015). However, despite the knowledge of cooperativity for inhibitor affinity, it was not known whether the oligomeric organization of DAT is required for the proper uptake of dopamine or whether the individual protomers function independently to translocate dopamine across the plasma membrane (Zhen and Reith, 2018). Zhen and Reith demonstrated, through cross-linking agent Copper Phenanthroline (CuP), that only one protomer of DAT is active at a time, thus a dimer of DAT functions only at 75% of what a monomer (protomer) functions at. Therefore, the oligomeric state of DAT regulates the function of the protein (Zhen and Reith, 2018).

2.5.4 DAT Regulation

DAT protein regulation is well established as it is an important feature for the modulation of dopamine clearance (Vaughan and Foster, 2013). The activity of the transporter is regulated at post-translational levels. Through various post-translational modifications (PTMs) including N-linked glycosylation- PTMs can modulate the rate at which the transporter moves dopamine or causes the internalization of the transporter (Torres *et al.*, 2003; Vaughan and Foster, 2013; German *et al.*, 2015). DAT may also form protein interactions which are involved in the

regulation and trafficking of DAT to the plasma membrane (German *et al.*, 2015). Multiple kinases play important roles in the regulation of DAT; these include protein kinase C (PKC), $Ca2^{+}$/calmodulin-dependent kinase II (CaMKII), Mitogen Activated Protein Kinase (MAPK) and ERK (Pramod *et al.*, 2013; Vaughan and Foster, 2013) as seen in figure 8. Although multiple phosphorylation sites are structurally situated on the intracellular domains, it appears these sites are not directly phosphorylated, instead, DAT is phosphorylated at non-consensus sites (Chen and Reith, 2000). Many of the DAT regulatory mechanisms overlap, as protein-protein interactions often result in PTMs and the localization of DAT on the plasma membrane is dictated by the various PTMs (German *et al.*, 2015). A brief description of some of the important regulatory mechanisms is described below in Table 1. “DAT regulation mediated by signalling systems is complex, with some functions regulated by more than one pathway and some pathways regulating more than one function, indicating the presence of convergent and divergent mechanisms.” (Vaughan and Foster, 2013).

Table 1: Brief description of DAT regulatory mechanisms

	Regulatory mechanism	**Mechanism of action and Effect on DAT**	**Citation**
N-linked glycosylation	Post Translation modification	Stabilizes DAT location on plasma membrane and increase dopamine transport rate	(Lew *et al.*, 1992) (Nguyen and Amara, 1996) (Li *et al.*, 2004)
PKC	Post Translation modification	Downregulation of dopamine transport capacity, enhancement of dopamine efflux and regulation of endocytosis of DAT	(Daniels and Amara, 1999) (Melikian and Buckley, 1999) (Vaughan and Foster, 2013) (Foster and Vaughan, 2017)
ERK	Post Translational modification	Upregulates dopamine transport capacity and increases DAT surface levels.	(Morón *et al.*, 2003) (Bolan *et al.*, 2007) (Vaughan and Foster, 2013) (Foster and Vaughan, 2017)

CAMKII	Post Translational modification	Enhances dopamine efflux through mechanisms involving phosphorylation of serine residues on the distal portion of the N-terminus	(Fog *et al.*, 2006) (Gorentla *et al.*, 2009) (Vaughan and Foster, 2013)
MAPK	Post Translational modification	Regulates the transport capacity and intracellular trafficking of DAT	(Morón *et al.*, 2003) (Vaughan and Foster, 2013)
TAAR1	Protein-Protein Interaction	Downregulation of DAT by activation of TAAR1 through mechanisms involving PKC and PKA activation, causes efflux of dopamine	(Xie and Miller, 2007) (Miller, 2011) (Asif-Malik, Hoener and Canales, 2017) (Leo and Espinoza, 2016)
D2 Auto receptors	Protein-Protein Interaction	Upregulation of DAT to surface membranes by mechanisms involving ERK1/2 activation,	(Brami-Cherrier *et al.*, 2002) (Bolan *et al.*, 2007) (German *et al.*, 2015)
Drug exposure	Transporter localization	Acute exposure to AMPH or METH upregulates DAT and increases DAT surface levels. Longer exposure downregulates DAT and promotes efflux and endocytosis, through mechanisms involving the activation of PKC and CaMKII.	(Fog *et al.*, 2006) (Vaughan and Foster, 2013) (German *et al.*, 2015) (Foster and Vaughan, 2017)

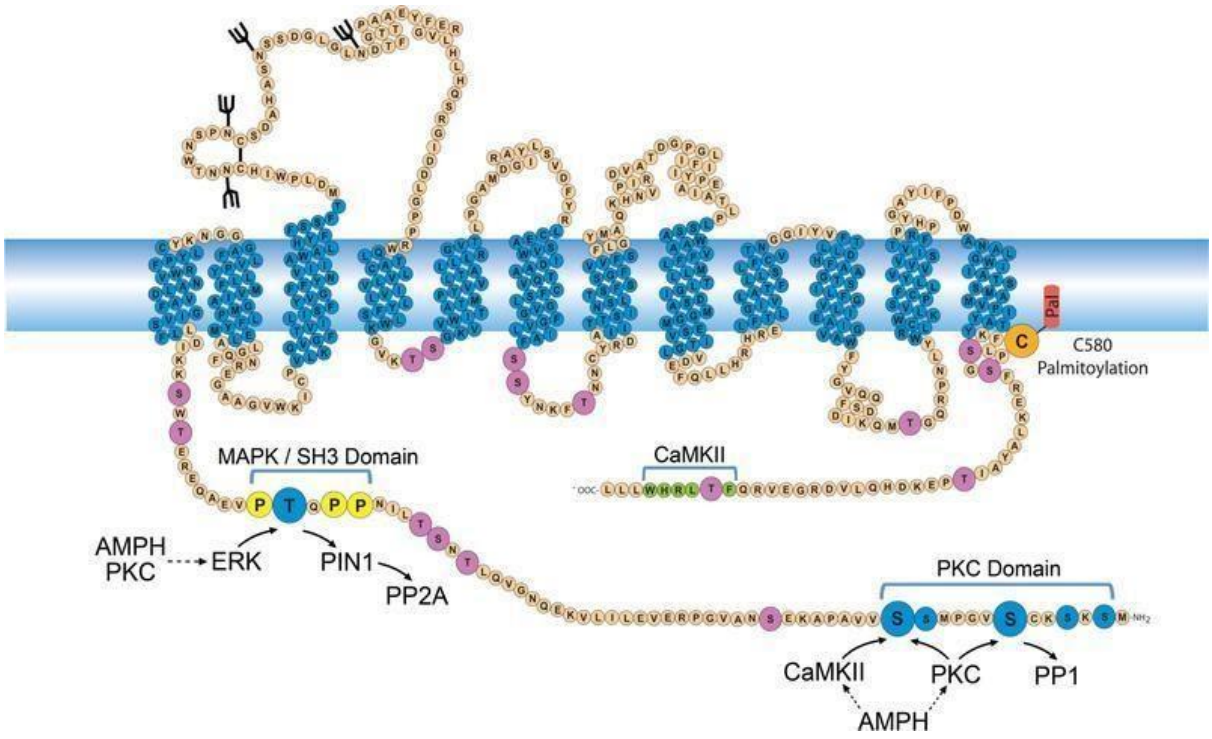


Figure 8: Schematic diagram of rat DAT (rDAT) showing the phosphorylation characteristics of DAT. PKC and MAPK domain phosphorylation sites in blue, with Ser7, Ser13, and Thr53 residues highlighted with large circles. Other intracellular Ser (S) and Thr (T) residues are shown in mauve, prolines flanking Thr53 that constitute an SH3 binding domain are shown in yellow (P), the CAMKII binding domain in the C-terminus is shown in green, and palmitoylation site Cys580 is shown in orange. Known and suspected kinase, phosphatase, and PIN1 inputs into Ser7, Ser13 and Thr53 are indicated with arrows, and dashed lines show indirect AMPH and PKC inputs into each site *(Foster and Vaughan, 2017).*

2.6 Effect of Dopamine on Male Reproduction

It is believed that the peripheral catecholaminergic system plays a role in the homeostasis of numerous organ functions (Peyrin and Dalmaz, 1975; Goldstein, 1997). In the periphery the major source of catecholamines is the sympathetic nervous system (Peyrin and Dalmaz, 1975). As previously stated, dopamine found in peripheral tissues is released from neuronal cells or it may be synthesized within specific parenchyma of these tissues i.e., hepatocytes and the renal medulla via APUD cells (Rubí and Maechler, 2010). The adrenal glands are an alternative source of catecholamine production, as chromaffin cells are APUD cells because of their ability to store and synthesize neurotransmitters (Brainard and Mandell, 2009; Carbone *et al.*, 2019). In the adrenal medulla, dopamine can be transformed into other catecholamines such as norepinephrine and epinephrine (Rondou, Haegeman and Van Craenenbroeck, 2010). The activity of sympathetic nerves predominantly controls the levels of dopamine found in the plasma. It may be released as the precursor molecule, L-DOPA, as well as norepinephrine into the parenchyma of target organs (Goldstein and Holmes, 2008; Rubí and Maechler, 2010). Exceptionally, in spite of the absence of specific dopamine sources in peripheral tissues, the

concentration of this catecholamine in the plasma is similar to that of epinephrine (Rubí and Maechler, 2010). It has been shown in humans and rats that the cells of the adrenal medulla and sympathetic nerves express the L-DOPA decarboxylase enzyme, which can synthesize dopamine from L-DOPA (Wahbe *et al.*, 1982; Mappouras, Stiakakis and Fragoulis, 1990; Rubí and Maechler, 2010). During times of stress or exercise, the sympathetic nervous system and the adrenal glands are activated to release catecholamines such as norepinephrine and epinephrine for the classic fight or flight response (Mcgrady, 1984; Rubí and Maechler, 2010). In human plasma dopamine levels may also increase under conditions of stress and exercise, as it is recognized as a modulator of peripheral organ function (Otth *et al.*, 2007).Blood plasma levels of circulating dopamine was found to be ~2.29 ng/mL in normal fertile males (Shukla *et al.*, 2009). Autonomic innervation, (sympathetic and parasympathetic innervation) in the reproductive system is of great significance as it regulates a wide variety of functions, such as vascular dilation, smooth muscle contraction and the stimulation of prostatic or vaginal secretions (Andersson, Hedlund and Alm, 2000; Purves *et al.*, 2001). Autonomic innervation has been well characterised in neurovascular and neuromuscular events, however the effects of autonomic innervation on reproductive capacity and fertility are not well established (Kempinas *et al.*, 1998).

The reproductive system is under the control of the hypothalamic-pituitary-gonadal (HPG) axis, however non-endocrine regulatory influences such as neurotransmitters, are likely to play a pivotal part via a “neuroendocrinotropic stimulatory complex” (Mayerhofer *et al.*, 1996; Gnessi, Fabbri and Spera, 1997). During the course of the 1970s and 1980s, the interest in the function of catecholamines on male fertility piqued, as it was shown that they may have an effect on hamster sperm function (Adeoya-Osiguwa, Gibbons and Fraser, 2006). Experimentation to discover the causation of alterations in sperm functions *in vitro*; including, capacitation, motility, and acrosome reaction, lead researchers to identify catecholamines as a key modulator of these events. It has also been shown that both adrenal gland extracts, which include catecholamine precursors, as well as individual catecholamines, were found to stimulate events required for hamster sperm functions (Bavister, Yanagimachi and Teichman, 1976; Cornett and Meizel, 1978; Bavister, Chen and Fu, 1979; Meizel and Working, 1980). It is thought that the high levels of catecholamines found in the reproductive tracts of mammals arise from the abundant autonomic innervation of the gonads or from the adrenal glands via the bloodstream (Kempinas *et al.*, 1998; Otth *et al.*, 2007). An alternative source of these catecholamines was suggested to be the interstitial fluid within the testis, and the ovaries of human and non-human primates. Neuron-like cells expressing neuronal proteins and catecholamine-biosynthetic enzymes are present in these organs (Dees *et al.*, 1995; Mayerhofer *et al.*, 1996, Mayerhofer *et al.,* 1998; Mayerhofer *et al.*, 1999a; Mayerhofer *et al.*,

1999b). It was demonstrated that human Leydig cells within the testes express four enzymes which are critical components involved in the biogenesis of catecholamines, namely, TH, AADC, dopamine beta-hydroxylase (DBH) and phenylethanolamine- N-methyltransferase (PNMT) (Romeo *et al.*, 2004; Davidoff *et al.*, 2005). TH is specifically the rate-limiting enzyme involved in the production of dopamine and norepinephrine, and thus it increases the probability that catecholamines may be synthesized from other sources, in addition to the innervation of reproductive organs by the sympathetic nerves (Urra *et al.*, 2014). These intratesticular and ovarian sources of catecholamines, in concert with bloodstream sources could potentially modulate reproductive capacity and therefore play a role in fertility (Mayerhofer *et al.*, 1998; Romeo *et al.*, 2004). In 2001, Fait and colleagues conducted a study to determine the seminal concentrations of different catecholamines and their association with semen quality in human semen. They concluded that although the concentrations of norepinephrine and DOPA were much higher than that of the normal plasma values, they were not associated with semen quality (Fait *et al.*, 2001). High concentrations of catecholamines were detected in the oviduct luminal fluid of humans, pigs, rabbits, and cows. Catecholamine concentrations varied in the different regions of the oviduct and they were differentially expressed during the different stages of the oestrous cycle. It was thus suggested that catecholamines may modulate tubal motility (Helm *et al.*, 1982; Chaud *et al.*, 1983; Khatchadourian *et al.*, 1987; Kotwica *et al.*, 2003).

Considering the sizeable effects that catecholamines seemed to play in male fertility, it was a natural step to begin searching for the presence of receptors for these catecholamines in the reproductive system. Previously it was thought certain plasma membrane receptors were neuron-specific and only found in the brain, however, these “neural” receptors were detected in other somatic cells. The mammalian spermatozoa appear to possess many of these neuronal receptors, and they play a role in sperm function (Meizel, 2004). In 1990, Young and Laing suggested that rabbit spermatozoa must possess catecholaminergic binding sites after they tested the effect of the antipsychotic drug, Spiperone, on sperm. It bound to the both the heads and tails of the spermatozoa (Young and Laing, 1990). Nevertheless, the first concrete evidence suggesting a catecholaminergic presence in the reproductive system came in 2006 with the identification of functional α- and β-adrenergic receptors in mammalian spermatozoa (Adeoya-Osiguwa, Gibbons and Fraser, 2006). This discovery was not surprising as catecholamines were detected in large quantities within the reproductive tracts of mammals (Khatchadourian *et al.*, 1987; Fait *et al.*, 2001) however, the resultant impact of this discovery was controversial as it meant that the use of amphetamine-related compounds (ecstasy, Ritalin, Benzedrine) could possibly have a positive impact on fertility *in vivo*, especially on females (Adeoya-Osiguwa, Gibbons and Fraser, 2006).

In 2007 the novel identification of a peripheral dopaminergic receptor, D2, in rat male germ cells lead to new and unexpected roles for dopamine signalling in the male reproductive tract (Otth *et al.*, 2007). Rat germ cells including spermatids and, mature spermatozoa and testes were found to express both the D2L and the D2S receptors, which were located via immunofluorescence as well as immunoblotting (Otth *et al.*, 2007). Spermatozoa from mouse, bull, and humans were also shown to express these proteins (Ramírez *et al.*, 2009). Analysis with a polyclonal antibody detected two bands with an apparent molecular weight of 47-50kDa for sperm and higher molecular weight bands in the brain and testis (Otth *et al.*, 2007; Ramírez *et al.*, 2009). This observed heterogeneity could be explained by homodimerization or hetero oligomerization of the D2 receptor (Canals *et al.*, 2003; Guo, Shi and Javitch, 2003; Lee *et al.*, 2003; Kearn *et al.*, 2005; Otth *et al.*, 2007). A functional D2 receptor (DRD2) was found to be present in boar sperm. It was positively identified and localized to the acrosomal region of boar sperm after 2 hours of incubation in capacitation media. This study proved that the dopamine receptor D2 is highly conserved across mammalian species and that localization is dynamic as it depends on the capacitation status of the sperm (Ramírez *et al.*, 2009).

Capacitation is required for spermatozoa to be able to fertilize the oocyte. It is defined by a series of changes that the sperm undertake during their transit through the female reproductive tract (Chang, 1984; Yanagimachi, 1994; Leahy and Gadella, 2011; Wertheimer *et al.*, 2013). The main aim of capacitation is to ensure that the sperm reach the oocyte at the appropriate time and in the proper state to begin fertilization (Chang, 1984; Yanagimachi, 1994). The finely tuned complex procedure involves the loss of seminal fluid, alterations in the sperm membrane fluidity caused by ion fluxes and biochemical modifications, the development of hyperactive motility and the initiation of signal transduction pathways (Yanagimachi, 1994; Schuh *et al.*, 2006; Schuh, Hille and Babcock, 2007; Wertheimer *et al.*, 2013). All these steps prepare the spermatozoa for the induction of the acrosome reaction which is required for the spermatozoa to bind to the zona pellucida and ultimately fertilize the oocyte (Chang, 1984; De Lamirande, Leclerc and Gagnon, 1997; Visconti and Kopf, 1998; Guraya, 2000). Capacitation *in vitro* can be achieved by simply washing the spermatozoa and removing the seminal fluid, as a first step (Leahy and Gadella, 2011). Capacitation may also be induced, *in vitro,* by adding media designed to mimic the electrolytic composition of the female oviduct, this media consists of energy substrates such as glucose, pyruvate, and lactate (Hoppe, 1976). Bicarbonate (HCO_3^-) is also an important component of the capacitating media as the influx of HCO_3^- into sperm induces soluble AC (sAC) to increase cAMP levels, thereby activating PKA to phosphorylate certain proteins in control of flagellar movement (P.E. Visconti *et al.*, 1995; Breitbart, 2002; Wennemuth *et al.*, 2003; Wertheimer *et al.*, 2013; Soriano-Úbeda *et al.*, 2019). In addition changes in sperm swimming trajectories have been observed during capacitation, these

changes have been defined as hyperactive motility (Aitken *et al.*, 1985; Morales, Overstreet and Katz, 1988; Kay and Robertson, 1998). Bovine Serum Albumin (BSA) is also added to capacitating media and induces Ca^{2+} influx into sperm, although the mechanism underlying the influx is not well understood (Xia and Ren, 2009). The Ca^{2+} influx is required for the increase in motility during capacitation and the destabilization of the membrane which prepares sperm for ultimately undergoing the acrosome reaction (Chang, 1984; Breitbart, 2002; Carlson, Hille and Babcock, 2007; Xia and Ren, 2009).

Protein function is controlled by many factors - phosphorylation and dephosphorylation are mechanisms utilized to alter protein expression and functionality (Hunter, 2000). Although serine and threonine residues may get phosphorylated, tyrosine phosphorylation may be the exclusive indicator of a signal transduction pathway in a cell (Naz and Rajesh, 2004; Sepideh *et al.*, 2009). Tyrosine phosphorylated proteins within spermatozoa play an important role in sperm functioning. They have been proposed to initiate and regulate sperm motility, sperm capacitation and the acrosome reaction (Visconti *et al.*, 1995a; Visconti *et al.*, 1995b). Tyrosine phosphorylation seems to be regulated by cAMP and PKA (Visconti *et al.*, 1995a; Visconti *et al.*, 1995b; Bajpai and Doncel, 2003). As cAMP is synthesized by AC it is interesting to note that sAC, found in mammalian sperm, is insensitive to metabotropic receptors in contrast to transmembrane AC (tmAC), instead sAC is said to be regulated by HCO_3^- and Ca^{2+} ions (Jaiswal and Conti, 2003; Litvin *et al.*, 2003; Wertheimer *et al.*, 2013). Subject to controversy, however, is the existence of tmAC in sperm, where some studies have proved their existence and others have failed to identify them (Monks, Stein and Fraser, 1986; Livera *et al.*, 2005; Strünker *et al.*, 2011; Brenker *et al.*, 2012). Wertheimer *et al.,* has demonstrated that tmAC are present on mouse spermatozoa and play important roles in the regulation of sperm functions (Wertheimer *et al.*, 2013). The first event of capacitation is illustrated by the elevation of intracellular $Ca^{2+}$and HCO_3 which activate AC, thus producing more cAMP. The cAMP activates PKA which has been shown to be involved in the phosphorylation of multiple sperm proteins during capacitation, thereby leading to hyperactivation and eventually AR (Breitbart, 2002; Schuh *et al.*, 2006; Carlson, Hille and Babcock, 2007; Schuh, Hille and Babcock, 2007). Low (100 nM) concentrations of dopamine have been shown to affect capacitation, specifically tyrosine phosphorylation. This was suggested to be through D2DR activation; however, high (1 mM) concentrations of dopamine significantly decreased the tyrosine phosphorylation pattern and sperm motility (Ramírez *et al.*, 2009). It was postulated that the inhibitory effects of dopamine on tyrosine phosphorylation and motility may be mediated by an excess of dopamine oxidation products as these are known to be toxic both *in vitro* and *in vivo* (Berman and Hastings, 2001; Ramírez *et al.*, 2009).

Catecholamines such as dopamine and norepinephrine were shown to exhibit a biphasic effect at different concentrations in sperm. Literature on seminal plasma levels of dopamine is scarce, however Shukla *et al.,* found via reverse-phase high pressure liquid chromatography, that ~4.34 ng/mL is found in the seminal plasma of normal fertile men (Shukla *et al.*, 2009). Low (100 nM) concentrations of dopamine treatments were shown to be protective, by increasing the sperm viability, whereas high concentrations (1 mM) negatively affected sperm parameters such as motility and acrosomal integrity (Way and Killian, 2002; Ramírez *et al.*, 2009). Numerous studies have evaluated the effect of known DAT inhibitors, such as cocaine and amphetamines, on spermatozoa or testis and hypothesized that since these molecules elicit some effect on sperm and testes, a mechanism for transport must exist (Yazigi, Odem and Polakoski, 1991; Yelian *et al.*, 1994; George *et al.*, 1996; Li *et al.*, 1997, 1999). Taken together these findings suggest that catecholamine transporters are present in sperm.

Ramírez *et al.,* investigated the presence of DAT on spermatozoa of mammalian species such as human, mouse, bull, and horse, however, they only reported on the identification thereof in boar sperm (a supplementary IF micrograph alludes to the presence of DAT on the other mammalian species mentioned above). These same authors document the presence of DAT in boar sperm, on WB membranes with an approximate molecular weight of 75-80 kDa (Ramírez *et al.*, 2009). This was the first evidence pointing to mammalian sperm possessing a catecholaminergic phenotype and therefore could possibly be sensitive targets for addictive drugs and antipsychotics such as cocaine and amphetamine (Ramírez *et al.*, 2009).

Monoamine Transporters DAT, NET and SERT were identified in equine spermatozoa. Immunofluorescence assays depicted- NET distribution within sperm seems to be localized to the acrosomal region. A similar pattern was observed for SERT, however a weak signal detected in the mid-piece and the tail was also present for SERT (Urra *et al.*, 2014). The presence of these monoamine transporters was further established using Western Blotting techniques; NET migrated to a band of ~70kDa and SERT to ~58kDa (Urra *et al.*, 2014). Most importantly a functional DAT was localized via indirect immunofluorescence on stallion spermatozoa, with a strong immunoreaction at the acrosomal region and weaker immunoreactivity in the principal piece of the sperm tail (Urra *et al.*, 2014). The presence of this protein was further proved by western blotting experiments. Rat brain extracts were used as positive controls. Analysis with a specific anti-DAT antibody revealed a band at ~70kDa for the equine sperm and several bands ranging between 45-120kDa for the positive control, which is due to the degree of glycosylation and the formation of homo-oligomers (Urra *et al.*, 2014). Urra and colleagues focus was on the identification of DAT in equine spermatozoa, but similarly to Ramírez *et al.,* 2009, they also investigated the presence of DAT in boar, human and bull sperm with WB analysis techniques (supplementary data of WB membrane suggest

DAT appears at ~70 kDa for all the species mentioned above) (Urra *et al.*, 2014). A functionality assay using fluorochrome 4-[4-(*Dimethylamino*)styryl]-N-methylpyridinium iodide (ASP^+) as a substrate demonstrated that DAT is functional in equine sperm. The transport activity (Km) of the DAT found in equine sperm was shown to be 3 times that of DAT in HEK293 (cultured human embryonic kidney cells, commonly used in cell culture experiments) cells. The higher Km could suggest that equine DAT has a lower affinity for dopamine and could require higher levels of substrate to operate. It would seem that equine DAT is insensitive to low doses of dopamine, but adversely higher doses would impact negatively on sperm functions, as previously mentioned. The negative effects of high doses of dopamine were partially reversed by incubating equine sperm with the highly selective DAT inhibitor, Vanoxerine (GRB12909). High (1 mM) concentrations of dopamine were shown to significantly decrease total motility after 1 hour of incubation, however upon the addition of Vanoxerine, this effect was partially reversed (Urra *et al.*, 2014).

In summary all the evidence taken together suggests dopamine's modulatory role on sperm physiology is complex. Sperm functions are regulated by signals present in the male and female reproductive tracts. Sperm functions associated with neural signals have predominantly been studied in a unidimensional approach, where one ligand activates one receptor or transporter thus altering sperm functions. However, sperm functioning *invivo* is completely different as they are exposed to multidimensional signalling complexes (Ramírez-Reveco *et al.*, 2017).

Therefore, the study aims to answer the problem statement- are DATs present on human sperm membranes and what are the effects of different concentrations of dopamine on human sperm parameters? By utilising WB and IF techniques, the study aims to locate DAT and thereafter determine the effects of different concentrations of dopamine on human sperm parameters.

Chapter 3

Materials and Methods

3.1 Introduction

This chapter provided an overview of the materials used in the study as well as where they were obtained from. It also details all the methods employed for each experiment. The study comprises of rat tissue collection for use as positive controls and human semen collection, to identify DAT and analyse the effects of dopamine on human sperm functions.

3.2 Ethical Clearance

Participants from the Faculty of Medicine and Health Sciences (FMHS) were recruited via a flyer and voluntarily agreed to participate in the study as sperm donors. Institutional Permission was obtained from Stellenbosch University (service desk ID: IRPSD-1982) (see Appendix A). All participants were informed of the full research protocol and signed an informed consent form (ICF), wherein it was made clear that they would be guaranteed anonymity and the samples would solely be used for research purposes as stated in the ICF. Participants were assigned a unique donor code and referred to by it throughout the entire study. Ethical clearance for use of the human semen samples in this study was granted by the Health Research Ethics Committee (HREC) of Stellenbosch University. Ethics Reference number: S/20/08/200 (see Appendix A).

The use of the animal tissue was approved by the Research Ethics Committee: Animal Care and Use. The animals collected for this study form part of a larger study titled: *The vascular and endothelial effects of HIV & antiretroviral therapy – functional effects and mechanisms*, with Ethics Reference number ACU-2019-8939. An amendment to the original ethics application was granted and 20 animals were collected as part of the current study. Ethics Reference number ACU-2019-10563 (see Appendix A).

3.3 Study Setting

The study was performed at the Stellenbosch University Reproductive Research Group (SURRG) laboratory in the Division of Medical Physiology, FMHS, Stellenbosch University, Tygerberg.

3.4 Study Design

The study was designed to be accomplished by firstly collecting rat tissue to serve as control and thereafter collecting human semen samples for experimentation. The animal tissue was collected and stored away for further analysis. Figure 9 provides a brief description of how the rat tissue was collected. A total of 20 rats were utilized for the current study and rat brain tissue was collected and used as positive controls. For the human semen collection, a total of 58

human semen samples were collected (n=58). Twenty-four samples were collected and frozen away for Western Blot (WB) analysis; a further 10 samples were collected and analysed with Immunofluorescence (IF) and 24 samples were collected and used for the dopamine treatment experiments. A brief study design of the experimental procedure followed can be seen in figure 10.

