Gut-brain axis: Central impact of gut peptides and metabolic drug on monoamine neurotransmission

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Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

August 2019

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Abstract

Gastrointestinal peptides like insulin and glucagon-like peptide are not only essential metabolic hormones but can also modulate important brain function, unrelated to feeding behaviour and glucose homeostasis. Different evidence suggests that these peptides, as well as synthetic insulin secretagogue such as, tolbutamide, can affect cognition, motivation, addiction as well as neuronal survival.

In this thesis, we have investigated in rats whether insulin, or the insulin-releasing agent tolbutamide, and the stable glucagon-like-peptide-1 (GLP-1) receptor agonist exendin-4 as well as putative GLP-1 congener geniposide, can affect the electrical activity of dopamine neurons in the ventral tegmental area (VTA) and of pyramidal neurons in the prefrontal cortex (PFC), using single-unit activity recording techniques. We have also investigated, whether these peptides can alter some dopamine-dependent behaviours such as, D-Amphetamine (a dopamine-releasing agent) -induced motor activity, quinpirole (a dopamine D_2/D_3 receptor agonist) and pramipexole (a dopamine D_3 preferred receptor agonist) -induced yawning, pica eating and pelvic grooming activities, as well as phencyclidine (an *N*-Methyl-D-aspartate receptor antagonist)-induced cognitive deficit. We also examined the pharmacological mechanism of these drugs on radiometric *in vitro* [DA] release and uptake assays and investigated whether GLP-1 can modulate the effect of diet modification in form of chronic sucrose intake on behavioural and electrophysiological parameters.

Interestingly, in uptake assay, exendin-4 and insulin weakened dopamine D_2/D_3 agonist (pramipexole and quinpirole)-induced [DA] uptake which suggests a potential interaction with D_2/D_3 receptor signalling. In addition, insulin and tolbutamide similarly reduced basal [DA] in striatal synaptosomes.

Our electrophysiological data shows that GLP-1 receptor activation can change the excitability of prefrontal cortex neurons, an effect that can be associated to the putative pro-cognitive action of some GLP-1 analogues. Interestingly, on VTA dopamine neurons, GLP-1 receptor activation potentiated a moderate inhibitory action of insulin on firing activity. However, a slight progressive decrease in the firing activity of ventral tegmental area dopamine neurons was observed, when insulin was administered through an electrode to allow for local diffusion into the brain region. The insulin secretagogue agent tolbutamide exerts biphasic or excitatory effects on VTA dopamine and PFC neurons, probably via K^{+}_{ATP} channels blockage through sulphonylurea receptors activation, indicating that most dopamine neurons and PFC neurons can be metabolic-sensitive. On sucrose treated rats, we noticed that sucrose treatment induced a partial but significant decrease in the sensitivity of dopamine autoreceptors which was surprisingly exacerbated by exendin-4 co-administration, indicating that exendin-4 may interact negatively with both pre- and post-synaptic dopamine receptors.

Our behavioural data shows that exendin-4 and insulin alter dopamine-dependent behaviour with a remarkable inhibitory effect on D-amphetamine-induced motor activity and pramipexole and quinpirole-induced yawning, pica eating, and pelvic grooming activities. As this behaviour is mainly mediated by dopamine D_3 receptors our data suggest the existence of an interaction between GLP-1 and dopamine D_3 receptors. This may implicate GLP-1 mediated neuronal processes as a particularly interesting therapeutic target for disorders involving dopamine D_2/D_3 receptors and dopamine transporter malfunction. GLP-1 also exhibited a pro-cognitive effect on PCP-induced cognitive impairment, through the enhanced glutamatergic transmission. Finally, data on sucrose revealed that prolonged ad libitum access to sucrose by adolescent rats may alter brain circuits related to dopamine neurotransmission. It increases the behavioural responses of dopamine agonists, and is possibly associated with hypersensitivity of some postsynaptic dopamine receptors. These effects were partially prevented by exendin-4, which may elicit some protective effects on dopamine receptor function. In succinct, gut peptides impact on neurotransmission as well as behavioural activities and potentially modulate psychostimulant effects.

Acknowledgements

First, I would like to thank the almighty God for the grace given me to start and completed this Ph.D. program. Despite the challenges, I still consider it amazing for such mental capacity and emotional stability granted to me by God. All praise is unto Him!

I would like to sincerely thank Dr. Benjamin Simon Gronier, my supervisor, for allowing me to work with him on such an interesting project, which is recently receiving a lot of attention. I thank him for always being there and ready to share with me, his research-based knowledge and for influencing my research ability.

I would also like to show profound gratitude to my second supervisor, Dr. Tyra Zetterström and other lecturers in the department, for their support and helpful ideas on this Ph.D. program.

My thanks go to all the members of staff responsible for radioisotope monitoring: Liz O'Brien and Dr. Paul Ainsworth, for all their help regarding radioisotope regulatory procedures and due diligence, risk assessment as well as radioisotope health and safety. I also appreciate the radiation technical team; Nazmin Juma, Dr. Ketan Ruparelia and Zahoor Ul-Haq for their tolerance in collection and disposal of radioactive wastes and from time to time indicating irregular entries as well as balancing radioisotope inventory log book.

Anita O'Donoghue was indispensable in maintaining the BSU and provided technical support for the use of animals, including maintainance of high standards within the animal unit. I want to thank all the technicians in room HB 2.17 for their immense technical support, in particular David Reeder (Mr fix it), Amrat Khorana, Claire West, and Jo Tonkin, for providing gallons of purified water and many consumables, fixing damaged equipment, as well as assisting in all manners of ways, this work could not have been smoothly undertaken without your day-to-day support.

Finally, my health felt gratitude and thanks go to my ever supporting amiable wife for her assistance, tenacity, encouragement, and motivation. Indeed, we achieved it together!

Abbreviations

ADHD	Attention Deficit and Hyperactivity Disorder
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
AgRP	Agouti gene-related peptide
ANS	Authonomic nervous system
ARC	Arcuate nucleus
APO	Apomophine
AP	Area postrema
CA	Caudal area
CCK	Cholecystokinin
CNS	Central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2-3-dione
cAMP	Cyclic adenosine monophosphate
D-AMP	Dextro-amphetamine
^{o}C	Degree Celsius
DZ	Diazoxide
DI	Discriminating index
DAT	Dopamine transporter
DMH	Dorsal medium hypothalamus
DMH	Dorsal motor nucleus
DRG	Dorsal root ganglion
DA	Dopamine
EC_{50}	Dose inducing 50% of maximum response

ENS	Enteric nervous system
EPSCs	Excitatory post-synaptic currents
Ex-4	Exendin-4
fMRI	Functional magnetic resonance imaging
FOS	Fructooligosaccharide
g / kg	Gram - kilogram
GABA	γ-amino-butyric acid
GOS	Galacto-oligosaccharides
GBA	Gut-brain axis
GIT	Gastrointestinal tract
$GlyT_1$	Glycine reuptake transporter 1
GLP-1	Glucagon-like-peptide-1
GPO	Geniposide
HPA axis	Hypothalamic–pituitary–adrenal axis
IC_{50}	Dose inducing 50% of maximum inhibition
IRS-2	Insulin receptor substrate-2
IML	Intermediolateral nucleus
IPSCs	Inhibitory post-synaptic currents
K_{ATP}	ATP-gated potassium channel
K_i	Inhibitory constant, binding affinity
K_{ir}	Inwardly rectifying potassium channel

K_M	Michaelis constant
Kv_1	Voltage-gated potassium channel, family 1
LHA	Lateral hypothalamic area
l	Litre
LTD	Long-term depression
LTP	Long-term potentiation
mEq	Milliequivalent
MCH	Melanocortin- concentrating hormone
MCAO	Middle cerebral artery occlusion
AP	Area postrema
mm	Millimetre
mM	Millimolar
MΩ	Megaohm
MPH	Methylphenidate
ms	Millisecond
MSN	Medium spiny neurons
nA	Nanoampere
NET	Norepinephrine transporter
NPY	Neuropeptide Y
nM	Nanomolar
NMDA	N-methyl-D-aspartate
NOR	Novel object recognition
NTS	Nucleus tractus solitaries

NAc	Nucleus accumbens
OGD	Oxygen-glucose deficiency
PVN	Paraventricular nucleus
%	Percentage
PYY	Peptide YY
PCP	Phencyclidine
PI3K	Phosphoinositide 3-kinase
PDK	Phosphoinositide-dependent protein kinase
PPX	Pramipexole
PFC	Prefrontal cortex
PPG	Preproglucagon
PKB	Protein kinase B
PCR	Polymerase chain reaction
QNP	Quinpirole
rpm	Rotations per minute
s - sec	Second
SERT	Serotonin transporter
SHR	Spontaneously hypertensive rat
SNPs	Single nucleotide polymorphism(s)
SNc	Substantia nigra pars compacta
SUR1	Sulfonylurea receptor 1
Syt7	Synaptotagmin-7
TTX	Tetradoxin
TBT	Tolbutamide
µm	Micrometre
µM	Micromolar
VSG	Vertical sleeve gastrectomy
V_{MAX}	Maximum velocity
$VMAT_2$	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
v/v	Volume to volume ratio
w/v	Weight to volume ratio

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Introduction

The concept of the gut-brain axis

The concept of the gut-brain axis (GBA) has typically been studied in connection with intestinal motility, secretion, and vascularity (Track, 1983). The earlier researchers have viewed this paradigm in the circumstance of behavioural eating and appetite alteration (Leclercq et al., 2014). However, brain imaging and electrophysiology techniques have further provided deeper insight into this phenomenal association between the central nervous system (CNS) and gastrointestinal tract (Ringel, 2002). A shred of more compelling evidence that underscores the role of GBA in brain function is its involvement in neuromodulation and neurotransmission processes as well as homeostasis balancing (Cryan, 2016). The term as coined, GBA exists due to biochemical signaling phenomenon between the gut and the central nervous system (Wang and Kasper, 2014). GBA involves a bidirectional communication between the CNS and gastrointestinal tract (GIT) impacting on cognitive and reward centers of the brain, with influence on enteric nervous system functions (Mayer *et al.,* 2014). However, it was originally thought to be limited to physiologic maintenance of GIT homeostasis but, recent advances in research has shown the involvement of this phenomenon in more complex brain communications involving motivation and reward (Strandwitz, 2018). In a more holistic approach, the GBA encompasses the CNS, neuroendocrine and neuroimmune systems, extending to the hypothalamic–pituitary–adrenal axis (HPA axis) and the gut microbiota (Dinan and Cryan, 2015).

The morphological basis of the gut-brain axis

The GBA includes a connection of autonomic neurons that link the central nervous system CNS specifically, the caudal brainstem and spinal cord—to the esophagus, gastrointestinal

tract, liver, and pancreas (Furness, 2006; Jänig, 1996). The axonal extensions connect via the vagus, splanchnic, mesenteric and pelvic spinal nerves to innervate the abdominal viscera (Figure 1).

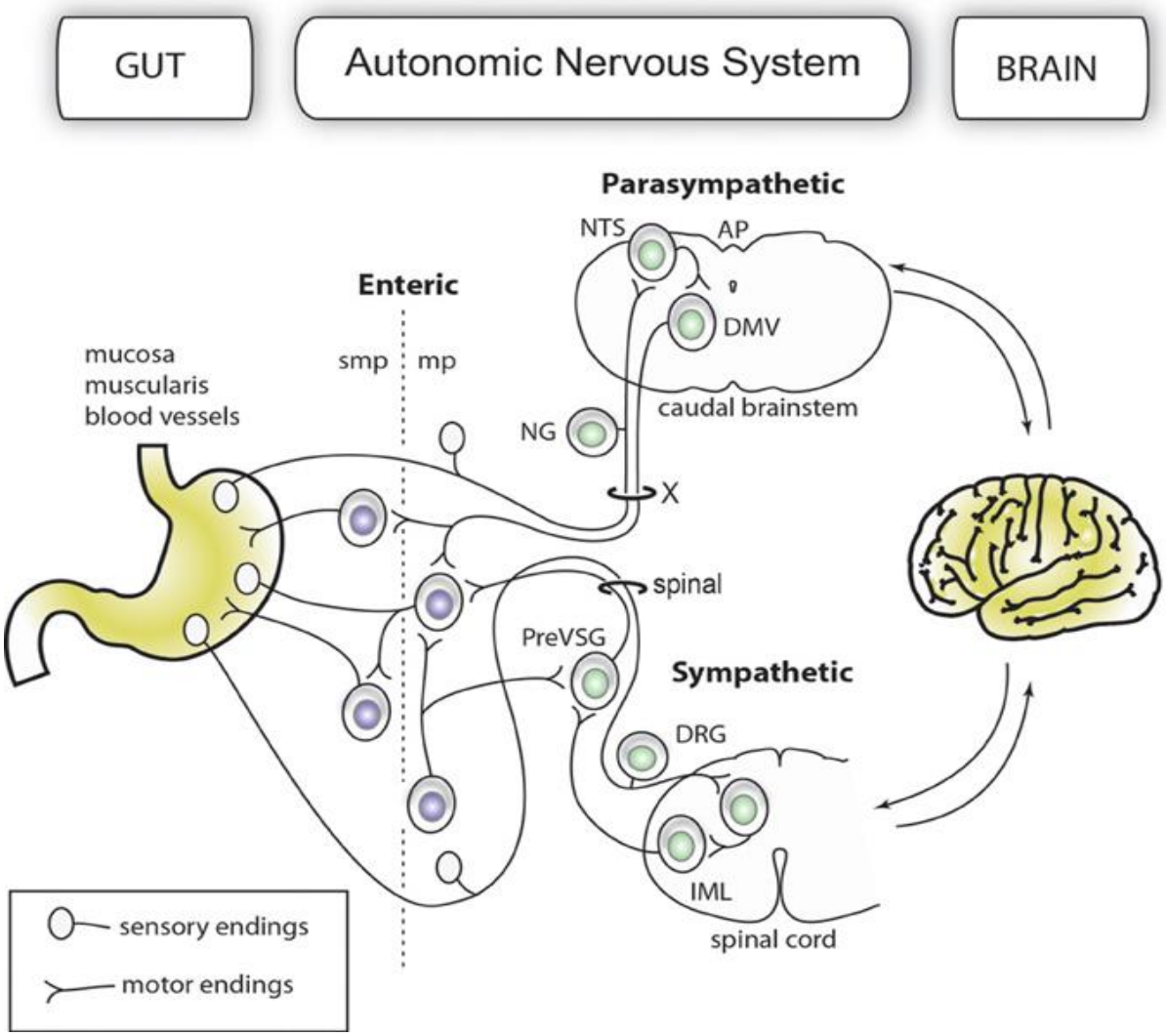


Figure (Fig.) 1: Anatomic formation of the gut-brain axis.

The principal anatomical and functional sub-classifications of a mammalian GBA are denoted with neurons associated with the enteric and extrinsic autonomic systems in contrasting colours. The enteric nervous system is entirely accommodated within the gastrointestinal wall. Abbreviations: NTS, nucleus tractus solitaris; AP, area postrema; DMV, dorsal motor nucleus; DRG, dorsal root ganglion; IML, intermediolateral nucleus; VSG, vertical sleeve gastrectomy(Gautron, 2013).

It is worth noting that the morphological examination of the gut-brain axis has a phenomenal long history. Claudius Galenus (Galen), circa A.D. 130–200 discovered the vagus nerve, as reported by Ackerknecht, (1974). However, the full anatomy of the GBA remained not available to scientists for certain period due to indistinction within the nerve cells as they move into the peripheral organs. Hence, it was not until the late 19th century that postganglionic neurons located in the gastrointestinal wall (also known as enteric neurons) were discovered by Auerbach and Meissner (Furness, 2008). Enteric neurons, together with postganglionic neurons in the gallbladder and pancreas, are the portion of the gut-brain axis, as they allow direct input from, and send information to, the rest of the autonomic nervous system (ANS). Nevertheless, enteric neurons are also able of operating autonomously of the gut-brain axis (Morris *et al.*, 1985; Powley, 2000). Functionally, GIT receives a two-way external innervation from ANS. This includes parasympathetic (cholinergic); vagal and pelvic nerves and sympathetic (noradrenergic): splanchnic nerves. The GIT accepts both excitatory and inhibitory innervations, the first is provided mainly by the parasympathetic nerves and last, by the sympathetic nerves (Dockray, 2009). The ENS has about 100 million neurons working as local sensory neurons, interneurons, and effector motor neurons. ENS is thought to be a part of CNS that has been replaced during development from the brain to the gut but preserving with brain two-way communication pathways (parasympathetic and sympathetic), each together with efferent and afferent nerves. The efferent extrinsic parasympathetic nerves are pre-ganglionic fibers terminating on cholinergic ENS neurons either of the myenteric plexus to control motor activity of the gut or in the submucous plexus to regulate the secretory activity of glandular cells or visceral circulation (Konturek *et al.*, 2004). The efferent extrinsic sympathetic (mainly noradrenergic) nerves are postganglionic

and some of them terminate on postganglionic cholinergic neurons of ENS to inhibit acetylcholine release via activation of their alpha-presynaptic receptors or directly on intestinal smooth muscle cells to affect motility of the gut or of vasoactivity of visceral vessels to influence the visceral circulation (Langley, 1994).

Features of the enteric nervous system (ENS)

The special feature of ENS from extrinsic autonomic nerves is its network of diverse neurons and potential to detect and relay sensory information; it can also function autonomously alongside with CNS. However, GIT may function without extrinsic innervation such as pyloric dilatation. The independent activity of ENS is known as "gut-brain" that is exhibited over GIT homologous with that of CNS exercising control over the somatic part of the body (Langley, 1994). Subsequently, ANS which comprises of sympathetic (regulates cardiovascular, reflex and stress activities) and parasympathetic nervous system (controls visceral and skeletal activities), including ENS originates from the neural crest from which the cells migrate to the gut during development. The moving cells occupy the gut wall to form its intrinsic innervation (Baynash*et al.,* 1994; Trupp *et al.,* 1996).

Microbiota-gut-brain axis

The microbiome colonizing the gut includes all microbes together with their genomic material residing the intestinal tract. This is the hallmark of two-way GBA that metamorphoses through initial habitation in which the brain and gut are linked together in an organism's health (Sarkar *et al.,* 2016). The central research that formed the basis of this paradigm was established when mice groomed in sterile conditions devoid of residence bacteria (bacteria-free mice) exhibited an escalated physiological reaction to stress as compared to control mice. These abnormal reactions were reversed when probiotic was ingested leading to bacterial recolonization of the gut system (Sudo et al., 2004). The

outcomes of this study established the microbiome's as functional biomarkers in the development of the hypothalamic–pituitary–adrenal (HPA) axis**.** In view of this background, gut bacteria have since been found to have overwhelming influence in controlling various and vital physiological activities, including immunomodulation, neuromodulation, homeostasis, drugs, and nutrients metabolizing, synthesis of vitamins and bioactive molecules and electrophysiological activity of the ENS (Kau et al., 2011; Hooper et al., 2012; Le Chatelier et al., 2013)**.** A recent advance in this area of research has in no doubt considered the presence of gut bacteria as the major factor in GBA. This reservoir has been the abode to over billion numbers of microbes heterogeneously inhabiting the gut, out-numbering the number of human cells and influencing almost all aspects of human biology (Cryan, 2016). In the light of this background, two important food agents that that are related to microbiota and provide vital physiologic effects in the gut have been identified namely; probiotic and prebiotic.

Probiotics are useful microbes that when administered possess favourable effect in the prevention and management of definite pathologic conditions leading to a positive health outcome (Chow, 2002; Lin et al., 2018; Sanders, 2015). These bacteria produce therapeutic effects through manipulation and colonization of resident gut bacteria, maintenance of human homeostasis leading to a therapeutic condition whereby the indigenous anaerobic flora limits the population of potentially pathogenic (mostly aerobic) flora in the digestive tract (Chow, 2002; Mogilnicka and Ufnal, 2018). The bacteria of popular concern are the Gram-positive *Bifidobacterium* and *Lactobacillus* families (Burnet and Cowen, 2013; Mayer *et al., 2014).* The ingestion of these microorganisms is followed by the immunological response in which pro and anti-inflammatory cells are differentiated, the antigenic cells being the pro-inflammatory (Sansonetti and Medzhitov, 2009).

Prebiotics, on the other hand, are non-digestible food materials that advantageously affect host well-being by particularly enhancing the growth and/or activity of 1 or a finite number of bacteria in the colon (Chow, 2002). They achieve this activity by eliciting compositional changes on intrinsic commensal bacteria when fermented in the gut (Gibson *et al.,* 2010). Fructooligosaccharide (FOS), is a prebiotic found naturally in wheat, onions, bananas, honey, garlic, or leeks. It has been found in chicory root and can be produced in the laboratory with sucrose as the substrate (Linardi *et al.*, 2001). Important prebiotic of vital relevance in the pharmaceutical formulation used as a daily supplement is Galacto-oligosaccharides (B-GOS). Gronier et al., (2018), demonstrated the effect of B-GOS on an in vivo iontophoresis electrophysiology on the rat frontal cortex and effect on the performance of the attentional set-shifting task. The study revealed increased neuronal responses to N-Methyl-D-aspartic acid (NMDA) in the rat frontal cortex and greater cognitive flexibility in the animals administered with B-GOS, an effect reported to be associated with an increased NMDA receptor function in the cortical brain region. A more recent finding has adopted new term called psychobiotics for prebiotics and probiotics, due to well-established evidence of impact on neurotransmission, reward, social behaviour, motivation, cognitive ability, systemic and central processes in animals and humans (Sarkar *et al.*, 2018).

Gut-brain axis signalling

Specific physiologic activities such as digestion, circulation, endogenous, and exogenous secretion and peristaltic movement are controlled by the preganglionic vagal and pelvic nerves hence, these processes are mediated by the ENS via CNS (Fox *et al.*, 1983). The overall signalling pathway is aided by the efferent fibers in this line. However, the postganglionic efferent neurons control the sympathetic outflow from the CNS via the gut which is active during trauma, stress or external stimulation (Jänig and Morrison, 1986). On the other hand, afferent vagal and sympathetic (spinal) nerves are connected to the afferent

fibers of the gut-brain mediating the signalling pathway that is transmitted to the CNS (Jänig and Green, 2014). This responds to specific physiologic processes such as muscular contraction and dilatation, secretion of various chemical substances like acids and nutrients in the gut, neuro-endocrine agents such as gut hormones, gut peptides, neurotransmitters, neuromodulators, cytokines and other agent of inflammation (Langley, 1994; Sharkey and Savidge, 2014; Holzer and Farzi, 2014).

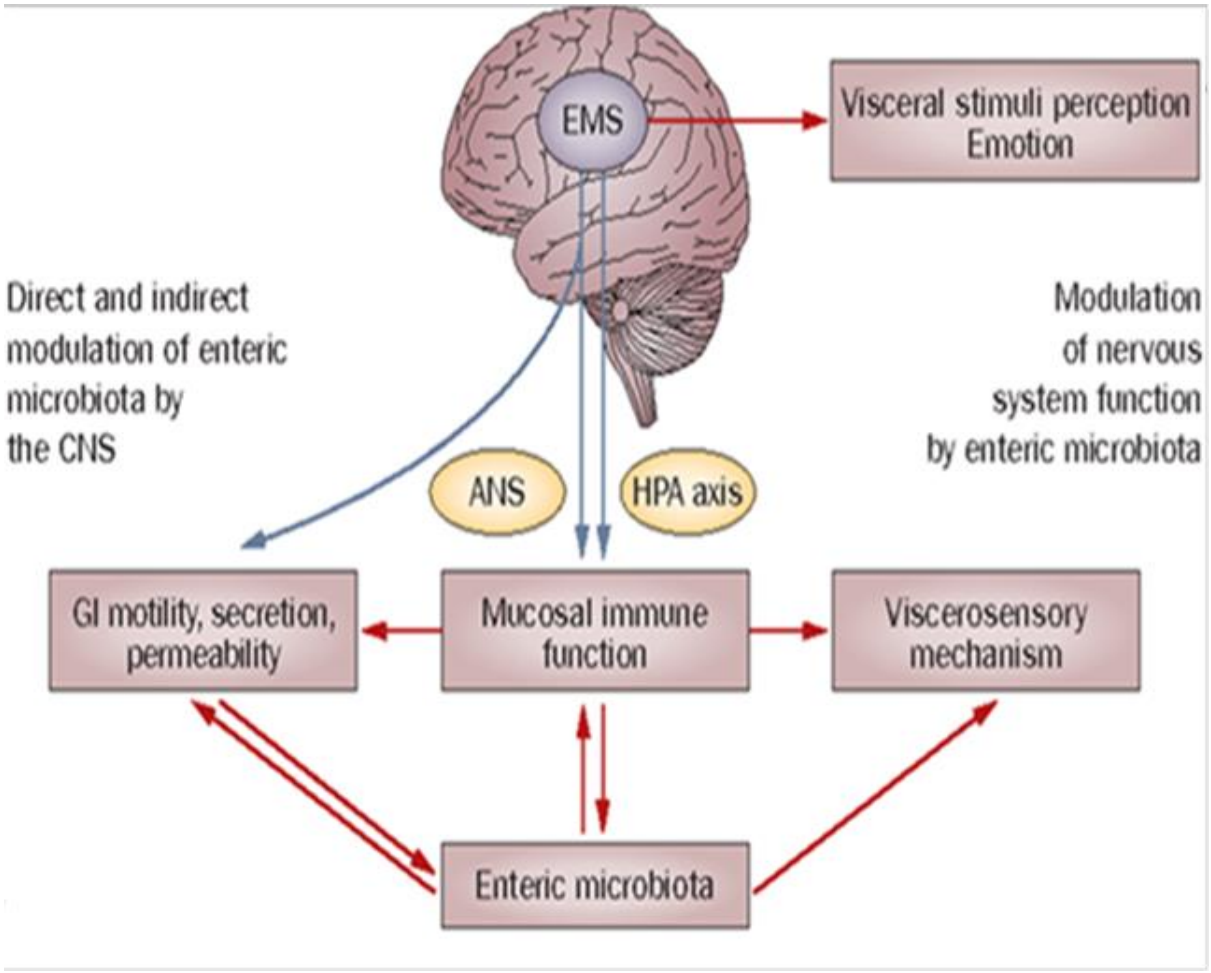


Figure. 2: Schematic illustration of bidirectional signaling of GBA

The nervous system can regulate many functions of the gut and alters the recognition of gut impulse through a set of similar outflows circuit called eMs, which consists of the parasympathetic and sympathetic divisions of the ANS, the HPA. The stimulation of eMs takes place through interoceptive and exteroceptive stressors. The enteric microbiotas are likely to interact with gut-based effector systems and with visceral afferent pathways, which establish a bidirectional brain –gut-enteric microbiota axis. Abbreviations: ANS, authonomic nervous system; CNS, central nervous system; eMs, emotional motor system; Gi, gastrointestinal; HPA, hypothalamus-pituitary-adrenal (Rhee et al., 2009).

In general, vagal nerves stimulation exerts anti-inflammatory effect (Thán *et al.*, 2000), improves depression (Jarczok *et al.*, 2018), alters feeding behavior (Pelot and Grill, 2018), ameliorates pain (Chakravarthy et al., 2015; Usichenko et al., 2017; Zhu and Marmura, 2016), calms anxiety (Fanselow, 2013; Genheimer *et al.*, 2017), and therapeutically used in seizure management (Chen et al., 2012a; Connor et al., 2012; Lisowska and Daly, 2012). It is noteworthy, to emphasize the importance of gut bacteria, as

they have a pronounced effect on the electrophysiological balance connected with ENS neurons (McVey Neufeld *et al.*, 2013). A classic example is myenteric neurons, which when incubated with *Bifidobacterium longum* NCC3001-fermented substances exhibited a decrease in action potentials after electrical stimulation (Bercik et al., 2011). Kunze *et al.*,(2009), reported increased excitability when *colonic* AH-neurons (the chief sensory neurons in the colon) were treated with *Lactobacillus rhamnosus*, a response as a result of the closing of calcium-controlled potassium gates. A study by Collins *et al.*,(2014) established an intestinal neural abnormality in the jejunum and ileum of germ-free mice in comparison to controls. However, in signaling related to neurotransmission through gut bacteria activity, dopamine and noradrenaline are produced by members of the *Bacillus* family, gamma-aminobutyric acid(GABA) by the *Bifidobacteria* family, serotonin by the *Enterococcus* and *Streptococcus* families, noradrenaline and serotonin by the *Escherichia* family, and GABA and acetylcholine by the *Lactobacilli* family (Lyte, 2011; Barrett *et al.*, 2012; Dinan *et al.*, 2015). Though there is a paucity of data to adduce these claims, these neurotransmitters modulate synaptic activity in the proximal neurons of the ENS, an important area future research opportunity.

Gut peptides

Gut peptides refer to a group of molecules with vital physiological activities, in the endocrine, nervous, digestive, circulatory and other systems (Abdulla *et al.*, 2014). They were first discovered as hormone over a century ago, being a factor originating from the duodenum that could stimulate pancreatic fluid production (Kreymann et al., 1987). Secretin, the peptide hormone was eventually discovered to be responsible for this activity (Bayliss and Starling, 1902). After these discoveries, more than 50 regulatory peptides have been established and the majority of which are present in both endocrine and paracrine cells and/or the intrinsic

nerves of the gastrointestinal tract (Harmar, 2004). The physiological functions mediated in the gut by these peptides include the control of motility (Avau *et al.,* 2013), influence secretion of fluid (Jin *et al.,* 1994; Johard *et al.,* 2003), regulate metabolic and enzymatic activities (Seufert, 2017; Wang *et al.,* 2017), modulate cell proliferation and survival (Parthsarathy and Hölscher, 2013; Khan *et al.,* 2016), maintain vascular and immune functions (Guéniche et al., 2006; Bellin et al., 2017) and perform inflammation role (Dandona*et al.,* 2018). In general, gut peptides elicit their effects through G protein-coupled receptors, a family of numerous membrane proteins, 87 of which are presently known to have peptide ligands (Williams and Schonbrunn, 1994; Al-Sabah *et al.,* 2014). The GPCRs has three division with a distinct molecular structure consisting of seven transmembrane domains, three extracellular loops, three intracellular loops, an amino-terminal extracellular domain and an intracellular carboxyl terminus (Wu *et al.,* 2017).

Appetite and satiety signalling

Gut peptides hormones are linked to many food-related gastric and intestinal signaling pathways(Read *et al.,* 1994). In recent times, researches have tried to establish the relationship between gastric signaling and release of gut peptides as it affects appetite, particularly hunger with satiety (Maljaars*et al.,* 2007; Gribble, 2012; Stengel and Taché, 2012; Sirohi *et al.,* 2017). In (Fig. 3), Orexigenic (appetite stimulant) property expressed by gut peptides hormone is predominantly due to ghrelin and is produced solely in the stomach, belonging to ligand growth hormone secretagogue receptor (Kojima et al., 2011; Nakazato et al., 2001). There is a surge in plasma ghrelin peaks before a regular meal, then reduces later to increase to another peak just before the next meal, suggesting that ghrelin may be a meal inducer (Lutter et al., 2008). It is also suggested to play a role in long-term regulation of energy balance as continuous ingestion of ghrelin in rodents results in hyperphagia and

weight gain without growth hormone (Karra et al., 2013; Uchida et al., 2013). The effects of ghrelin on eating behavior are mediated *via* the infundibular nucleus and the solitary tract nucleus of the hypothalamus, connected with vagal afferent loops thereby, opposing the actions of leptin through disinhibition of second-line neuropeptides such as neuropeptide Y (NPY) and agouti gene-related peptide (Abbott et al., 2005).

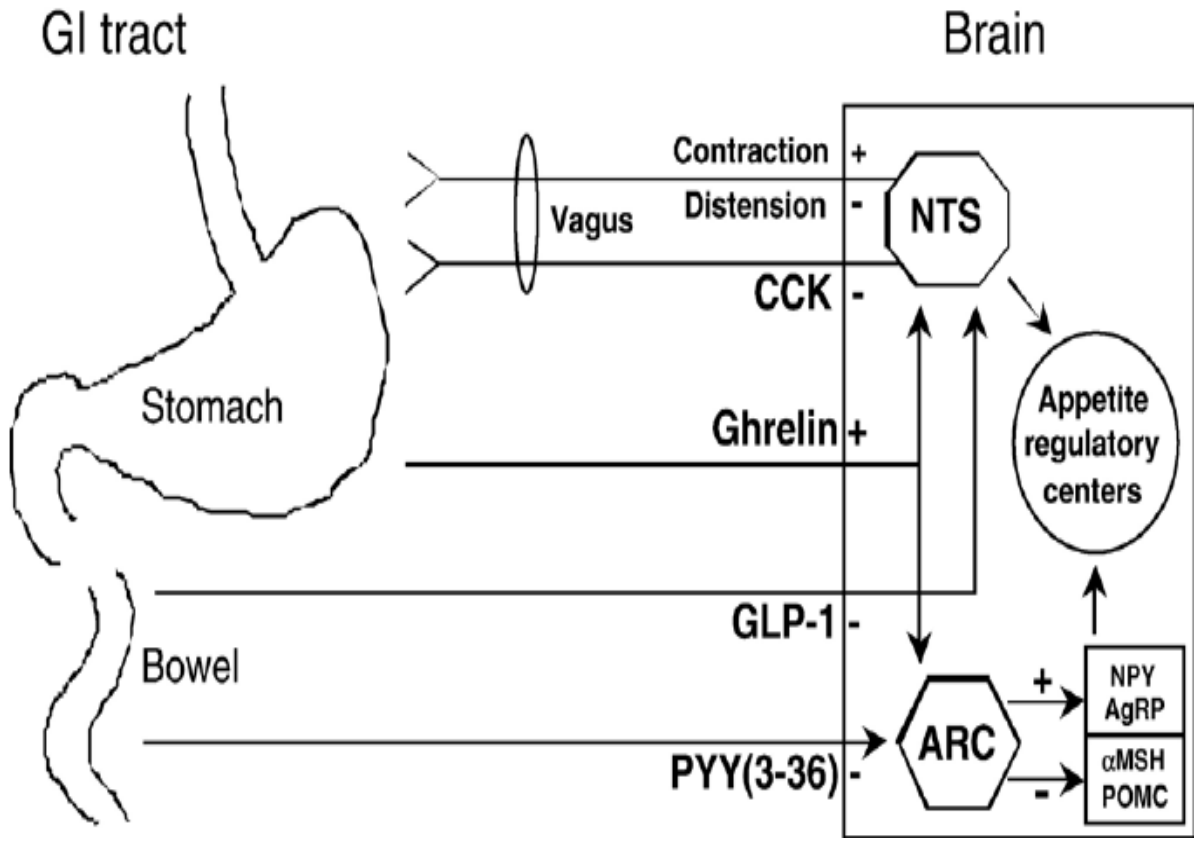


Figure 3: Appetite signaling from gut peptides and enteric nerves to Brain

Afferent gastrointestinal signals controlling food intake. CCK, cholecystokinin; NPY, neuropeptide; AgRP, Agouti gene-related peptide; α-MSH, α-melanocyte stimuli; ARC, arcuate nucleus; NTS, nucleus tractus solitarius; GLP-1, glucagon-like peptide-1; PYY, peptide tyrosine tyrosine (Näslund and Hellström, 2007).

In starvation, there is ghrelin increase which boosts appetite and reduced levels of ghrelin in obesity may be a secondary response to over-eating (Scott et al., 2012). The overall finding with ghrelin is a negative correlation with percentage body fat, fasting insulin, and fasting leptin, all of which are elevated in obesity (Nowroozi-Asl et al., 2016; Kyrgios et al., 2013; Amini et al., 2012; Cakiroglu et al., 2013). In converse, many among the gut peptides are anorexigenic (appetite suppressant) namely; cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY(3-36) (PYY(3-36)), which all are recognized as physiologic regulators of food intake (Muurahainen et al., 1988; Flint et al., 1998; Beglinger and Degen, 2006). The most studied of all gut peptides is CCK, regulating control of food intake and this has gathered extensive review of researches over the years(Yu and Smagghe, 2014; Kaloudi et al., 2015; Boyce et al., 2016). PYY, PYY(3-36), NPY and NPY(3-36) are released after a meal from the GI tract and induce satiety effect mediated through different Y receptors (Moriya et al., 2009, 2010). Specifically, the antisecretory mucosal mechanisms by which these peptides exert their effects are the same in human and mouse colon, with Y_1 receptor-mediated responses being solely epithelial, while Y_2-mediated effects are neuronal in origin (Hyland et al., 2003; Cox, 2007).