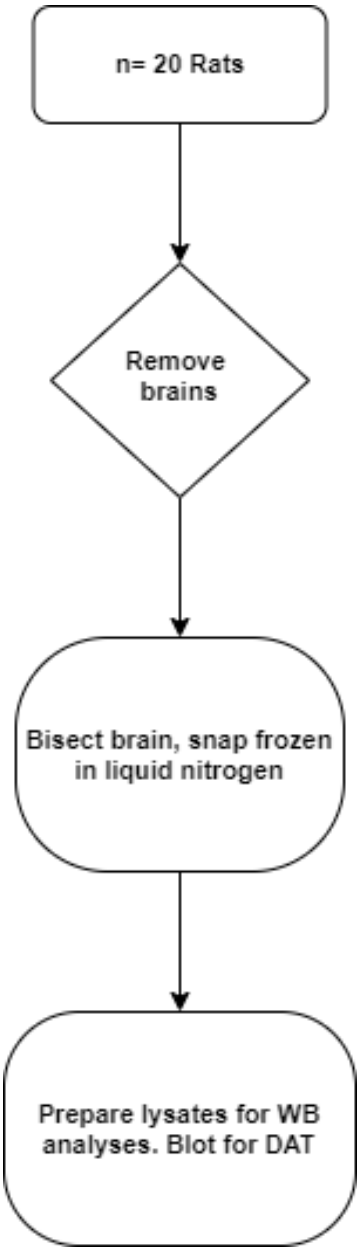


Figure 9: Brief description of animal tissue collection. (DAT- Dopamine Transporter, WB- Western Blot)

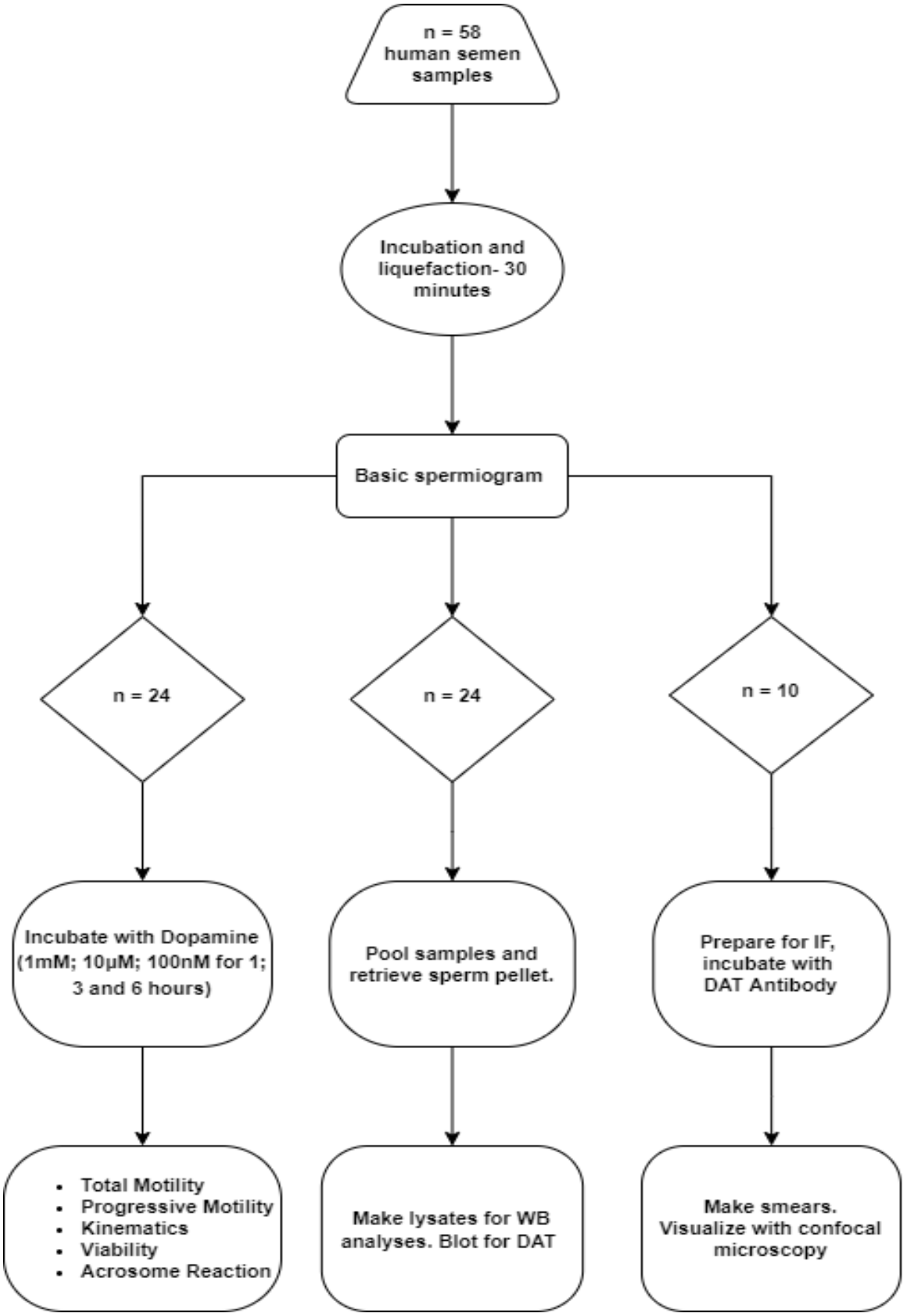


Figure 10: Brief description of the experimental design. (DAT- Dopamine Transporter, IF- Immunofluorescence, WB- Western Blot)

3.4.1 Animal tissue collection

All animals were raised under the same conditions and following 16 weeks of being kept on a control diet they were sacrificed. Before sacrifice, the animals were weighed, and the end body weights recorded. Twenty control male Wistar rats were sacrificed by means of a sodium pentobarbitone injection and exsanguination.

After exsanguination, the animals were decapitated, the skull was cracked open and the whole brain was removed. The brain was subsequently bisected and kept on ice in a petri dish. Thereafter the brain halves were immediately snap frozen in liquid nitrogen in foil envelopes and stored at -80°C until further analysis.

3.4.2 Human semen collection

Semen samples were collected from volunteer donors from the FMHS. The participants recruited were healthy donors aged between 18 and 25 years. Exclusion criteria included:

- heavy smokers (3-10 cigarettes a day)
- frequent alcohol consumption (>2 units per day)
- recreational drug users (e.g., marijuana; LSD and ecstasy)
- psychostimulant drug use of any kind (e.g., Ritalin, Concerta and Adderall).

All donors were required to abstain from any sexual activity for 2-7 days and samples were collected according to the guidelines outlined by the World Health Organization (WHO, 2010). Semen was collected via masturbation into a wide-mouthed sterile plastic container, provided by the Stellenbosch University Reproductive Research Group (SURRG). The samples were immediately placed in an incubator (Heal Force®) set at 37°C, with 5% CO_2 and 95% humidity. All samples were allowed to liquefy for 30 minutes prior to any analysis. Semen was used for the spermiogram and analyses to be completed on day of collection. Sperm samples were also centrifuged (Hermle Z 326 K, Lasec, South Africa), the pellets were collected and snap frozen in liquid nitrogen before being stored away at -80°C until further analysis.

3.5 Materials

Dopamine Hydrochloride (5g) obtained from Sigma-Aldrich, South Africa. Dulbecco' Modified Eagles Medium (DMEM), Phosphate Buffered Saline (PBS), Foetal Bovine Serum (FBS), Bovine Serum Albumin (BSA), Triton x-100, Fluorescein-Isothiocyanate Pisum Sativum Agglutinin (FITC-PSA), DPX Mountant and Dako Fluorescent Mounting medium were obtained from Sigma-Aldrich (South Africa). Eosin and Nigrosin were obtained from Fertipro (NV, Belgium). Anti-Dopamine Transporter antibody [ab111468] and Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488) [ab150073] secondary antibody was obtained from Abcam (Biocom, South Africa). Hoechst 33342, nuclear stain, was obtained from Thermo Fisher Scientific (South

Africa). Anti-Rabbit IgG HRP-linked secondary antibody was obtained from Cell Signalling technology (South Africa).

3.6 Standard Semen Analysis (Spermiogram)

The standard semen analysis, also known as the spermiogram, is used to test the semen quality and basic sperm parameters. The spermiogram provides a baseline of information before any treatments to the sperm samples and is also referred to as the neat analysis. The basic semen analysis was assessed in terms of the guidelines defined by the WHO (WHO, 2010). The basic semen analysis consisted of macroscopic and microscopic evaluations. The macroscopic evaluation included measuring the volume, pH, and viscosity of the semen sample, while also noting the colour of the sample. The microscopic evaluation included assessing sperm motility as well as sperm concentration with Computer-Aided Sperm Analysis (CASA).

3.6.1 Volume

The volume of an ejaculate is most commonly the first parameter assessed during the basic semen analysis. It is important to quantify the volume of the entire ejaculate and get a precise measurement as this is indicative of all the cellular and non-cellular elements contained within the sample. The WHO sets a lower reference limit of 1.5 mL (5^{th} centile, 95% confidence interval (CI) 1.4-1.7 mL) for semen samples to be regarded as normal (WHO, 2010). Once liquefied, semen samples were transferred with a Pasteur pipette directly from the sample container into a graduated 15 mL falcon tube, and the volume was measured on the balance scale (Kern, Sigma-Aldrich, South Africa) and recorded in millilitres as per WHO guidelines. Samples with a volume lower than the reference limit was excluded from the study. All collected samples passed the lower reference limit and were included in the study.

3.6.2 pH

The pH of an ejaculate is an important parameter to measure as it gives a reflection of the various accessory gland secretions (AGS). According to the WHO a normal pH falls within a range of 7.2-7.8 (WHO, 2010). Values outside of the normal pH range could be indicative of sperm alterations or infections. The pH was measured after liquefaction by means of litmus pH strips and a colour wheel (pH- indicator paper, Merck Millipore). A 2µL drop was pipetted onto a small strip of litmus paper and the resultant colour change compared to the colour wheel. Samples with pH measurements outside the normal range were excluded from the study. All samples collected fell within the normal pH range and none were excluded.

3.6.3 Viscosity

Viscosity measures the seminal fluids resistance to natural flow. It is important to measure viscosity as a high degree of viscosity could interfere with sperm motility and concentration determination. The viscosity of the semen sample was measured by the duration of time it took the sample to fill up a chamber slide. Leja® 8 Chamber slides (2153 GN Nieuw Vennep, The Netherlands) with a 20-micron depth chamber were used to assess the viscosity. Two microlitres was pipetted into the chamber and the time it took to fill up was measured. The sample was deemed too viscous if it took >10 seconds to fill the entire chamber. If the sample was too viscous it was diluted in a 1:1 ratio with DMEM.

3.6.4 Colour

Semen normally displays a white to opaque colour. It is important to note the colour as varying from the norm could indicate an infection. Brownish or red colour indicates that there is blood in the semen, this is known as haemospermia. Semen appearing yellow or green is likely due to medication, however brown semen is indicative of infections including sexually transmitted infections (STI's) such as, gonorrhoea or chlamydia. The colour of the semen was determined visually and classified either normal or abnormal. Only those classified as normal were included in the study, all samples collected were classified as normal and included in the study.

3.6.5 CASA-related analysis

The CASA system comprises of a computer with the appropriate software, and a microscope with a camera attached. The Sperm Class Analyser®(SCA®) software system, version 6.3.0.59 (Microptic, S. L., Barcelona, Spain) is an automated software system used to track and analyse spermatozoa and take images thereof. The CASA system is comprised of a Nikon Eclipse 50i Microscope (IMP, Cape Town, South Africa), a temperature regulated microscope stage and Basler A312fc digital colour camera (Microptic, S. L., Barcelona, Spain). The microscope was set to phase-contrast (Ph1); brightness, 100; 10x objective; the green filter and the temperature of the stage set to 37°C.

3.6.5.1 Motility

The most significant parameter assessed during a basic semen analysis is the sperm motility. Spermatozoa require the ability of motility to be able to travel along the female reproductive tract to reach the oocyte. Sperm motility is measured as the percentage of moving spermatozoa in a semen sample. The WHO classifies a total motility of 40% (38-42%) and progressive motility of 32% (31-34%) as normal (WHO, 2010).Sperm motility is assessed by means of the CASA aided by the SCA® automated software system. The SCA® system

analyses a wide range of parameters including total motility, progressive motility, and various kinematics of the sperm head. The “Human50” setting was selected on the SCA® software, which is optimized for human sperm. A 2µL drop of semen is pipetted into a 20 µm Leja® chamber slide and observed under the microscope. A minimum of 5 randomly selected fields on the slide were recorded and 50 images per second captured. At least 1000 spermatozoa were examined.

3.6.5.2 Concentration

Sperm concentration is an essential parameter in determining fertility and it is routinely measured during a basic semen analysis. Sperm concentration is the number of spermatozoa per millilitre in a semen sample. Total sperm count represents the concentration of spermatozoa per ejaculate. WHO sets a lower limit of 15 million spermatozoa per millilitre (M/mL) (12-16 M/mL) and 39 million spermatozoa per ejaculate (33-46 M/mL) (WHO, 2010). Men with a total sperm count <39 M/mL are considered oligozoospermic. The concentration of a sample is measured in conjunction with the motility using the CASA and a 20µm Leja® chamber slide.

3.7 Sample preparation

Once the sample has liquified, undergone the basic semen analysis and adhered to the guidelines prescribed by WHO including- a volume of ≥1.5 mL, a concentration of ≥15 x 10^6 spermatozoa per mL, a total motility of 40% and a progressive motility of 32%, it has passed the neat analysis. A single wash was employed to remove the seminal plasma and all the cellular debris. The single wash includes adding an equal volume of DMEM, to the semen sample in a 1:1 ratio. The sample was then centrifuged at 1500 rpm for 15 minutes at room temperature (RT) (22°C). The supernatant was discarded using a Pasteur pipette and the pellet was resuspended in 2-3 mL of DMEM. The pellet is disturbed and dissolved in the DMEM. The washed sample was measured for motility and concentration once again. The sample was used for further experimentation if it had a concentration of ≥15 x 10^6 spermatozoa per mL.

3.8 Immunofluorescence (IF)

For immunodetection by fluorescence, an indirect immunofluorescence analysis was utilized. Indirect Immunofluorescence, also known as secondary immunofluorescence, is a technique employed to detect targets of interest using two antibodies. The primary antibody binds to the target of interest and the secondary antibody, which carries the fluorophore, recognizes, and binds to it (Wardyn and Jeyasekharan, 2018). For the detection of DAT, an Anti-Dopamine Transporter antibody was utilized as the primary antibody and a donkey Anti-Rabbit IgG H&L

(conjugated to Alexa Fluor® 488) as the secondary antibody. Hoechst 33342 was employed as a counterstain for the nucleus.

For the indirect immunofluorescence analysis, we utilized a modified protocol initially described by Ramírez *et al.,* (2009). The samples are prepared as described in section 3.7. The sperm samples were diluted in 5 mL round bottom Falcon tubes (Corning, USA) with 1% BSA-PBS (wash buffer- see Appendix B) to attain the appropriate number of spermatozoa, 20 M/mL, and then centrifuged (Hermle Z 326 K, Lasec, South Africa) for 10 minutes at 500 x *g* force (relative centrifugal force- rcf). The supernatant was decanted, and the spermatozoa were fixed by resuspending in 100 µL of 2% paraformaldehyde and incubating at RT for 15 minutes, whereafter they were washed twice. The wash step includes topping up to 1 mL with wash buffer and centrifuging for 10 minutes at 500 x *g*. The sperm were then permeabilized in 100µL of 0,3% Triton X-100 in 5% BSA-PBS (see Appendix B) at RT for 10 minutes, whereafter they were washed twice. The spermatozoa were blocked for non-specific binding in 500µL 10% FBS at RT for 40 minutes, after which, the sperm were washed twice- the tubes were left to stand for 5 minutes on the bench top and thereafter centrifuged for 10 minutes.

For staining, the spermatozoa were incubated overnight in the fridge at 4°C with 10µL of Anti-Dopamine Transporter antibody (Anti-DAT) to a final concentration of 5µg/mL. After incubation with the primary antibody, the sperm were washed twice, 5 minutes on the benchtop and 10 minutes in the centrifuge to remove the unbound primary antibody. The secondary antibody, Donkey Anti-Rabbit conjugated to Alexa Fluor® 488, was prepared in a 1:1000 dilution and 100µL of secondary antibody and 1µL of 1µg/mL Hoechst, was added to the appropriate tubes and allowed to incubate for 40 minutes in a humidified chamber. Hoechst is a nuclear stain that is highly cell membrane permeant and is routinely used as a nuclear counterstain as it is less toxic than other dyes, which ensures a higher viability of stained cells (BD Biosciences-Europe, 2009). The sperm were then washed twice, 5 minutes on the benchtop and 10 minutes in the centrifuge to remove the unbound secondary antibody. Finally, the sperm were resuspended in 100µL 1%BSA-PBS buffer and mixed well. A 10µL drop was added to a Superfrost Plus positively charged slide (Adkin Med-Lab Supplies, South Africa) and a smear was made. The smear was allowed to air dry for 1-4 hours, whereafter it was mounted with Dako fluorescent mounting medium.

Confocal microscopy was performed for the analysis of the samples using the Carl Zeiss LSM780 with ELYRA PS1 Super-resolution platform (Carl Zeiss, Germany). An Alpha Plan-Apochromat 100x/1.46 Oil DIC M27 Elyra objective, along with the 405 nm laser (for the detection of Hoechst 33342), 488 nm laser (for the detection of DAT-Alexa Fluor® 488) and

the GaAsP detector 32+2 PMT and the Transmitted light detector T-PMT was utilized for imaging. Alexa Fluor® 488 was detected in the range 499-543 nm and nuclear stain Hoechst, was detected in the range 415-460 nm. For data collection, Z-stacks were acquired with a variable step width for each image. Maximum intensity projections of Z-stacks were processed and generated in the Zeiss Zen Blue 2012 software (Carl Zeiss, Germany) and utilized for subsequent analysis.

Figure 11 depicts the controls used for the experimental setup; the negative controls were obtained when sperm were incubated with only secondary antibody.

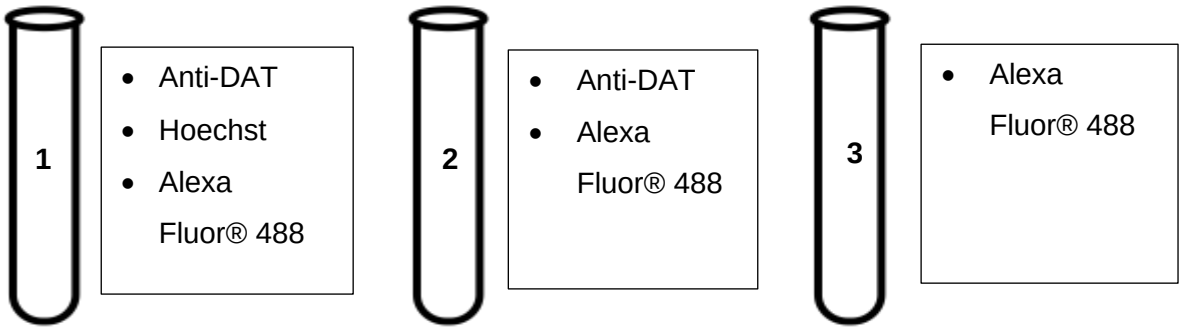


Figure 11: Schematic of the experimental setup for immunofluorescence assay. Tube 1 represents the positive control, containing the primary and secondary antibodies as well as the nuclear stain. Tube 2 represents another positive control without the nuclear stain and tube 3 represents the negative control, containing only the secondary antibody.

3.9 Western Blotting Analyses (WB)

Western Blotting is an analysis technique for the detection of immobilized specific proteins (antigens), conjugated with HorseRadish Peroxidase (HRP) antibodies. The procedure involves the electrophoretic transfer of proteins from Sodium Dodecyl Sulphate (SDS)-polyacrylamide gels to a Polyvinylidene Fluoride (PVDF) transfer membrane. Polyacrylamide gel electrophoresis (PAGE) is utilized to separate denatured proteins based on their size (molecular weight). Visualization on a membrane after the transfer provides further detail on protein expression. Western Blotting is a useful analysis tool that not only confers information about protein size (in comparison to a size marker in kDa) but also protein expression (in comparison to a control sample or tissue). Western blot analysis is a time-consuming experimental procedure that may take up to 3 days to complete. On day 1 the lysates are prepared, and the protein concentration is determined by the Bradford Protein Assay. On day 2 the gels are made, transferred to membranes, and incubated with primary antibody overnight. Finally, on day 3 the membranes are incubated with secondary antibody and visualized by chemiluminescence. For the detection of the protein of interest, DAT, an Anti-Dopamine Transporter antibody was used as the primary antibody and an Anti-Rabbit IgG HRP-linked antibody was used as the secondary antibody. Rat brain tissue was used as the positive control (previously shown to express DAT).

3.9.1 Protein extraction- Brain Tissue

The rat brains were collected as described in section 3.4.1 and were used as the positive control for the detection of DAT protein. Whole brains were removed from -80°C storage and pulverised. The pulverization step was done using a mortar and pestle, pre-cooled in liquid nitrogen. Approximately 0.5-0.6g of tissue was weighed (Mettler Toledo, Switzerland) out after pulverization. Only two samples were utilized for the final WB analysis.. Brain tissue from control rats 1 and 2 were labelled C1 and C2 respectively.

C1= 0.53g

C2= 0.52g

3.9.2 Protein extraction- Sperm

Twenty-four human sperm samples were collected for Western Blot analysis and used for the detection of DAT protein. Five donors donated samples to reach n=24. Semen was collected and prepared as described in section 3.7 and stored at -80°C. On the day of the lysate preparation the samples were taken out of the freezer and incubated at 37°C for 30 minutes to thaw. Twelve samples were pooled and represent one sample, therefore from 24 donors two samples were generated, namely S1 and S2. The samples were centrifuged at 1500 rpm for 10 minutes and the pellets were collected.

S1 (485.70 M/mL) = Donor 1 (6 sperm samples) + Donor 2 (6 sperm samples)

S2 (724,37 M/mL) = Donor 3 (7 sperm samples) + Donor 4 (3 sperm samples) + Donor 5 (2 sperm samples)

3.9.3 Lysate preparation and Bradford Protein Assay

To prepare the lysates the pulverized tissue and the sperm pellets were first homogenized to break apart the cells. On ice, 700 µL of Lysis Buffer (LB), prepared as described in Table 2, was added to labelled Eppendorf's, containing 7 stainless steel beads (1.6 mm) (Next Advance, Inc, USA). The samples were homogenized using the Bullet Blender at 4°C, set at speed eight for one-minute, this was repeated three times with five-minute intervals in between. The samples were then centrifuged (BKC-TH16R High Speed Refrigerated centrifuge, Axiology Labs, South Africa) for 20 minutes at 15000 rpm at 4°C. The supernatant was collected and transferred to new labelled Eppendorf's.

Table 2*:* Lysis Buffer

Reagent	Final concentration in buffer	Amount of 10X stock for 10 mL	Action
20 mM Tris-HCl [pH 7.5]	200 mM	1 mL	Buffer
1 mM EGTA [ethylene glycol tetra-acetic acid]	10 mM		Chelating agent
1 mM EDTA [ethylene diamine tetra-acetic acid]	100 mM	100 µL	Chelating agent (metalloprotease inhibitor)
150 mM NaCl [Sodium Chloride]	1 M	1,5 mL	Salt
1 mM β- glycerophosphate		0,002 g	Serine/Threonine Phosphatase Inhibitor
2,5 mM tetra-Na-Pyrophosphate		0.01 g	Serine/Threonine Phosphatase Inhibitor
1 mM Na_3VO_4 (0,18 g/10 mL H_2O) [Sodium Orthovanadate]	10 mM	1 mL	Tyrosine and Alkaline Phosphatase Inhibitor
SDS [Sodium Dodecyl Sulphate]	1%	1 mL	Detergent
50 µg/mL PMSF [Phenyl Methyl Sulfonyl Fluoride]	100 mM	30 µL	Serine protease inhibitor
PI [Protease Inhibitor] cocktail: • ABESF [4-(-Aminoethyl) benzenesulfonyl fluoride hydrochloride] • Aprotinin • Bestatin hydrochloride • E-64 [N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide • Leupeptin hemisulfate salt • Pepstain A		50 µL	• Serine protease inhibitor • Serine protease inhibitor • Aminopeptidase inhibitor • Cysteine protease inhibitor • Serine and Cysteine protease inhibitor • Acid protease inhibitor
1% Triton X-100	10%	1 mL	Detergent
Fill up to 10 mL with distilled water			

***PMSF has a very short half-life and is therefore added to the buffer last.*

The Bradford Protein Assay is used to determine the concentration of protein in a sample compared to a BSA standard, using a spectrophotometer. The BSA standards were used to generate a standard curve, using the optical density (OD) readings and the protein concentration of the standards (known) see Appendix C. The Bradford reagent was prepared in a 1 in 5 dilution, 20 mL of Bradford reagent was added to 80 mL of dH_2O and filtered. Figure 12 depicts how the samples and standards were prepared - BSA was used to prepare Bradford standards in duplicate (C); a 10x dilution was made with the supernatant (A), whereafter a 20x dilution was made in duplicate and thoroughly vortexed (B); Filtered Bradford reagent (900 µL) was added to the BSA standards (C) and the duplicate samples (B). Using a spectrophotometer, the OD were recorded. From the standard curve (generated from the BSA standards), (see Appendix C), a line of best fit was plotted, and an equation generated. The equation and the OD readings of the samples (B) was used to determine the protein concentration in each sample. .

The lysate contained the sample, the sample buffer (SB) and the lysis buffer. SB accounts for a third of the volume of the lysate and therefore a calculation determined the amount of sample and LB to be added. SB was prepared by adding 850 µL Laemmli Buffer (Table 3) to 150 µL β-mercaptoethanol.

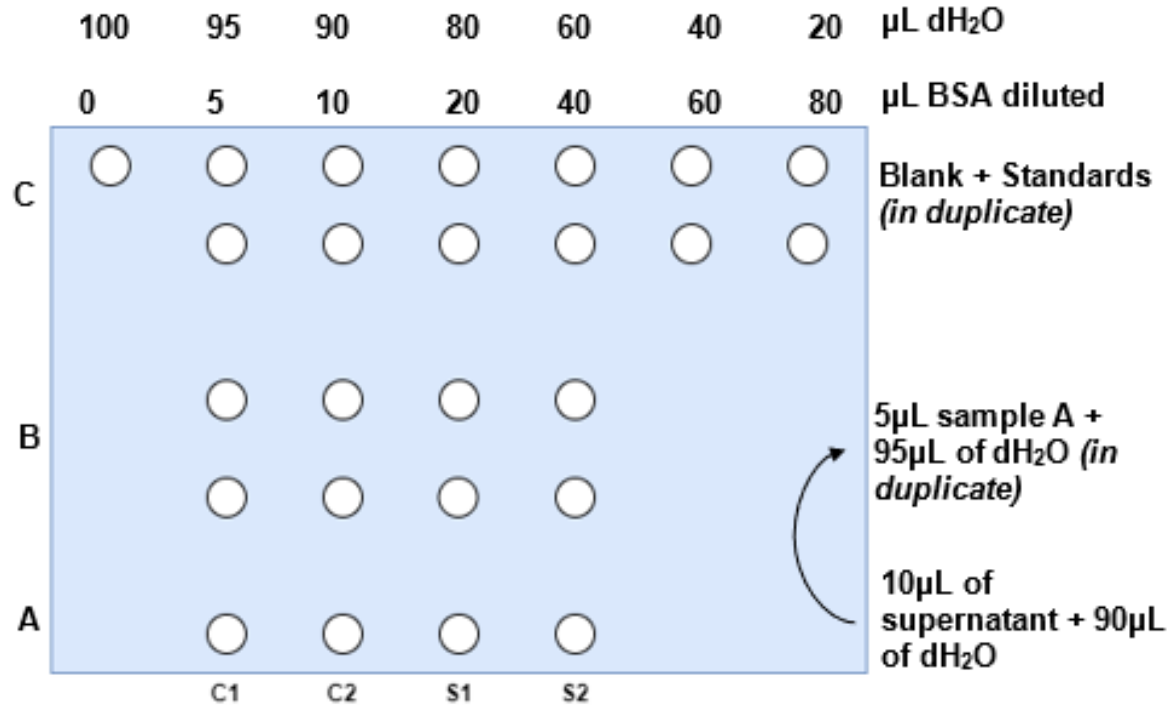


Figure 12: Bradford Protein Assay- setup

Table 3: 2X Laemmli Buffer (Laemmli, 1970)

Reagent	Concentration
SDS	4%
Glycerol	20%
β-mercaptoethanol	10%
Bromophenol Blue	0.004%
Tris-HCl	0.125 M

The lysates were prepared accordingly, containing the SB, LB, and sample (supernatant) ensuring a final protein concentration of 27.5 µg per 12 µL of lysate (refer to Appendix C).. Lysates were frozen away at -80°C until further analysis.