The traditional role of Glucagon-like peptide-1 (GLP-1)

Glucagon-like peptide(GLP-1) and glucagon-like peptide-2 (GLP-2) are produced and secreted from endocrine L-cells in the mucosa of the ileum and colon (Gil-Lozano et al., 2014). After food ingestion, GLP-1 and GLP-2 are released in equal concentration into the systemic circulation (Elliott et al., 1993). GLP-1 is a major contributor to the ileal brake mechanism of the upper GI tract, thereby modulating gastric emptying and acid secretion (Giralt and Vergara, 1999). Two vital physiologic effects of GLP-1 is its insulinotropic and glucagonostatic actions (Holst, 2007). It stimulates not only insulin gene transcription but

also all steps of insulin biosynthesis (Nathan et al., 1992). GLP-1 is capable of inducing new β cells in subjects with an insufficient number of these cells (Buteau et al., 1999). Drucker, (2003) and Farilla et al., (2003) reported the actions of GLP-1as a stimulant of β cell differentiation and proliferation and inhibit apoptosis in β cells, the processes that lead to an eventual increase in the β cell mass and function over a long-term period. In addition to its effect on the β cells, GLP-1 reduces glucagon secretion, all the overall effects qualify it as a suitable therapeutic agent for type 2 diabetes (Avgerinos et al., 2018; Patel et al., 2018c). For this thesis, we have concentrated on describing physiological and therapeutic effects of exendin-4 (Ex-4) a GLP-1 agonist and geniposide (GPO) an iridoid glycoside compounds with a putative GLP-1effect. Also, tolbutamide a sulphonylurea with Kir6.2/K_{atp} channels blocking activity use in treating type 2 diabetes mellitus as well as insulin, an hormonal peptides were the focus of our study.

The central functions of Glucagon-like peptide-1

GLP-1 modulates hippocampal neuronal activity

Looking beyond energy homeostasis, GLP-1 has assumed new roles in food and drug reward (Dickson et al., 2012a). It has been reported that hippocampal GLP-1R activation signals food reward behavior (Hsu et al., 2015a). The receptor activation consequently triggers neuronal spontaneous firing of the CA1 region of the hippocampal cell. This effect can be inhibited by 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX), a glutamate antagonist and demonstrated that GLP-1R signaling facilitates excitatory synaptic transmission in the hippocampus (Oka et al., 1999). This study further reported a biphasic response to GLP-1 agonist starting with increased and followed by reduced single-unit activity in the CA1 region of the hippocampus (Oka et al., 1999). Similarly, GLP-1 prevents Aβ1-40-induced attenuation in the occurrence of excitatory post synaptic currents in the hippocampal neurons

(Wang et al., 2013). There is an abundance of GLP-1R in the caudal area of CA1 and CA3 which may suggest the involvement of GLP-1 in volume transmission (Cork et al., 2015; Hamilton and Hölscher, 2009; Hsu et al., 2015b). The CA3 pyramidal neurons are also controlled by GLP-1R signalling and activation by Ex-4 an analogue of GLP-1, enhances both the frequency and amplitude of spontaneous inhibitory postsynaptic currents (IPSCs); nonetheless, these actions are inhibited by tetrodotoxin (TTX), which is indicating that action potentials are necessary (Korol et al., 2015). Another credible report is that GLP-1Ractivation facilitates depolarization of presynaptic inhibitory neurons which eventually enhance the inhibitory tone on the postsynaptic CA3 neurons of the hippocampus. GLP-1R activation has also been reported to stimulate $GABA_A$ receptors through endogenous synaptic GABA even when neurotoxin, tetrodotoxin was injected (Korol et al., 2015). Consequently, GLP-1 amplifies $GABA_A$ signalling in the CA3 region of hippocampus via both pre- and postsynaptic channels. It was reported however, that in the dentate gyrus (DG) region of the hippocampus GLP-1R blocker (Ex-9) facilitates the input-output relation and lowers the paired-pulse ratio (PPR) of excitatory postsynaptic potentials (EPSPs), indicating that endogenous GLP-1R inhibition enhances excitatory synaptic transmission in animal model(Kobayashi et al., 2013). The effects of GLP-1R activation or blockage in the hippocampus can be summed up to depend on the neuronal subtypes that are engaged, which can either be G_s or $G_{i/o}$ of the G protein signaling cascades to produce its effect (Montrose-Rafizadeh et al., 1999; Hällbrink et al., 2001). A recent study of GLP-1 relating to CNS disorders reported that, the drug improves repeated restraint stress-induced depression-like behaviour in mice by a reduction in neuronal apoptosis through GLP-1R/AKT signaling pathway (Zhao et al., 2018).

GLP-1 influences the hypothalamic function

Beyond the homeostatic function of the hypothalamic nuclei, dysfunction in this centre may lead to alteration in feeding behaviour (Hetherington and Ranson, 1942). The expression of GLP-1Rs are ubiquitously present in the paraventricular nucleus (PVN), arcuate nucleus (ARC), dorsal medium hypothalamus (DMH) and lateral hypothalamic area (LHA) (Cork et al., 2015). It is of interest to know that, NTS GLP-1 neurons present on the NTS are only connected to the PVN and DMH in hypothalamus, but not in the ARC (Gu et al., 2013), indicating that the PVN and DMH receives an indirect synaptic control by GLP-1 produced from the NTS, while ARC neurons are modulated by volume GLP-1 input, either from NTS or from circulating GLP-1. It was also reported by (Acuna-Goycolea and van den Pol, 2004a), that activation of GLP-1R in the orexin LHA neurons causes an enhanced firing frequency of neuronal activity, but does not effect on melanocortin- concentrating hormone (MCH)-expressing neurons. The increase in firing frequency of action potential in orexin neurons may be dependent on both the facilitation of excitatory synaptic transmission and direct postsynaptic depolarization of the cell membrane (Acuna-Goycolea and van den Pol, 2004a). The study further reported that promotion of excitatory synaptic transmission by GLP-1 may also be mediated by the afferent nerve terminal, as only the frequency but not the amplitude of mEPSCs in the presence of TTX is increased. Hence, blockages of synaptic inputs to LHA orexin neurons are also positively controlled by GLP-1R activation via presynaptic regulation. These inputs to LHA, which can be regulated by GLP-1, may come from PVN or ARC, as both nuclei have abundant GLP-1 receptor expression (Acuna-Goycolea and van den Pol, 2004a). More study by Calvo et al., (1995), showed an increase in endogenous glutamine (a precursor for GABA) concentration after treatment with GLP-1. Astrocytes, notwithstanding are responsible for the production of this amino acid. Therefore, GLP-1 may regulate astrocyte functions through a glutamine-dependent pathway in the

VMH. A more recent study suggested that GLP-1R activation in the astrocytes may also be responsible for its anorexic property of (Reiner et al., 2016). In summary, GLP-1 controls hypothalamic neuronal activity via presynaptic regulation and a recent study has suggested the involvement of synaptotagmin-7 (Syt7) in phosphorylation by GLP-1 (Wu et al., 2015) and Syt7 is abundantly expressed in the CNS including hypothalamus (Sugita et al., 2001).

GLP-1 regulates reward through the mesolimbic system

The mesolimbic dopamine (DA) pathway is an important system that is responsible for food and drug reward signalling (Kenny, 2011a; Volkow et al., 2011a). GLP-1 receptors are abundantly expressed in reward neural centres, which is made up of nucleus accumbens (NAc) and the ventral tegmental area (VTA) (Merchenthaler et al., 1999a), and the activation of GLP- 1R in both regions results in decreased food intake (Mietlicki-Baase et al., 2013a, 2014a). Both the NAc and VTA receive projections from NTS GLP-1 neurons although this does not take place in the hippocampus (Gu et al., 2013a). Inhibition of GLP-1R increases highly palatable high-fat food consumption, which shows that these relationship are biochemically relevant for the control of feeding behaviour (Alhadeff et al., 2012a) GLP-1R activation enhances excitatory synaptic inputs to medium spiny neurons (MSNs) in the NAc core of rats (Mietlicki-Baase et al., 2014a) likely via increase of presynaptic release probability (increased mEPSC frequency, no change in mEPSC amplitudes, and a reduction in PPR (Mietlicki-Baase et al., 2014a). However, administering GLP-1 to the NAc core has no effects on DA release, indicating no presynaptic regulation on DAergic nerve terminals. It is worthy of note that, GLP-1 seems to cause hyperpolarization in MSN cells and suppresses the input–output necessary for the generation of action potentials when injecting currents (Mietlicki-Baase et al., 2014a). The overall results from these data indicate that within the NAc, the functions of GLP-1 in regulating MSN functions are of many features. The effect of

GLP-1 in the VTA has been studied. GLP-1 analogue applied to the VTA of a rat was reported to enhance excitatory synaptic VTA DA neurons through the presynaptic pathway, an effect likely to reduce the PPR and enhance the frequency of sFPCs (Mietlicki-Baase et al., 2013a). However, a more recent study has revealed that the effect of GLP-1 on DA and non DA neurons in the VTA is cell-type dependent. While GLP-1R activation enhances excitatory synaptic VTA DA neurons, such activation causes depression of synaptic impulse of non-DA neurons that are projected to the NAc (Wang et al., 2015a). The two reports indicate the paradoxical effect of GLP-1 activity on VTA, which is dependent on cell type and further suggest that VTA DA neurons extended to both NAc and prefrontal cortex, response differently to drug and food rewards (Lammel et al., 2011, 2012).

The roles of GLP-1 in caudal hindbrain neurons

Preproglucagon (PPG) neurons producing GLP-1 are widely present in the NTS part of the brain region (Gu et al., 2013b). Direct vagus inputs are received by the NTS and have been found to play a key function in food intake reward. GLP-1R activation in the NTS reduces intake of palatable high-fat food, operant responding for sucrose under a progressive ratio schedule of reinforcement, and the conditional place preference for a palatable food (Alhadeff and Grill, 2014). However, using a transgenic mouse model, it has been shown that GLP-1-producing PPG neurons are not affected by GLP-1 itself but generates fast electrical impulse by leptin (Hisadome et al., 2010). More studies found out that GLP-1Rs are not present in the preproglucagon neurons in the nucleus of the solitary tract (NTS-PPG neurons), PPG-GFP but a reduction in leptin receptors on GLP-1 producing neurons causes hyperphagia (Hayes et al., 2010, 2011; Scott et al., 2011). Base on this premise, GLP-1 signaling in the NTS is likely controlled by non- PPG neurons located in the NTS. Part of the brain stem that provides the preganglionic motor fibres is dorsal motor nucleus of the vagus

(DMV) which is connected to the visceral organs (Travagli et al., 2006), innervates the intrapancreatic ganglia, and controls pancreatic secretory functions, including insulin secretion and glucose homeostasis (Sakaguchi et al., 1994; Trapp et al., 1994a). About fifty-percent of the pancreas-projecting neurons in the DMV respond specifically to GLP-1 (Wan et al., 2007), showing that regulation of this system is mediated through some specific population of neurons in the DMV. Activation of GLP-1R in DMV depolarizes a subpopulation of neurons through inhibiting K+currents, which is not affected by TTX, suggesting a postsynaptic inhibition of K+-conductance, similar as reported previously (Acuna-Goycolea and van den Pol, 2004b). Taken together, GLP-1 treatment activates DMV preautonomic neurons, which project to the pancreas and results in insulin exocytosis and/or inhibition of glucagon release.

Functions of GLP-1 in behavioural paradigm

Recent studies have reported the role of GLP-1 agonist in drug-mediated behavioural activities with an animal model. These studies report that, treatment with Ex-4 attenuates the rewarding properties of amphetamine as well as cocaine-induced locomotor stimulation (Egecioglu et al., 2013a; Graham et al., 2013), impaired alcohol-induced behaviours in rodents (Egecioglu et al., 2013b) and attenuates nicotine-induced locomotor stimulation as well as accumbal dopamine release (Egecioglu et al., 2013c), all suggesting that GLP-1R signaling is largely implicated in behavioural and neurochemical processes. Further research has demonstrated that, intestinal L cells express VGLUT2 and secrete glutamate along with GLP-1 (Zheng et al., 2015a). Because of this, GLP-1-positive neurons within the caudal (visceral) nucleus of the solitary tract (cNST) also are glutamatergic and may enhance glutamate release when stimulated (Zheng et al., 2015b). All these suggest a role of GLP-1R

agonist in neurotransmission and potentially play a novel function in motivation, cognition and reward behaviours.

Geniposide, a putative GLP-1R agonist action on the CNS

Geniposide is a water-soluble iridoid glycoside, a major constituent in the fruits of *Gardenia jasminoides* (Gardenia fruits), which is a popular Chinese herb widely used in the traditional management of liver disorders, inflammatory disorders, contusions and brain disorders (Chen et al., 2010; Wang et al., 2012). More researches have recently developed attention on neuroprotective (Lv et al., 2014a), antitumor (Hsu et al., 1997), modulation of DNA expression (Gálvez et al., 2005), analgesic (Gong et al., 2014a), anti-inflammatory, coloretic and hepatoprotective effects (Chou et al., 2003; Liu et al., 2010; Ma et al., 2011) of geniposide. The drug confers protection on memory impairment and maintenance of cognitive responses, exhibited in animal behavioral studies (Gao et al., 2014; Lv et al., 2014b). Molecular screening via a high throughput for GLP-1 receptor agonists shows that geniposide is an agonist for the GLP-1 receptor with Kdof ~6.1″μM (Liu et al., 2006). The GLP-1 receptor belongs to the class B family of G protein-coupled receptors (GPCRs GLP-1 just like insulin and IGF-1 activates second messenger signaling pathways that are commonly linked to growth factor signaling (Hölscher, 2014a). Liu et al., (2012) also demonstrated that stimulation of GLP-1 receptor by geniposide promotes neurotrophic and neuroprotective effect in cells.

Insulin action in the brain

Insulin, on the other hand, is an important hormone for controlling peripheral glucose homeostasis. The brain is now regarded as insulin-sensitive organ due to abundance presence

of both insulin receptors (IRs) and insulin, alongside with series of a cascade of signaling processes that are associated with many brain regions (Havrankova et al., 1978). The group also described the relative abundance of brain's insulin, that it is independent of peripheral insulin, because plasma insulin levels did not affect the brain's insulin concentration at any time (Havrankova et al., 1979). Also, escalated insulin levels had been reported both in the human brain and in many rodents models (Dorn et al., 1983). Meanwhile, the availability of huge concentrations of insulin in the brain has become a debatable concept, raising the question about its source. Interestingly, the presence of insulin in the cerebrospinal fluid (CSF) could not be solely ascribed to its ability to cross the blood-brain barrier (BBB), but thought to be partly due to receptor-mediated transport system for circulating peptides (Banks et al., 1997a; Pardridge, 1999). The IRs identified has been linked with important physiological activities on the CNS such as, neuronal cell differentiation, glucose homeostasis, feeding trends, additive behaviour and body mass control, as well as cognitive processes which include memory and learning (Derakhshan and Toth, 2013). However, in addition to these metabolic roles of the brain insulin receptor, a recent study further demonstrated that insulin resistance in brain induces dopaminergic dysfunction leading to anxiety and behavioural disorders (Kleinridders et al., 2015a) this shows a new function for insulin signaling in neuronal control. Further study with rodents without brain insulin receptor substrate 2 (IRS2), suggests a potential role of IRS2 in the modulation of hippocampal synaptic function and plasticity in mice, which could be mediated via the N-methyl-D-aspartate (NMDA) receptor and the phosphoinositide 3-kinase (PI3K) signalling pathway (Costello et al., 2012). This implies that, dysfunctional insulin signalling in the brain is one of the major indicators in neurodegenerative and cognitive disorders. Apart from regulating neural circuits and involved in maintaining energy homeostasis, insulin also affects cognitive functions through its actions on synaptic plasticity and long-term potentiation in the

hippocampus and other brain regions responsible for learning and memory (Stranahan et al., 2008). More studies also have shown a strong correlation between Alzheimer's disease and CNS insulin resistance (Zhao et al., 2008). Alzheimer's disease is a neurodegenerative disease leading to progressive decline of memory and cognitive function and is the commonest form of dementia, accounting for more than 50% of cases (Querfurth and LaFerla, 2010). Though aging is the most leading risk factor, now there is ample evidence that people with glucose intolerance, insulin resistance and metabolic syndrome are at higher risk for cognitive impairment and dementia compared to age- and gender-realated controls (Arvanitakis et al., 2004; Whitmer et al., 2008). A meta-analysis and a large-scaled pooled analysis demonstrate that diabetes is associated with an approximately 60–70% increased risk of all types of dementia (Chatterjee et al., 2016). Therefore, Alzheimer's disease is sometimes referred as type 3 diabetes, a brain specific impairment of insulin signaling (de la Monte and Wands, 2008). Multifactorial pathogeneses that can be linked to brain insulin signaling defects, such as oxidative stress due to hyperglycemic toxicity, chronic inflammatory processes, mitochondrial dysfunction, abnormal cholesterol metabolism, adverse vascular changes and severe hypoglycemia are thought to trigger the development of dementia in people with metabolic disturbances (Blázquez et al., 2014).

` Diet alteration may also play an important part in the development of insulin resistance in the brain. Indeed, diet-induced changes such as, high liquid sugar and high fat diet in the peripheral insulin sensitivity has been shown to contribute to alterations in brain signalling and cognitive fuctions (Kothari et al., 2017). In hamsters, a diet high in fructose induces peripheral as well as neural insulin resistance, as evidenced by decreased insulin-mediated IR, IRS1, and Akt phosphorylation and elevated protein-tyrosine phosphatase 1B (PTP1B) expression in the cerebral cortex and hippocampus (Mielke et al., 2005).

This in turns potentially led to cognitive impairment due to insulin resistance. In recent time the potential impact of diets rich in free sugar such as sucrose has been found to be associated with excess energy intake rather than having a direct detrimental effect on metabolism in human (Macdonald, 2016a). Insulin dysfunction such as insulin resistance or low production has also been investigated to cause a long-term depression, a functional measure of synaptic plasticity, suggesting that brain insulin resistance may contribute to cognitive impairment (Mielke et al., 2005). High fat feeding of rats also impaired neuronal insulin signaling and long-term depression in the CA1 region of the hippocampus (Pratchayasakul et al., 2011). Furthermore, insulin treatment rescued impaired hippocampal neuron proliferation in mice with experimental diabetes Taken together, insulin's action in the CNS is crucial in maintaining cognitive function in animals (Ho et al., 2012).

Metabolic drug effect on the CNS

In addition to gut peptides, this Ph.D. research also investigates metabolic agents such as sulphonylureas, a prototype of which is tolbutamide. These are molecules primarily responsible for controlling blood sugar levels. Tolbutamide is a first generation sulfonylurea, an oral-hypoglycemic agent used in the management of type-2 diabetes (Rennie and Anderson, 1963). It stimulates insulin secretion by binding to a high-affinity subunit (SUR1) of the beta-cell potassium-sensitive ATP channel (K channel). Binding results in blocking of K^+ efflux through the Kir6.2 channel, depolarization of the beta cell, the opening of voltage-sensitive Ca^{++} channels, an influx of Ca^{++}, and insulin secretion (Bryan and Aguilar-Bryan, 1999). However, recent evidence has demonstrated a possible CNS and neuronal effect of this agent, which may influence cognition and neurotransmission, through K^+ sensitive ATP modulation, particularly on $K_{6.2}$ channel. It is widely accepted that an opening of K_{ATP} channels causes hyperpolarization of the cell membrane by increasing the K^+-conductance

and the K^+-efflux (Roeper et al., 1990). On the other hand, blockade of these channels decreases K^+-conductance and K^+-efflux causing depolarization of the cell with a consecutive increase of cytosolic $Ca^{2+}$and secretion of hormones or transmitter substances (Schmid-Antomarchi et al., 1990). Furthermore, a previous investigation shows that K_{ATP} channel blockade with the sulfonylurea derivative glibenclamide provided robust protection to dopamine neurons undergoing spontaneous and selective degeneration in midbrain cultures (Toulorge et al., 2010). Thus, in the presence of diazoxide, a K_{ATP} channels opener, the signalling process is reversed as the propagation of action potential initiation is shut down resulting in inhibition of insulin secretion. Also, some studies have shown the important modulatory effect of the channels on the excitability of substantia nigra neurons (Peltier and Vecchio, 1961; Röper and Ashcroft, 1995; Schiemann et al., 2012a) and dopaminergic midbrain neurons (Liss et al., 1999). Interestingly, Liss et al., (1999) further revealed that dopaminergic substantia nigra (SN) neurons express K-$_{ATP}$ channels subtypes with different sulphonylurea-sensitive receptors. These channels are configured in pore-forming subunits namely, Kir6.1 and Kir6.2 as well as a sizeable supplementary subunit, the sulfonylurea receptor (SUR1, SUR2A, and SUR2B) (Tinker et al., 2018). Thus, the Kir6.0 series is part of the inwardly rectifying member of potassium channels (K_{ATP}) while; the SUR is part of the adenosine triphosphate (ATP)-binding class of proteins.

Objectives

Gut peptides are primarily considered an important hormone for glucose and lipid homeostasis. However, recent studies over the years have shown that insulin and newer insulin-releasing antidiabetic agents such as glucagon-like peptide (GLP-1), as well as synthetic insulin secretagogue like tolbutamide, may be involved in regulation of dopamine transmission, influence addictive behaviour, and also affect cognition, motivation, and neuronal survival (Calsolaro and Edison, 2015a; Mansur et al., 2018a). However, most of the mechanisms underlying the central effect of these peptides are yet to be completely understood. Thus, interaction with dopamine transporters (DAT) (Egecioglu et al., 2013b, 2013a; Jones et al., 2017), dopamine signalling processes (García-Tornadú et al., 2010; Anderberg et al., 2014; Stouffer et al., 2015), glutamatergic AMPA/kainite signaling (Gheni et al., 2014; Mietlicki-Baase et al., 2014a; Nampoothiri et al., 2014) as well as mesolimbic reward system (Davis et al., 2010; Dickson et al., 2012b; Tiedemann et al., 2017; Wakabayashi et al., 2018) have been hypothesized to be associated with the central activity of these peptides.

Here, using radiometric [DA] release and uptake assays, we will test the *in vitro* activities of insulin, exendin-4, geniposide and tolbutamide on the striatal slices of rats. We will also confirm the outcomes of these peptides in the regulation of the *in vivo* electrical activities of ventral tegmental area dopamine neurons and prefrontal cortex pyramidal neurons, using single cell extracellular electrophysiology technique. We will also examine whether these peptides can alter some dopamine-dependent behaviours such as, D-Amphetamine-induced motor activity, quinpirole (a dopamine D_2/D_3 receptor agonist) and pramipexole (a dopamine D_3 preferred receptor agonist) -induced yawning, pica eating and pelvic grooming activities, as well as phencyclidine (an *N*-Methyl-D-aspartate receptor

antagonist)-induced cognitive deficit and motor activation. In another series of behavioural experiment, we will test whether GLP-1 can modulate the effect of diet modification in form of chronic sucrose intake on body weight, feeding pattern, and depressive phenotype, as well as assess the behavioural and electrophysiological impacts of continuous sucrose consumption.

Chapter I- Behavioural, neurochemical and electrophysiological responses following acute and repeated administration of dopaminergic agonists

I-1- Introduction

Psychostimulants acting as indirect non-selective agonists, used in the management of ADHD, like d-amphetamine (D-AMP), methamphetamine and methylphenidate, cause, when acutely administered, a wide range of behavioural changes (Archer et al., 1988; Leng et al., 2004). These involve a robust increase in horizontal motor activity accompanied by an increase in rearing trait (Russell et al., 1987), the latter reflecting a specific activation of dopaminergic neurotransmission (Kozler et al., 2017). On the other hand selective or preferential dopamine D_2/D_3 receptor agonists (like quinpirole (QNP), a dopamine D_2 selective and pramipexole (PPX), a D_3 preferred agonist) unveil some specific behaviours such as yawning, penile erection (pelvic grooming), hypothermia and pica eating, which are either D_2 or D_3 receptors mediated (Zharkovsky et al., 1993; Ohmura et al., 2011). D-AMP and related psychostimulants, exert their action by blocking DA reuptake to enhance DA release via competitive interaction with DAT. As a result of stimulating effect on DA autoreceptors and negative feedback mechanisms from DA-triggered regions, the increased DA release causes an inhibition of midbrain DA neurons' firing (Bunney et al., 1973; Einhorn et al., 1988), through the of activation of dopamine autoreceptors found on the cell bodies of the dopamine neurons (Ford, 2014). Previous studies have provided evidence that this DA-mediated response could be altered, after chronic administration with psychostimulants and have been implicated to play a principal role in behaviours associated with abuse of psychostimulants (Kamata and Rebec, 1983; White and Wang, 1984). Further works have shown that firing and burst activity of DA neurons in DA-rich brain region such

as the VTA has a direct correlate with behavioural modification during a reward-associated experience in humans and animal subjects (Hollerman and Schultz, 1998; Paladini and Roeper, 2014). A recent study from our laboratory has also demonstrated that chronic methylphenidate treatment during early but not during late adolescence enhanced D-amphetamine-induced rearing activity, as well as midbrain dopamine cell excitability (firing, burst, and population activity). Furthermore, this effect was associated with partial desensitization of dopamine D_2 auto-receptors (Di Miceli et al., 2019a). On the other hand, pramipexole (PPX), a D_3 preferring dopamine receptor agonist primarily used in the management of Parkinson's disorder and restless legs syndrome, has been reported to inhibit DA neuronal firing in the VTA (Guttman and Jaskolka, 2001; Reichmann et al., 2006; Chernoloz et al., 2009a). This drug is clinically used as monotherapy for Parkinson disease and has demonstrated antidepressant and antimanic clinical effects (Salawu, 2012). Interestingly, the long-term administration of PPX has been reported to desensitize dopamine cell bodies D_2/D_3 autoreceptors, thereby preventing inhibition of the basal firing activity of DA neurons in the VTA during its chronic administration (Chernoloz et al., 2009a). On the other hand, the effects of an administration (chronic or sub-chronic) of D-AMP on D_2 /D_3 autoreceptors have not been clearly characterized.

The present chapter addresses some vital issues related to the effects of D-amphetamine on dopamine neurotransmission using different paradigms (neurochemistry, electrophysiology, behaviour). It is crucial to this thesis, as it provides the basis as well as the template for our other studies on GLP-1 and insulin on brain functions particularly on dopamine neurotransmission. We have attempted to identify for the first time, possible electrophysiological effects of PPX on dopamine neurons in naïve rats and rats previously exposed twice to D-AMP on a week interval. This is to generate new data on DA D_2/D_3 sensitivity subsequent to two doses of D-AMP administration. Also, we have studied

behavioural, as well as neurochemical activities of PPX and QNP. In particular, we have assessed radiometric release and uptake processes of dopamine in striatal brain slices and synaptosomes respectively.

I-2-Materials and Methods

I-2-A-Subjects

Adult male Sprague-Dawley rats were purchased from Charles River, UK. Animals were then housed in groups of 4 animals per cage, maintained at 20-22^{o}C with humidity rates above 40% under a 12:12 L/D cycle with lights on at 07h00 AM (Ante Meridiem). Animals were kept in polypropylene cages measuring 56x38x17 cm. Food and water were provided ad libitum. Rats were allowed a 3-day acclimatization period after taking the delivery into the animal house. All experiments were performed during the light phase and with permission from the UK Home Office and De Montfort University Ethics Committee under the Project License 10B9FE34 and with the Personal License 128F91DAAQ.

I-2-B- Drugs

All drugs were purchased from Sigma (Sigma-Aldrich, UK), except for PPX in dose of 0.1 mg/kg given intraperitonially (i.p.) (Hellobio, UK), apomorphine hydrochloride (i.p.) (Tocris, UK) as well as NGB 2904 and QNP in dose of 0.05 mg/kg (i.p.) (Tocris, UK). The drugs were dissolved into Krebs (release assays) and HEPES (uptake assays) buffers, as appropriate. The drugs were dissolved in distilled water or saline for studies involving behavioural and electrophysiological profiling.

I-2-C- *In vitro* ^{3}H-dopamine release assay

Male Sprague-Dawley rats were sacrificed by cervical dislocation and the brains were then quickly removed and placed on an ice-cold platform for further dissection. Tissue from the striatum (Fig. 4) was rapidly dissected out and placed in ice-cold oxygenated Krebs buffer(NaCl 125 mM, MgSO4 1.2 mM, KCl 2.5 mM, CaCl2 2.5 mM, KH2PO4 1.2 mM, NaHCO3 25 mM, glucose 10 mM, pargyline 10 µM -in order to reduce dopamine metabolism-, pH 7.4). Slices were then cut into 350×350 µm prisms using a McIlwain tissue chopper, washed three times in fresh, oxygenated Krebs buffer and allowed to recover for 30 min at room temperature. After this stage, all experiments were made under constant oxygenation and at 37 °C. Tissue prisms were loaded with ^{3}H-dopamine (1.0 µCi/mL, specific activity 30-40 Ci/mmol) for 40 mins, then washed three times with fresh Krebs buffer to allow a washout period. Tissue prisms were then separated into 6 even portions, each one being loaded into one perfusion chamber. An equilibrating period of 40 mins started with superfusions (0.6 ml/min) of either normal (125 mM) or low Na+ (25 mM by substitution of equimolar NaCl with Choline Chloride) Krebs buffers passing through all perfusion chambers. Four samples (2.4 ml/sample) were collected at 4-min intervals to determine basal 3H-dopamine outflow for each. At the end of the experiment, tissue prisms were collected and dissolved in 1 ml of tissue solubilizer. All samples were analyzed by liquid scintillation spectrometry. Drugs were diluted in the appropriate modified or unmodified Krebs medium. In this chapter, brain slices were prepared from the striatal region of the brain throughout the study. However, in this chapter, a dopamine agonist, QNP 10µM and a powerful dopamine-releasing drug, D-AMP 10µM were investigated to determine their capacity to induce tritiated dopamine release on striatal brain slices.

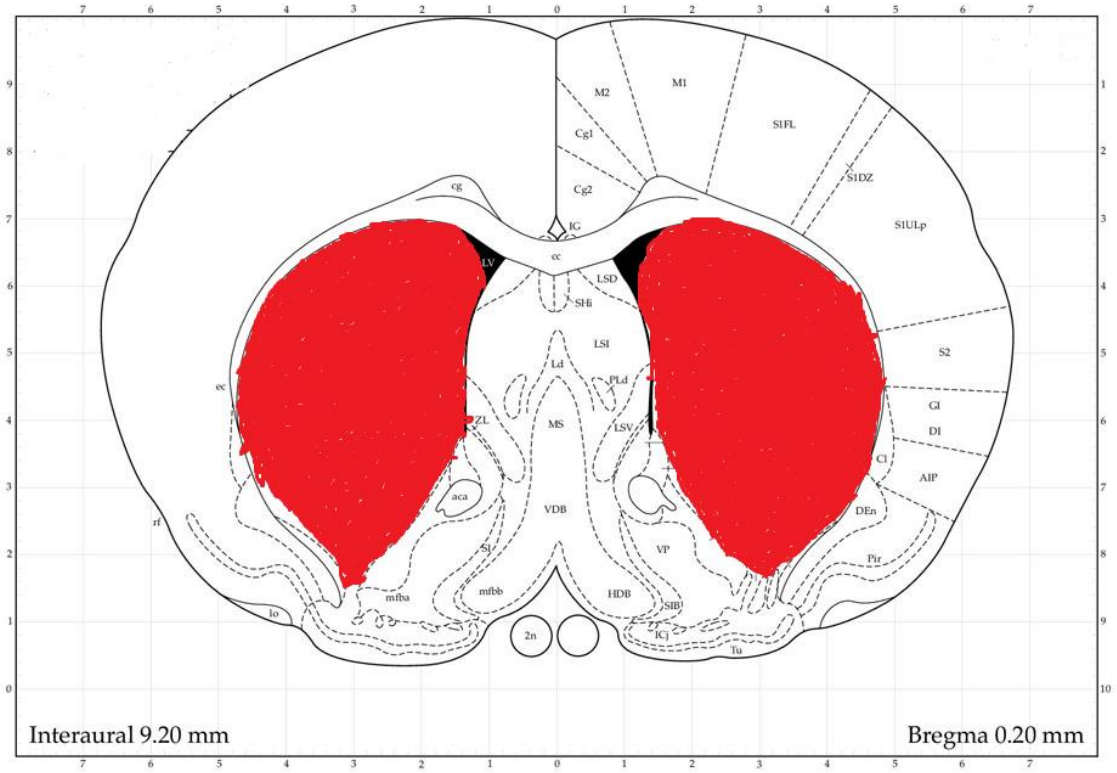


Figure 4: Region of interest for *in vitro* neurotransmitter efflux experiments.
Striatum location is represented by shaded area which corresponds to regions of interest for *in vitro* neurotransmitter release. Scales represent distances (in mm) from the midline and the surface of the brain. Coronal slices adapted from Paxinos and Watson (1997).

I-2-D-*In vitro* ^{3}H-dopamine uptake assay

A range of male Sprague-Dawley rats weighing 200-350g was used. Animals were sacrificed by cervical dislocation and the brains were then quickly removed and placed on an ice-cold platform for further dissection. Tissue from the striatum was quickly removed and placed in ice cold sucrose homogenization buffer (320 mM sucrose, 5 mM HEPES, pH 7.4). The tissue is then homogenized (5 strokes) on ice in 5 mls of fresh oxygenated sucrose buffer using a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 1000g for 10 min at 4°C to remove nuclear debris. The supernatant was then centrifuged again at 16,000g for 20 min at 4°C. The supernatant was discarded and the pellet was washed

twice, by resuspending the pellet in 15 ml ice cold synaptosome uptake buffer (HBSS: 0.137 M NaCl, 5.4 mMKCl, 0.25 mM Na2HPO4, 10 mM D-glucose, 4.2 mM NaHCO3, 0.44 mM KH2PO4, 1.3 mM CaCl2, 1.0 mM MgSO4 supplemented with 10 µM pargyline (a monoamine oxidase B (MAO-B) inhibitor) and 100 mM ascorbic acid, PH 7.4) and recentrifuging at 14,000g, 4°C for 20 min. The final pellet is resuspended in 8ml of HBSS. This is referred to as synaptosome, subsequently 250µl of synaptosome preparation was incubated with either 50µl of HBSS buffer or 50µl of methylphenidate 10µM (dopamine uptake inhibitor) or the tested drugs (for 4 minutes in a 37^0C water bath or at 4^0C. The uptake is initiated by adding 50µl of ^{3}H-Dopamine) in 10 seconds intervals and the specific dopamine uptake at different second's intervals. The incubation is left for 5 minutes and is terminated by the addition of 5mls of ice cold HBSS buffer also in 10 seconds intervals between each tube. The samples are then immediately filtrated through GF/B Whatman filters on a vacuum filtration manifold. The filters were washed twice with HBSS buffer and were transferred to scintillation vials. 1ml of filter count was added to each vial and was left overnight to dissolve, scintillation liquid is then added and the radioactive counting is recorded. The total uptake is measured from the samples containing buffer, whereas the unspecific uptake is measured from the samples with methylphenidate. The specific dopamine uptake at different concentrations is calculated by subtracting nonspecific binding counts from the total count concentrations is calculated by subtracting nonspecific binding counts from the total count and expressed as a fractional percentage.

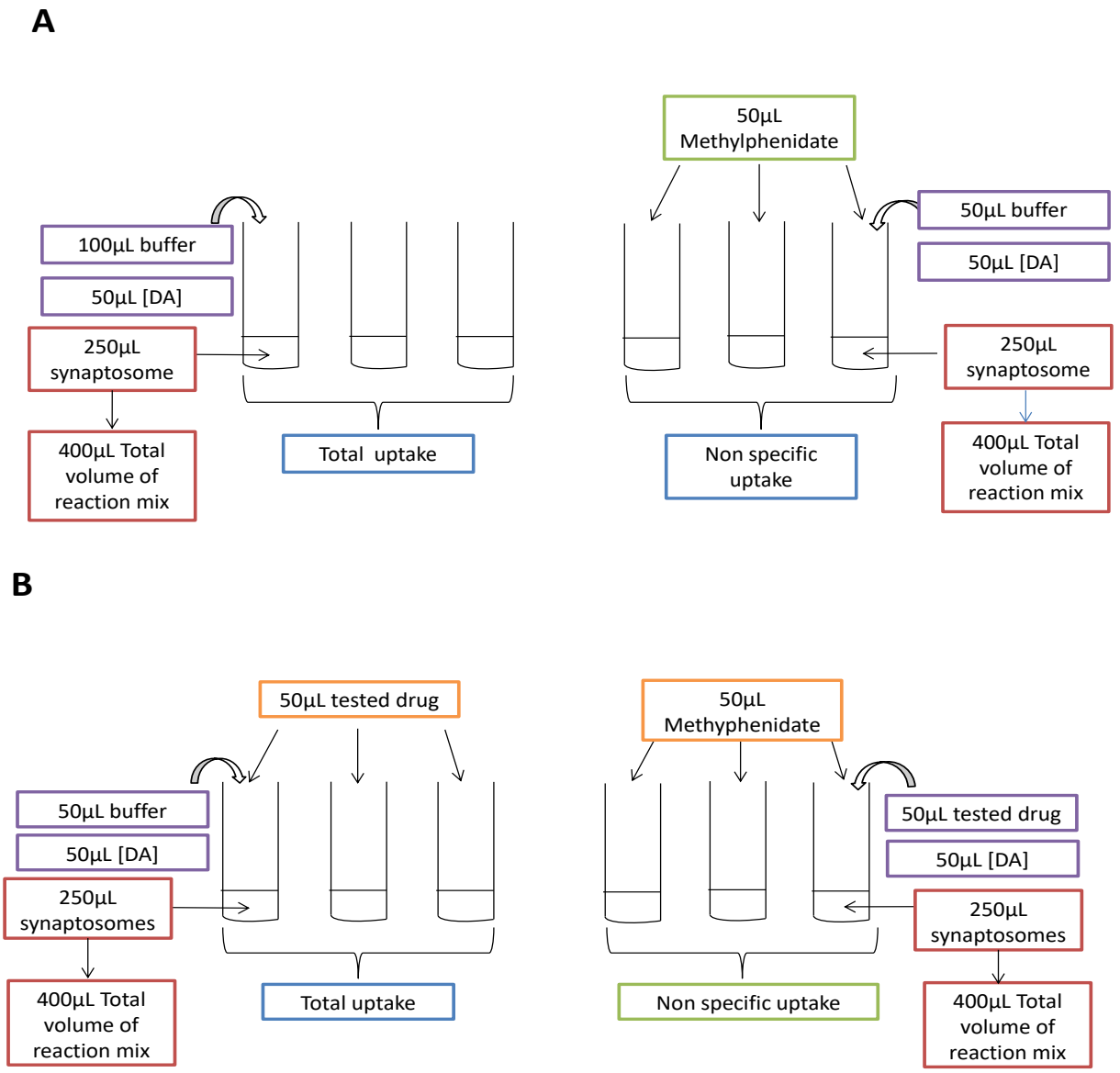


Figure 5: Radiometric uptake protocol for control (A) and tested samples (B).
After the brain was isolated, 250µl of synaptosomes preparation was incubated with either 50µl of HBSS buffer or 50µl of methylphenidate 10µM (dopamine uptake inhibitor for determining nonspecific uptake), and the tested drugs or buffer (50µl) for 4 minutes in a 37^0C water bath or at 4^0C. The uptake is initiated by adding 50µl of ^{3}H-Dopamine. The total reaction mix was 400 µl in each test tube.