3.9.4 Separation and Transfer of Proteins

The proteins were now ready to be separated by gel electrophoresis. The Bio-Rad Mini Gel Protean System (Bio-Rad, CA, USA) was assembled with a 1 mm thick spacer glass plate. A 10% stain-free running gel was prepared as described in Table 4. Since the protein of interest has a molecular weight of 60-80 kDa, a 10% gel is optimal to separate proteins. Immediately after pouring the running gel into the cast, a few drops of butanol was added to prevent oxidation of the gel, remove bubbles and to ensure a straight-line formed. The gel was allowed to set for 30 minutes whereafter the butanol was washed off with dH_2O. A 4% stacking gel was prepared as described in Table 5. Once the stacking gel was poured onto the running gel, the combs were inserted, they formed the wells. The stacking gel was allowed to set for 15 minutes whereafter the combs were removed.

Table 4*:* Running Gel- stain free

Reagent	Stock	10%
dH_2O		4,85 mL
Stain Free		50 µL
Tris-HCl (pH 8.8)	1,5 M	2,5 mL
SDS	10%	100 µL
Acrylamide	40%	2,5 mL
*APS	10%	50 µL
*TEMED	99%	20 µL

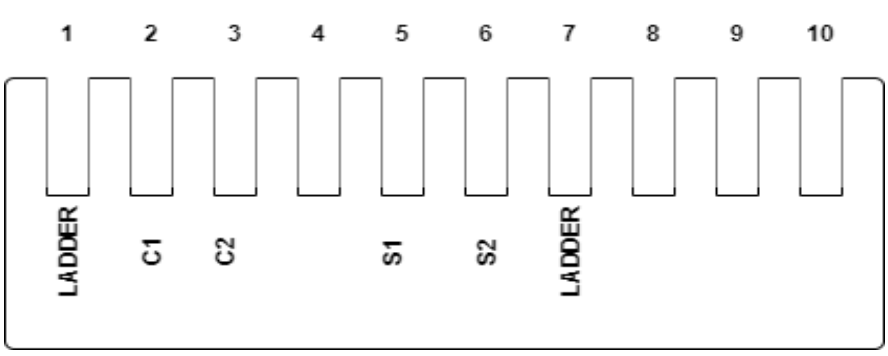
* APS and TEMED react fast to set the gel, add last

Table 5*:* Stacking Gel

Reagent	Stock	10%
dH_2O		3,05 mL
Tris-HCl (pH 6.8)	0,5 M	1,25 mL
SDS	10%	50 µL
Acrylamide	40%	500 µL
*APS	10%	50 µL
*TEMED	99%	10 µL

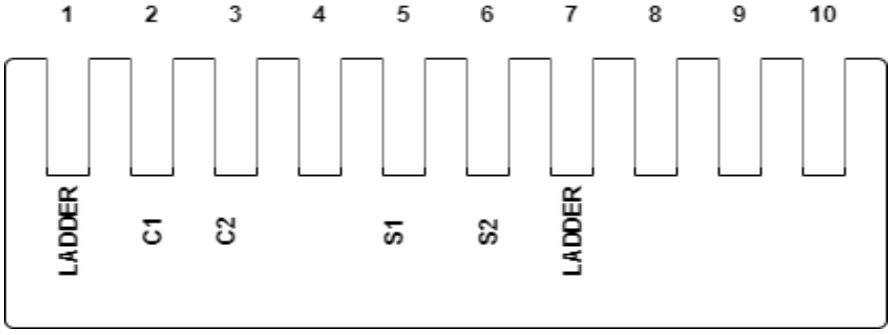
* APS and TEMED react fast to set the gel, add last

The lysates were removed from the -80°C storage and thawed on ice. The samples were boiled for 5 minutes and centrifuged for ~30 seconds to remove condensation. The gels were placed into the electrophoresis tank and topped up with running buffer (see Appendix B). A Hamilton pipette was used to load the samples and the molecular weight marker (ladder), into the wells of the gel. The samples to be loaded are the brain samples C1 and C2, which serve as the positive controls, and the two sperm samples S1 and S2. Two gels were prepared and loaded as described in figures 13 and 14.



- Lane 1- 5 µL
- Lane 2- 12 µL
- Lane 3- 12 µL
- Lane 5- 12 µL
- Lane 6- 12 µL
- Lane 7- 5 µL

Figure 13: Gel 1, stain-free



- Lane 1- 2.5 µL
- Lane 2- 7.5 µL
- Lane 3- 7.5 µL
- Lane 5- 15 µL
- Lane 6- 15 µL
- Lane 7- 2.5 µL

Figure 14: Gel 2, stain-free

Proteins were separated according to size. The 1st run was set for 10 minutes at 100 and 200 mA, the 2nd run was set for 50 minutes at 200 V and 200 mA. Once the running process was completed the gels were removed from the glass plates and activated using the Chemidoc™ MP Imager System with Image Lab™- 5 software (Bio-Rad, CA, USA) to ascertain whether protein loading was equal for all the samples. Hereafter the separated proteins on the gels were transferred to membranes. The Trans-Blot® Turbo™ Transfer System (Bio-Rad, CA, USA) was utilized to transfer the separated proteins on the gels to Millipore Immobilon®-P transfer membranes cut to the appropriate size of the gel. The membranes were sandwiched with the gels and placed into the transfer tank with transfer buffer (see Appendix B). The transfer took place on ice at 200 V and 200 mA for 55 minutes. Subsequently, the membranes were visualized on the ChemiDoc™ to ensure all the proteins had transferred correctly to the membranes. Proteins on membranes were then fixed in Methanol for 30 seconds.

3.9.5 Immunodetection of Proteins

The membranes were placed on a shaker and blocked for non-specific binding at RT for 2 hours. Blocking buffer consisted of 95 mL of TBS-Tween (10 % Tris-buffered saline, 0.1% Tween- see Appendix B) with 5 mL long life fat-free milk. After blocking, the membranes were washed three times using TBS-Tween for 5 minutes each, on the shaker at RT. The membranes were then cut and incubated with the primary antibody overnight on the shaker at 4°C. Anti-DAT was prepared in a 1:1000 dilution with 5%BSA-TBS-Tween (see Appendix B). The next day the membranes were washed and incubated with the secondary antibody. The membranes were placed on the shaker at RT for 1 hour in anti-Rabbit IgG HRP-linked secondary antibody diluted in 5%BSA-TBS-Tween in a 1:2000 dilution. Hereafter the membranes were once again washed to remove any unbound antibody and incubated with Clarity™ ECL for 5 minutes at RT. Finally, the membranes were exposed using the ChemiDoc™. Exposure times varied for sperm and brain samples.

3.10 Dopamine Treatment

The effect of dopamine was tested on human sperm parameters *in vitro*. The effect of high (1 mM), medium (10 µM) and low (100 nM) concentrations of dopamine compared to a control (no dopamine) was tested on human sperm over time. Parameters tested included: Total Motility, Progressive Motility; Kinematics, Viability and Acrosome Reaction. Kinematic parameters included- curvilinear velocity, VCL, (µm/s); straight-line velocity, VSL, (µm/s); average path velocity, VAP, (µm/s); straightness index, STR, (%); linearity index, LIN, (%); oscillation index, WOB, (%); amplitude of lateral head displacement, ALH, (µm); and beat-cross frequency, BCF, (Hz). A total of 24 human semen samples were collected and prepared as described in section 3.7.

3.10.1 Preparation of Dopamine concentrations

Dopamine Hydrochloride is received in powdered form and diluted with PBS to create Stock solutions as described in Appendix B . The stocks were aliquoted and stored in Eppendorf's at 4°C.

For experimental procedures, the sample was diluted with correct stock solution to obtain the desired final treatment concentrations, in correspondence with what has been used in previous studies (Ramírez *et al.*, 2009; Urra *et al.*, 2014), see Appendix B for preparation of dopamine treatment concentrations. Dopamine treatment concentrations were, 1 mM, 10 µM and 100 nM, while the control was PBS with no treatment. The control and treatment groups were incubated for 1, 3 and 6 hours. The total motility, progressive motility, kinematics, viability, and acrosome reaction was analysed at each time point. An additional time point, 0 minutes, was added to record the baseline parameters upon the addition of treatment (see Appendix B).

3.10.2 Assessment of Motility and Kinematic parameters

Sperm motility is arguably the most important functional parameter of sperm. Spermatozoa need to be motile to swim through the female reproductive tract and reach the oocyte. Sperm are powered by the flagellum-like tail to propel it towards the egg. Sperm kinematics concern the sperm head and its motions during motility. The pattern of velocity of sperm head motions are due to the flagellar beat, and therefore may be measured as an assessment of motility. Motility parameters was assessed as described in section 3.6.5.1. The CASA analyses a range of motility parameters and various Kinematic parameters. At each time point the motility and kinematic parameters were measured with the CASA and SCA® system. Total Motility and Progressive Motility was measured and expressed as a percentage of Total Motility and a percentage of Progressive Motility, respectively. At each time point the CASA and SCA® system also automatically captured images of individual spermatozoan head movements and

digitally reconstructed their trajectories. The kinematics reported on, includes VCL; VSL; VAP; STR; LIN; WOB; ALH and BCF.

3.10.3 Assessment of Viability

The viability of spermatozoa is an important parameter of sperm function as it is indicates how many live spermatozoa are in a sample or treatment group. The WHO sets a lower reference limit of 58% for viable sperm (WHO, 2010). A dye-exclusion staining technique was employed to assess the viability of the spermatozoa. Once sperm die, they lose the structural integrity of their membranes, therefore they become permeable to stains. Viable sperm remained unstained and appeared white under the microscope while non-viable sperm were stained pink. Sperm motility also correlates to another parameter, the sperm viability or vitality, consequently motile sperm equals viable sperm.

A mixture of 20 µL Eosin and 30 µL Nigrosin was pipetted into Eppendorf tubes. At each time point 10 µL of the sperm sample (control and treatment groups) was added and the solution mixed after which smears were created (in duplicate), by pipetting 10 µL of the mixed sample onto labelled frosted slides. The slides were allowed to air-dry overnight. Once dried a small drop of DPX Mounting medium was added to the slide and a cover slip mounted on top. The Nigrosin provides a background stain while the eosin targets damaged (dead) sperm by penetrating the membrane. A Nikon Eclipse E200 light microscope was set to 40x magnification with a blue filter and contrast setting A. Both the viable and non-viable sperm were counted and a minimum of 100 spermatozoa were counted per slide, using a desk counter for each group and the results expressed as a percentage of viability.

3.10.4 Assessment of the Acrosome Reaction

The acrosome reaction (AR) is a functional parameter of sperm that is crucial for fertilization to occur, therefore, it is required to take place at the appropriate time. The use of fluorescein-conjugated Pisum sativum agglutinin (FITC-PSA) is utilized for its ability to distinguish between acrosome intact and acrosome reacted sperm. At each time point 10 µL of the sample was pipetted onto labelled frosted slides and smears were made (in duplicate) to dry overnight. Once dried the slides were fixed in 95% Ethanol for 30 minutes at 4°C, they are then left to air-dry.

In a dark room 450 µL of PBS was added to 50 µL of FITC-PSA and vortexed thoroughly. 10 µL of this mixture was pipetted onto the air-dried slides and a smear was made. The slides were kept in the dark for 45 minutes at RT whereafter they were briefly washed in distilled water. The slides were left to air-dry once more. A drop of Dako Fluorescence Mounting Medium was placed on the slide and a cover slip mounted on top. The slides were analysed with fluorescent microscopy. The slides were viewed under the Nikon Eclipse 50i microscope

with the green filter, at 40x magnification, dark field with the FITC filter selected. A minimum of 100 spermatozoa were counted per slide. Spermatozoa were classified as either intact (when the acrosome fluoresced bright green) or reacted (when no acrosome was detected, and the spermatozoa appeared dull and empty). Both the acrosome intact and acrosome reacted sperm were counted and expressed as a percentage of Acrosome Reacted.

3.11 Statistical Analysis

GraphPad™ Prism Version 5.03 (San Diego, USA) was used for all the statistical analyses. Results are expressed as the mean ± Standard Deviation (SD). Statistical significance was set at $P < 0,05$. A Two-Way ANOVA was utilized to compare the effects of time and drug concentrations on specified variables. Ordinary One-Way ANOVAs were performed, to distinguish if the concentration of dopamine influenced sperm parameters over time. The recommended normality test's- Shapiro-Wilk's and D'Agostino and Pearson omnibus, was utilized to determine normality whereafter a One-Way ANOVA or Kruskal Wallis test was applied appropriately.

Chapter 4

Results

4.1 Introduction

The results are presented in chronological order, commencing with the indirect immunofluorescence images, followed by the western blot images and data. Finally, the dopamine treatment's effect on the various sperm parameters is described in section 4.4. The dopamine treatment results are represented graphically while the IF and WB results are presented as images.

4.2 Indirect Immunofluorescence

The DAT protein was detected using an anti-DAT primary antibody, Hoechst as a nuclear stain and Alexa Fluor 488 (AF488) as a secondary antibody. As seen in Figure 15E, DAT was found to be strongly present in the midpiece region and principal piece of the sperm tail. Furthermore, lower, but still visible, DAT fluorescence was observed in the acrosomal region of the spermatozoa. When looking at the sperm which were incubated with secondary antibody only, (Figure 15A), we observe DAT weak signal in this negative control. This weak signal can be attributed to non-specific binding of the AF488. However, the DAT fluorescence in the positive control observed (Figure 15D) is strong and thus cannot wholly be attributed to non-specific binding. Taking all these factors into account, we can therefore, conclude with a fairly high confidence that DAT is found to be present on human sperm membranes (See Appendix D for enlarged images).

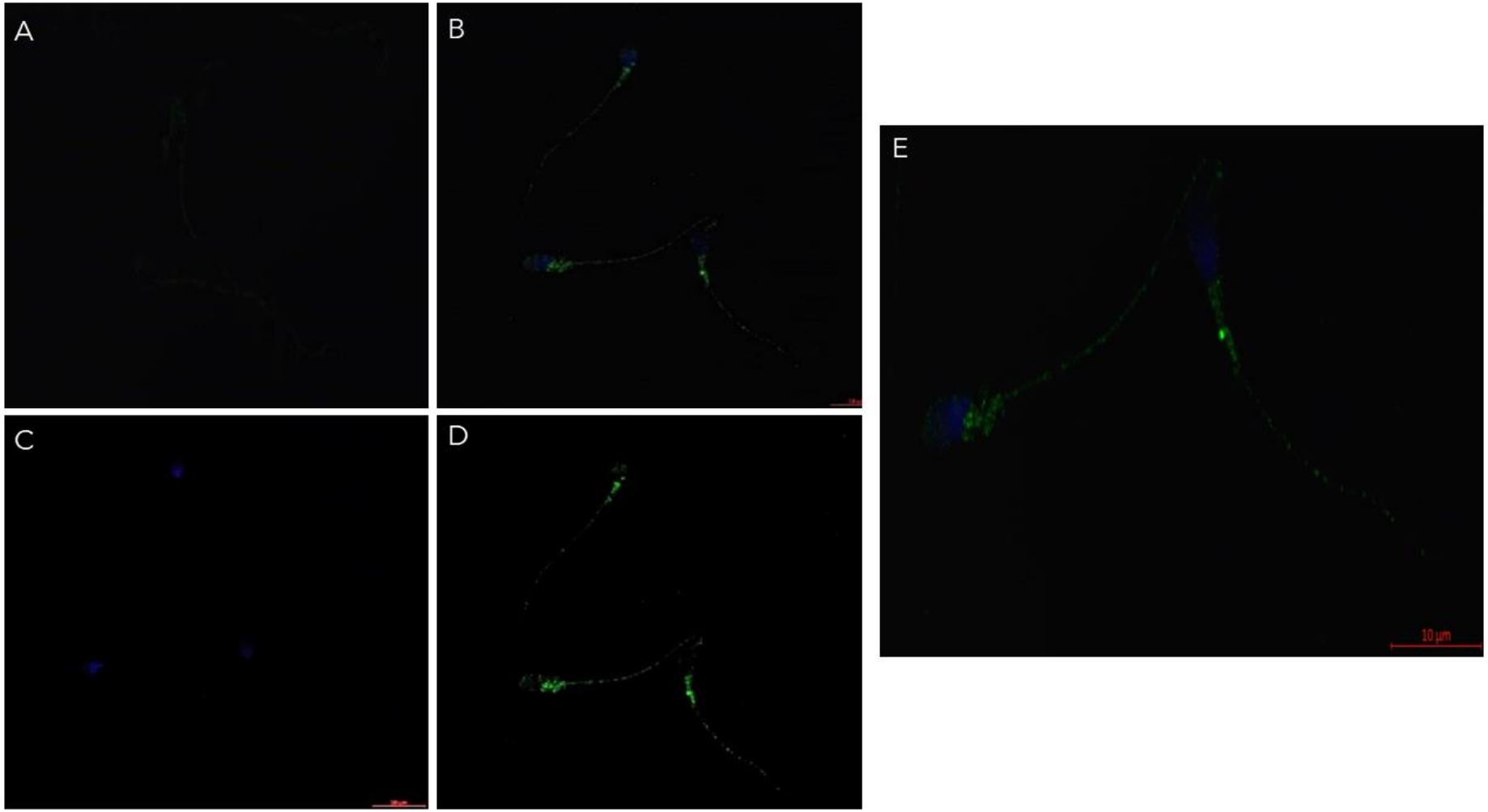


Figure 15: Localization of the Dopamine Transporter (DAT) in human sperm, via indirect immunofluorescence (IF) assay. A). Negative control containing only AF488 B) Merged image representing Hoechst, DAT and AF488. C) Nuclear stain Hoechst, D). DAT and AF488. E) Magnification of merged image, bar scale is 10 µM. Images are representative of 10 individual donors

4.3 Western Blotting

Utilization of western blotting techniques generated the following images. For the detection of DAT, an anti-DAT primary antibody was used against an anti-Rabbit IgG HRP-linked secondary antibody.

Figure 16 represents the total protein transferred to membrane 1 from gel 1. As seen in Table 6, lanes 1 and 2 which represent the control samples C1 and C2, respectively, have a much higher protein content than lanes 4 and 5 which represents S1 and S2, respectively. This discrepancy is noted even though equal loading took place, and all lanes contain equal protein content (27 µg) (refer to figure 13).

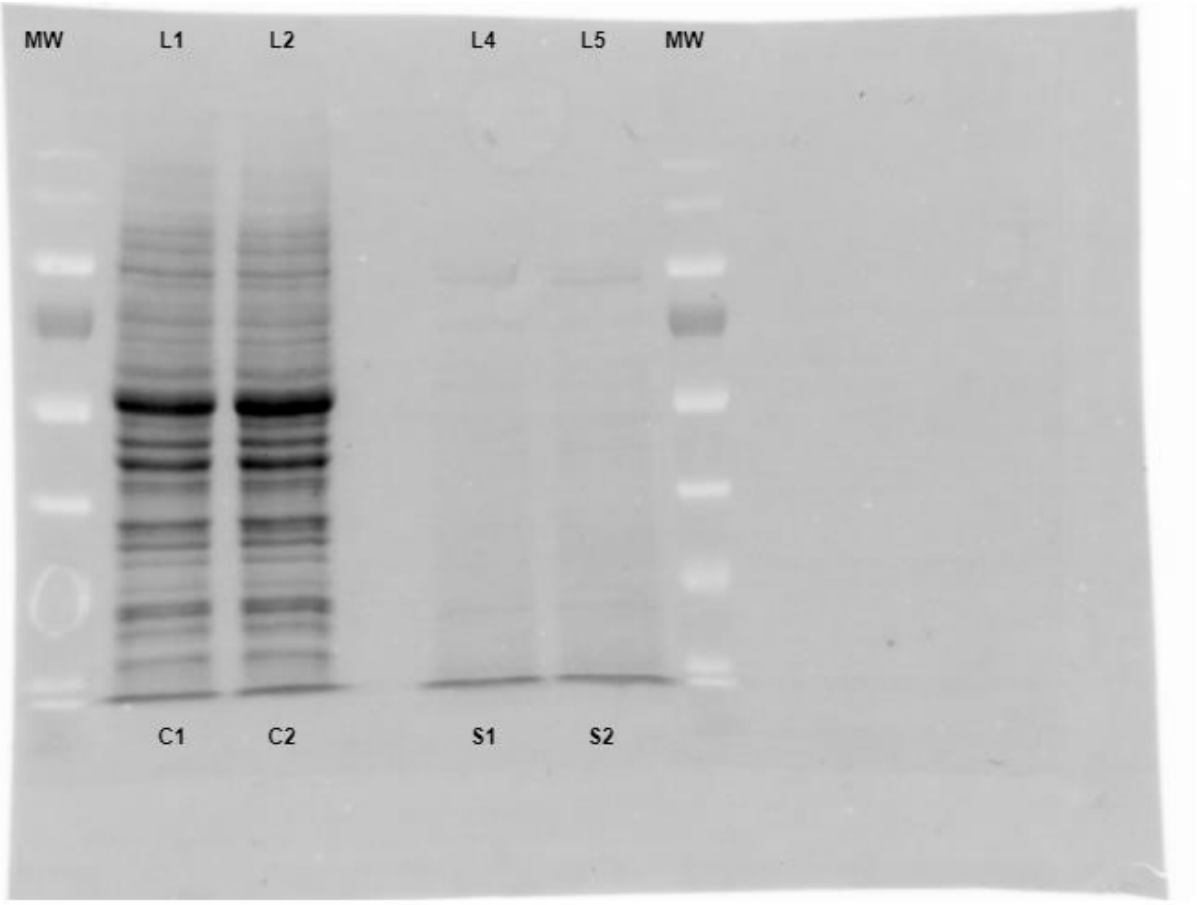


Figure 16: Membrane 1- Total Protein transferred to membrane. Lane 1 (L1) = C1 brain sample, Lane 2 (L2) = C2 brain sample, Lane 4 (L4) = S1 sperm sample, Lane 5 (L5) = S2 sperm sample.

Table 6: Total Protein on Membrane 1 (Pixel Intensity)

Lane No.	Adjusted Total Lane Volume (Int)	Total Lane Volume (Int)	Background Volume (Int)
1	640326050	1655075760	1014749710
2	722842500	1692226250	969383750
4	74851006	1061528992	986677986
5	74796909	1080210654	1005413745

Figure 17 depicts DAT protein expression from both brain (controls) and sperm samples after 2 seconds of exposure in the ChemiDoc™. Several bands were detected in the positive control C1 and C2, with prominent bands at 69 kDa and ~50 kDa. No bands were detected for sperm, in both samples, at 2 seconds of exposure.

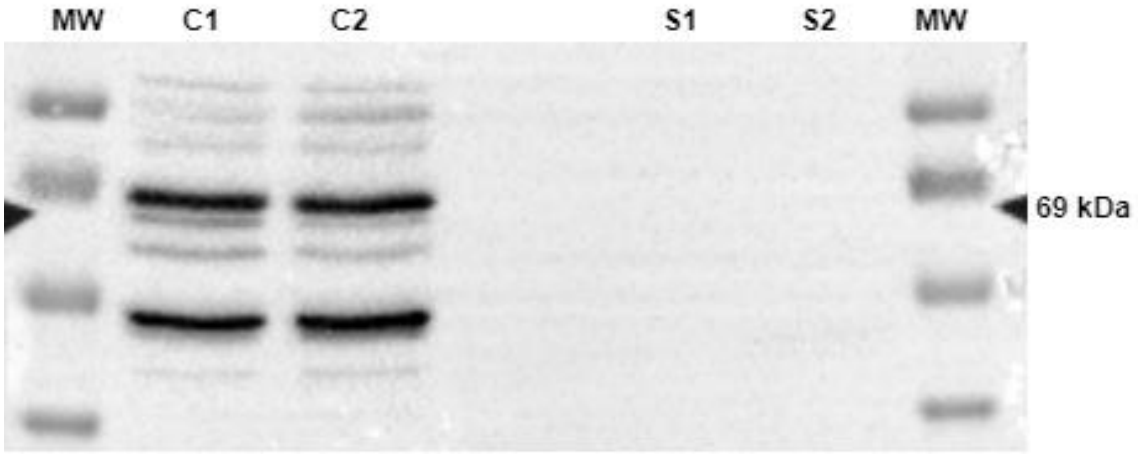


Figure 17: SDS PAGE and Western Blot analysis for total protein extract (27.5 µg). Membrane 1- DAT expression after 2 seconds of exposure. Rat brain (C1 and C2) and human sperm (S1 and S2) with anti-DAT antibody.

Figure 18 depicts DAT protein expression from both brain (controls) and sperm samples after 80 seconds of exposure in the ChemiDoc™. Several bands are detected in the positive controls, albeit over-exposed, with prominent bands at 69 kDa, 62 kDa and 48 kDa. A faint band was detected in S2 at 62 kDa and a darker band at 48 kDa. S1 failed to produce any bands after 80 seconds of exposure. The band detected at 62 kDa and 48 kDa in S2 correspond to the bands in the positive controls C1 and C2.

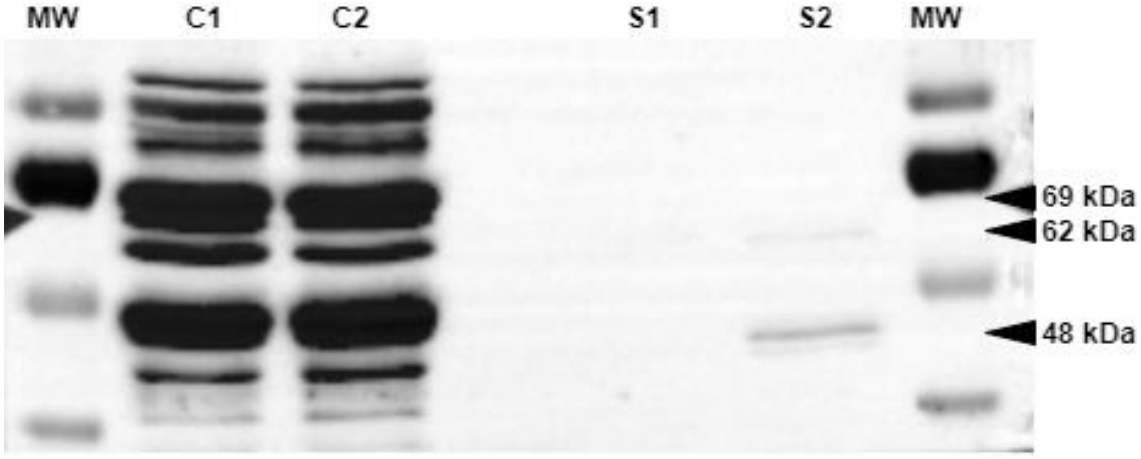


Figure 18**: SDS PAGE and Western Blot analysis for total protein extract (27.5 µg). Membrane 1-DAT expression** after 80 seconds of exposure. Rat brain (C1 and C2) and human sperm (S1 and S2) with anti-DAT antibody

Figure 19 represents the total protein transferred to membrane 2 from gel 2. As seen in Table 7, lanes 1 and 2 which represent the control samples C1 and C2, respectively, have a much higher protein content than lanes 4 and 5 which represents S1 and S2, respectively. This discrepancy is noted even though double the volume of sperm sample was loaded, compared to the brain sample (refer to figure 14).