I-2-E-Behavioural assessments

Psychostimulants such as D-AMP are known to alter locomotion (Amini et al., 2004; Paulus and Geyer, 1991;Fang and Tan, 1998), grooming (Taylor et al., 2010; Wooters and Bardo, 2009), scratching (Antoniou et al., 1998; Malin and Goyarzu, 2009), stereotypes and rearing activities (Bonasera et al., 2008; Rebec et al., 1997). The consequences of D-AMP on such behaviours were assessed. Male Sprague-Dawley naïve rats weighing 300 grams were housed individually at the beginning of the behavioural experiments and allowed to acclimatize with the environment for 30 days. All drugs were dissolved into saline. Animals received a single intraperitoneal injection of either: 0.8 ml/kg of saline (NaCl 147 mM) or 1.0 mg/kg D-amphetamine. Animals were then scored for behavioural parameters during a 15mins-time period and up to a total of 60 minutes. Counting of well-defined behavioural traits such as rearing, scratching, grooming, jumping, running, climbing, catalepsy and stereotypical movements were done manually through physical scoring. In another series of experiments, we assessed the behavioural parameters induced by dopamine agonists such as QNP and PPX. In these experiments, animals received a single intraperitoneal injection of either 0.2ml/kg of saline (NaCl 147 mM), 0.1 mg/kg QNP or 0.05 mg/kg PPX. Animals were then scored for behavioural manifestations during a 15mins period and up to a total of 60 minutes. Counting of well-defined activities such as yawning, pelvic grooming (an index of penile erection) and pica eating were conducted manually. Further experiments on the effect of D_2 and D_3 receptor antagonists were studied on D-AMP induced locomotor activity. While additional groups of animals were exposed to a single intraperitoneal D-AMP 1 mg/kg once weekly for 2 weeks; after behavioural experiments, animals were evaluated for electrophysiological profiling.

I-2-F- *In vivo* extracellular single-unit electrophysiology

Following behavioural experiments, animals were classified based on the degree of locomotor activity exhibited. In this study, we adopted a modified categorization of motor activity according to Nakamura et al., (2014) as, low-(10-30 counts/15 min), moderate-(40-99 counts/15 min) and high-(over 100 counts/15 min) activity phenotypes. Two of the phenotype group (moderate and high) were subjected to electrophysiological recording in the ventral tegmental area. Animals were initially deeply anaesthetized with chlorhydrate secured to a stereotaxic frame and maintained at 36–37 °C with a heating pad. A catheter was inserted into the lateral tail vein to perform systemic drug administration and an incision was made across the top of the head and the edges of the skin drawn back to reveal the cranium. Bregma was marked and a hole was drilled through the bone at the coordinates of the ventral tegmental area according to the atlas of Paxinos and Watson (1997). Electrodes were manufactured in house from borosilicate capillaries (1.5 mm, Harvard Apparatus Ltd., UK) pulled on PP-830 electrode puller (Narishige, Japan) and filled by hand with an electrolyte solution (NaCl 147 mM). The tip of the electrode was broken down under a microscope to an external diameter of 1–1.5 μm. Typical resistance was in the range 4–8 MΩ. Single-unit recordings with the tested drugs were applied. Outputs from the electrode were sent to a Neurolog AC pre-amplifier and amplifier (Digitimer, UK). Signals were filtered and sent to an audio amplifier, a Tektronix 2201 digital storage oscilloscope and a 1401 interface connected to a computer running Spike 2 (CED, Cambridge, UK) for data capture and analysis. Descent of the electrode was carried out using a hydraulic micromanipulator (Narishige). Coordinates for the ventral tegmental area were: anteroposterior -4.5 to -5.5 mm, lateral 0.3–1.2 mm, dorsoventral 7.5–9 mm below the cortical surface. Drugs were administered i.v. *via* the lateral tail vein after stable recording. In this chapter, the number of putative ventral tegmental area dopamine neurons per track was recorded by recording the

total number of active neurons defined as a neuron firing more than 5 spikes per 10s encountered during one electrode descent within the region of interest. Midbrain dopamine neurons were identified according to electrophysiological criteria summarised by Ungless and Grace, (2012): always a low pitch sound, occasionally a notch in the rising phase, usually a prominent negative phase and always a long triphasic action potential (>1.5 ms) with a time greater than 1 ms from the start of the depolarization to the end of the repolarisation (mostly > 1.5 ms). Only presumed dopaminergic neurons were selected in our study (Chenu et al., 2013; Grace and Bunney, 1984; Ungless et al., 2004; Valenti et al., 2011). A burst activity in such neurons is defined as two spikes occurring at an interval of 80 ms or less, followed by a silence period of at least 160 ms (Grace and Bunney, 1984; Overton and Clark, 1997; Paladini et al., 2003). By adopting this identification criteria, we may exclude a small proportion of dopamine neurons, particularly some PFC-projecting and amygdala-projecting midbrain dopamine neurons, which may have slightly different electrophysiological characteristics, (though it is still disputed) like a shorter action potential (Chieng et al., 2011; Ford et al., 2006; Hnasko et al., 2012; Lammel et al., 2008; Margolis et al., 2006, 2008; Marinelli and McCutcheon, 2014; Zhang et al., 2010). However, our strict criteria allow for the recording of a rather homogenous population of midbrain dopamine neurons. Coordinates for the ventral tegmental area were: anteroposterior -4.5 to -5.5 mm to Bregma, lateral 0.3-1.2 mm, dorsoventral 7.2-9.5 mm below cortical surface. In another series of electrophysiological studies, we assessed the impact of intravenously administered PPX on midbrain dopamine firing activity both in naïve group as well as D-AMP pre-exposed group.

I-2-G- Data analysis

All data are expressed as the mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using paired or unpaired Student's t-tests or one/two-way analysis of variance (ANOVA), followed by appropriate post-hoc *Neuman–Keuls* (one-way ANOVA) or Bonferroni tests (two-way ANOVA). In radiometric release assays, the mixed model two-way ANOVA was used when sampling size varied across groups. Probabilities smaller than 0.05 were considered to be significant; *n* values refer to the number of samples used. Fractional efflux for each superfusate sample was calculated by dividing the amount of tritium in each sample by the total tritium left thereafter. The effect of a tested condition was assessed on at least 3 subsequent sample collections and averaged. Normalized efflux values are calculated for each chamber as the ratio between the mean tested values (generally from at least 3 collections) and average baseline values (usually 3-4 collections). All of the experiments presented were repeated on at least 3 animals.

In radiometric uptake assays, the total uptake is measured from the samples containing buffer, whereas the unspecific uptake is measured from the samples with methylphenidate. The specific dopamine uptake at different concentration is calculated by subtracting nonspecific binding counts from the total count and expressed as a fractional percentage.

For behavioural experiments, the sum of each behavioural trait occurring during 15 minute interval periods was plotted against the type of drug administered. Results are expressed as the mean±standard error of the mean (SEM) of counts for each trait per group. Repeated 2-way ANOVA was also conducted when a significance difference was tested.

For electrophysiology, the mean basal firing activity was evaluated after the neuron had attained a stable firing rate, generally after at least 5 min of recording. Pre-drug values of firing rate were obtained by averaging the firing rate over at least 4 min immediately before

the intravenous administration while post-drug values were obtained by averaging the firing over 5 min following drug administration. Individual change in basal firing activity was considered significant following drug (or saline) administration when the post-drug value was significantly different from the pre-drug value (determined as indicated above, paired Student's t-test analysis). For pre-treated animals, population activity of the neurons were expressed as mean±standard errors and compared within the group. When neurons with very low spontaneous activity were recorded (baseline activity below 4 spikes per 10 seconds), a change of more than 5 spikes per 10 seconds was considered significant, or if the analysis of 100 spikes before and after drug administration led to significant unpaired Student's t-test for each neuron tested this way. These two methods of analysis did not differ in the results obtained. Proportions of a specific type of response in two different groups of animals were also compared using Fisher's exact test (comparing proportions of responses and no response/opposite responses in two groups). All burst results are reported as the % of spikes in burst.

I-3-Results

I-3-A- D-AMP and QNP differentially induce dopamine efflux

Fig. 6A shows the time course of dopamine efflux following the application of D-AMP in the superfusion medium, confirming that the drug causes a massive dopamine release. When applied at the same concentration to the prism chamber, it induced a significant increase ($t=5.538$ $df=10$) in dopamine efflux (Fig. 6B). As could be expected QNP 10 μM elicited a significant ($t=2.023$ $df=14$) slight reduction in dopamine efflux on the striatal slices (50% below baseline, Fig. 6C).

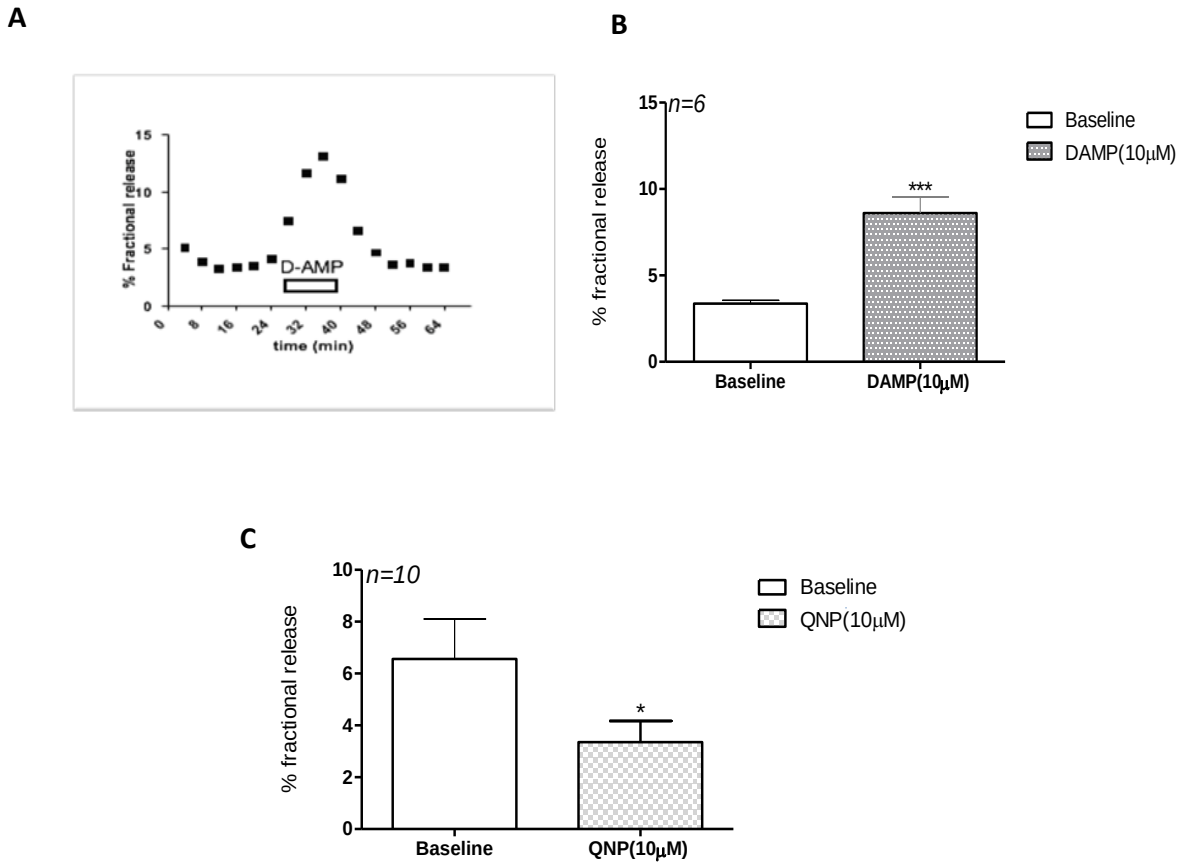


Figure 6: Dopaminergic drugs effect on striatal ^{3}H-dopamine release.
(**A**): Typical time course profile from an individual experiment showing ^{3}H-dopamine release from striatal slices before, during and after exposure to 10 µM D-AMP. Data shows the average fractional efflux from 3 perfusion chambers over time. (**B**): Superfusion of D-AMP (10 µM) significantly induced dopamine efflux (**C**): Effect of quinpirole (QNP, 10 µM), a D_2/D_3 receptor agonist on baseline dopamine efflux, showing a significant reduction from the baseline. In this and the following figures, n values indicate the number of tissue samples which have been tested (from which an average fractional efflux is calculated from 3-4 subsequent collections). *P<0.05, ***P<0.0001 *vs.* baseline, paired Student's t-test.

I-3-B- Dopamine agonists PPX and QNP increased DA uptake

Uptake process is sensitive to certain conditions such as temperature and time of incubation. We tested the impact of pramipexole (PPX, 10µM, and 100µM), a D_3 preferred dopamine agonist and quinpirole (QNP, 125 nM and 1µM) a D_2 preferred dopamine agonist on ^{3}H-dopamine uptake a measure of dopamine transporter (DAT) activity. The time of

incubation of the reaction mix on the synaptosome was kept constant at 5 mins in all tubes. PPX (10µM) did not show any effect on ^{3}H-dopamine uptake (Fig. 7A). However, at a higher dose of 100 µM, ^{3}H-dopamine uptake was increased significantly (20% increase over the control, Fig. 7B), Student's t-test **p<0.05 *vs* control (t=4.367 df=10). When tested at both concentrations of 10µM and 100 µM, a dose dependent-significant F (5, 5) =5.180 increase in DA uptake was observed. This represents an enhanced activity of DAT in the presence of PPX. (***p<0.001, *Neuman-Keuls* test after ANOVA, Fig. 7C). Conversely, QNP, at lower concentration (125 nM) caused a significant increase (t=3.131 df=21) in ^{3}H-dopamine uptake (Fig. 8A), while at higher concentration of 1µM; QNP did not show any impact on ^{3}H-dopamine uptake (Fig. 8B). Both doses were tested under the same condition and the difference did not show any dose-response relationship. QNP 125nM in this condition still exhibits significant increase (30% increase over control, Fig. 8C). The DAT activity is said to be optimum at a dose of 125 nM (***p<0.001, Dunnett Multiple Comparison Test after ANOVA).

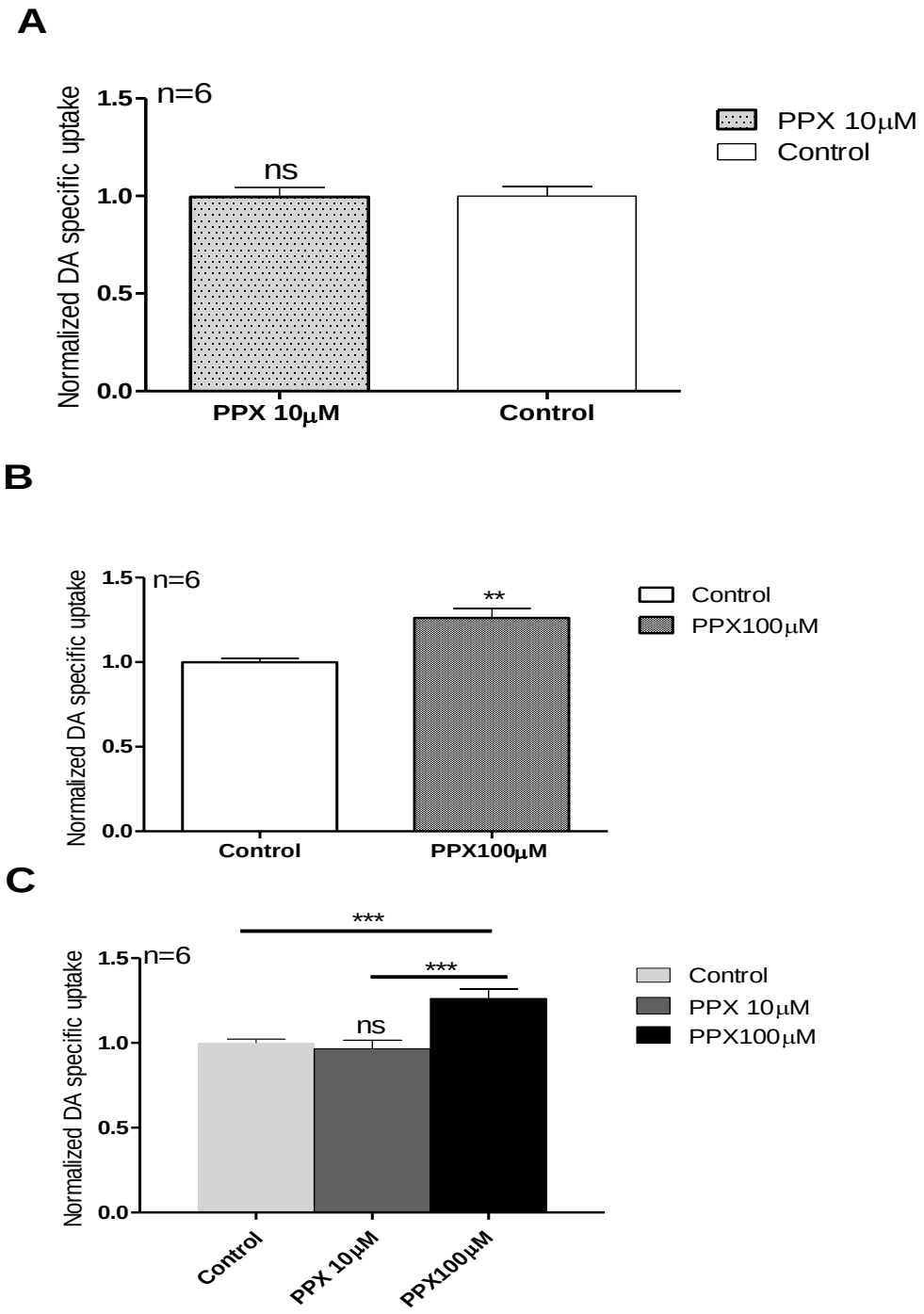


Figure 7: Effect of pramipexole (PPX) on ^{3}H-dopamine uptake activity in synaptosomes (**A**): Incubation of PPX 10 µM shows no effect on the DA uptake activity. (**B**): PPX at 100 µM significantly increased DA uptake activity. **P<0.05 *vs* baseline Student's test (**C**): Dose- dependent effect of PPX on the striatal synaptosome PPX 100 µM, ***P<0.001 *vs* PPX 10 µM and *vs* control; *Newman-Keuls* post hoc tests, after significant one way ANOVA.

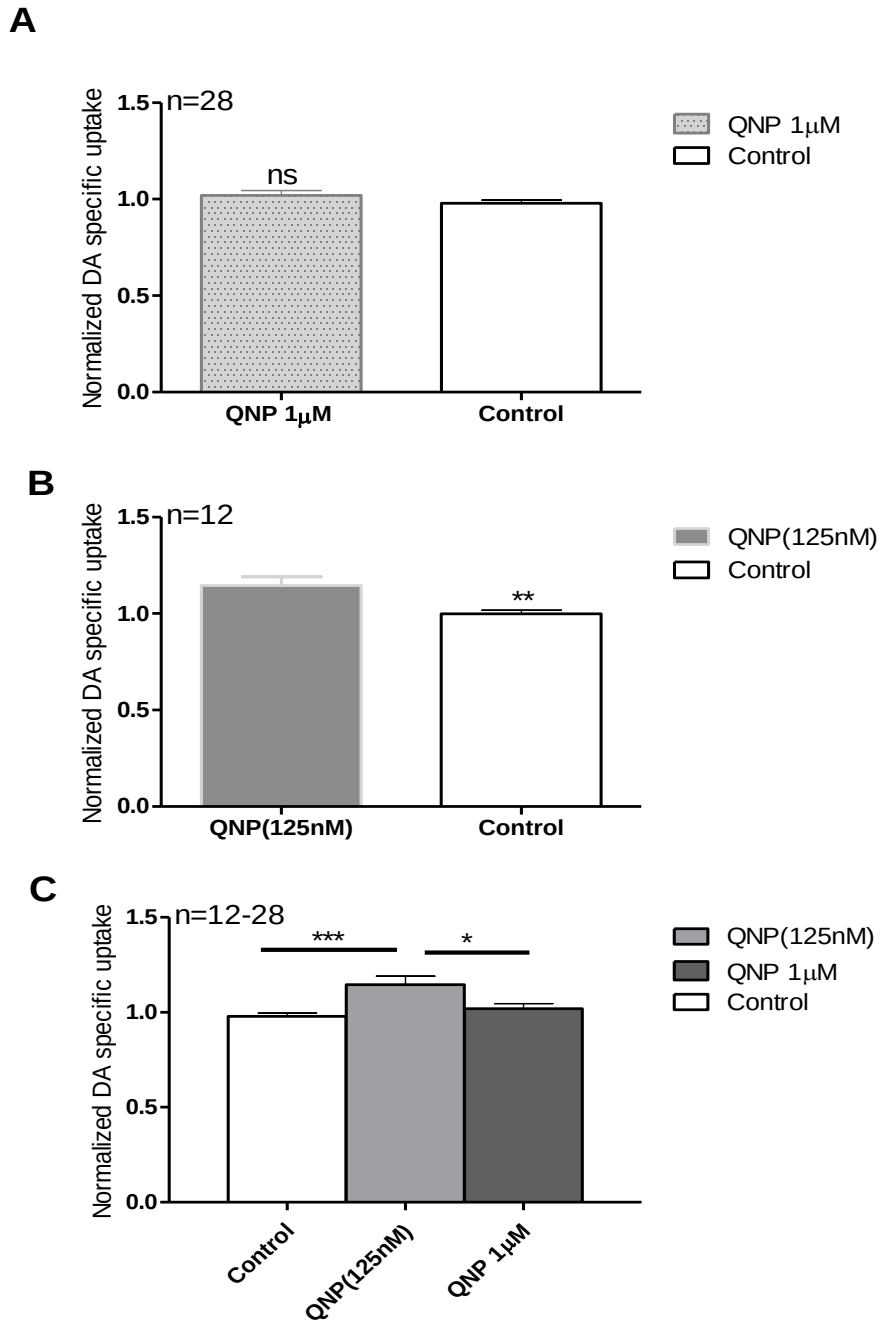


Figure 8: Effect of quinpirole on ^{3}H-dopamine uptake activity on striatal synaptosomes (**A**): Incubation of QNP 125 nM exhibited a significant increase in DA uptake activity; **P<0.005 *vs.* baseline, Student's t-test. (**B**): QNP at 1 µM did not show an effect on DA uptake activity. (**C**): QNP did not show a dose-dependent relationship when both doses were tested. ns: not significant, *P<0.05, ***P<0.0001 *vs.* specific conditions, *Newman-Keuls* post hoc tests, after significant one-way ANOVA.

I-3-C- Acute psychostimulant and dopamine agonists' administration exhibited varied behavioural effects

I-3-C1- Acute D-AMP administration augments motor activity

Behavioural characteristics such as rearing, upright scrambling and climbing were assessed in a single-housed cage of dimension 600 cm x453 cm during 60 minutes after a single D-amphetamine of 1 mg/kg or 3mg. The initial basal activities of the rats were assessed by administering distilled water. Then, scoring began after 5 mins of the administration of D-AMP. D-AMP-induced hyperactivity in the form of rearing, upright scrambling, and climbing activity have been identified as DA-dependent behaviour (Avdelidis and Spyraki, 1986). For the purpose of this thesis, we sum up these three traits as a single type of motor activity for statistical analysis. We assessed a dose-response relationship between the two concentrations, compared with the baseline locomotor activity of the rats. We tested 1mg/kg of D-AMP which produced a relatively moderate increase in locomotor activity. Then, at a dose of 3 mg/kg, D-AMP strongly increased the frequency of all the behavioural traits by 15000% over the baseline and 100% over the initial dose. Both doses (D-AMP 1mg/kg or 3 mg/kg) show a significant increase $F(6, 35) = 0.21$ in locomotor activity from the baseline.

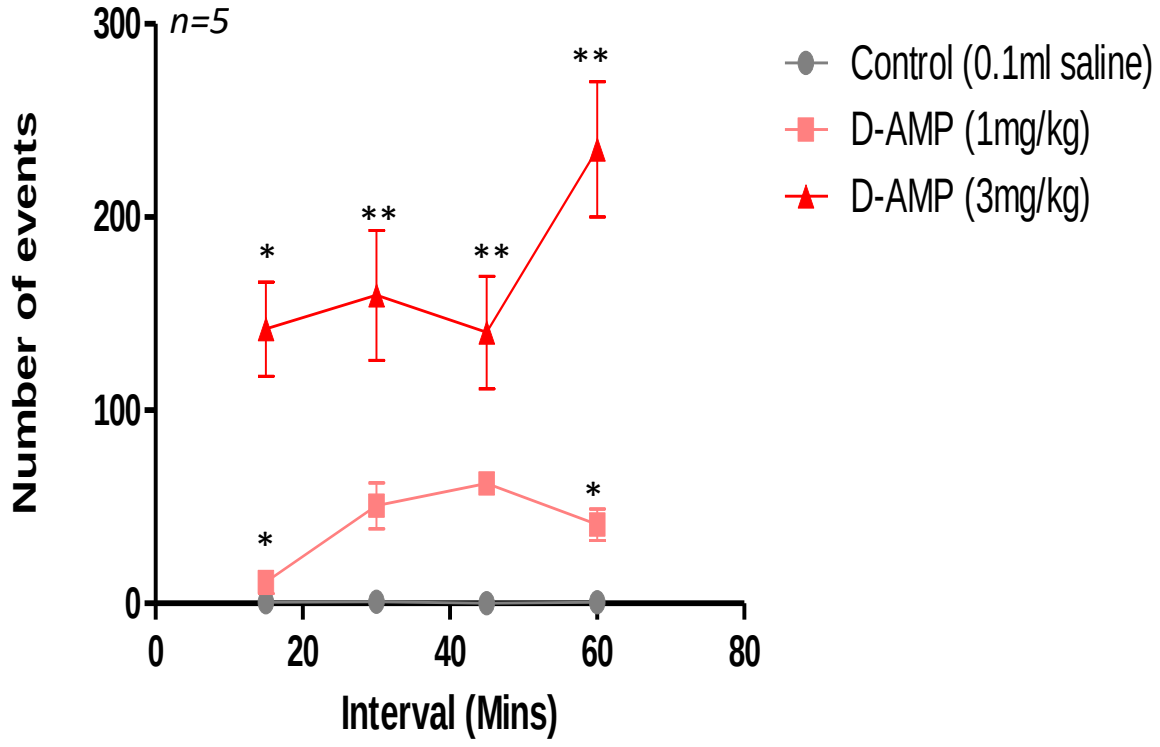


Figure 9: Dose-response relationship of acute D-AMP on motor activity
In this experiment, the 3 behavioural characteristics (rearing, upright scrambling, and climbing) were combined and analysed during the first 60 minutes following intraperitoneal drug exposure. Exposure to D-AMP (1 mg/kg), significantly increased the cumulative number of behavioural events during the entire observation period, D-AMP 3mg/kg strongly increased the number of motor events throughout the recording period. There were significant differences at 15 and 60 mins between 1 and 3 mg/ kg D-AMP treated groups.*p<0.05, **p<0.001 *vs.* vehicle, *Bonferroni* test after significant repeated measures two-way ANOVA.

I-3-C2- Single D-AMP administration at an interval of a week induces escalated motor activity and phenotypic behaviours

For this thesis, we used D-AMP 1 mg/kg throughout, a dose that is sufficient enough to induce motor activity in rats. Motor activity was scored by counting the number of rearing and upright scrambling activities according to (Avdelidis and Spyraki, 1986). We then study behavioural activity at two different stages within an interval of a

week. This is done to evaluate alterations in motor activity and sensitivity, when there is an initial exposure to the drug. There was a significant increase $F(3, 56) = 0.63$ in motor activity after the 2nd challenge with D-AMP 1 mg/kg (242% above the 1st challenge, Fig. 10A). Next, we studied certain phenotypic idiosyncrasies in motor activity. Out of 24 animals tested with D-AMP 1 mg/kg acute single dose, 4 showed extremely low motor activity phenotype, 3 exhibited moderate motor activity phenotype and 4 displayed high motor activity phenotype. In fig, 10B, we later studied the electrophysiological responses of these phenotypes, in relation to VTA DA neurons firing activity. There were significant $F(6, 36) = 0.89$ changes in motor activity within the phenotypic groups.

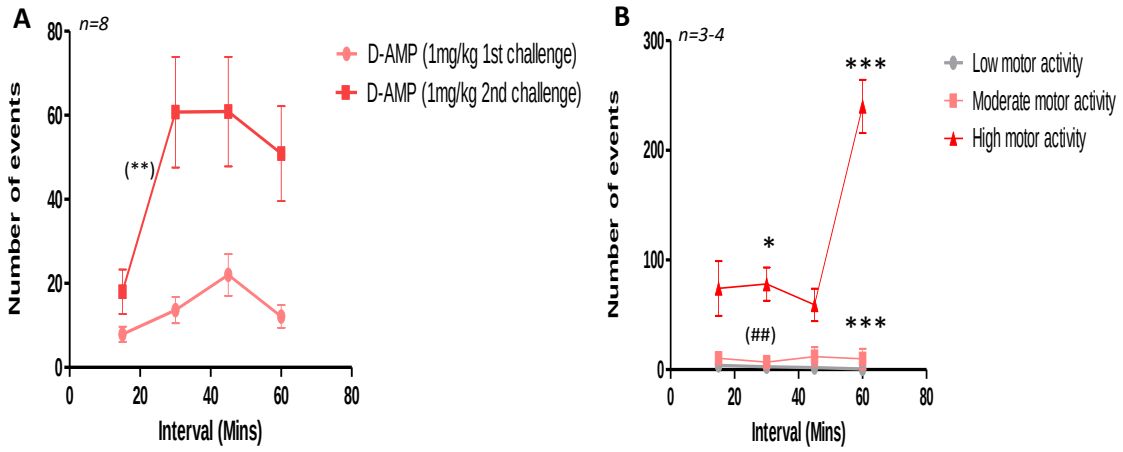


Figure 10: D-AMP induces behavioural phenotype and increases motor activity in pre-treated rats

(A): Rats acutely pre-exposed to D-AMP (1 mg/kg), significantly increased rearing activity during the entire observation period following the 2nd challenge over the 1st challenge, **P<0.001 *vs.* 1st challenge at all time. *Bonferroni* after significant repeated measures two-way ANOVA. **(B):** Rats treated with D-AMP (1 mg/kg) that showed high motor activity phenotype exhibited a significant increase in rearing activity particularly during the 15-30 mins and during the last 15 mins observation periods, compared to low and moderate motor activity phenotypes. *P<0.05, *vs.* low and moderate motor activity groups; ***P<0.0001 *vs.* low and moderate motor activity groups (45-60 mins period) and *vs.* low motor activity throughout the 60 mins duration. *Bonferroni* after significant repeated measures two-way ANOVA.

I-3-C3- D-AMP-induced motor activity inhibited by D_2 receptor antagonist and partially affected by D_3 receptor antagonist

In this experiment, we investigated the role of D_2/D_3 receptors signalling in D-AMP induced locomotor activity. Here, we tested the impact of selective D_2 receptor antagonist eticlopride 50 µg/kg on dopamine-mediated motor activity. Intraperitoneal pre-treatment with eticlopride 50 µg/kg applied 15mins earlier before D-AMP 1mg/kg (i.p). There was complete significant inhibition F (3, 8) =0.35 of locomotor activity accompanied by stereotypic manifestation such as frozen behaviour. This indicates that eticlopride can modulate the effect D-AMP-induced motor activity (Fig.11A). In converse, we tested selective D_3 receptor antagonist NGB 2904 1 mg/kg. There was no effect on locomotor activity, although the animals became restless and exhibited moderate to high rearing activity like for the D-AMP treated control (Fig. 11B). The controls are done at the same time as the treated (cage by cage) and also 3 treated rats done at the same time as 3 controls.

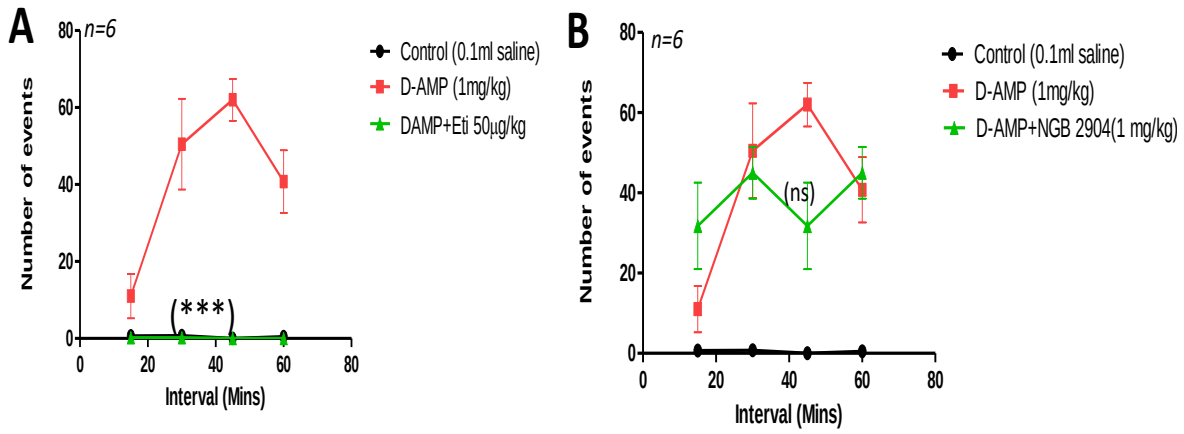


Figure 11: Effect of D_2/D_3 receptor antagonist on D-AMP induced motor activity
In (**A**) following 15mins pre-treatment with 50 µg/kg eticlopride, a D_2 selective antagonist, there was significant complete inhibition of motor activity during the 60 mins observation. ***P<0.0001 *vs*. D-AMP control throughout the 60 mins observation. *Bonferroni tests* after significant repeated measures two-way ANOVA. (**B**) However, the selective D_3 antagonist NGB 2904 1 mg/kg, did not have significant effect on D-AMP induced motor activity P>0.05 *vs*. DAMP control.

I-3-C4- pramipexole and quinpirole differentially induce yawning, pica eating and pelvic grooming effects

In these experiments, we studied 3 classical behavioural characteristics exhibited by PPX and QNP. The well-defined behaviours are yawning, pelvic grooming which is an index of penile erection and pica eating. These traits are to some degree dependent on selectivity on D_2/D_3 dopamine receptors (Andersson, 2001; Collins et al., 2005a). Dose-response relationship of PPX on yawning activity was determined with PPX 0.05, 0.1 and 0.15 mg/kg (Fig. 12A) following an i.p. administration over 60 mins observation. PPX at a dose of 0.05 mg/kg shows the highest yawning activity. This dose forms the basis in determining optimum yawning concentration for subsequent studies in the thesis. We subsequently accessed the responses to other behavioural traits (pelvic grooming and pica eating, Fig. 12B) at 0.05mg/kg.