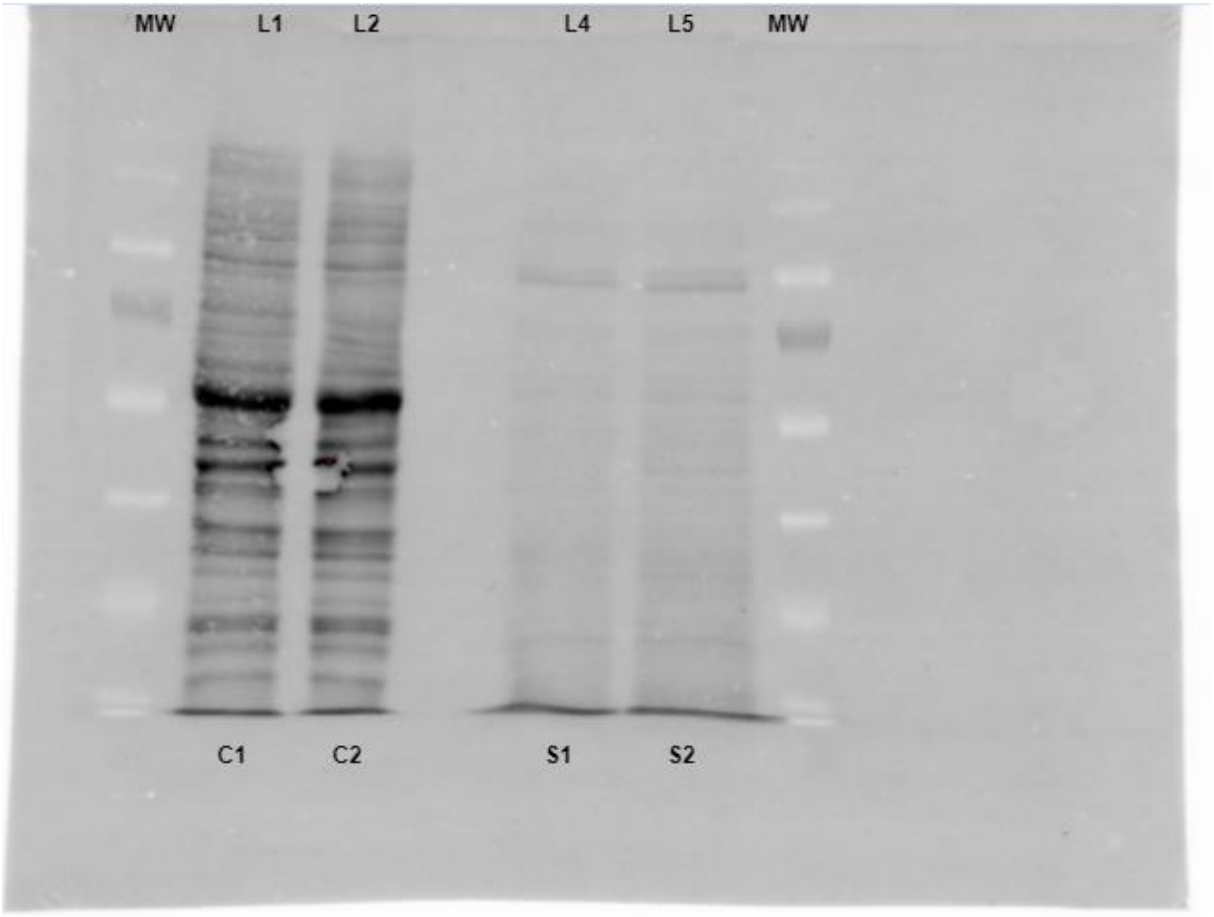


Figure 19: Membrane 2- Total protein transferred to membrane. Lane 1 (L1) = C1 brain sample, Lane 2 (L2) = C2 brain sample, Lane 4 (L4) = S1 sperm sample, Lane 5 (L5) = S2 sperm sample.

Table7: Total Protein on Membrane 2 (Pixel Intensity)

Lane No.	Adjusted Total Lane Volume (Int)	Total Lane Volume (Int)	Background Volume (Int)
1	490286500	1711259250	1220972750
2	476212170	1500240525	1024028355
4	144302528	1280192752	1135890224
5	164248605	1251198348	1086949743

Figure 20 depicts DAT protein expression from both brain (controls) and sperm samples after 2 seconds of exposure in the ChemiDoc™. Several bands were detected in the positive controls C1 and C2, with prominent bands at 69 kDa and ~50 kDa. No bands were detected for sperm, in both samples, at 2 seconds of exposure.

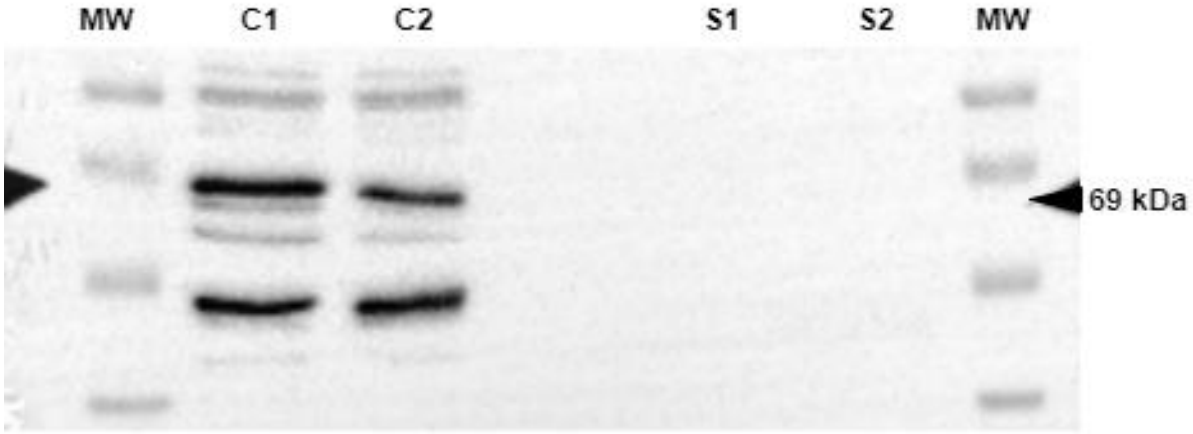


Figure 20: SDS PAGE and Western Blot analysis for total protein extract (27.5 µg). Membrane 2- DAT expression after 2 seconds of exposure. Rat brain (C1 and C2) and human sperm (S1 and S2) with anti-DAT antibody.

Figure 21 depicts DAT protein expression from both brain (controls) and sperm samples after 80 seconds of exposure in the ChemiDoc™. Several bands are detected in the positive controls, albeit over-exposed, with prominent bands at 69 kDa, 62 kDa and 48 kDa. Two faint bands are detected in S1 at 62 kDa and at 48 kDa. A faint band at 62 kDa is detected in S2 and a darker band is detected at 48 kDa. The band detected at 62 kDa and 48 kDa in the sperm samples correspond to the positive controls C1 and C2.

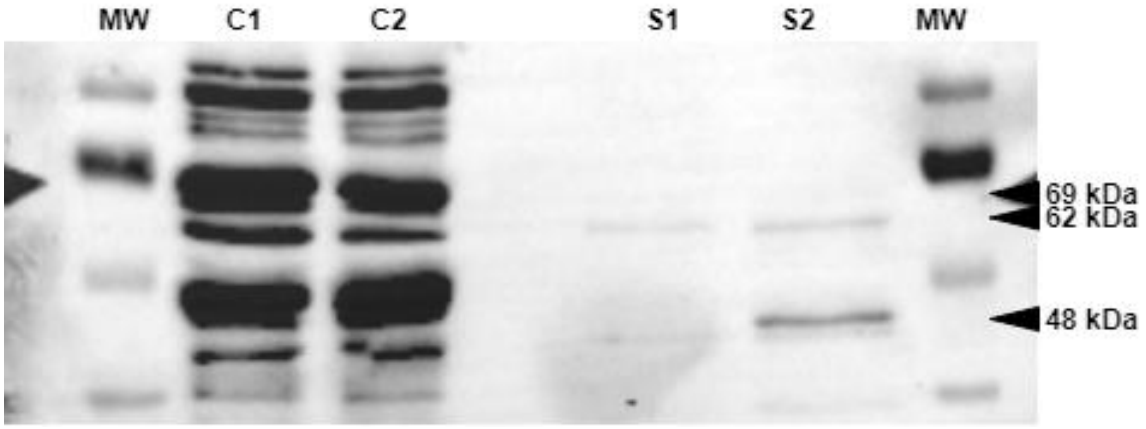


Figure 21: SDS PAGE and Western Blot analysis for total protein extract (27.5 µg). Membrane 2- DAT expression after 80 seconds of exposure. Rat brain (C1 and C2) and human sperm (S1 and S2) with anti-DAT antibody.

4.4 Dopamine Treatments effect on Functional parameters

The results of the dopamine treatments effect on functional human sperm parameters are described below. A total of 24 sperm samples were collected, treated, and analysed. Total Motility, Progressive Motility, Kinematics, Viability and Acrosome Reaction was analysed.

4.4.1 Total Motility

Figure 22 depicts a Two-Way ANOVA performed on the Total Motility data; it represents the effect of Dopamine on Total Motility of spermatozoa expressed as a percentage over time. The WHO sets a lower reference limit of 40% for Total Motility. An expected time dependent decrease of Total Motility is observed, this is an expected observation in *in vitro* experiments, however this is greatly exacerbated by the addition of the high concentration of dopamine. As shown in figure 22, dopamine had a significant effect on total motility specifically at the high concentration of 1 mM. The high concentration of dopamine is shown to significantly decrease Total Motility as early as 60 minutes when compared to the control (51.99% ± 15.93 vs. 18.10% ± 8.75). The low and medium concentrations did not significantly affect Total Motility at any time point when compared to the controls.

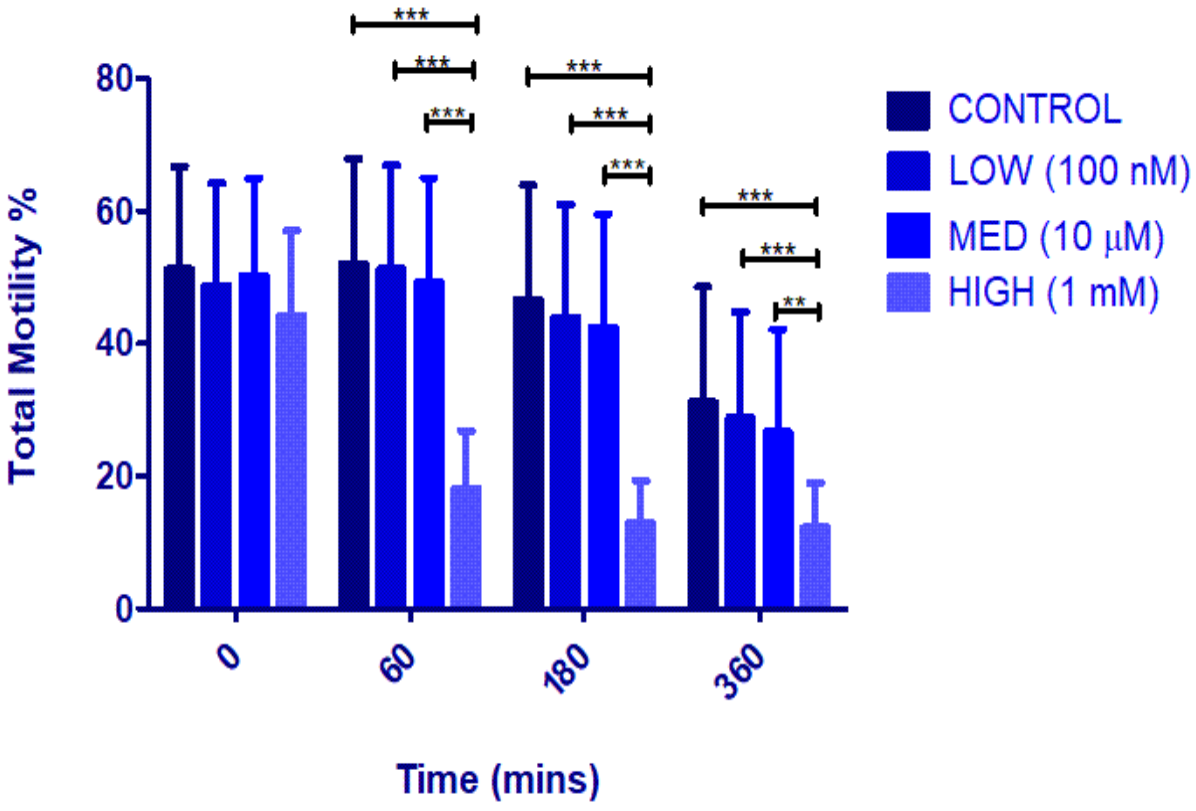


Figure 22: Effect of different concentrations of dopamine on Total Motility over time. n = 24 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 23 depicts the effect of Dopamine at different concentrations on Total Motility expressed as a percentage over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. In Figure 23A, it is clear to see that Total Motility decreases significantly as time progresses without any treatment present (51.27% ± 15.44 vs. 31.14% ± 17.47), however this is expected as sperm lose motility over time. Figure 23B depicts the effect of the low (100 nM) concentration of dopamine over time. Time significantly affected the decrease of Total Motility. Figure 23C depicts the effect of the medium (10 µM) concentration of dopamine over time. Once again, a significant decrease is observed when comparing all the time points to 360 minutes. Figure 23D depicts the effect of the high concentration of dopamine over time. The high concentration significantly decreased the total motility at 60 minutes (44.30% ± 12.72 vs. 18.75% ± 8.75), at 180 minutes (44.30% ± 12.72 vs. 12.83% ± 6.498) and at 360 minutes (44.30% ± 12.72 vs. 10.46% ± 6.715) when compared to time 0.

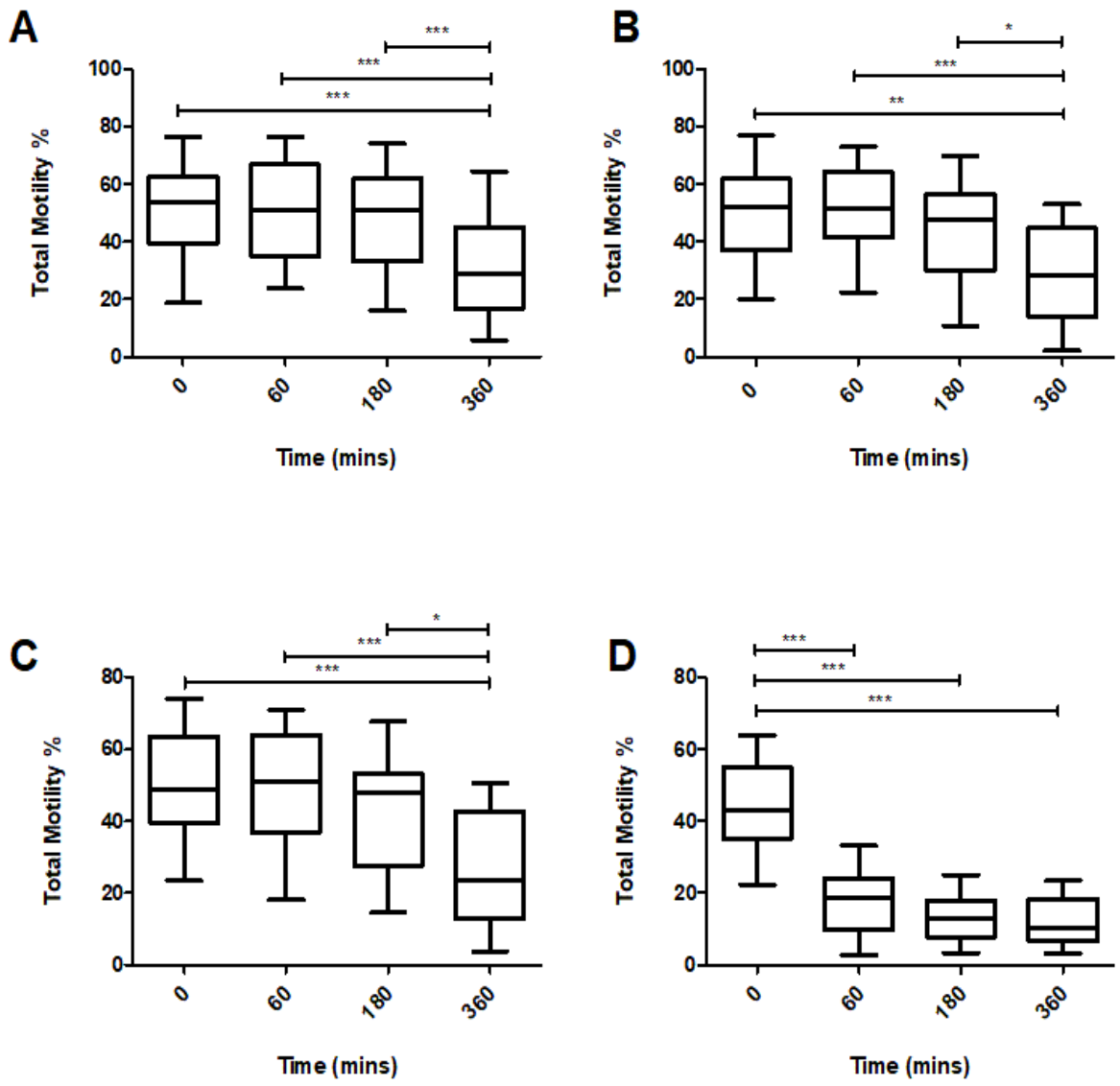


Figure 23: Effect of time at different concentrations of dopamine on Total Motility (A) Total Motility of Control. (B) Total Motility at a Low (100 nM) concentration of dopamine. (C) Total Motility at a Medium (10 µM) concentration of dopamine. (D) Total Motility at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.2 Progressive Motility

Figure 24 depicts a Two-Way ANOVA performed on the Progressive Motility data; it represents the effect of Dopamine on Progressive Motility of spermatozoa expressed as a percentage over time. The WHO sets a lower reference limit of 32% for Progressive Motility. Similarly to Total Motility a time-dependent decrease is observed for Progressive Motility, which is significantly exacerbated by the high concentration of dopamine. The high concentration of dopamine is shown to significantly decrease Progressive Motility as early as 60 minutes when compared to the control (39.03% ± 13.50 vs. 7.296% ± 4.578). Both the low and medium

concentrations of dopamine do not significantly affect the Progressive Motility at any time point when compared to controls.

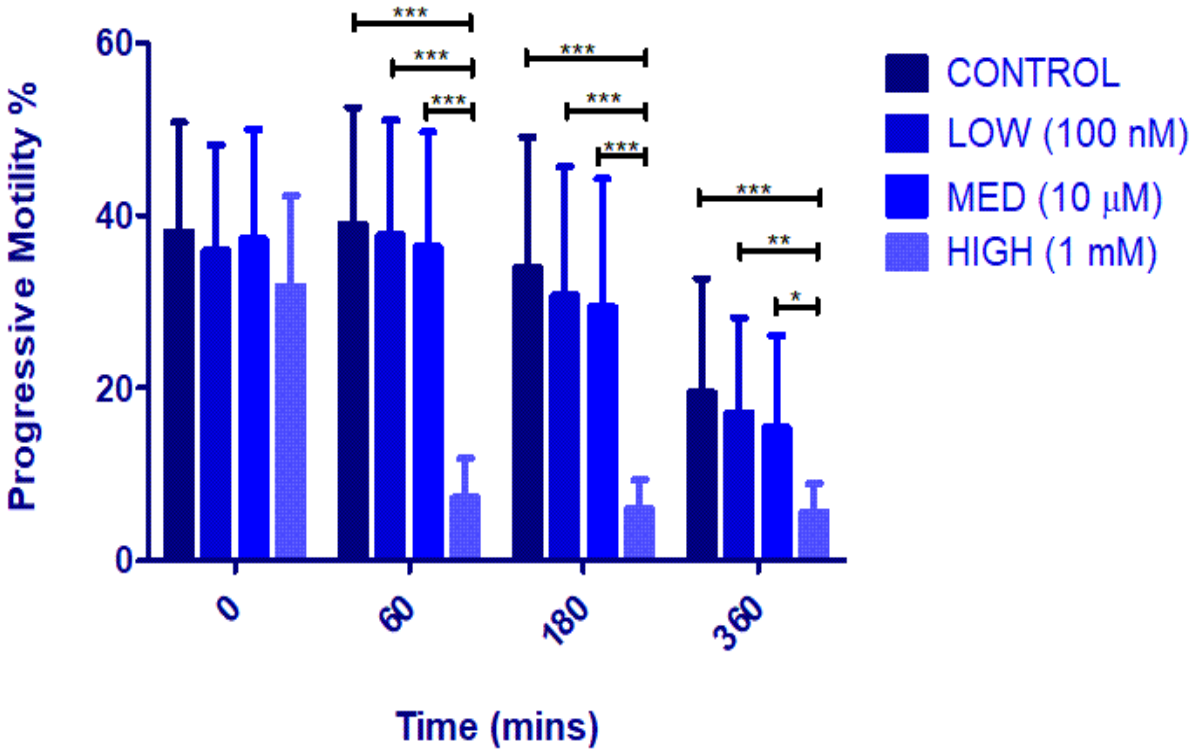


Figure 24: Effect of different concentrations of dopamine on Progressive Motility over time. n = 24 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 25 depicts the effect of Dopamine on Progressive Motility expressed as a percentage over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. In Figure 25A, it is clear to see that Progressive Motility decreases significantly as time progresses without any treatment present (31.12% ± 12.66 vs 19.47% ± 13.24). Figure 25B depicts the effect of the low (100 nM) concentration of dopamine over time. Progressive Motility was significantly affected by time. Figure 25C depicts the effect of the medium (10 µM) dopamine concentration over time. At 6 hours the Progressive Motility was significantly decreased. Figure 25D represents the % Progressive Motility over time at the high dopamine concentration. The high concentration significantly decreased the Progressive Motility as early as 60 minutes (29.05% ± 10.48 vs 6.150% ± 4.578) when compared to time 0. At 180 minutes the decrease was also significant (29.05 ± 10.48 vs 5.945 ± 3.416). The high concentration decreased the Progressive Motility from 29.05% at 0 minutes to 4.82% at 360 minutes.

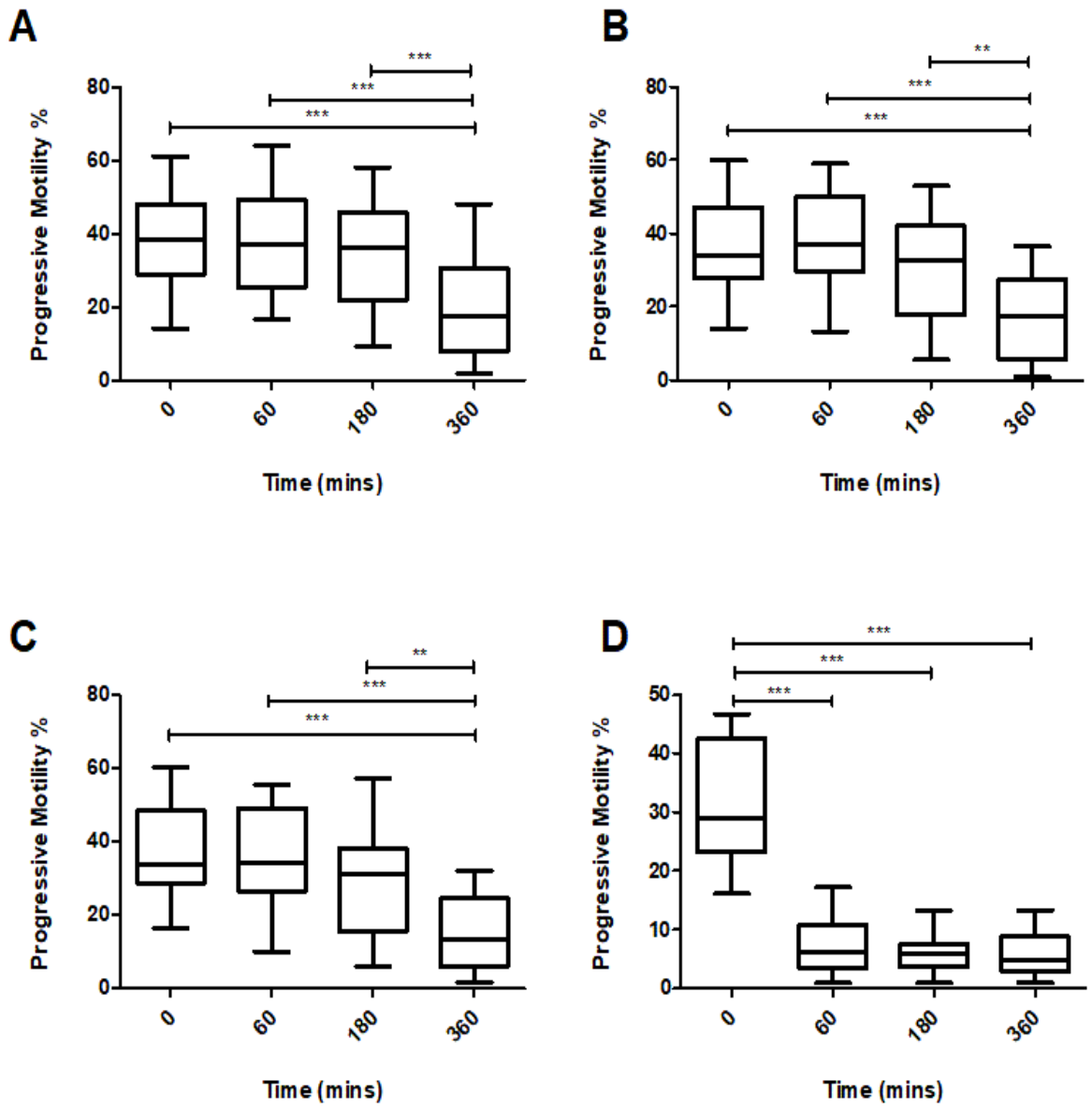


Figure 25: Effect of time at different concentrations of dopamine on Progressive Motility(A) Progressive Motility of Control. (B) Progressive Motility at a Low (100 nM) concentration of dopamine. (C) Progressive Motility at a Medium (10 µM) concentration of dopamine. (D) Progressive Motility at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.3 Kinematics

Kinematic analysis of sperm movement within a sample measures the velocity, acceleration, and displacement of spermatozoa. The velocity and trajectory of the sperm determine motility and are therefore important to characterise. The velocity parameters include the Curvilinear Velocity (VCL), the Straight-line Velocity (VSL), and the Average Path Velocity (VAP), these are all measured as speeds (µm/s). The trajectory of a spermatozoa can influence the velocity values; therefore, the velocity values are compared by ratios that have been derived (Mortimer and Mortimer, 1990). The Linearity Index (LIN %) is derived by (VSL/VCL) x 100; the Straightness Index (STR %) is calculated as (VSL/VAP) x 100; and the Oscillatory Index (WOB %) is calculated as (VAP/VCL) x 100. The Amplitude of Lateral Head Displacement (ALH) measures the degree of lateral displacement of the sperm head around its average path and is measured in µm (Ayad, Van der Horst and Du Plessis, 2018). The Beat-Cross Frequency (BCF) describes the frequency at which the sperm head crosses the average path, it is measured in Hz (Ayad, Van der Horst and Du Plessis, 2018). Since the pattern of velocity of sperm head motion is due to the flagellar beat, it may therefore be measured as an assessment of motility (Kay and Robertson, 1998).

4.4.3.1 VCL

Figure 26 represents a Two-Way ANOVA performed on the Kinematic parameter Curvilinear Velocity (VCL). It depicts the effect of dopamine on VCL over time expressed as speed per second (µm/s). As seen in figure 26 the VCL is significantly affected by the high concentration of dopamine. VCL decreases gradually over time but the decrease is significantly exacerbated by the high concentration of dopamine at each time point when compared to the controls, time 0 (101.2 ± 12.18 vs. 91.42 ± 10), 1 hour (102.8 ± 12.67 vs. 61.66 ± 7.073), 3 hours (92.73 ± 15.97 vs. 67.23 ± 5.297) and 6 hours (78.08 ± 10.95 vs. 68.45 ± 5.129).

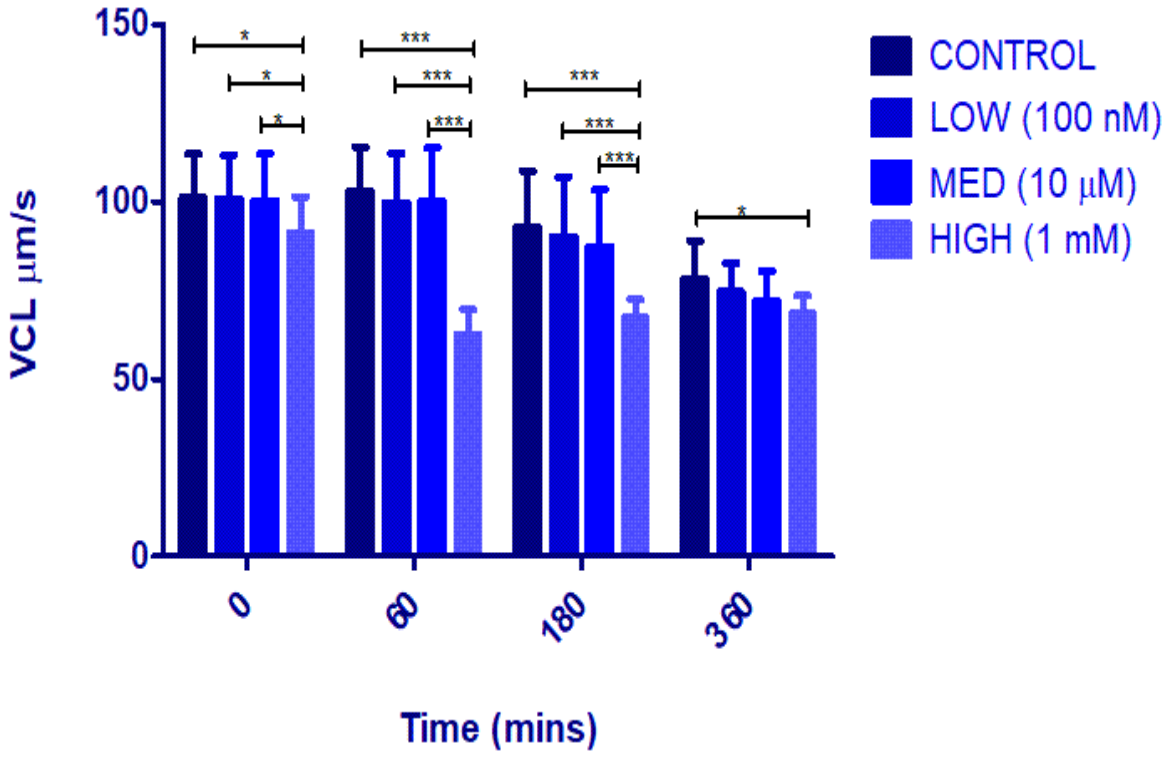


Figure 26: Effect of different concentrations of dopamine on Curvilinear Velocity (VCL) over time. n= 24 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 27 depicts the effect of Dopamine on VCL expressed as a speed (µm/s) over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. Figure 27A represents the effect of the control on VCL, as seen VCL significantly decreases over time (101.2 ± 12.18 vs 78.08 ± 10.95) when no treatment is present. Figure 27B depicts the effect of low dopamine concentration on VCL. Time significantly affected the decrease of VCL. Figure 27C depicts the effect of the medium concentration of dopamine on VCL. A time-dependent decrease is observed from time 0 to 6 hours; however, the decrease is significantly exacerbated after 3 hours (87.29 ± 16.16 vs. 71.94 ± 8.601). Figure 27D depicts the effect of the high concentration of dopamine on VCL. As in figure 28D the high concentration of dopamine significantly decreases VCL from as early as 60 minutes (102.8 ± 12.67 vs. 62.66 ± 7.073) when compared to the control at 60 minutes.

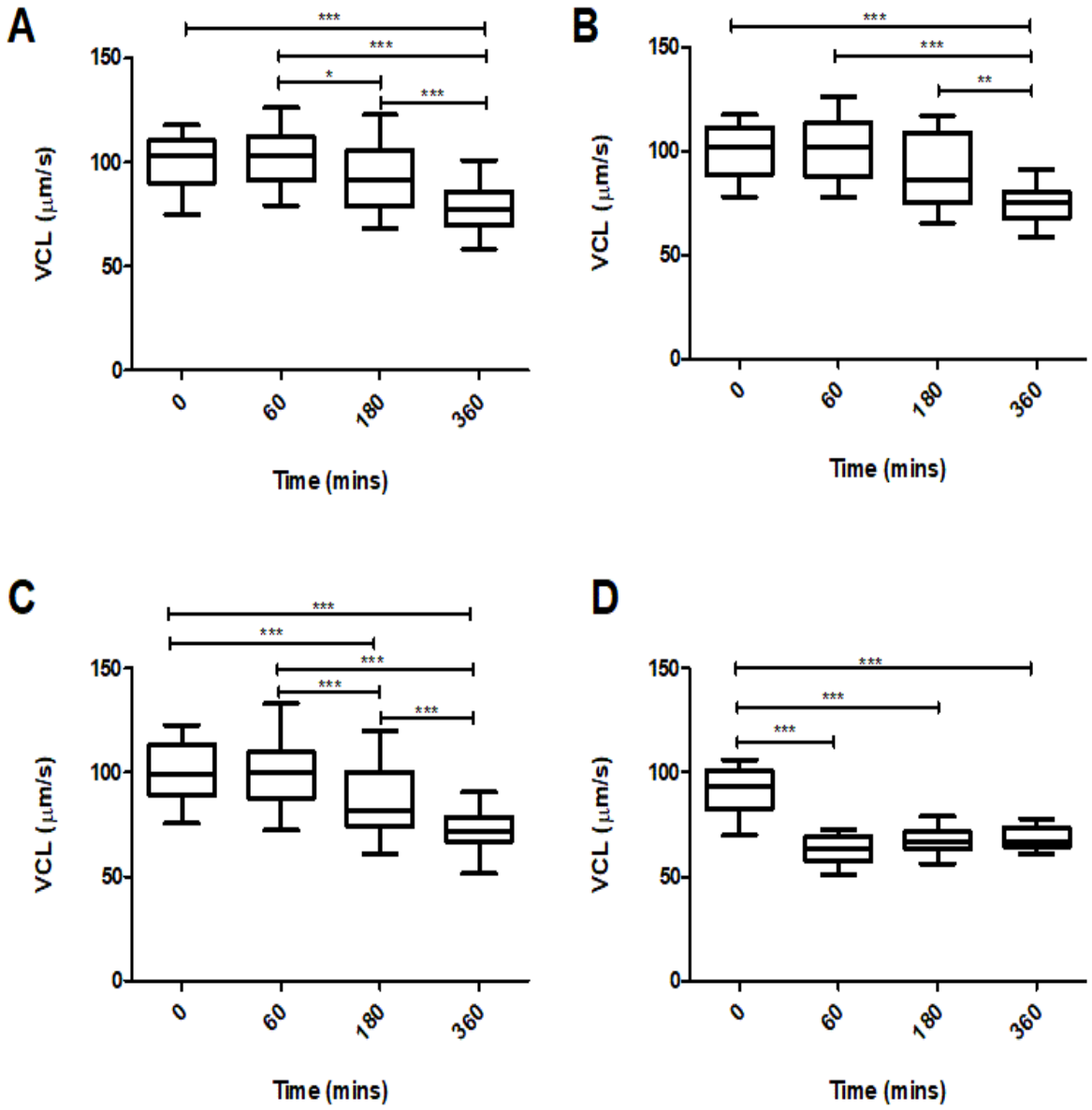


Figure 27: Effect of time at different concentrations of dopamine on Curvilinear Velocity (VCL) (A) VCL of Control. (B) VCL at a Low (100 nM) concentration of dopamine. (C) VCL at a Medium (10 μM) concentration of dopamine. (D) VCL at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.3.2 VAP

Figure 28 represents a Two-Way ANOVA performed on the Kinematic parameter Average Path Velocity (VAP). It depicts the effect of dopamine on VAP over time expressed as speed per second (μm/s). Figure 28 shows that the high concentration of dopamine significantly decreased the VAP at 1, 3 and 6 hours when compared to the control, low and medium concentrations of dopamine. After 1 hour of incubation the high concentration decreased the VAP (control) from 61.25 ± 8.283 to 30.89 ± 5.233.

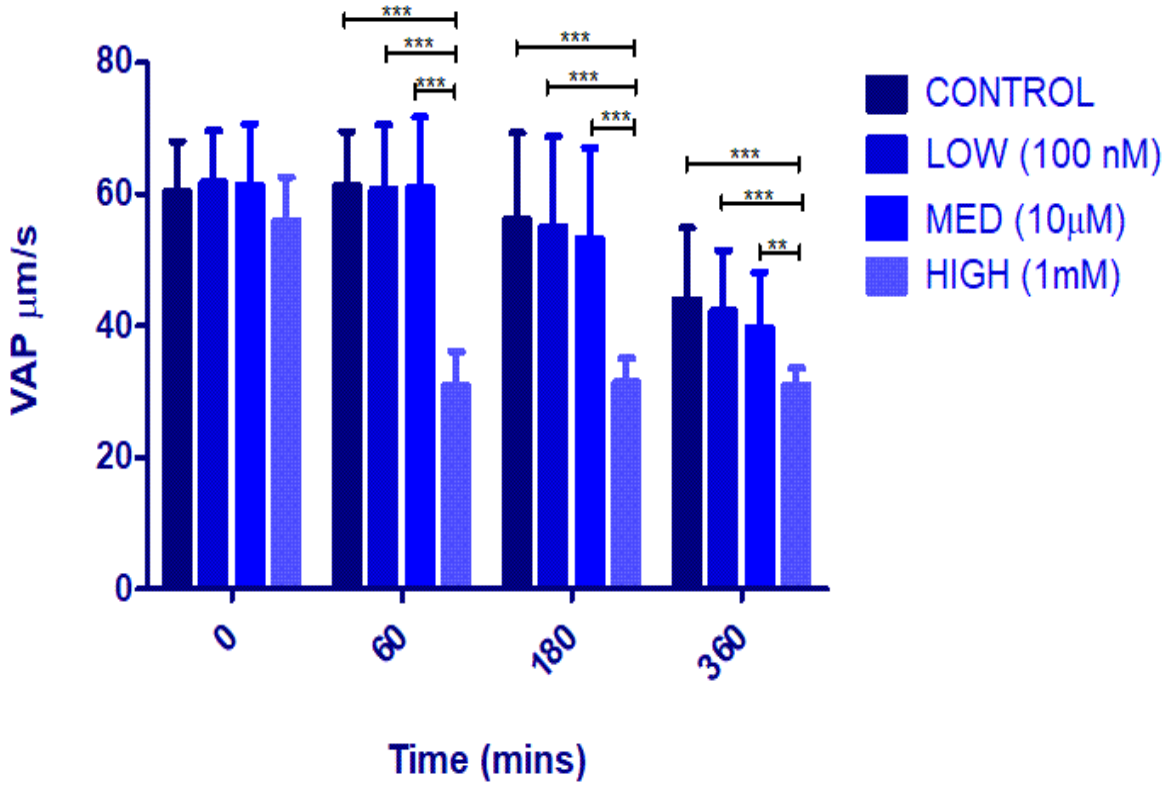


Figure 28: Effect of different concentrations of dopamine on Average Path Velocity (VAP) over time. n = 24 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 29 depicts the effect of dopamine on VAP expressed as a speed (µm/s) over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. Figure 29A depicts the effect of the control on VAP. An expected time-dependent decrease is observed in the absence of treatment. Figure 29B and 29C depicts the effect of the low and medium concentrations, respectively of dopamine on VAP. A significant time-dependent decrease is observed for VAP for both the low and medium concentrations of dopamine. Figure 29D depicts the effect of the high concentration of dopamine on VAP. As seen in figure 29D the high concentration of dopamine significantly decreases VAP after 1 hour of incubation (61.25 ± 8.283vs.30.89 ± 5.233) when compared to the control at 60 minutes.

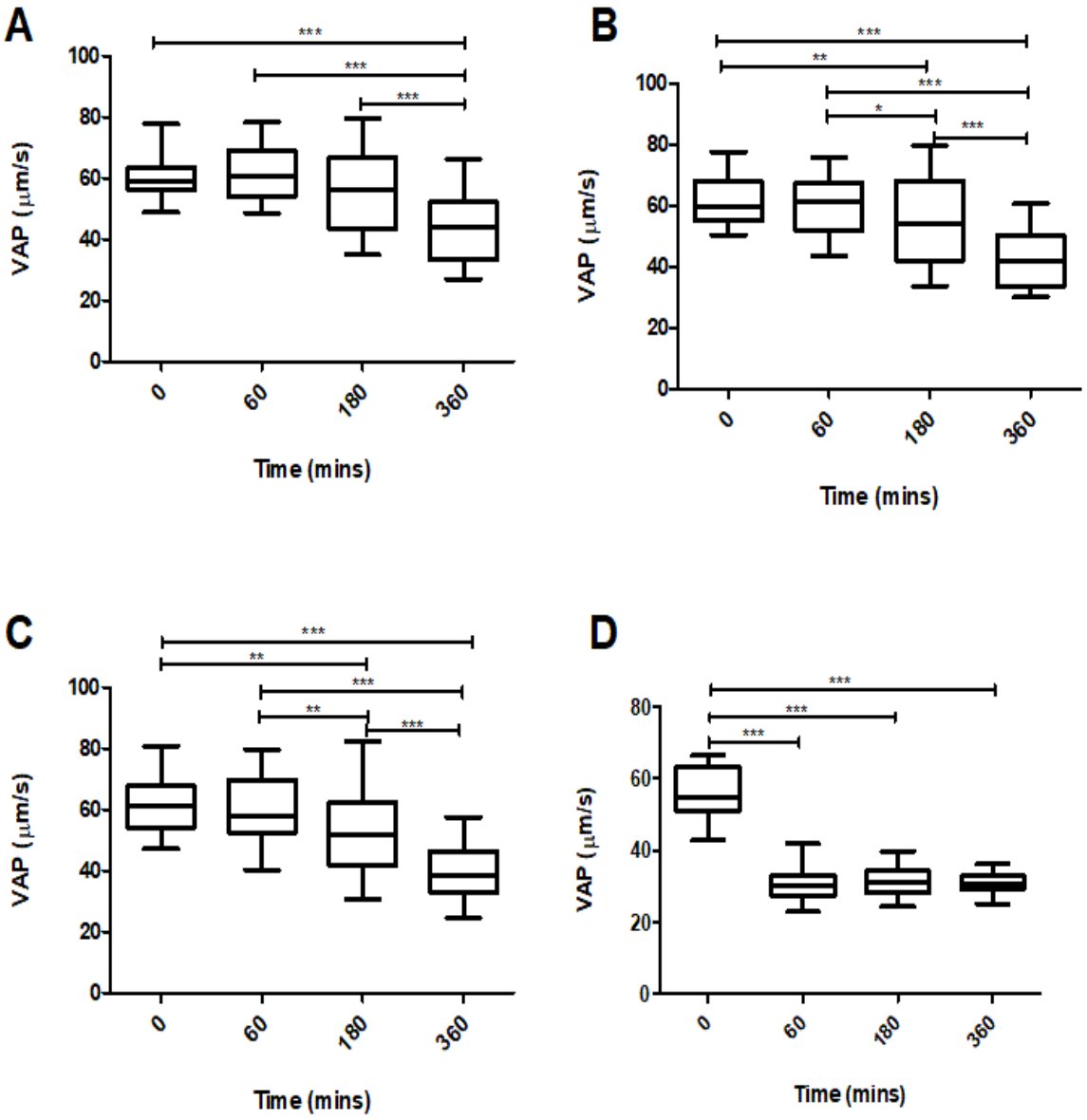


Figure 29: Effect of time at different concentrations of dopamine on Average Path Velocity (VAP) (A) VAP of Control. (B) VAP at a Low (100 nM) concentration of dopamine. (C) VAP at a Medium (10 µM) concentration of dopamine. (D) VAP at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.3.3 VSL

Figure 30 represents a Two-Way ANOVA performed on the Kinematic parameter Straight-Line Velocity (VSL). It depicts the effect of dopamine on VSL over time expressed as speed per second (µm/s). From figure 30 it can be seen that high concentrations significantly decreased the VSL at 1, 3 and 6 hours, when compared to the control, low and medium concentrations of dopamine. After 1 hour of incubation with the high concentration of dopamine the VSL (control) was decreased from 42.92 ± 7.838 to 13.07 ± 4.379.

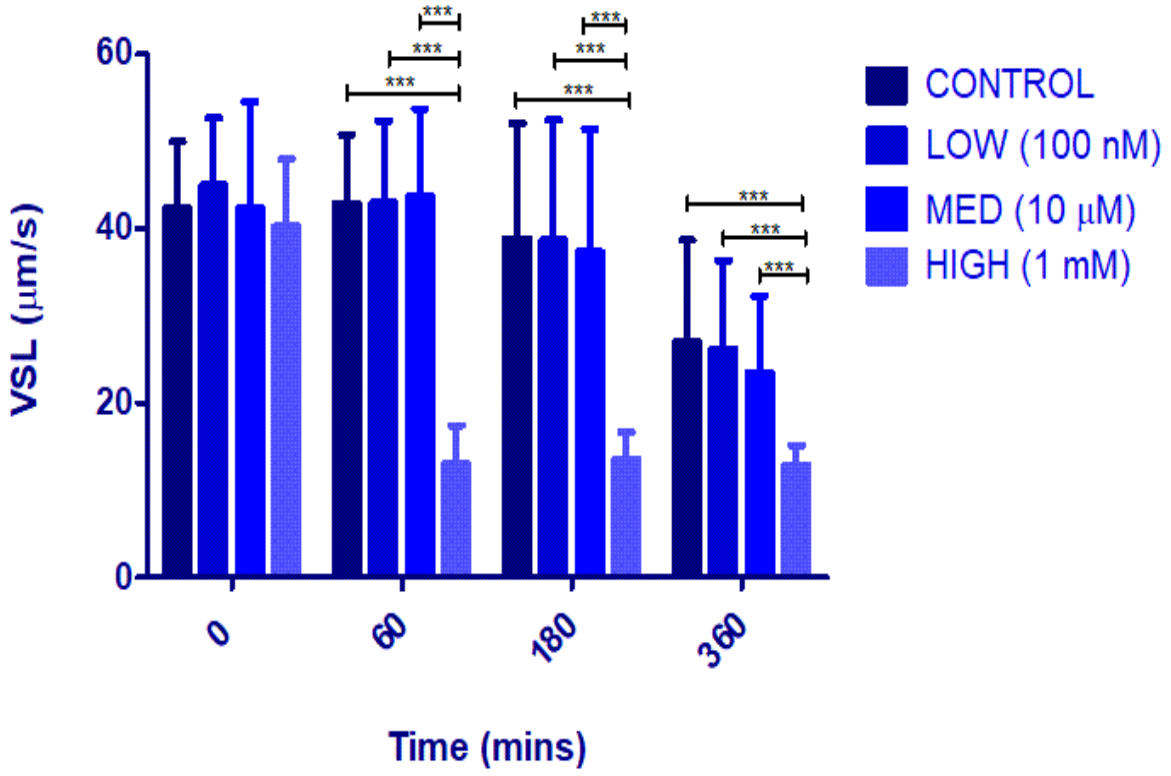


Figure 30: Effect of different concentrations of dopamine on Straight-line Velocity (VSL) over time. n = 24 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 31 depicts the effect of dopamine on VSL expressed as a speed (µm/s) over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. Figure 31A represents the effect of the control on VSL, as seen VSL decreases significantly as time progresses (42.26 ± 7.716 vs. 27.05 ± 11.59). Figures 31B and 31C depicts the low concentration and medium concentrations of dopamine on VSL, respectively. The low and medium concentrations did not significantly affect the VSL, however, a time-dependent decrease is observed. Figure 31D depicts the high concentration of dopamine on VSL over time. As seen, VSL is significantly decreased after 1 hour of incubation with the high concentration (40.26 ± 7.68 vs. 13.07 ± 4.379) when compared to time 0.

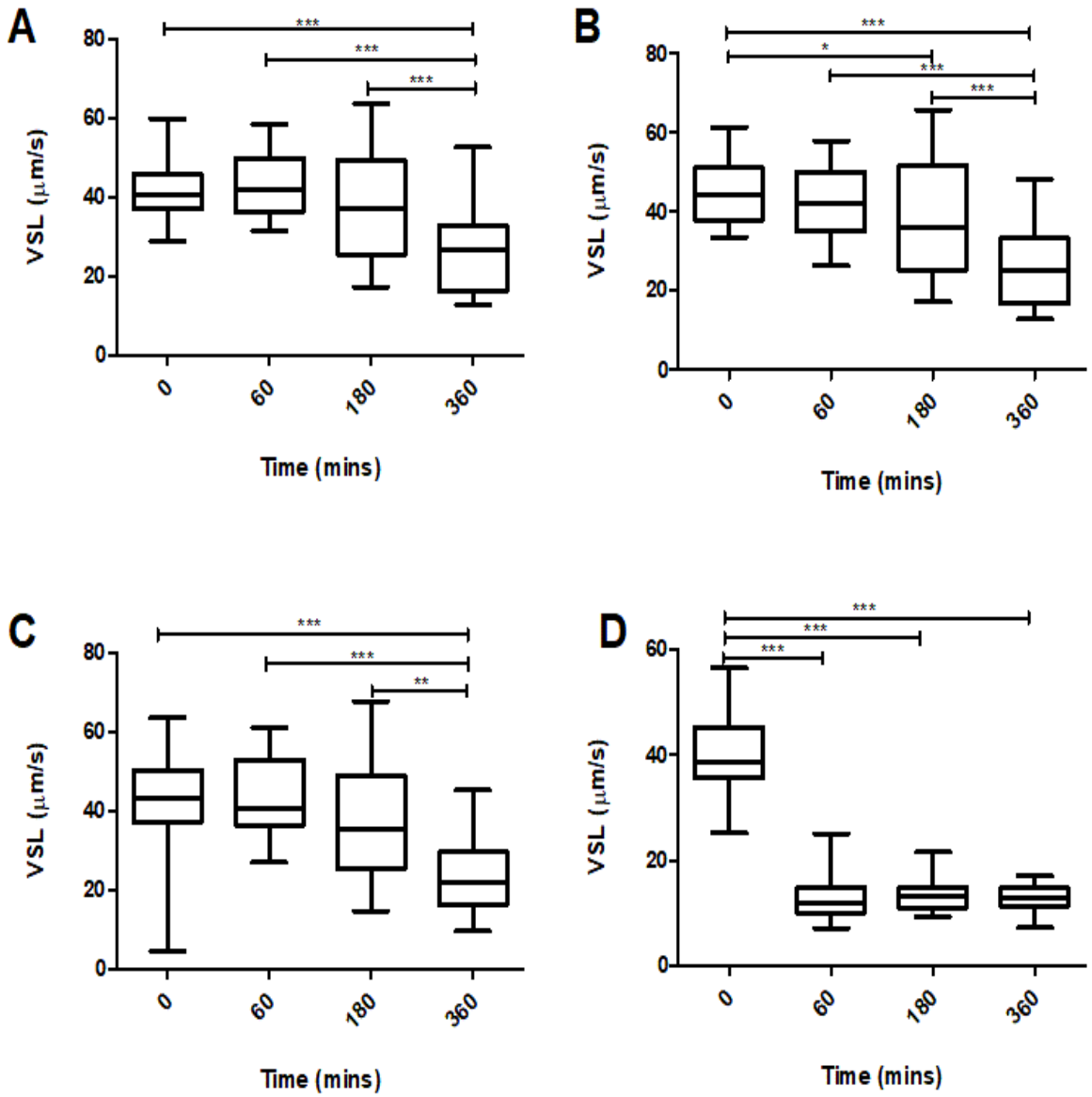


Figure 31: Effect of time at different concentrations of dopamine on Straight-line Velocity (VSL). (A) VSL of Control. (B) VSL at a Low (100 nM) concentration of dopamine. (C) VSL at a Medium (10 µM) concentration of dopamine. (D) VSL at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.3.4 STR

Figure 32 represents a Two-Way ANOVA performed on the Kinematic parameter Straightness Index (STR). It depicts the effect of dopamine on STR over time expressed as a percentage over time. From figure 32 it can be seen that the high concentration significantly decreases the STR % at 1, 3 and 6 hours when compared to the control, low and medium concentrations of dopamine. After 1 hour of incubation with the high concentration of dopamine the STR (control) decreased from 62.23% ± 4.622 to 39.63% ± 6.781.

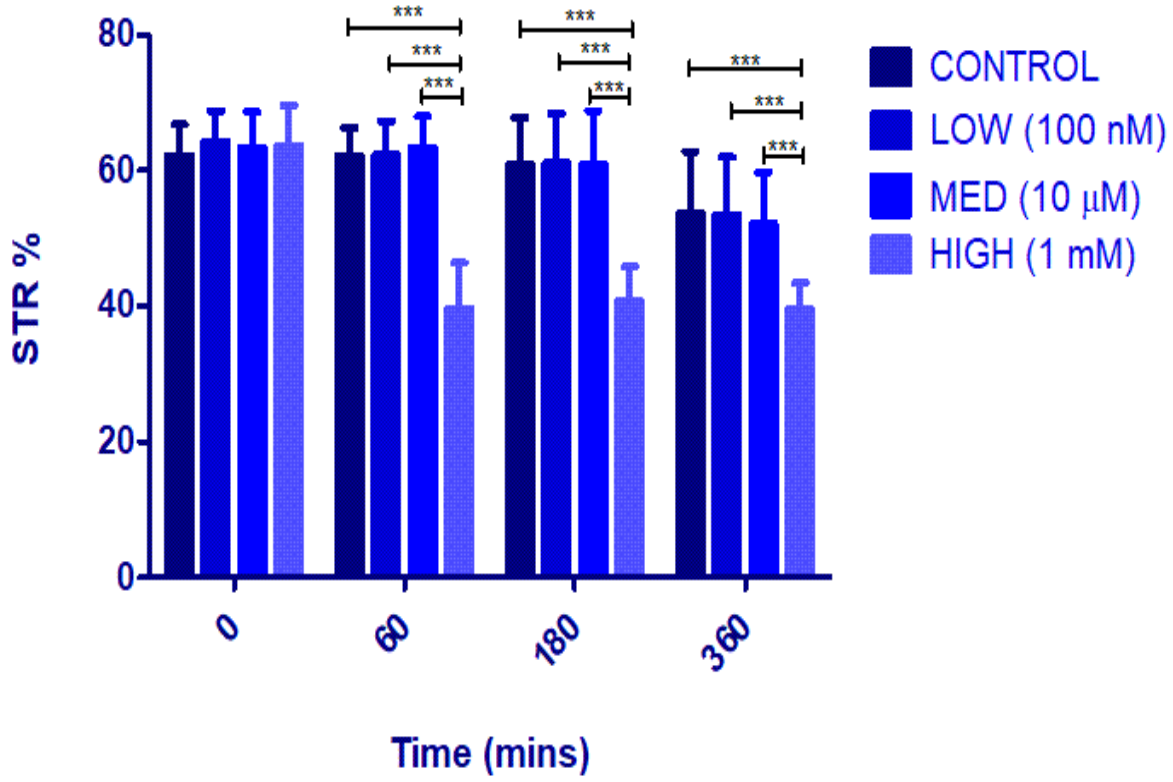


Figure 32: Effect of different concentrations of dopamine on Straightness Index (STR) over time. n = 24 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 33 depicts the effect of dopamine on STR expressed as a percentage over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. Figure 33A depicts the effect of the control on STR. As seen in Figure 33A the STR decreases significantly over the 6 hours, without treatment present (62.23% ± 4.081 vs.53.70% ± 9.129). Figures 33B and 33C depict the effect of the low and medium concentrations of dopamine of STR, respectively. As seen in these figures, a significant time-dependent decrease is observed. However, as seen in Figure 33D, the high concentration significantly decreases STR from as early as 1 hour when compared to time 0 (63.71% ± 5.962 vs. 39.63% ± 6.781).