Dose-response relationship with QNP at 0.1 and 0.05 mg/kg on yawning activity was determined. The dose at 0.1 mg/kg exhibited a more pronounced yawning effect than 0.05 mg/kg (Fig. 12C). The two other behavioural characteristics (pelvic grooming and pica eating) were also determined at this dose (Fig. 12D)

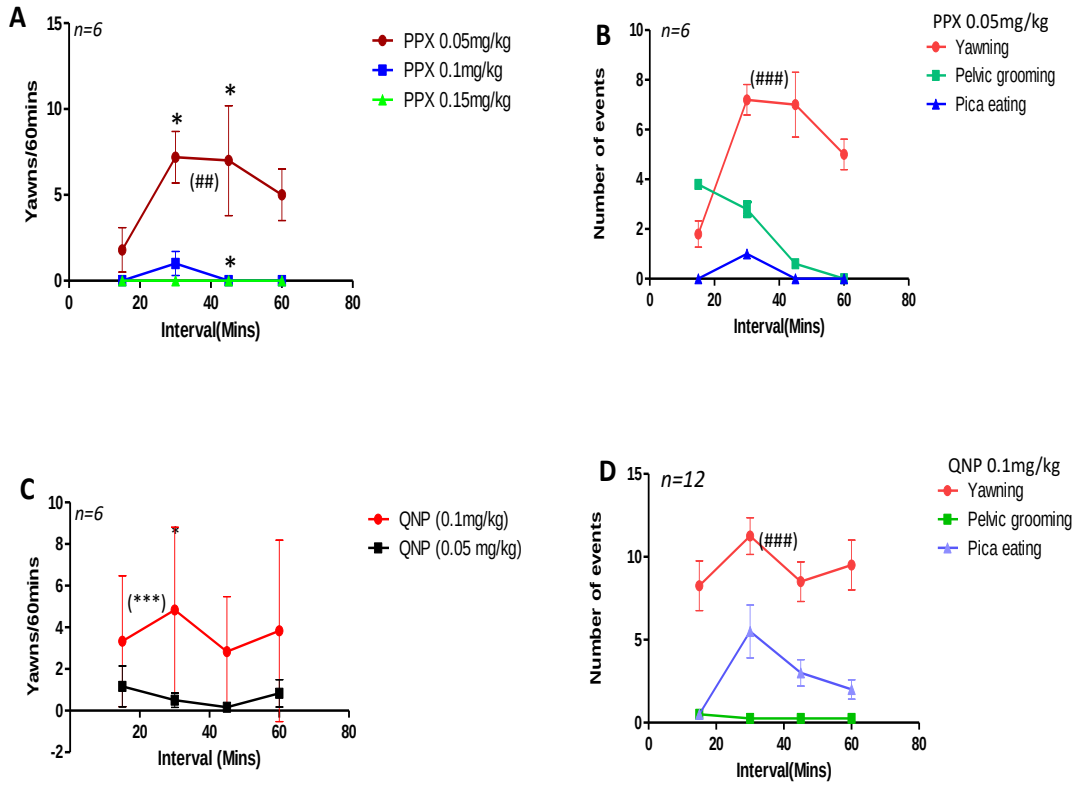


Figure 12: Effect of D_2/D_3 receptor agonists on yawning, pelvic grooming and pica eating behavioural traits

(**A**): PPX at a dose of 0.05 mg/kg exhibited a significant increase in yawning activity over 0.1 mg/kg and more significant yawning activity at 30 mins and 45 mins observation period. ##P<0.001, *P<0.05 *vs.* 0.1 mg/kg PPX. *Bonferroni* tests after significant repeated measures two-way ANOVA (**B**): PPX at an optimum dose (0.05 mg/kg) elicits more significant yawning activity than all the 2 behavioural traits; pelvic grooming and pica eating, throughout the 60mins observation time. ###P<0.0001 vs. each trait. *Bonferroni* tests after significant repeated measures two-way ANOVA (**C**): Conversely, in QNP dose-response 0.1 mg/kg QNP i.p administration exhibited a significant increase in yawning than 0.05 mg/kg for all time ***P<0.0001 *vs.* 0.1 mg/kg PPX (**D**): Yawning effect with QNP pooled controlled was more significantly pronounced than other behavioural effects, ###P<0.0001 vs. pelvic grooming and pica eating. *Bonferroni* tests after significant repeated measures two-way ANOVA.

I-3-D1–Rats exposed to repeated intermittent administration of DAMP induces desensitization of midbrain DA neurons to PPX

Fig. 13A illustrates the electrophysiological characteristics of a typical VTA DA neuron while fig. 13B displays an area of the VTA where our electrodes were placed. In this experiment, two groups of animals (*n=9*) were used. Each group either received 1 mg/kg D-AMP (ip.) or corresponding vehicle, once a week in a total of 2 weeks. In other words, the treated rats received D-AMP challenge twice with one-week interval. We then investigated the effect of D-AMP challenge treatment on the ability of the preferential dopamine D_3 receptor agonist PPX (cumulative doses 20-100 µg/kg, iv.) to alter the firing activity of VTA dopamine neurons (to test dopamine autoreceptor sensitivity). First, we tested this effect on the naïve rats. The naïve rats displayed a complete inhibition/sensitivity to PPX at the 4^{th} dose administered causing a decrease in firing activity followed by decrease in burst activity (not shown). The firing rate was reversed by eticlopride 0.2 mg/kg to initial baseline firing (Fig. 13C, D). However, few cells (about 22%) displayed moderate desensitization effect to PPX in naïve rats. Conversely, in D-AMP challenged rats the mid-brain DA neurons exhibited a significant dose-response desensitization of D_2 autoreceptor to PPX (Fig.13 C, E). There is a statistical significant difference F (3, 28) =9.92 within the group. In the same manner, desensitized neurons were reversed by eticlopride 0.2 mg/kg to initial baseline firing with decreased firing and burst activities (up to 96% reduction). We also observed that in about 60% of the neurons, apomorphine a D_1/D_2 receptor agonist exhibited desensitization to that population of cells and only a population of 20% of the neurons increased the firing rate after apomorphine administration.

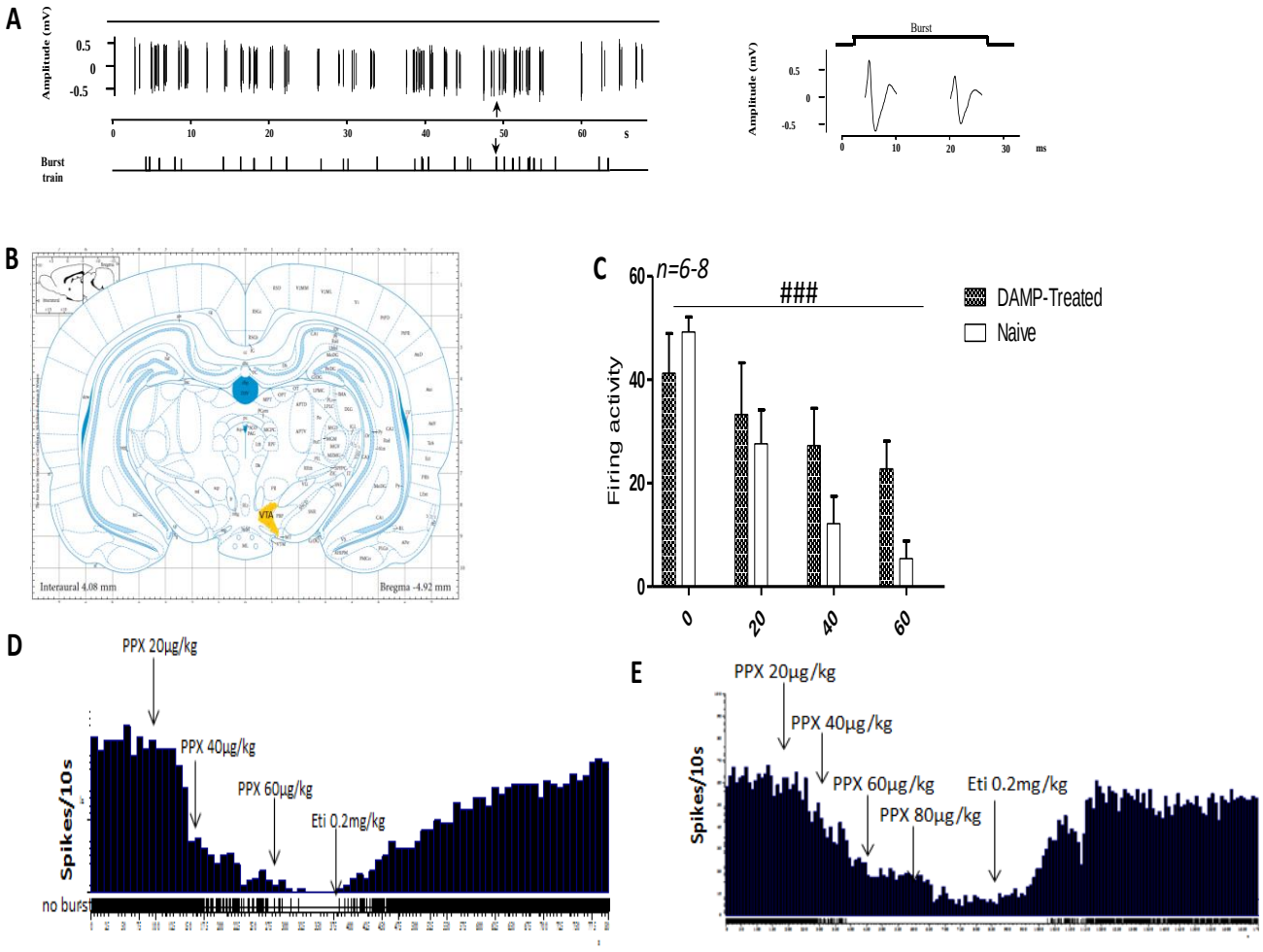


Figure 13: VTA DA neurons' responses to pramipexole administration on autoreceptors sensitivity

(**A**): Electrophysiological criteria of VTA DA neurons, showing burst train and shape of the neuron (**B**): Histological illustration of electrode placement in VTA DA neuron recording (**C**): D-AMP treated rats exhibited significant desensitization of VTA DA autoreceptors to PPX when compared to naïve rats. Two-way ANOVA (###P<0.0001). **(D):** In control naïve rats, the VTA DA neurons exhibited a complete inhibition to PPX acute dose. The cell was reactivated by eticlopride 0.2mg /kg to its original firing rate. **(E):** D-AMP treated rats showed pronounced desensitization in firing to PPX acute administration.

I-3-D2– D-AMP-induced behavioural phenotypic variability correlates with electrophysiological studies

Data from (Fig. 10B) on behavioural phenotypes induced by D-AMP 1mg/kg were subjected to electrophysiological study. Briefly, after 1mg/kg D-AMP administration in a single dose/week for 2 weeks, the animals were grouped into major two phenotypes, low motor activity and high motor activity. The classification was based on the degree of behavioural traits (climbing, rearing, and upright scrambling) exhibited by each group. The population activity was then assessed. We observed a slight but significant difference between the two phenotypes, the rats that exhibited high motor activity showed VTA neurons with increased firing activity, than the rats classified as low motor activity (Fig. 14). However, the population size needs to be increased for more reliable statistics.

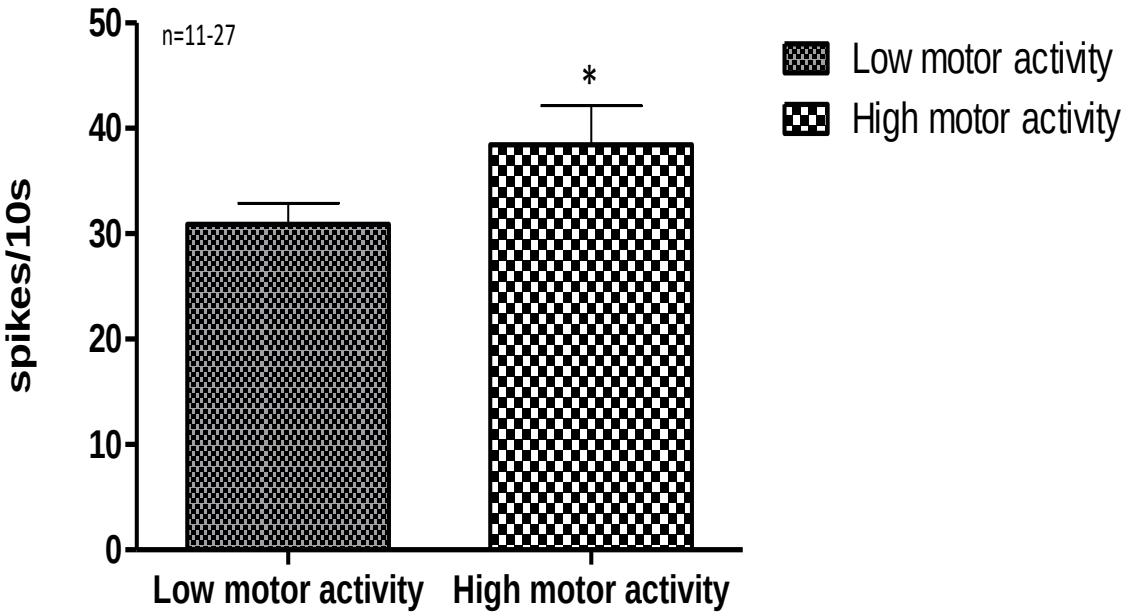


Figure 14: D-AMP-induced behavioural phenotypes correlates electrophysiological outcome.
There exists between the low motor activity and high motor activity rats, a slightly significant difference in VTA DA firing activity. *P=0.056, Student's t-test.

I-4-Discussion

In this study, we have confirmed the capacity of dopamine-releasing drugs to differentially modulate dopamine transmission. While D-AMP 10 µM significantly increased ^{3}[H] dopamine efflux, QNP 10 µM molecularly lowered the release. We also observed increases in basal ^{3}[H] uptake induced by QNP and PPX in striatal synaptosome preparations. In behavioural experiments, 50µg/kg eticlopride completely blocked D-AMP-induced motor activity. A slight correlation was noticed between DA neurons firing activity in the VTA and behavioural phenoytypes of motor activity according to the adopted classification. Though, this must be confirmed on a larger population of neurons In parallel to an increased motor response to D-AMP following a second challenge with this drug, we observed a notable desensitisation of the D2/3 dopamine autoreceptor PPX. The above findings indicate an interesting interaction between the dopamine transporter and D2/D3 receptors at the level of the dopamine terminals.

D-AMP is a powerful dopamine-releasing agent in the striatum when given at the lowest concentration of 10 µM (Fig. 6A). This concentration is found to be equivalent to the therapeutic dose range (0.5-2 mg/kg) in the clinical management of ADHD (Wallace, 2012). D-AMP exhibits its powerful dopamine releasing effect by competitively displacing dopamine at the dopamine transporter site, prevents DA uptake and blocks vesicular monoamine transporter 2 ($VMAT_2$), that is essential for DA reuptake from the cytosol back into the synaptic vesicles (Schwartz et al., 2006; Partilla et al., 2006). The inhibitory action of D-AMP on DAT causes a spontaneous dopamine release from the neuron by making the transporter to reverse it action for more dopamine output (Brown, 2000; Nickell et al., 2014). In addition to homeostatic regulation of vesicular release of dopamine via $VMAT_2$ (Mundorf et al., 2000), one crucial regulator of DA signaling is the DA transporter (DAT), which swiftly transfers extracellular DA into intracellular content for either vesicular

storing or releases of DA through reversal of DAT-mediated transport (Jaber et al., 1997; Sitte et al., 1998). More recent preclinical studies have demonstrated that repeated exposure to D-AMP for 6 days can induce damage to nerve terminals of DA neurons and alters dopaminergic responsiveness (Schrantee et al., 2017), which could be detected by pharmacological magnetic resonance imaging (Schrantee et al., 2017). Interestingly, repeated D-AMP administration at both toxic dose and lower dose as that used in the management of ADHD, potentially cause a long-lasting decrease in striatal dopamine and dihydroxyphenylacetic acid (DOPAC); a major metabolite (McCann and Ricaurte, 2004; Ricaurte et al., 2005). Apart from DOPAC, two other active metabolites of D-AMP namely, 4-hydroamphetamine and methyl-p-tyrosine, do accumulate in the brain cells after both acute and chronic D-AMP administrations (Cho et al., 1975; Dougan et al., 1986). Besides lowering of dopaminergic presynaptic markers, repeated intermittent administration of D-AMP can cause an escalated DA output (Boileau et al., 2006), which is hypothesized to be enhanced by increased DA release and/or reductions in DA reuptake and metabolism (Pierce and Kalivas, 1997). Altogether, the disturbances in the brain monoamine homeostasis impel hypersensitivity to later drug administration (Smith et al., 2016), which may potentially enhance locomotor activity (Antoniou et al., 1998; Kameda et al., 2013) and stereotyped behaviour in rats (Feier et al., 2012). We found from this study that D-AMP superfused at a concentration of 10 µM on striatal slices significantly increased dopamine release over the baseline (Fig. 6B), an investigation consistent with previous studies by Kantor et al., 1999, (2002).

Conversely, quinpirole, a dopamine D_2/D_3 receptor agonist caused a significant decrease in dopamine release compared to the baseline when superfused at a concentration of 10 µM in striatal brain slices (Fig. 6C). There is substantiated evidence that dopamine D_2 receptor stimulation controls presynaptic dopamine release (Zetterström et al., 1986; Schmitz

et al., 2001). These data are consistent with studies that demonstrated that selective D_3 receptor agonist PPX and QNP D_2/D_3 increase vesicular dopamine uptake (Truong et al., 2003). Our data is also in line with previous studies that showed that both QNP and PPX enhance dopamine uptake and promote eventual redistribution of intracellular dopamine, an effect that is reversible and linked with the homeostasis of $VMAT_2$ within the axonal terminals (Brown et al., 2001).

It is also noteworthy, that presynaptic dopamine D_2/D_3 autoreceptors are involved in the regulation of dopamine uptake processes. The dopamine agonists (QNP and PPX), according to our data, cause the activation of dopamine D_2/D_3 mediated uptake and may be involved in the homeostasis of membrane DAT into different sub compartmentalized regions in the brain. Clinically, this data is of relevance in the pharmacologic management of Parkinson disease. In this condition, dopamine receptor agonists are used to treat symptoms emanating from Parkinson disorder. Moreover, the neuroprotective ability is potentially possessed by these molecules (Motyl et al., 2018; Tozzi et al., 2018; Wang et al., 2018). PPX, for instance, protects the basal ganglia of the brain against loss of nigrostriatal dopamine neurons, associated with dopaminergic degeneration in Parkinson disease mouse model (Hall et al., 1996; Motyl et al., 2018). We then hypothesize that neuroprotective effect possessed by these agents is partially due to dopamine D_2/D_3 receptor-modulated reorganization of vesicles, leading to enhancement in cytoplasmic DA segregation and thereby causing less DA to be released for oxidative stress that would have produced lethal neurotoxic radicals; an hallmark of Parkinson disease (Fleckenstein and Hanson, 2003; Kita et al., 2003).