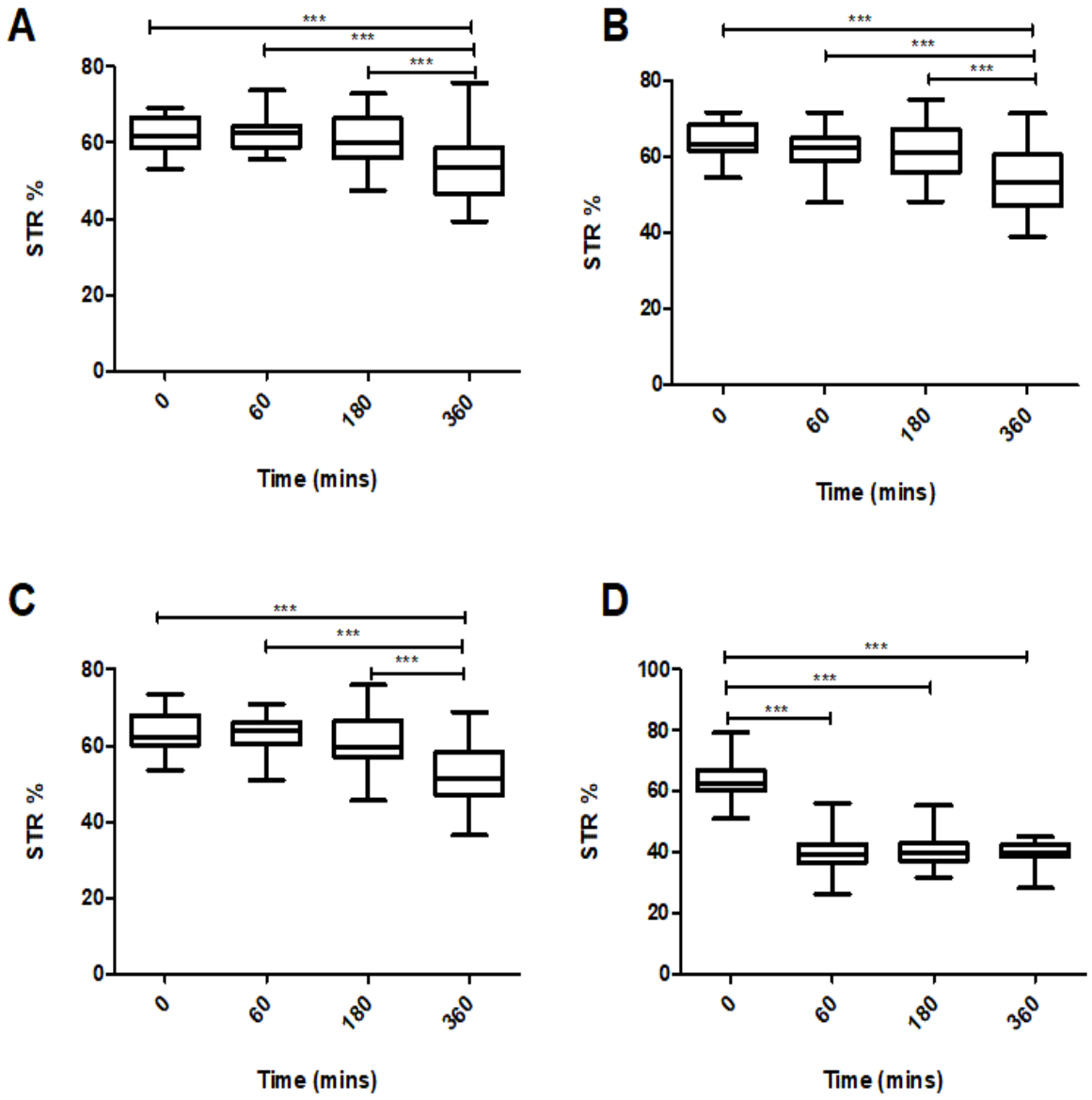


Figure 33: Effect of time at different concentrations of dopamine on Straightness Index (STR). (A) STR of Control. (B) STR at a Low (100 nM) concentration of dopamine. (C) STR at a Medium (10 µM) concentration of dopamine. (D) STR at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.3.5 LIN

Figure 34 represents a Two-Way ANOVA performed on the Kinematic parameter Linearity Index (LIN). It depicts the effect of dopamine on LIN over time expressed as a percentage over time. As seen in figure 34 the high concentration significantly decreases the LIN % at 1, 3 and 6 hours when compared to the control, low and medium concentrations of dopamine. After 1 hour of incubation with the high concentration of dopamine the LIN is significantly decreased when compared to the control at 60 minutes (38.79% ± 4.230 vs. 21.07% ± 5.510).

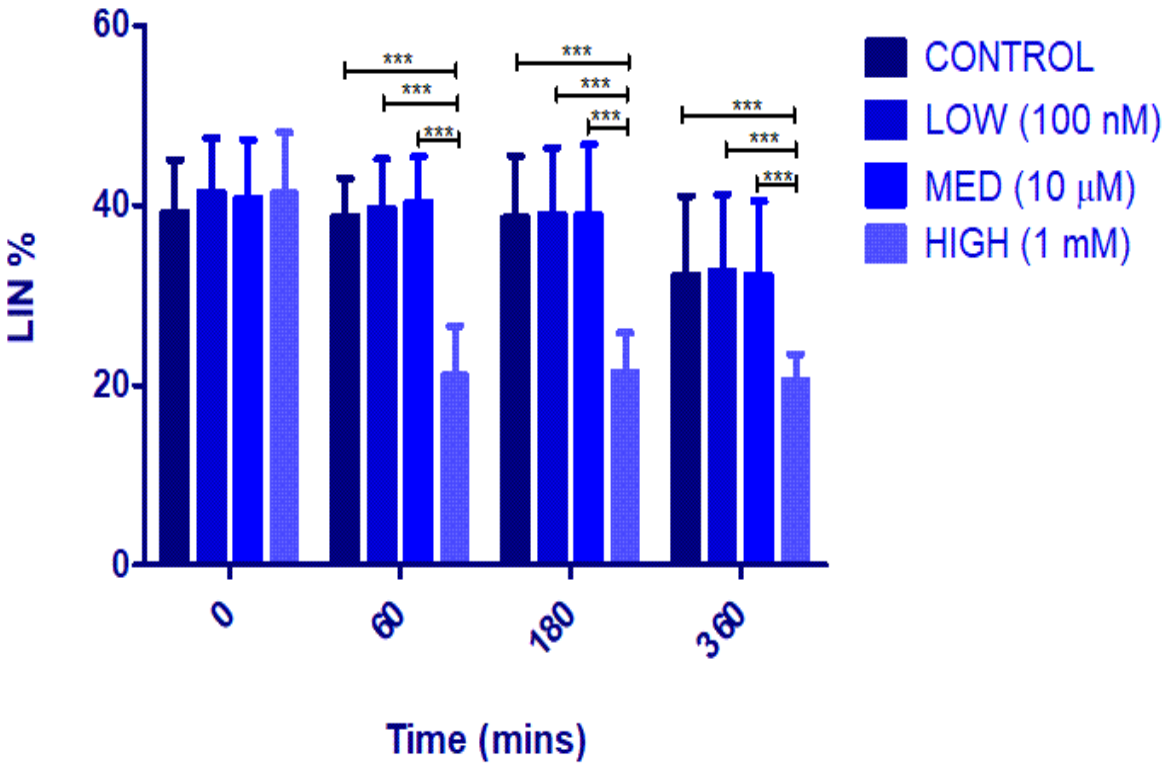


Figure 34: Effect of different concentrations of dopamine on Linearity Index (LIN) over time. n = 24Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 35 depicts the effect of dopamine on LIN expressed as a percentage over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. Figure 35A depicts the effect of the control on LIN over time. As seen a significant time-dependent decrease is observed in the absence of treatment (39.24% ± 5.826 vs. 32.28% ± 8.784). Figures 35B and 35C depict effect of the low and medium concentrations of dopamine on LIN, respectively. Once again, a time-dependent decrease is observed. However, in Figure 35D, which depicts the effect of the high concentration of dopamine on LIN over time, a significant decrease is observed as early as 1 hour when compared to time 0 (41.33% ± 6.796 vs. 21.07% ± 5.51).

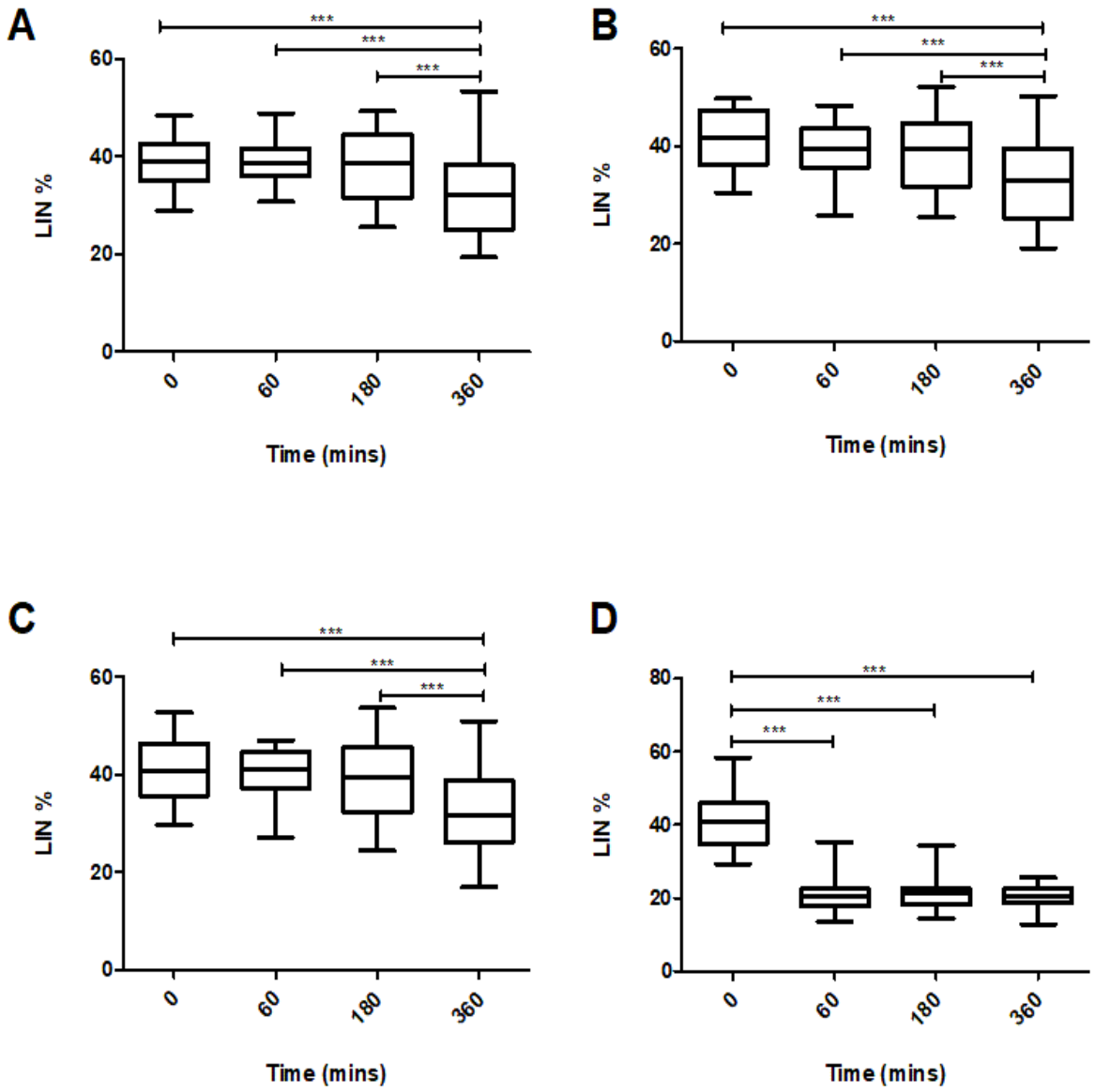


Figure 35: Effect of time at different concentrations of dopamine on Linearity Index (LIN). (A) LIN of Control. (B) LIN at a Low (100 nM) concentration of dopamine. (C) LIN at a Medium (10 µM) concentration of dopamine. (D) LIN at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.3.6 WOB

Figure 36 represents a Two-Way ANOVA performed on the Kinematic parameter Oscillatory Index (WOB). It depicts the effect of dopamine on WOB over time expressed as a percentage over time. From figure 36 it can be seen that the high concentration significantly decreases the WOB % at 1, 3 and 6 hours when compared to the control, low and medium concentrations of dopamine. As seen in Figure 36 the high concentration of dopamine significantly decreases

the WOB from as early as 1 hour when compared to the control at 60 minutes (59.02% ± 3.8 vs. 49.66% ± 5.09).

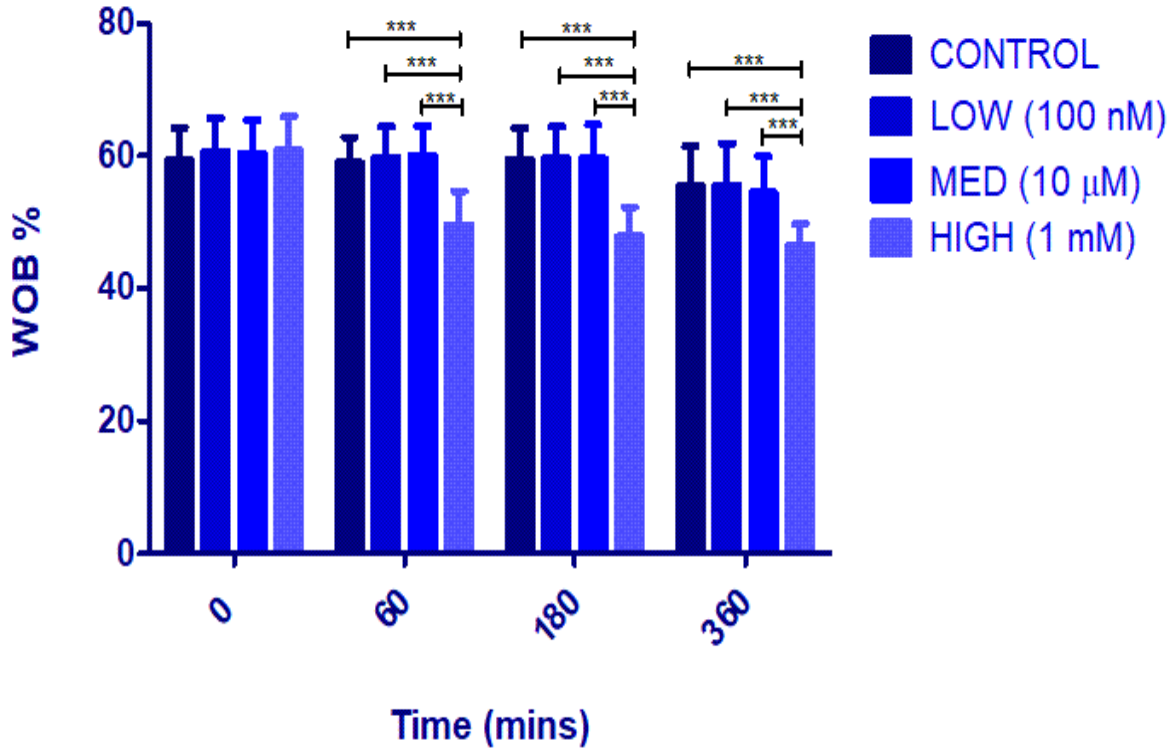


Figure 36: Effect of different concentrations of dopamine on Oscillatory Index (WOB) over time. n = 24 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 37 depicts the effect of dopamine on WOB expressed as a percentage over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. Figure 37A depicts the effect of the control on WOB over time. As seen in Figure 37A, a significant time-dependent decrease is observed in the absence of treatment after 3 hours (59.55% ± 4.629 vs. 55.42% ± 6.042). Figures 37B and 37C depicts the effect of the low and medium concentrations of dopamine on WOB, respectively. As seen in these figures time significantly decreases the WOB, although after 3 hours the decrease is exacerbated. Figure 37D depicts the effect of the high concentration of dopamine on WOB over time, and as seen WOB is significantly decreased after 1 hour of incubation when compared to time 0 (60.80% ± 5.112 vs. 49.66% ± 5.09).

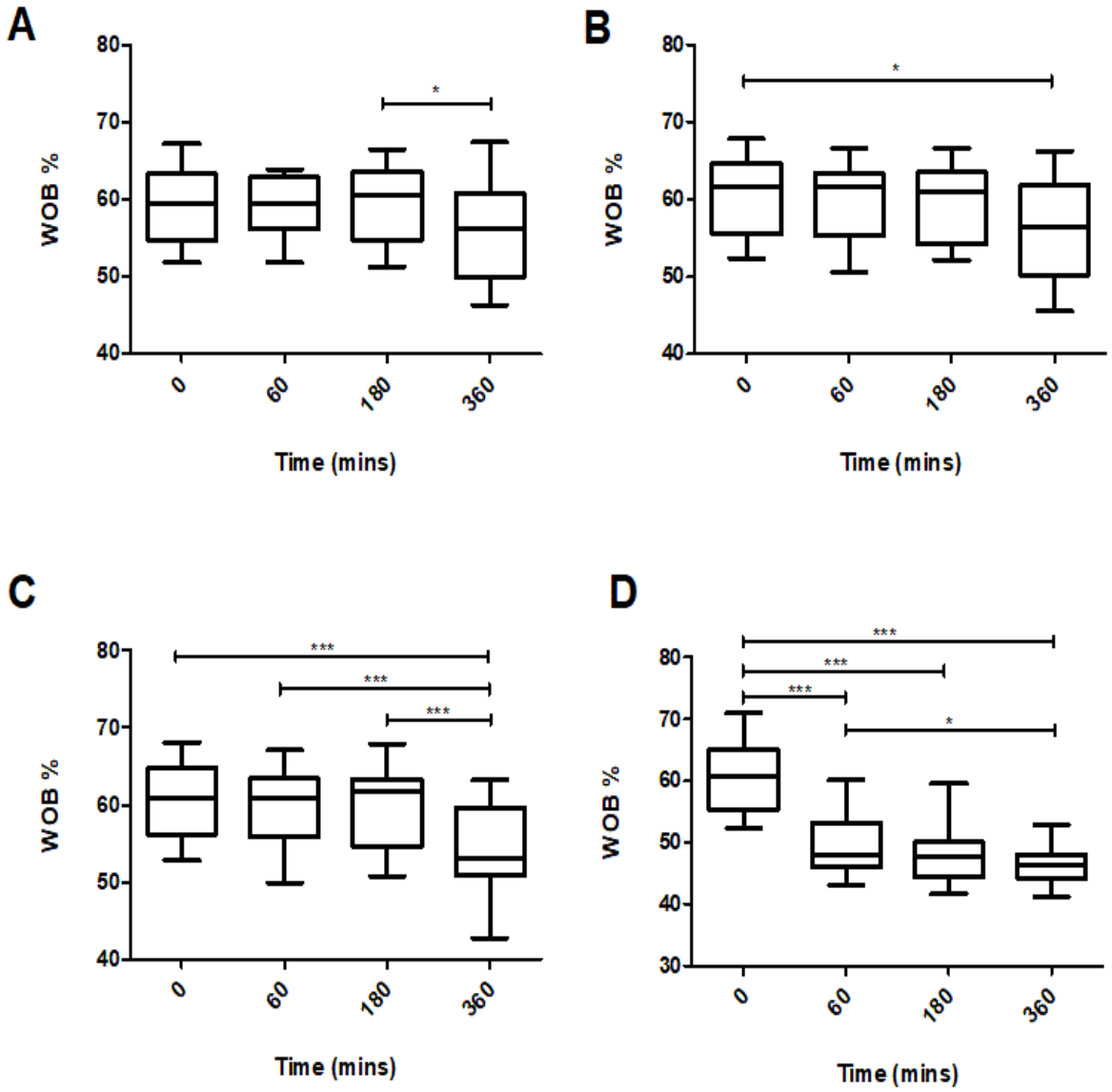


Figure 37: Effect of time at different concentrations of dopamine on Oscillatory Index (WOB). (A) WOB of Control. (B) WOB at a Low (100 nM) concentration of dopamine. (C) WOB at a Medium (10 µM) concentration of dopamine. (D) WOB at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.3.7 ALH

Figure 38 represents a Two-Way ANOVA performed on the Kinematic parameter Amplitude of Lateral Head Displacement (ALH). It depicts the effect of dopamine on ALH over time expressed as distance covered over time (µm). It is seen in figure 38 that the high concentration significantly decreases the ALH at time 0, 1 hour and 3 hours, when compared to the control, low and medium concentrations of dopamine. At 6 hours the ALH is not significantly affected. After the addition of dopamine, at time 0, the high concentration

significantly decreases the ALH when compared to the control (2.547 ± 0.2968 vs. 2.313 ± 0.2475).

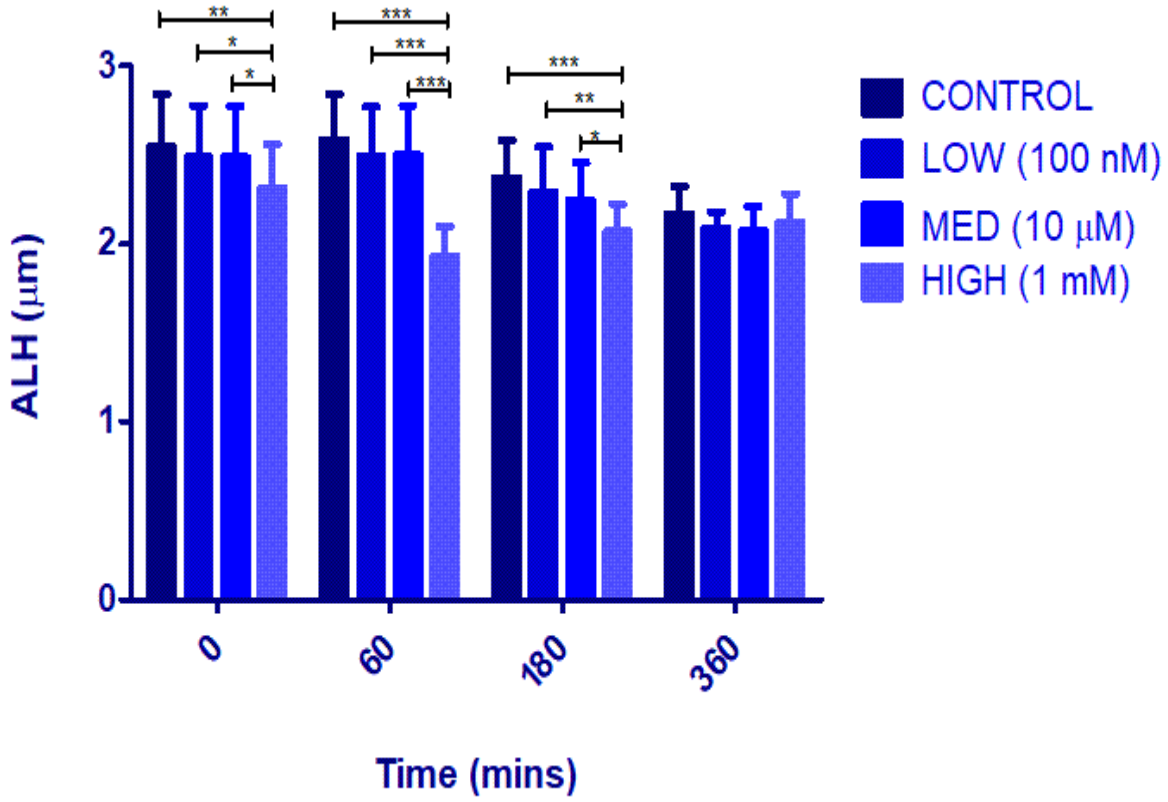


Figure 38: Effect of different concentrations of dopamine on Amplitude of Lateral Head Displacement (ALH) over time. n = 24 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 39 depicts the effect of dopamine on ALH expressed as a distance covered over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. Figure 39A depicts the effect of the control on ALH, as seen in this figure ALH is significantly decreased in the absence of treatment, however the decrease is exacerbated after 3 hours of incubation (2.371 ± 0.2123 vs. 2.169 ± 0.1522). The same effect is observed in Figures 39B and 39C, which depict the effect of the low and medium concentration of dopamine on ALH, respectively. Figure 39D depicts the effect of the high concentration of dopamine, an initial significant decrease is observed after 1 hour of incubation when compared to time 0 (2.313 ± 0.2475 vs. 1.931 ± 0.1681), however the ALH increases significantly after 1 hour of incubation (1.931 ± 0.1681 vs. 2.119 ± 0.1622).

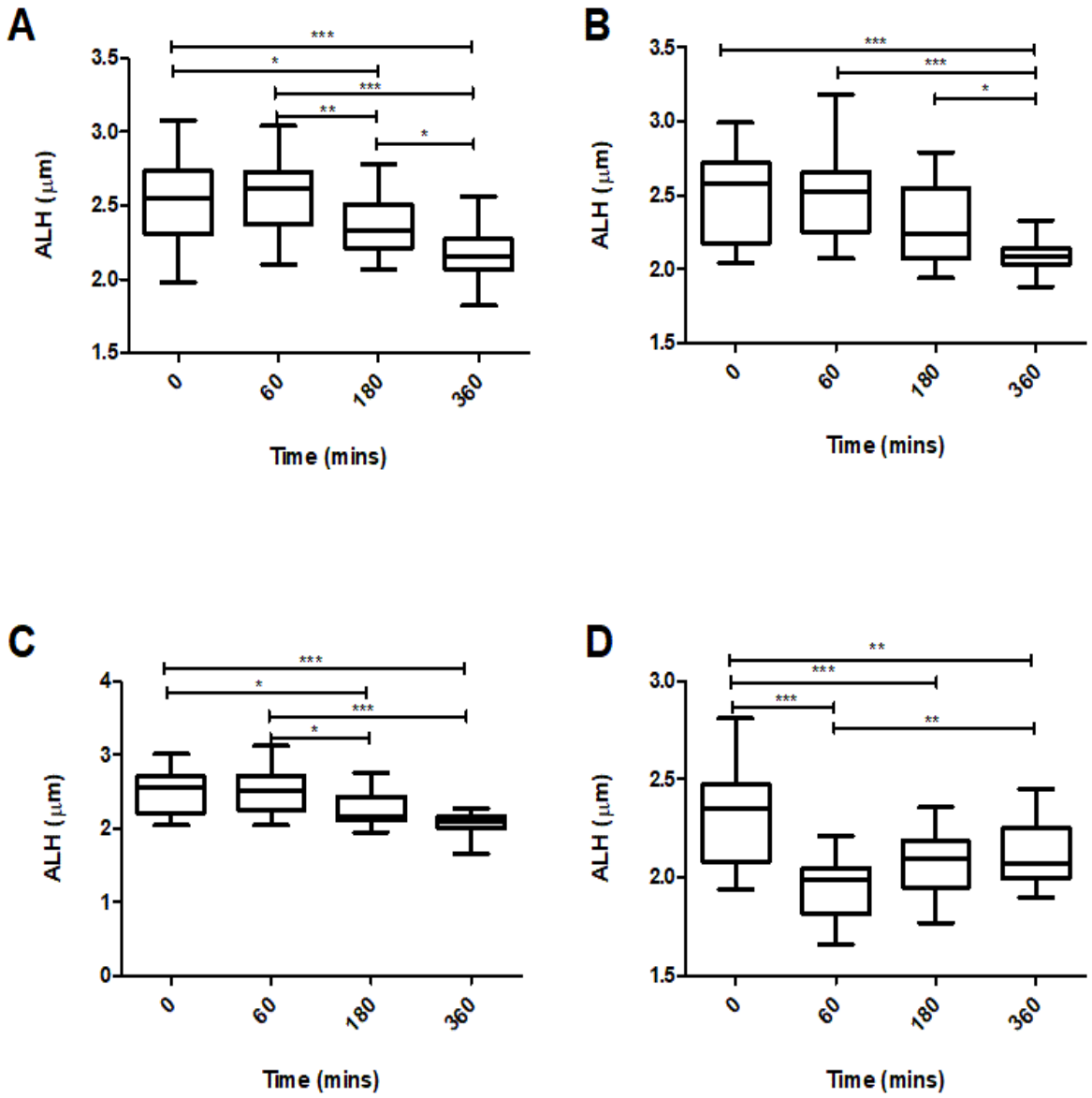


Figure 39: Effect of time at different concentrations of dopamine on Amplitude of Lateral Head Displacement (ALH). (A) ALH of Control. (B) ALH at a Low (100 nM) concentration of dopamine. (C) ALH at a Medium (10 μM) concentration of dopamine. (D) ALH at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.3.8 BCF

Figure 40 represents a Two-Way ANOVA performed on the Kinematic parameter Beat-Cross Frequency (BCF). It depicts the effect of dopamine on BCF over time expressed as a frequency over time (Hz). From figure 40 it can be seen that the high concentration significantly decreases the BCF at 1, 3 and 6 hours when compared to the control, low and medium concentrations of dopamine. After 1 hour of incubation the high concentration of dopamine significantly

decreases the BCF when compared to the control at 60 minutes (12.8 ± 1.569 vs. 5.54 ± 1.675).

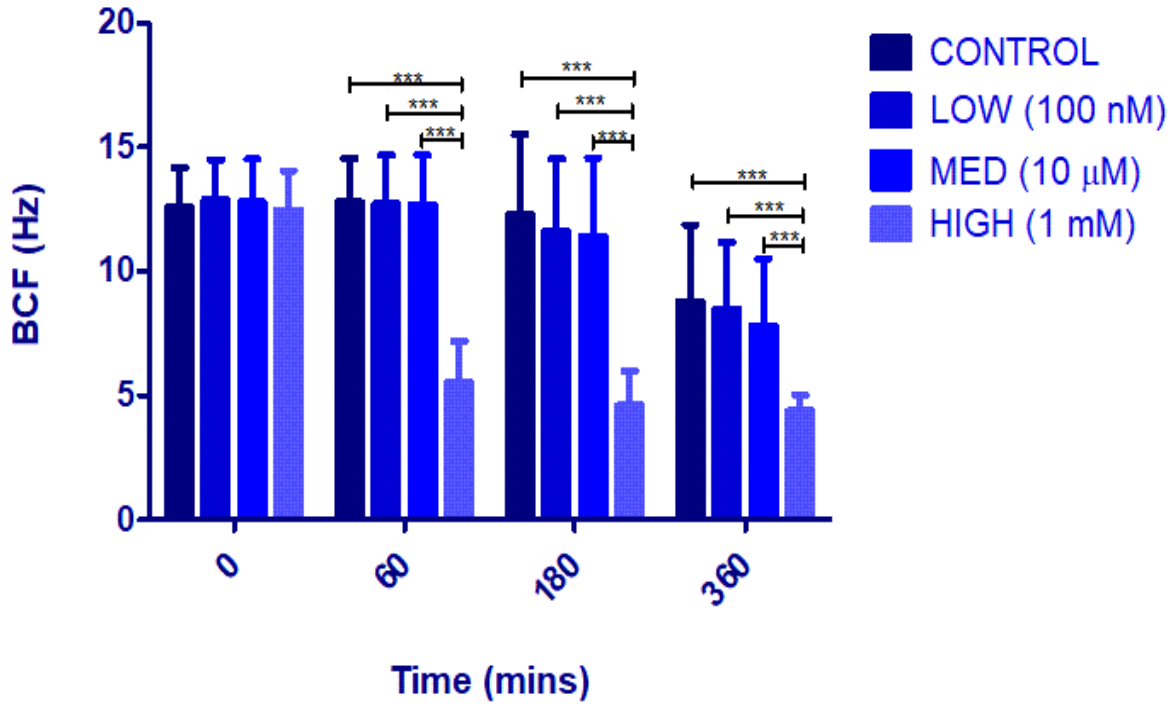


Figure 40: Effect of different concentrations of dopamine on Beat-Cross Frequency (BCF) over time. n = 24 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 41 depicts the effect of dopamine on BCF expressed as a frequency over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. Figure 41A depicts the effect of the control on BCF over time, as seen in this figure a significant time-dependent decrease is observed in the absence of treatment, however after 3 hours this decrease is exacerbated (12.29 ± 3.227 vs. 8.803 ± 3.085). The same effect is observed in Figures 41B and 41C, which represent the effect of the low and medium concentrations of dopamine on BCF, respectively. However, as seen in Figure 41D, which depicts the effect of the high concentration of dopamine on BCF over time, a significant decrease can be observed as early as 1-hour when compared to time 0 (12.49 ± 1.58 vs. 5.54 ± 1.675).

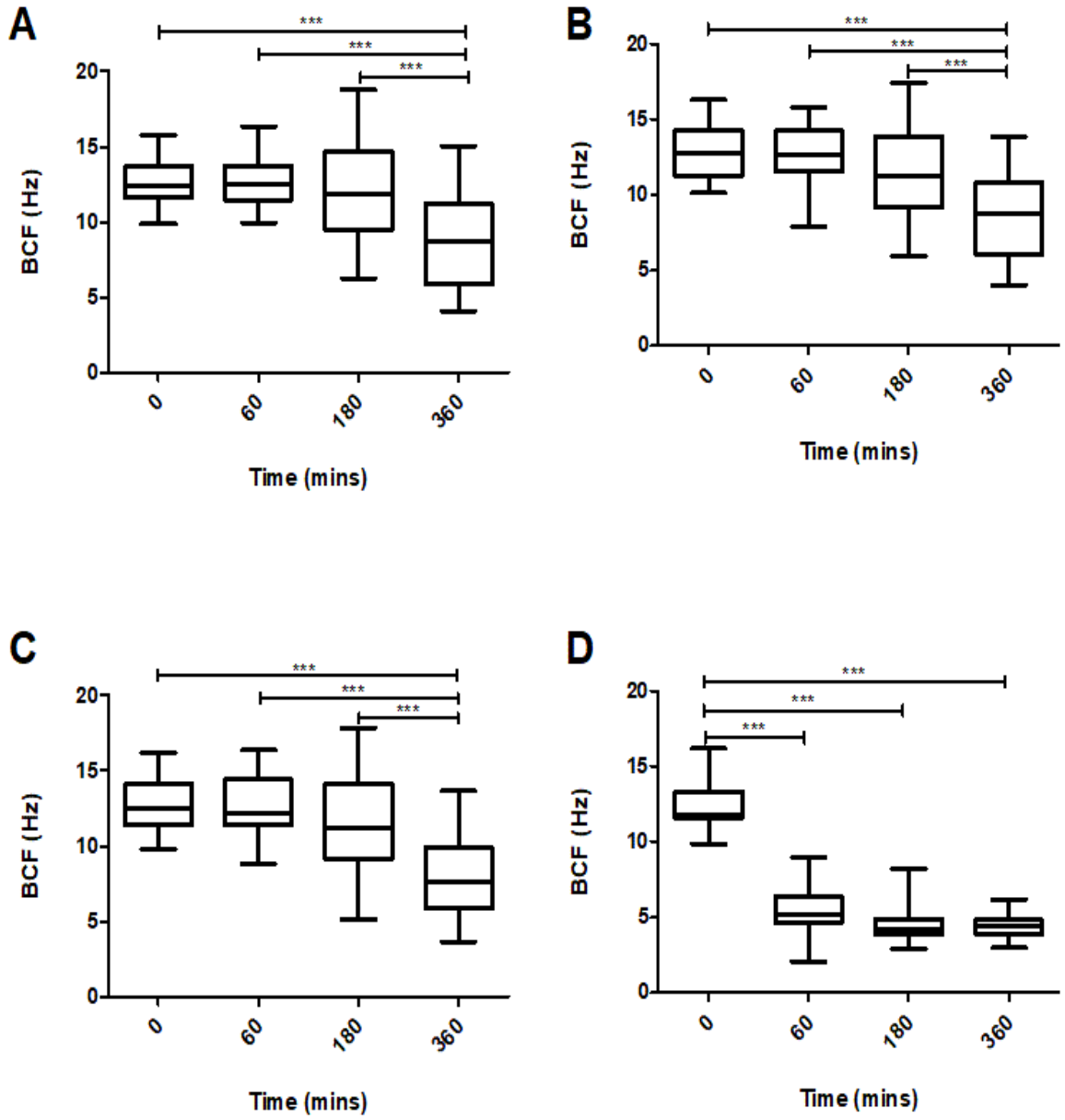


Figure 41: Effect of time at different concentrations of dopamine on Beat-Cross Frequency (BCF). (A) BCF of Control. (B) BCF at a Low (100 nM) concentration of dopamine. (C) BCF at a Medium (10 µM) concentration of dopamine. (D) BCF at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 24

4.4.4 Viability

The sample size for viability (performed via a dye-exclusion technique) was 22, as 2 donors viability data (slides) could not be analysed due to damage. Figure 42 depicts a Two-Way ANOVA performed on the Viability data; it represents the effect of dopamine on Viability of spermatozoa expressed as the percentage of viable sperm over time. As seen in figure 42 Dopamine had a significant effect on viability specifically at the high concentration of 1 mM.

The high concentration of dopamine is shown to significantly decrease viability as early as 60 minutes, when compared to the control (55.78% ± 4.491 vs. 49.03% ± 7.704). At 3 and 6 hours the viability is significantly decreased by the high concentration of dopamine when compared to the control, low and medium concentrations. At 6 hours the viability of the sperm, when exposed to the high concentration of dopamine, is decreased to 17.89% ± 14.82 from 46.20% ± 8.510.

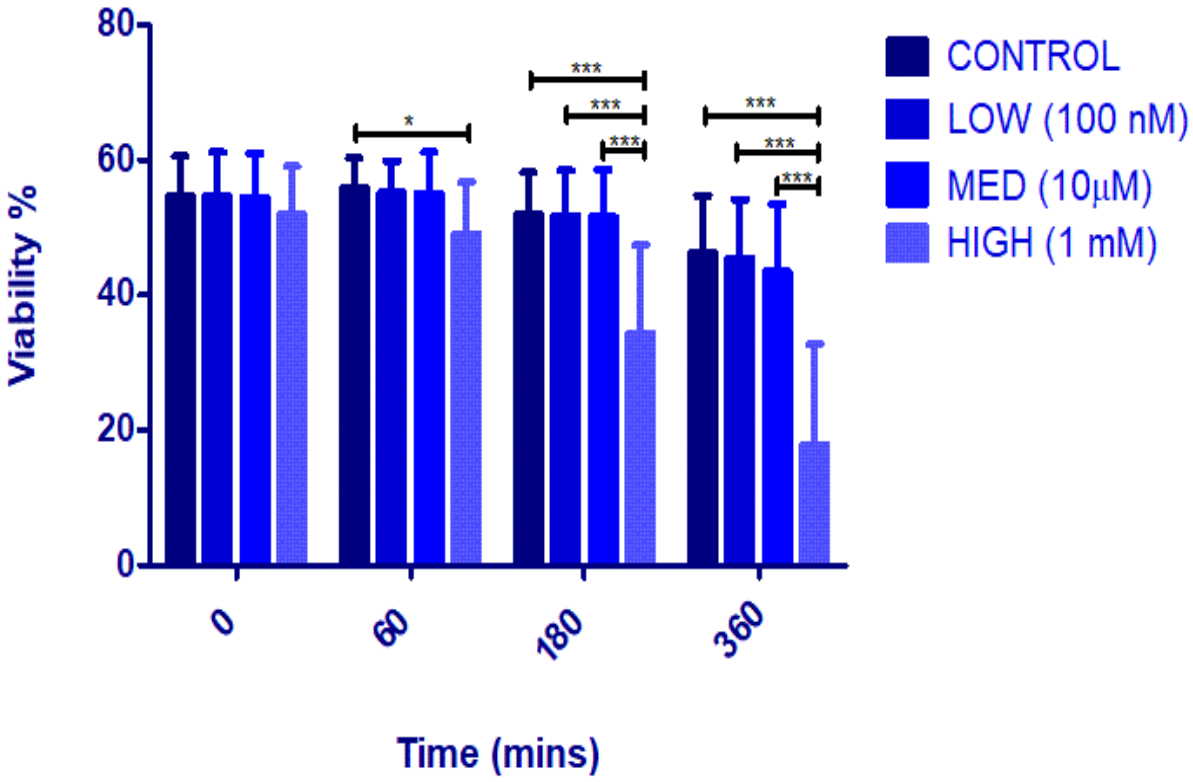


Figure 42: Effect of different concentrations of dopamine on Viability over time. n = 22 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 43 depicts the effect of dopamine at different concentrations on Viability expressed as a percentage over time. One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. In Figure 43A, it is clear to see that Viability decreases significantly as time progresses without any treatment present (54.57% ± 6.030 vs 46.20% ± 8.510), however this is expected as sperm die over time. Figure 43B depicts the effect of the low concentration of dopamine over time. Time significantly affected the decrease of viability. Figure 43C depicts the effect of the medium concentration of dopamine over time. Once again, a significant decrease is observed when comparing the all the time points to 360 minutes. Figure 43D depicts the effect of the high concentration of dopamine over time. The high concentration significantly decreased the viability at 180 minutes

(51.86% ± 7.220 vs 34.36% ± 13.03), and at 360 minutes (46.20% ± 8.51 vs 17.89% ± 14.82) when compared to the controls.

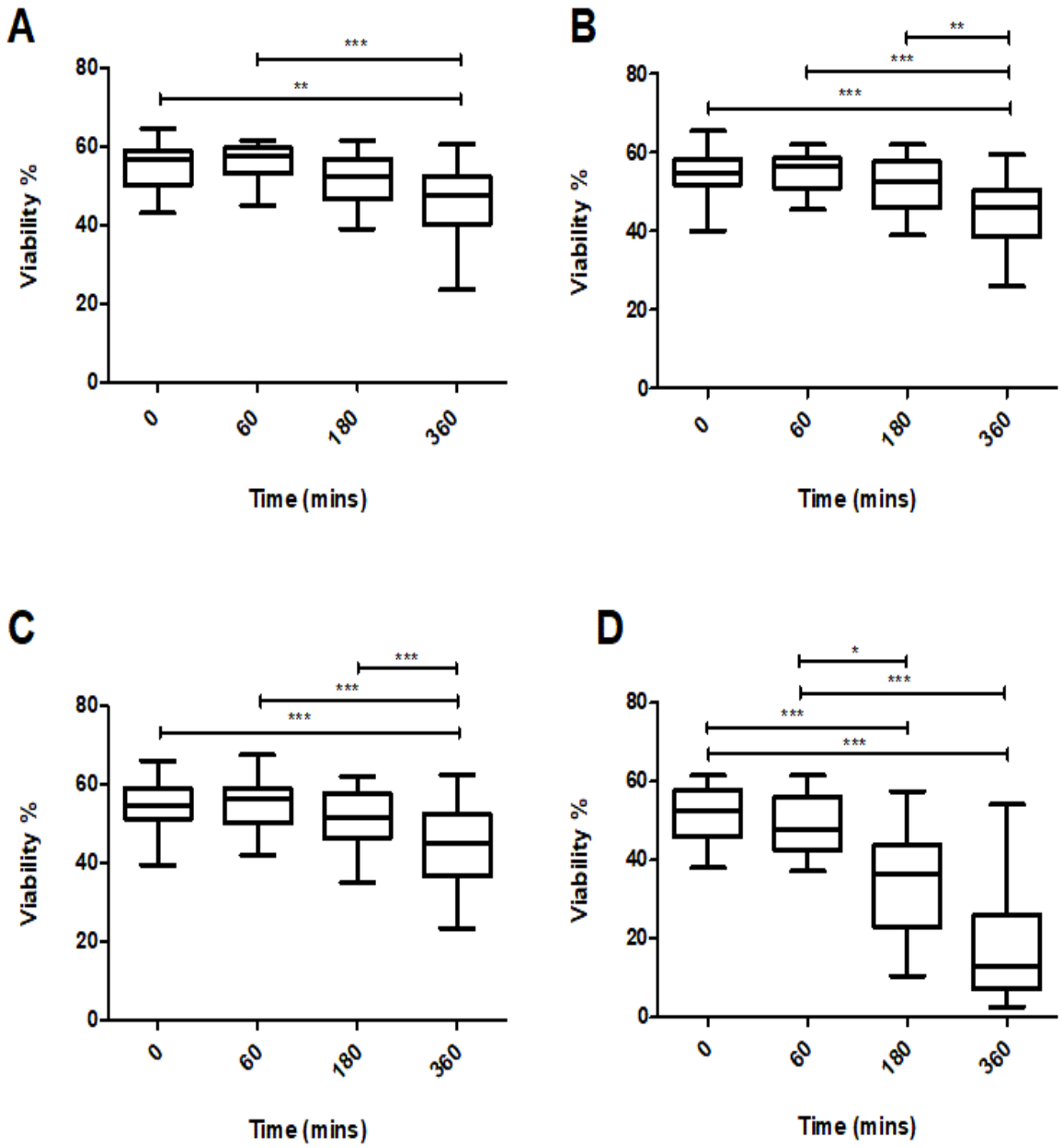


Figure 43: Effect of time at different concentrations of dopamine on Viability. (A) Viability of Control. (B) Viability at a Low (100 nM) concentration of dopamine. (C) Viability at a Medium (10 µM) concentration of dopamine. (D) Viability at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 22

4.4.5 Acrosome Reaction

The sample size for exploring the acrosome reaction was only 17, as 7 donors acrosome data (slides) could not be analysed due to damage. Figure 44 depicts a Two-Way ANOVA

performed on the Acrosome Reaction data, expressed as a percentage of acrosome reacted sperm over time. As seen in figure 44 the acrosome reaction is elicited prematurely as time progresses, however, at time 0, upon addition of the treatment, both the medium (26.88% ± 3.199 vs. 31.21% ± 3.464) and high (26.88% ± 3.199 vs. 31.91% ± 2.852) concentrations of dopamine exacerbated the effect of premature acrosome reaction when compared to the control. At 3 hours the high concentration significantly increased the amount of acrosome reacted sperm compared to the control. At 6 hours the high concentration significantly affected the acrosome reaction compared to the control, low and medium concentration. The high concentration of dopamine elicited 45.37% ± 5.940 of acrosome reactions after 6 hours of incubation, much higher than the control at 6 hours (37.85% ± 5.358).

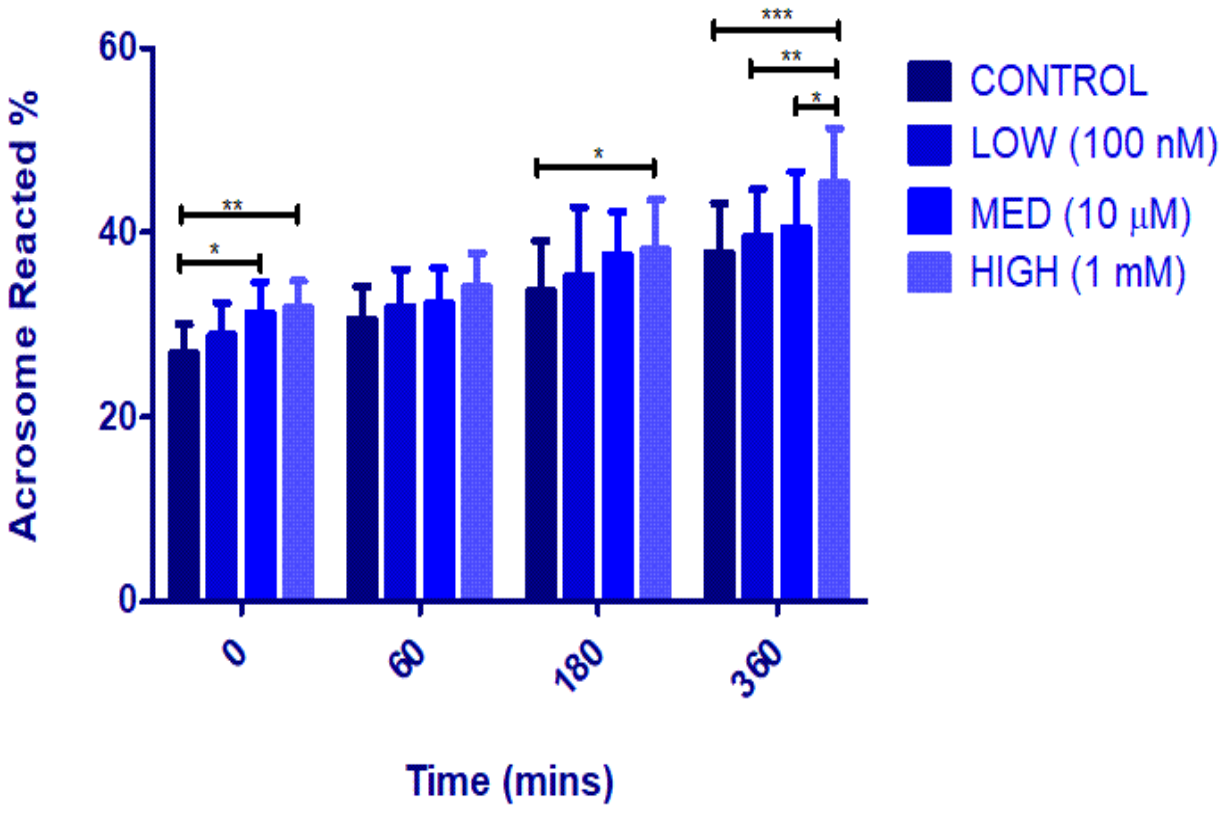


Figure 44: Effect of different concentrations of dopamine on Acrosome Reaction over time. n = 17 Two-Way ANOVA ***P<0.001, **P<0.01, *P<0.05

Figure 45 depicts the effect of different concentrations of dopamine on Acrosome Reaction expressed as a percentage of acrosome reacted sperm over time One-Way analysis of variance was performed and either Dunn's Multiple Comparison or Bonferroni's Multiple Comparison test was performed. Figure 45A depicts the baseline of the acrosome reaction when no treatment was added (control). As time progressed the acrosome reaction was

elicited, as expected. Figure 45B depicts the effect of low concentrations of dopamine on acrosome reaction, it would seem time significantly increased the percentage of acrosome reacted sperm. Figure 45C depicts the effect of the medium concentration of dopamine on acrosome reaction, the acrosome reaction is increased significantly from 3 hours onwards, when compared to time 0 (31.21% ± 3.464 vs. 37.53% ± 4.695) and 1 hour (32.29% ± 3.849 vs. 37.53% ± 4.695). Figure 45D depicts the effect of the high concentration of dopamine on acrosome reaction. The high concentration significantly increases the acrosome reaction at 3 hours (33.68% ± 5.434 vs 38.18% ± 5.391) and at 6 hours (37.85% ± 5.358 vs 45.37% ± 5.940) when compared to the control.

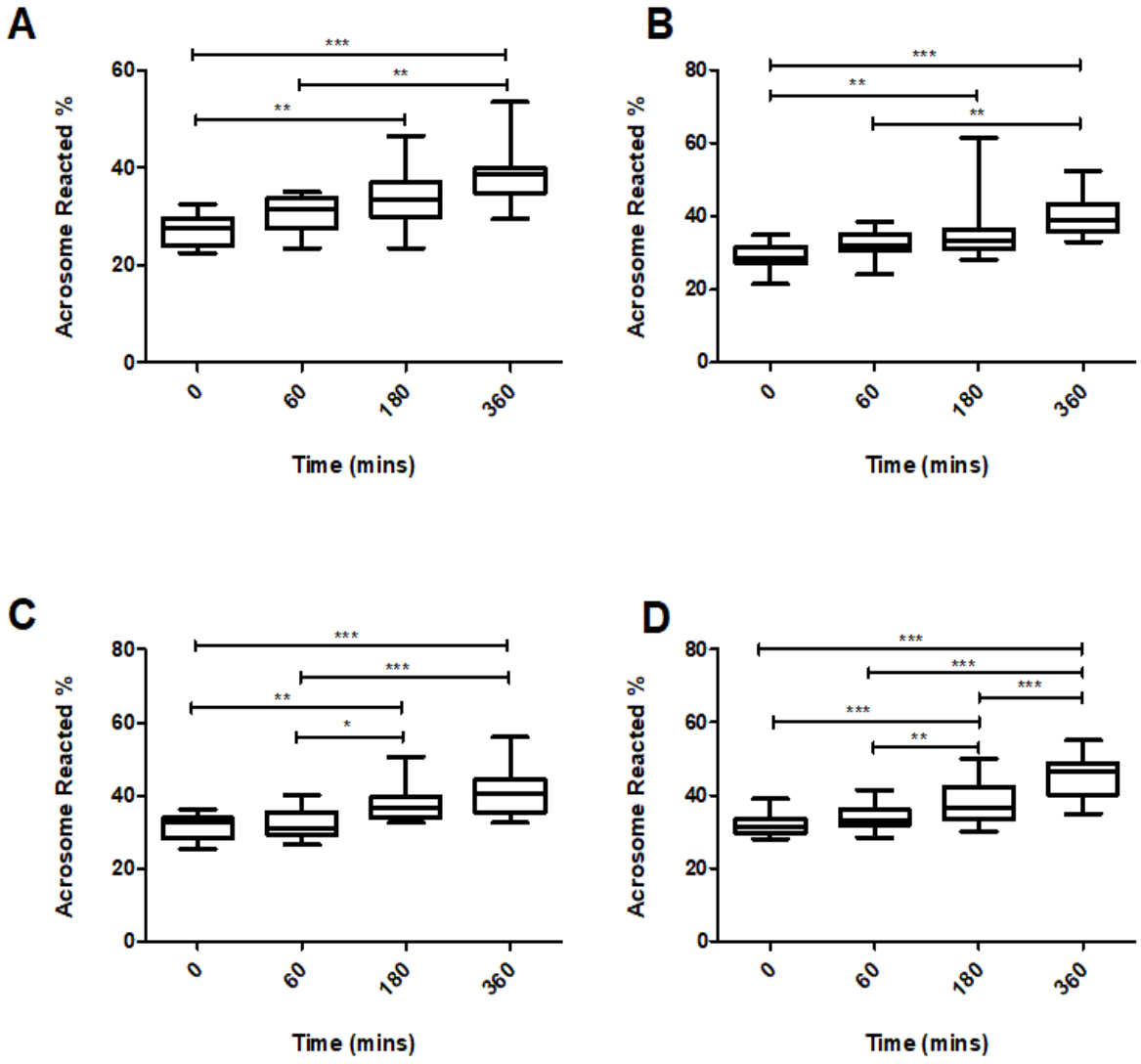


Figure 45: Effect of time at different concentrations of dopamine on Acrosome Reaction. (A) Acrosome Reaction of Control. (B) Acrosome Reaction at a Low (100 nM) concentration of dopamine. (C) Acrosome Reaction at a Medium (10 µM) concentration of dopamine. (D) Acrosome Reaction at a High (1 mM) concentration of dopamine. ***P<0.001, **P<0.01, *P<0.05 n = 17

Chapter 5

Discussion

The first aim of the current study was to identify the Dopamine Transporter (DAT) on human sperm membranes. The DAT was identified by an indirect immunofluorescence assay and its presence was confirmed using western blotting analyses. Secondly, the study aimed to investigate the effects of dopamine on human sperm parameters such as motility, viability, and acrosome reaction. This study aimed to focus on the effects of the dopaminergic pathway in the functions of human sperm and to elucidate possible mechanisms through which it may act to bring about any possible changes. Various objectives have been utilized to achieve the aims set out and the results thereof will be discussed in this chapter.

The presence of DAT on mammalian sperm membranes, including human, mouse, bull and horse has previously been investigated by indirect IF techniques, however, Ramírez *et al.,* (2009) focused on the discovery of DAT in boar spermatozoa and only included the other species mentioned above, including humans, in supplementary data. Boar sperm displayed a strong immunoreaction in the principal piece of the sperm (Ramírez *et al.*, 2009). Similarly, Urra *et al.,* (2014) positively identified a functional DAT in equine sperm via indirect IF, with a strong immunoreaction in the acrosomal region. From the immunofluorescence assay results as performed in the current study, a strong immunoreaction was found in the positive controls, while the negative controls elicited a rather weak immunoreaction. Thus, from the IF micrographs it is evident that the study wasable to identify and show the presence and localization, with fairly high certainty, of DAT on human sperm membranes. These findings are in accordance with previous studies mentioned above, with our findings being the first to focus on human spermatozoa.

Two studies previously investigated the presence of DAT on sperm membranes using WB techniques; Ramírez *et al.,* (2009) presented findings that boar sperm expresses DAT at ~75 kDa; Urra *et al.,* (2014) mainly focused on the identification of a functional DAT in equine spermatozoa, appearing on WB membranes at ~70 kDa. However, these authors also included supplementary data which suggests that boar, human, and bull spermatozoa also possess a DAT with a molecular weight of ~70 kDa (Ramírez *et al.*, 2009; Urra *et al.*, 2014). DAT is a glycoprotein located in cell membranes with a molecular weight of approximately 60-80 kDa when found in its native form. Our findings for the western blotting analyses showed that two protein bands with molecular weights of 62 kDa and 48 kDa are present in human sperm, which correlate to the bands found in the positive control (rat brain tissue). As seen in figures

18 and 21 of Chapter 4, the positive controls display numerous protein bands, with prominent bands at 69 kDa, 62 kDa and 48 kDa. DAT has been shown to form dimers, trimers, and tetramers in neuronal membranes of mammalian species such as canines, rats, and humans (Hastrup, Karlin and Javitch, 2001; Hastrup, Sen and Javitch, 2003; Pramod *et al.*, 2013; Cheng *et al.*, 2019). The heterogeneity observed within the brain could be due to homo-oligomerization, hetero-oligomerization of DAT with the D2 receptor or the different degrees of glycosylation of DAT. Photoaffinity labelling of neuronal DATs in canines showed that it presents as a polypeptide with an apparent weight of 62 kDa on WB membranes and possesses multiple glycosylation sites (Sallee *et al.*, 1989). However complete N-link deglycosylation of the neuronal DAT with PNGase-F, an endoglycosidase, decreased the molecular weight on WB membranes to approximately 48 kDa (Sallee *et al.*, 1989). It was suggested that the 48 kDa protein could represent the ligand binding subunit of DAT (Sallee *et al.*, 1989). Brain specific differences were observed in rats, where DAT was expressed at ~76 kDa in the striatum but presented with at slightly lower molecular weight band in the nucleus accumbens; this size difference in molecular weight was attributed to the degree of glycosylation in the different brain regions (Lew *et al.*, 1992). Since whole rat brain extracts were utilized as controls in this study it could be postulated that the high degree of heterogeneity observed could be attributed to the differential expression of DAT in various brain regions, as suggested by previous authors.

DAT structurally possess three N-linked glycosylation sites on the 2nd extracellular loop and Li *et al.,* (2004) observed through site-directed mutagenesis that N-linked mutant DATs from human substantia nigra migrate to different molecular weights on WB membranes. Upon removal of one of these glycosylation sites (treated with N-glycanase to yield a single mutant), the wild-type DAT from neuronal membranes migrated from ~75 kDa to a broad band of ~62 kDa. In addition, it displayed an additional discrete band at ~49 kDa. The double mutant DAT (after removal of two glycosylation sites) displayed a broad band at ~58 kDa and an additional discrete band at ~48 kDa. Finally, the triple mutant (removal of all three glycosylation sites) yielded a broad band at ~45 kDa and a more discrete band at ~39 kDa. These authors concluded that these results suggest that each glycosylation site for DAT is approximately 10 kDa in length, which falls within the range previously demonstrated for the norepinephrine transporter (Melikian *et al.*, 1996; Nguyen and Amara, 1996; Li *et al.*, 2004). Glycosylation patterns may vary substantially depending on the cell type, the glycoprotein is produced in, and the physiological state in which the cell is in (Lis and Sharon, 1993). The sperm samples S1 and S2 present faint bands at 62 kDa and 48 kDa as seen in Chapter 4 figures 18 and 21. We conclude that both these bands represent DAT, albeit differentially glycosylated. Thus,

from the WB results the study was able to confirm the results found in the IF assay, which show DAT to be present on human sperm membranes.

Sperm comprises of a head, and a flagellum. The flagellum can be divided into four distinct regions namely, the neck, the midpiece, the principal piece and the end piece (Cummins and Woodall, 1985; Gu *et al.*, 2019). The midpiece is comprised of the mitochondrial sheath, which contains numerous tightly packed mitochondria (Lindemann and Lesich, 2016; Gu *et al.*, 2019). The mitochondrial sheath is about 1.5 times as long as the sperm head; in bull spermatozoa, it is 11 µm in length (Lindemann and Lesich, 2016; Rothmann and Bort, 2018). The mitochondrial sheath is about 80 nm thick and lies directly beneath the plasma membrane. It is believed to be an important energy source, essential for sperm motility (Piomboni *et al.*, 2012; Briz and Fàbrega, 2013; Carrillo, 2016; Ayad, Van der Horst and Du Plessis, 2018). The principal piece is located just beneath the midpiece and comprises of a fibrous sheath which measures ~40 µm in length (Carrillo, 2016; Lindemann and Lesich, 2016; Rothmann and Bort, 2018). The fibrous sheath lies beneath the plasma membrane and acts as a scaffold for proteins involved in the regulation of sperm motility (Eddy, Toshimori and O'Brien, 2003; Turner, 2003). It was shown that the fibrous sheath also possesses glycolytic enzymes to supply energy for movement, implying mitochondrial oxidative phosphorylation within the sperm midpiece to not be the only mechanism behind sperm motility (Turner, 2006).

The human sperm head is approximately 4-5.5 µm long and 3 µm wide (Cummins and Woodall, 1985; Rothmann and Bort, 2018). The head comprises of the nucleus, a limited amount of cytoplasm a few cytoskeletal structures, and the acrosome (Toshimori and Ito, 2003). The acrosome, a Golgi-derived cap, sits on the anterior portion of the sperm head and it is surrounded by an outer and inner acrosomal membrane (Aguas and Da Silva, 1985; Toshimori and Ito, 2003; Fléchon, 2016). The acrosome contains specific enzymes such as the proteolytic enzyme Acrosin and plays a key role in gamete fusion by facilitating the spermatozoa to penetrate the zona pellucida and fuse with the oocyte (Schill, Töpfer-Petersen and Heissler, 1988; Brucker and Lipford, 1995; Breitbart and Spungin, 1997; Fléchon, 2016). The acrosome reaction is a Ca^{2+}-dependent exocytotic process and it is essential that the acrosome reaction does not occur before the required time, as spermatozoa whose acrosomes have reacted prematurely will not be able to fertilize an oocyte (Brucker and Lipford, 1995; Breitbart and Spungin, 1997; Yanagimachi, 2011).The acrosome reaction will only occur once the spermatozoa has undergone capacitation and it is the steps of the capacitation process which ultimately lead to the acrosome reaction taking place (Breitbart and Spungin, 1997; Breitbart, 2002; Carlson, Hille and Babcock, 2007). In the current study a weak immunoreaction was detected in the acrosomal region of the spermatozoa. Previously equine sperm displayed a strong DAT fluorescence in the acrosomal region (Urra *et al.*, 2014). The

acrosomal integrity of sperm was significantly decreased upon the addition of both the medium and high concentrations of dopamine as observed in figure 45 in Chapter 4. Furthermore after 3 hours of incubation with the high concentration of dopamine the acrosome reaction was significantly prematurely elicited when compared to the controls.

Ca^{2+}- mediated signalling mechanisms have been implicated in multiple functions within sperm. An increase in intracellular Ca^{2+}, through calcium channels situated on sperm membranes (as well as intracellular stores), increase cAMP production, and thus activates PKA to phosphorylate sperm tail proteins such as A-kinase anchoring protein 3 (AKAP-3), found within the fibrous sheath of the sperm tail (Darszon *et al.*, 2006; Carlson, Hille and Babcock, 2007; Leahy and Gadella, 2011; Correia, Michelangeli and Publicover, 2015; Vizel *et al.*, 2015; Carrillo, 2016). PKA phosphorylation of sperm tail proteins activate the ATPase, and the energy released from the ATP hydrolysis is transformed into movement (Tash, 1989; Turner, 2006). Tyrosine phosphorylation through activation by PKA has been shown to be crucial in the modulation and induction of sperm motility and the regulation of capacitation and acrosome reaction (Visconti *et al.*, 1995a; Visconti *et al.*, 1995b; Tash and Bracho, 1998; Turner, 2006; Vizel *et al.*, 2015). Sperm motility is generally accepted as the central component of normal male fertility as it is essential for the fertilization of oocytes (Turner, 2006; Malik *et al.*, 2011). Spermatozoa achieve motility once ejaculated and it is the presence of the HCO_3^- in the seminal plasma which activates the sAC (soluble Adenyl Cyclase) found within sperm, ultimately leading to increased cAMP production (Carlson, Hille and Babcock, 2007; Carrillo, 2016). The acrosome reaction is a complex process which occurs through the binding to, and activation of specific receptors located on the acrosomal cap, with the zona pellucida, resulting in the release of hydrolytic enzymes. However, it is the presence of Ca^{2+} that has been shown to be a prerequisite for the acrosome reaction to occur (Brucker and Lipford, 1995; Breitbart and Spungin, 1997; Breitbart, 2002; Darszon *et al.*, 2006). The initial influx of Ca^{2+} from extracellular stores via calcium channels in the membrane at the start of capacitation leads to cAMP-dependent intracellular Ca^{2+} increases, which causes membrane fluidity. This, along with an elevation of pH, culminates in acrosomal granule exocytosis (Brook *et al.*, 1996; Breitbart and Spungin, 1997; Darszon *et al.*, 2006; Correia, Michelangeli and Publicover, 2015).

Ramírez *et al.* (2009) reports that in the absence of capacitating media, low (100 nM) and medium (10 µM) concentrations of dopamine potentiates sperm tyrosine phosphorylation as well as sperm motility. However, at high (1 mM) concentrations of dopamine, in the presence of capacitating media, boar sperm displayed a marked decrease in tyrosine phosphorylation, total and progressive motility as early as 1 hour (Ramírez *et al.*, 2009). Additionally, it was shown that low (100 nM) concentrations of dopamine, when incubated alongside capacitating

media, increased boar sperm viability significantly after 3 hours of incubation; this was postulated to be through D2 receptor activation. Furthermore Urra *et al.,* (2014) substantiates these findings; after incubation with high (1 mM) concentrations of dopamine, total motility was significantly decreased as early as 1 hour (Urra *et al.*, 2014). In the current study both total and progressive motility were significantly decreased from as early as 1 hour in the presence of the high (1 mM) concentration of dopamine. Additionally, the high concentration of dopamine also significantly decreased all the kinematic parameters analysed after 1 hour of incubation. The low (100 nM) and medium (10 µM) concentrations did not significantly affect sperm motility parameters. This is in accordance with results of Ramírez *et al.,* (2009) and Urra *et al.,* (2014). The viability of spermatozoa incubated with the high concentration of dopamine decreased after 1 hour of incubation and significantly decreased after 3 hours, however the low and medium concentrations of dopamine did not affect sperm viability.

DAT was detected in both the sperm midpiece region, in the present study via IF, as well as the principal piece of the sperm tail. Thus, the inhibitory effects observed on motility parameters and viability when incubated with high concentrations of dopamine, is thought to be through mechanisms involving DAT. Although the viability results obtained herein do not exhibit the biphasic effects seen by Ramírez *et al.,* (2009) for boar sperm, it may still be postulated that the negative effects observed are occurring through dopamine uptake by DAT. It is also hypothesized that the plasma membrane location of DAT in the acrosomal region of the spermatozoa plays a role in the premature decrease of acrosomal integrity. Bolan *et al.,* (2007) reports that D2 receptors and DAT forms a complex hetero oligomer in neuronal membranes, but that this protein-protein interaction is not involved in the regulation of DAT. Instead D2 receptor regulates DAT through mechanisms involving ERK 1/2 activation. In heterologous expression systems the activation of D2 receptors by agonists, such as dopamine, induces phosphorylation of ERK1/2. This therefore upregulates DAT at the surface membrane, allowing a greater number of transporters to be available to translocate dopamine into the cell (Brami-Cherrier *et al.*, 2002; Bolan *et al.*, 2007). It is hypothesized that the continued activation of D2 receptors by dopamine results in the acquisition of more DATs to the surface membrane, thereby allowing the influx of a greater amount of dopamine into the spermatozoa.

High levels of dopamine are known to be toxic *in vitro* and *in vivo* and therefore the cytosolic accumulation of dopamine through DAT or the enzymatic degradation of dopamine consequently causes sperm motility, viability and acrosomal integrity to decrease (Berman and Hastings, 2001; Ramírez *et al.*, 2009). The presence of MAO has been established in both human and equine sperm (Dragatsis, Levine and Zeitlin, 2000; Urra, 2012). As seen in neuronal cells, the oxidation of dopamine (either intracellularly or extracellularly) via MAO (Monoamine Oxidase) leads to an accumulation of reactive oxygen species (ROS) such as

H_2O_2 and DOPAL which may lead to mitochondrial damage or the oxidation of membrane lipids eventually leading to cell death (apoptosis) (Li *et al.*, 2001). Oxidative stress caused by the accumulation of ROS in spermatozoa is considered to be the major contributing factor in limiting sperm functional capacities (Ramírez-Reveco *et al.*, 2017). ROS is shown to be beneficial to spermatozoa in low concentrations, by stimulating capacitation and acrosome reactions (Lamirande and Gagnon, 1993; Griveau and Le Lannou, 1997; Du Plessis, 2016). However, high concentrations of ROS in spermatozoa leads to sperm pathology via ATP depletion, leading to DNA damage, lipid peroxidation and loss of motility, viability, and induction of the premature acrosome reaction (Bansal and Bilaspuri, 2011; Du Plessis, 2016). Thus, through increased dopamine uptake by DAT, spermatozoa produce excess ROS and can subsequently have lower sperm parameters.

Considering that catecholamines are present throughout both the female and the male reproductive tracts and that sperm express a catecholaminergic phenotype, it is highly likely that they fulfil some role in sperm functionality and reproductive physiology. As previously mentioned, mammalian oviductal regions differentially express catecholamines, and in addition, the concentration of catecholamines in these regions are controlled by the different stages of the oestrous cycle (Helm *et al.*, 1982; Chaud *et al.*, 1983; Khatchadourian *et al.*, 1987; Kotwica *et al.*, 2003). Therefore, since high concentrations of dopamine decreases motility parameters, it could be presumed the mammalian oviduct preferentially selects the best sperm by immobilizing them, at the correct period of time i.e., ovulation (Urra *et al.*, 2014).

In summary the study demonstrated that human sperm express DAT on their membranes and that dopamine elicits a dose-dependent effect on functional sperm parameters including, total motility, progressive motility, kinematics, viability, and acrosome reaction. Therefore, we postulate that in humans dopamine is a physiological modulator of important sperm functions and thus ultimately involved in the control of *in vivo* fertilization.

Chapter 6

Conclusion

Multiple lines of evidence clearly support the presence of a dopaminergic phenotype in the reproductive system of mammals such as humans. Dopamine has been shown to modulate reproductive parameters through mechanisms not fully understood. Functional studies in animals, suggest DAT and DRD2 play a role in sperm functions such as capacitation, motility parameters and acrosome reaction. The identification of DAT on human sperm membranes adds to the current understanding of dopamine modulation within the reproductive system. It is evident that in human sperm D2 receptors are present by the effects observed upon stimulation with low doses of dopamine. The biphasic effect observed upon incubation with high doses of dopamine can now be explained by the presence of DAT on human sperm membranes. Considering that catecholamines are located throughout the entire passage of sperm along the male and female reproductive tracts it crucial to understand the role these catecholamines play in modulating reproductive capacities. Understanding the molecular events through which sperm function is key to understanding fertilization. Dopamine was found to modulate sperm functional parameters and can therefore be regarded as a physiological modulator of *in vivo* fertilization.

Limitations

- Due to the Covid-19 pandemic and accompanying lockdown restrictions, time constraints were a major limitation in this study. Due to this, experiments could not be repeated to confirm or disprove unexpected results.
- Covid-19 safety protocols greatly impacted on the number of donors the study was able to gather. Only 5 donors were able to donate for the WB analysis to reach n=24. This could be a limitation as the samples were pooled together. Therefore, a lack of variability within the sperm samples could possibly limit protein expression.
- We suspect the sample size of 24 also proved problematic for the WB analyses, as the protein content was extremely low for sperm compared to the brain controls, even though equal protein loading took place, therefore the protein bands appear very faintly on the membrane.

Future Directions

- An antibody with a high specificity may be utilized in future experiments or adjust the current antibody's concentration.
- The blocking step in the IF protocol may need to be adjusted. The current protocol used a 10% FBS blocking buffer for 40 minutes, this may need to be adjusted by increasing the blocking buffer concentration or incubating for a longer period in blocking buffer, in order to block non-specific sites.
- Functionality assays need to be performed to confirm that DAT in human spermatozoa is a functional protein. This may be done with the specific DAT inhibitor, Vanorexin.
- Future studies should confirm the presence of other monoamine transporters such as NET and SERT on human sperm membranes since they play a role in dopamine uptake.
- Considering that a DAT inhibitor may reverse the negative effects of high doses of dopamine and be protective to sperm, future studies could investigate the effects of psychostimulants such as Ritalin (a known DAT inhibitor) on sperm parameters.
- Future studies could investigate whether a signalling complex exits between human dopamine receptors and transporters.

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Appendix A

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INSTITUTIONAL PERMISSION:

AGREEMENT ON USE OF PERSONAL INFORMATION IN RESEARCH

Name of Researcher: Lisa Marie Ferguson

Name of Research Project: The Novel Identification of the Dopamine Transporter (DAT) and the Effects of Dopamine on Human Sperm Functions

Service Desk ID: IRPSD-1982

Date of Issue: 17 December 2020

The researcher has received institutional permission to proceed with this project as stipulated in the institutional permission application and within the conditions set out in this agreement.

1 WHAT THIS AGREEMENT IS ABOUT	
What is POPI?	1.1 POPI is the Protection of Personal Information Act 4 of 2013. 1.2 POPI regulates the entire information life cycle from collection, through use and storage and even the destruction of personal information.
Why is this important to us?	1.3 Even though POPI is important, it is not the primary motivation for this agreement. The privacy of our students and employees are important to us. We want to ensure that no research project poses any risks to their privacy. 1.4 However, you are required to familiarise yourself with, and comply with POPI in its entirety.
What is considered to be personal information?	1.5 'Personal information' means information relating to an identifiable, living, individual or company, including, but not limited to: 1.5.1 information relating to the race, gender, sex, pregnancy, marital status, national, ethnic or social origin, colour, sexual orientation, age, physical or mental health, well-being, disability, religion, conscience, belief, culture, language and birth of the person; 1.5.2 information relating to the education or the medical, financial, criminal or

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Approval Notice

New Application

20/01/2021

Project ID :16662

HREC Reference No: S20/08/200

Project Title: The Novel Identification of the Dopamine Transporter (DAT) and the effect of Dopamine on Human Sperm Functions

Dear Ms Lisa Marie Ferguson

The **Response** received on 08/01/2021 was reviewed and **approved** by members of **Health Research Ethics Committee** via **expedited** review procedures on 20/01/2021.

Please note the following information about your approved research protocol:

Protocol Approval Date: 20 January 2021

Protocol Expiry Date: 19 January 2022

Please remember to use your Project ID 16662 and Ethics Reference Number S20/08/200 on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Translation of the informed consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see *Forms and Instructions* on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Please note that for studies involving the use of questionnaires, the final copy should be uploaded on Infonetica.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website https://applyethics.sun.ac.za/ProjectView/Index/16662

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Mrs. Melody Shana

Coordinator

HREC1

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Animal Tissue Use Approval

04 June 2019

PI: Miss Bongekile Skosana

REC: ACU Reference #: ACU-2019-10563

Title: The Novel Identification of the Dopamine Transporter (DAT) and the effects of Dopamine and DAT Antagonist, Ritalin, on human sperm functionality

Dear Miss Bongekile Skosana

Your Notification with reference number #ACU-2019-10563 , was reviewed on 04 June 2019 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is valid for a period of five years. A new application must be submitted when the source of the material changes.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your REC: ACU reference number: # ACU-2019-10563 on any documents or correspondence with the REC: ACU concerning your research protocol.

If you have any questions or need further help, please contact the REC: ACU office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: ACU policy and procedures.

Sincerely,

Mr Winston Beukes

Coordinator: Research Ethics (Animal Care and Use)

Appendix B

Preparation of buffers

- **PBS** (Control diluent)

  10 mL Stock PBS (10X) + 90 mL dH_2O = 100 mL PBS (1X)

- Wash Buffer- **1%BSA-PBS**

  1g BSA + 100 mL PBS = 1% BSA-PBS

- Permeabilization buffer- **5% BSA-PBS, 0.3% Triton X-100**

  5g BSA + 100 mL PBS = 100 mL 5% BSA-PBS

  90700 µL 5% BSA-PBS + 300 µL Triton X-100 = 100 mL 5% BSA-PBS, 0.3% Triton X-100

- **Running Buffer** (10X Stock)

  60.6g Tris + 288g Glycine + 20g SDS + 2 L dH_2O = 2 L Running buffer (10X Stock)

  100 mL (10X Stock) + 900 mL = 1 L Running buffer (working solution)

- **Transfer Buffer**

  6.06g Tris + 28.83g Glycine + 1600 mL dH_2O = 1.6 L Transfer Buffer

  1.6 L + 400 mL Methanol (20%) = 2 L Transfer Buffer

- **TBS** (10X Stock)

  48.4g Tris + 160g NaCl + 1500 mL dH_2O = 1.5 L TBS (10X Stock)

  pH with 1 M HCl to pH 7.6 and top up to 2 L= 2 L TBS (10X Stock)

- **TBS-Tween** (working solution)

  100 mL (10X Stock TBS) + 900 mL dH_2O + 1 mL Tween = 1 L TBS-Tween

- **Blocking Buffer**- 5% Milk TBS-Tween

  5 mL long-life milk + 95 mL TBS-Tween = 100 mL 5% Milk TBS-Tween

- **Antibody diluent-** 5% BSA-TBS-Tween

  5g BSA + 100 mL TBS-Tween = 100 mL 5% BSA-TBS-Tween

Dilution of Dopamine Hydrochloride

Step 1: 5 mg + 1 mL PBS = 5 mg/mL (1 mL) (STOCK 1)

Step 2: C_1 x V_1 = C_2 x V_2

(5 mg/mL) x (V_1) = (1 mg/mL) x (1 mL)

(V_1) = 0.2 mL

(V_1) = 200 µL

∴ 200 µL (STOCK 1) + 800 µL PBS = 1 mg/mL (1 mL) (STOCK 2)

Step 3: C_1 x V_1 = C_2 x V_2

(1 mg/mL) x (V_1) = (0.1 mg/mL) x (1 mL)

(V_1) = 0.1 mL

(V_1) = 100 µL

∴ 100 µL (STOCK 2) + 900 µL PBS = 0.1 mg/mL (1 mL) (STOCK 3)

Step 4: C_1 x V_1 = C_2 x V_2

(0.1 mg/mL) x (V_1) = (0.01 mg/mL) x (1 mL)

(V_1) = 0.1 mL

(V_1) = 100 µL

∴ 100 µL (STOCK 3) + 900 µL PBS = 0.01 mg/mL (1 mL) (STOCK 4)

Step 5: C_1 x V_1 = C_2 x V_2

(0.01 mg/mL) x (V_1) = (0.001 mg/mL) x (1 mL)

(V_1) = 0.1 mL

(V_1) = 100 µL

∴ 100 µL (STOCK 4) + 900 µL PBS = 0.001 mg/mL (1 mL) (STOCK 5)

Preparation of Dopamine Concentrations

Final Treatment Concentrations:

a. 1 mM
b. 10 µM
c. 100 nM

Molecular Weight (MW) of Dopamine = 189.64 g/mol

Convert Molar to mg/mL

a. $189.64\ g/mol\ x\ (1\ x\ 10^{-3}) = 0.18964$

$\frac{0.18964}{1000} = 1.8964\ x\ 10^{-4}$

$\frac{1.8964\ x\ 10^{-4}}{0.001\ mL} = 0.18964\ mg/mL$

b. $189.64\ g/mol\ x\ (10\ x\ 10^{-6}) = 1.8964\ x\ 10^{-3}$

$$\frac{1.8964\ x\ 10^{-3}}{1000} = 1.8964\ x\ 10^{-6}$$

$$\frac{1.8964\ x\ 10^{-6}}{0.001\ mL} = 1.8964\ x\ 10^{-3} mg/mL$$

c. $189.64\ g/mol\ x\ (100\ x\ 10^{-9}) = 1.8964\ x\ 10^{-5}$

$$\frac{1.8964\ x\ 10^{-5}}{1000} = 1.8964\ x\ 10^{-8}$$

$$\frac{1.8964\ x\ 10^{-8}}{0.001\ mL} = 1.8964\ x\ 10^{-5}\ mg/mL$$

Experimental setup for dopamine treatments

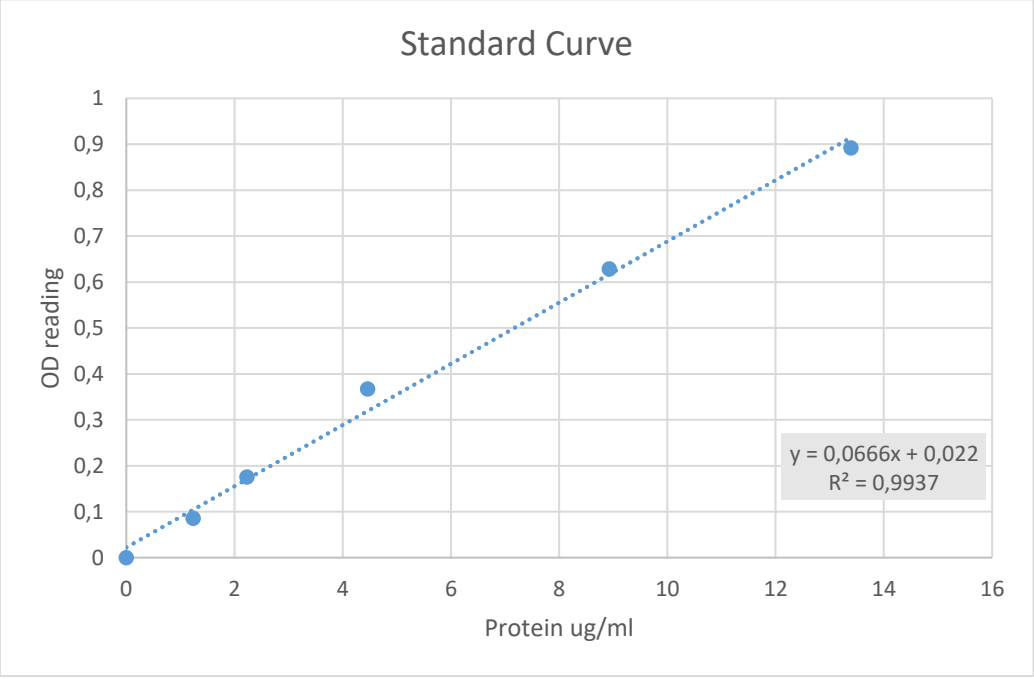
Time (minutes)	CONTROL [PBS]	LOW [100 nM]	MEDIUM [10 µM]	HIGH [1 mM]
0	Analyse Parameters	Analyse Parameters	Analyse Parameters	Analyse Parameters
60	Analyse Parameters	Analyse Parameters	Analyse Parameters	Analyse Parameters
180	Analyse Parameters	Analyse Parameters	Analyse Parameters	Analyse Parameters
360	Analyse Parameters	Analyse Parameters	Analyse Parameters	Analyse Parameters

Appendix C

BSA Standards for protein concentration determination

Diluted BSA (µL)	OD (nm)	Mean OD (nm)	Protein (µg/mL)
0	0	0	0
5	0.088	0.0855	1.2397
5	0.083		
10	0.169	0.1755	2.232
10	0.182		
20	0.356	0.367	4.464
20	0.378		
40	0.587	0.6285	8.928
40	0.67		
60	0.892	0.892	13.392
60	0.892		
80	1.037	1.0295	17.856
80	1.022		

BSA Standard curve- generated from the OD readings and the protein concentrations of the diluted BSA standards.



Lysate Preparation

Sample	Sample OD	Mean OD	Bradford [protein]/ 2nd Dilution (µg/µL)	[Protein] in 1 µL of 2nd dilution (µg/µL)	[Protein] in Sample (µg/µL)	27,5 µg Protein in lysate (µL)	Lysis Buffer (µL)	Sample Buffer 1:2 (µL)	Load Onto Gel (µL)
C1	0.878	0.862	12.60511	2.521021	25.21021	1.09	6.91	4	12
	0.845								
C2	0.736	0.74	10.78078	2.156156	21.56156	1.28	6.72	4	12
	0.744								
S1	0.216	0.194	2.582583	0.516517	5.165165	53.24	2.68	4	12
	0.172								
S2	0.269	0.276	3.813814	0.762763	7.627628	36.05	4.39	4	12
	0.283								

Appendix D