From behavioural point of view, D-AMP at low doses has a calming effect in ADHD patients and is a drug of choice that is frequently prescribed in such disorder (Castells et al., 2018). The increased motor activity exhibited in rodents (Fig. 9A) by this low dose (1-2 mg/kg) has been attributed to resulting from a behavioural manifestation of enhanced DA

activity in the mesolimbic dopamine pathway (Zetterström et al., 1983). However, at high dose (Fig. 9A) DAMP (>3 mg/kg) enhanced locomotor activity was observed with additional well-defined behavioural manifestations in the form of stereotyped and frozen behaviours. There is evidence that hyperactivity caused by D-AMP is fundamentally mediated through the reward pathway (ventral tegmental area, nucleus accumbens, and olfactory tubercle), while stereotypic manifestations are essentially through the nigrostriatal pathway (Creese and Iversen, 1974; Faraone, 2018; Asser and Taba, 2015). D-AMP together with other drugs of abuse has both short-term and long-term effects on the brain activity, and sensitization to the drug is consequential to dependence in form of behavioural adaptation (Robinson and Berridge, 2000). Sensitization to the drug in form of behavioural manifestation can be termed as an increase in behavioural reaction triggered by a challenge administration of a drug after repeated dosing (Chinen et al., 2006; Robinson and Berridge, 1993; Robinson and Becker, 1986a). In this study, before the initial single acute treatment of D-AMP 1 mg/kg, we then challenged with the same dose after a week. The significant change in locomotor activity could be related to neuroadaptation that underlays sensitization to the locomotor-stimulant action of D-AMP in rats (Robinson and Berridge, 2001). This further underscores the physiological process of drug dependence, drug-seeking and craving manifestation in the higher primate. (Solinas et al., 2008). This event has been evinced for most of the molecules that elicit abuse tendency particularly, the psychostimulants (Fukushiro and Frussa-Filho, 2011). The process of D-AMP-induced sensitization in rats has two main stages namely: the induction stage and manifestation stage. While the first stage is exhibited subsequent to rodents' responsiveness to initial exposure to the drug, the manifestation stage is characterized by unusual escalated responses when the subject is challenged by the drug, administered in the same dose (Kalivas and Weber, 1988). Studies have shown that these two stages stimulate different brain regions. While the induction stage sensitization is linked with

ventral tegmental area stimulation, the manifestation stage is connected to nucleus accumbens stimulation (Cador et al., 1995; Perugini and Vezina, 1994; Vezina and Stewart, 1990). In another study, Paulson and Robinson, (1991) described that a repeated administration of D-AMP directly administered into the nucleus accumbens resulted in locomotor agitation in rodents that are initially pre-exposed to intravenous administration of D-AMP. Similarly, repeated administration of D-AMP directly into the VTA resulted in increased hyper motor activity triggered by a challenge intravenous administration of D-AMP in rodents (Kalivas and Weber, 1988). In succinct, we can hypothesize that significant increase exhibited by the second challenge with D-AMP (Fig. 10A) is associated with activation of the cortico-limbic system, with initial propagation from the VTA to nucleus accumbens.

In Fig. 10B, we observed three well-defined behavioural phenotypes classified by Nakamura et al., (2014) with little modification. These are Low-(10-30 counts/15 min), moderate-(40-99 counts/15 min) and high-(over 100 counts/15 min) activity phenotypes according to the level of degree of locomotor activity. Adopting the same D-AMP dose of 1 mg/kg single acute intraperitoneal injection, preliminary data from our laboratory showed possible behavioural correlate in response to phenotypes with dopamine electrical activity in the mid-brain region. Numerous studies have established dopamine as a principal neurotransmitter modulating motivated responses such as reward and motor activity (Carlsson, 2001). Thus, DAT plays a fundamental role in dopamine homeostasis (Gainetdinov and Caron, 2003). However, we cannot eliminate the role of other transporters such as, serotonin transporter (SERT) and norepinephrine transporter (NET) in maintaining DA balance; all being a family member of Na+/Cl−-dependent transporters (Torres and Amara, 2007). Gainetdinov and Caron, (2003) also reported that transgenic mice devoid of DAT (DAT knockout mice) exhibited an increase in motor activity, due to enormous synaptic dopamine level leading to multiple behavioural anomalies associated with cognitive

impairment. In addition, blockade of NET may be responsible for DAMP increase activity in DAT KO mice. This indicates that other mechanisms may also be involved, such as blockade of NET. Since our studies were not carried out on transgenic mice, we can infer from the foregoing that even in the wild type rats, the genetic variability of DAT is responsible for varied behavioural phenotype responses to D-AMP.

Next, we investigated the functional role of dopamine D_2 and D_3 receptors dynamic in D-AMP induced locomotor activity. Dopamine D_2 receptors are reported to regulate DA transporter (DAT) activity, and mediate some behavioural effects of D-AMP (Sevak et al., 2007). A study by (Ralph et al., 1999) initially gathered that D_2 receptors modulation was mainly responsible for D-AMP-induced pre-pulse inhibition, in that, mice devoid of D_2 receptors exhibited less sensitivity to this trait. Further studies to corroborate these findings demonstrated that inhibition of D_2 receptors by a dopamine antagonist raclopride significantly reduced D-AMP triggered hyper locomotor activity (van den Boss et al., 1988). In Fig. 8A, B, and C, we have also demonstrated the effect of QNP a D_2 receptor agonist on the regulation of DAT via increased speed of dopamine uptake in synaptosome. Our finding is consistent with Meiergerd et al., (1993) study on activation of D_2 receptors that resulted in enhanced DA uptake *in vitro* and *in vivo* kinetic evaluations. In Fig. 11A, we have demonstrated an effect of eticlopride, a dopamine D_2 receptor antagonist on D-AMP-induced locomotor activity. There was a complete shutdown of motor activity when compared to D-AMP treated control. Our study is unequivocally consistent with all the findings enumerated earlier. However, more recently, attention is given to the role of dopamine D_3 receptors subtype in the modulation of D-AMP induced a locomotor response. This was investigated in Fig. 11B, by testing with selective dopamine D_3 antagonist NGB 2904 and the response showed no apparent significant change on the D-AMP treated control. Previous studies have shown that dopamine D_3 receptors play a vital function in the modulation of animals

locomotor behaviour, as a result, this dopamine subtype has been suggested as a therapeutic target for schizophrenia (Richtand et al., 2001), Parkinson disorder (Bézard et al., 2003), mania (Ashok et al., 2017) as well as management of addiction (Pritchard et al., 2007). In view of this background, more clarity is sought to unravel the behavioural dynamic of dopamine D_3 receptor activation and inhibition. More proof demonstrates that D_3 receptors activation plays an antagonistic function in motor response to D-AMP, potentially via inhibitory effect to D_1 receptor-modulated signal transduction (Xu, 1998; Zhang et al., 2004). However, there exists a conflict of findings between in vitro D_3 receptor screening and in vivo behavioural studies. Early works on selective D_3 antagonists such as, U99194A (14>30-fold selective D3 vs. D2) (Clifford and Waddington, 1998; Waters et al., 1994), nafadotride (9>10-fold selectivity) (Audinot et al., 1998; Sautel et al., 1995) and PD 152255 (45>fold selectivity) (Corbin et al., 1998) have generally shown to facilitate increase motor activity in rodents. Broadly, these molecules enhance rat's motor activity at reduced doses and antagonize motor activity at much larger strength. Hence, a biphasic action thought to be due to antagonism on dopamine D_3 receptors subtype with the reduced dose, while activation of D_2 receptors, at a much larger dose (Levant and Vansell, 1997). Selective D_3 blockers have also been shown to increase D-AMP-induced motor activity (Waters et al., 1993) and behavioural sensitivity to cocaine (Waters et al., 1993) at reduced doses. Further studies have been demonstrated on dopamine D_3 phenotype receptor variant of mice, in which few (Xu et al., 1997), except (Betancur et al., 2001) family demonstrated that DA D_3 mutant rodents are extremely active in a new environment. Hence, there are conflicting findings on the selectivity properties of the D_3 receptor antagonist family. Our aim in this study was to compare the behavioural response of selective D_3 blocker with D_2 receptor antagonist. We have observed that while dopamine D_2 antagonist eticlopride completely blocked the motor response, NGB 2904 a selective DA D_3 antagonist, augments the motor activity (Fig. 11B).

These findings support previous evidence on the modulatory impacts of dopamine D_2/D_3 effect on D-AMP-induced locomotor activity (Lapin and Rogawski, 1995; Calipari et al., 2014a).

Similarly, behavioural evaluation of dopamine D_2/D_3 receptor agonist was compared to selective DA D_3 agonist. Classically, apart from motor activity D_2/D_3 agonists are known to influence diversified heterogeneous behavioural responses such as, pelvic grooming (Yamada et al., 1986), which is a measure of penile erection hypothermia (Faunt and Crocker, 1987) and pica eating (Takeda et al., 1993a). Here, we determine the dose-response relationship between the classes of dopamine agonists being studied. The responses of interest in this study are yawning, pelvic grooming and pica eating behavioural traits; all mediated by either dopamine D_2 or D_3 stimulation. PPX at 0.05 mg/kg exhibited more efficacious responses for behavioural traits than higher doses (Fig. 12A). Our study is consistent with previous findings that reported that D_3-preffering agonists stimulate yawning activity at much lower doses (Collins et al., 2007). However, at higher dose of PPX, yawning is inhibited. This is hypothesized to be due to competing D_2 agonist activity as the dose becomes larger (Collins et al., 2005b). QNP is a non-selective dopamine D_2/D_3 receptor agonist that exhibited more behavioural traits at a much higher dose of 0.1 mg/kg (Fig.12C). However, even at this dose, the AUC of PPX at 0.05 mg/kg was 264 events while QNP 0.1 mg/kg exhibited an AUC of 168 events. Apparently, more behavioural activities particularly yawning and pelvic grooming were more expressed with PPX, a selective D_3-preffering agonist at lower doses. This is also in line with the study that induction of yawning and pelvic grooming by DA D_2/D_3 agonist is facilitated by a selective activation of dopamine D_3 receptor whereas, the inhibition of these two behavioural responses exhibited at high doses is modulated by agonistic activation on D_2 receptor subtype (Collins et al., 2005b, 2007).

The present electrophysiological investigation has elucidated the acute impact of PPX, a clinically used selective dopamine D_3 receptor agonist on mid-brain dopamine electrical activity. We tested this effect both in naïve and D-AMP intermittently treated rats. In the naïve rats, the mean firing activity of DA was significantly reduced with attenuated burst activity after the PPX second dose of 0.05 mg/kg (Fig. 13C, D). Physiologically, the VTA dopamine autoreceptors modulate spontaneous electrical discharge of dopamine neurons (Lacey et al., 1987; Mercuri et al., 1997) via enhancement of inwardly rectifying signaling of GIRK activity (Lacey et al., 1987; Kim et al., 1995). Dopamine neurons present in the VTA and substantial nigra (SNc) are found to co-express both Kir 3.2 (GIRK2) and Kir 3.3 (GIRK3) channels (Davila et al., 2003) in a sequence of cascade process, D_2 receptors essentially couple to GIRK2 channels (Beckstead et al., 2004). Therefore, the activation of signaling of GIRK pathway results in release of potassium, which inhibits the action potential (hyperpolization) due to increase K^+ outflow Presumably, the negative feedback effect of dopamine D_2 autoreceptors present in the midbrain dopamine neurons and the cascade of signaling processes activated by PPX ensued in the drastic reduction of DA firing rate with eventual complete shutdown of activity. Interestingly, the burst activity was also significantly attenuated whereas, some neurons didn't show any burst effect; the ones that exhibited it was reduced as the dose of PPX approaches 0.05 mg/kg. Centrally, the burst activity is responsible for a large output of transmitter equivalent to the same number of impulses present at regular interlude at the same period (Gonon, 1988). Another electrophysiological study reported that PPX administered for 2 days in rats, altered the VTA DA neurons firing rate and burst activity by 40% reduction in firing from the baseline and that this alteration in firing rats was restored after chronic treatment for 14 days (Chernoloz et al., 2009b). Millan et al., (2000), also reported two types of dopamine firing which are, tonic and phasic patterns, and can be induced by PPX which is associated with an agonistic effect on either D_2 or D_3

receptors respectively. We discovered from our findings that most DA neurons from naïve rats exhibited total inhibition/sensitivity to PPX at a low dose (60µg/kg). More studies on acute PPX administration reveals an inhibition on the neuronal firing in the NAcc (Piercey, 1998). Our finding on PPX as it relates to firing and burst activity attenuation is consistent with highlighted studies and further illustrated that low dose of PPX on naive rats abolishes desensitization process. In another series of electrophysiological study, we pre-treated rats (*n=9*) with once-weekly single intraperitoneal D-AMP 1 mg/kg injection for two weeks. Conversely, the DA neurons exhibited desensitization subsequent to low dose PPX administration (Fig. 13C, E). This raises the question of what mechanism can explain the D-AMP effect on D_2 autoreceptors. In animals pre-exposed to psychostimulant, the degree of sensitivity is principally dependent on the nature of the pretreatment frequency. Hence, sporadic treatment with fewer doses of drug induces more sensitization than persistent exposure to very large drug doses (Robinson and Becker, 1986b; Stewart and Badiani, 1993; Vanderschuren et al., 1997). We have previously highlighted the functional role of dopamine D_2 autoreceptor located on the VTA dopaminergic neurons. The neuromodulatory roles such as dopamine neuronal firing, dopamine synthesis, and release through negative feedback mechanism as well as activation of signaling of GIRK pathway has been reported (Calipari et al., 2014b). Thus, dysfunctional D_2 autoreceptors (Gorter et al., 2016; Keebaugh et al., 2017) associated with repeated D-AMP (once weekly for two weeks) led to abnormal dopamine activity in the VTA and NAc, this may be part of the basis for a drug-craving behavioural anomaly. Adopting the electrophysiological paradigm, we observed for the first time a profound influence of short time intermittent administration of D-AMP on VTA dopamine neuronal transmission.

Finally, on electrophysiological evaluation, we studied the potential correlation between VTA dopamine firing activity with behavioural phenotype of low as well as high

locomotor activity (Fig. 10B, 14). Although, the activity assessment method is limited in available sample size to precisely investigate correlation between behavioural and VTA DA firing activity. Behavioural modification in animals such as, hunger, emotion, motivation and sex drive, takes place both externally as well as an internal compensatory mechanism (Gorter et al., 2016; Keebaugh et al., 2017). The internalized behaviours are encrypted in diverse forms and are believed to be associated with neural activity in specific brain regions. Physiologically, voluntary events in a neuronal activity tend to exhibit momentous changes with or without external stimuli such as psychostimulant (Fox and Raichle, 2007). Here, we introduced an external stimulus D-AMP 1 mg/kg acute injection and reported a relative statistical significant association between low motor activity subjects with reduced VTA dopamine firing rate, as well as population activity and vice versa. We posit that a potential causal link exists between the behavioural traits that are DA-dependent and DA firing rate (Hamidovic et al., 2009). This may have a clinical implication on individualized medicine approach, based on varied unpredictable response to drug. More studies in humans have provided more evidence to support this phenomenon that, genetic variability in DA receptor is associated with behavioural and impulsivity in D-AMP treated healthy individual. Besides, Aumann and Horne, (2012) postulate that firing activity of DA neurons in the SNc potentially regulates DA gene expression. They concluded that neuronal activity in the brain controls DA behavioural phenotype, initiated from the SNc neurons. To some extent, our finding is in line with these studies, albeit, large samples are needed to appreciate and confirm the extent of this perceived association to give credence to our finding. In this chapter, we establish that DA receptors functionally regulate the activity of mesocorticolimbic and nigrostriatal pathways, performing a vital role in DA transmission. These studies have demonstrated that D-AMP administration in intermittent repeated doses modulate midbrain dopamine D_2/D_3 receptor function and signaling. Interestingly, autoreceptors dysfunctionality may potentially

result in conformational changes of pre and postsynaptic D_2/D_3 receptors with direct impact on drug-craving associated behaviours. Our investigation on neural circuit's impact of DA neuronal activity and interplay is censorious for understanding the neurochemical as well as the electrophysiological basis of diverse behavioural response. It was also important to report these data for better understanding of what will be presented later with GLP-1, insulin, and sucrose consumption studies.

Chapter II: Insulin modulation of dopamine signalling and dopamine-dependent behaviours

II-I- Introduction

Insulin is primarily considered an important hormone for blood sugar homeostasis (Lerner and Porte, 1972; Seltzer et al., 1967). Beyond this early findings, Kleinridders et al., (2014), have demonstrated with the aid of quantitative real-time polymerase chain reaction (PCR) that its receptors are widely distributed throughout the brain regions in proportionality to the subtypes (Fig. 15). These receptors are activated by insulin in the brain to signal vital physiological processes such as, appetite regulation (Perry and Wang, 2012), lipid metabolism (Koch et al., 2008), control of body temperature (Sanchez-Alavez et al., 2011), maintenance of body weight and reproduction (Brüning et al., 2000), as well as vital energy homeostasis (Burks et al., 2000). Also, recent studies over the years have shown that insulin may be involved in the regulation of DA transmission (Caravaggio et al., 2015; Patel et al., 2018a), DA gene expression and signalling (Mansur et al., 2018a), influence addictive behaviour (Dai et al., 2017; Daws et al., 2011), and is likely associated with pathology of neurodegenerative disorders (Calsolaro and Edison, 2015a). Insulin is suggested to have a profound effect on the activity of dopamine transporter (DAT) and drug that targets such site (Carvelli et al., 2002; Patterson et al., 1998). Thus, D-AMP, a psychostimulant and a classical candidate of a molecule that interacts with DAT activity, may potentially modulate insulin effect on DAT activity or vice versa. The investigation of pharmacological interaction between insulin and D-AMP may possibly help us in answering the question that is associated with the concept of drug-craving and various metabolic disorders emanating from insulin dysfunctionality. Owens et al., 2005, (2012), further demonstrated that disturbance to

DA haemostasis was exhibited when the rodent is made hypoglycaemic in the presence of streptozotocin. The study further implicates DAT clearance deficiency as a factor responsible for decreased responses in motor activity in streptozotocin-induced hypoinsulinemic rats (Owens et al., 2005a, 2012). Previously in chapter I, we established the potential role of dopamine D_2 receptor in modulating D-AMP-induced motor response (Fig. 11A), as well as the impact of D_2 receptor agonist in enhancing DA uptake rate, a function of DAT. All these findings together suggest that D_2 receptors can also modulate DAT activity. The vital role of D_2 is further substantiated by the evidence that D_2 receptors knockout mice significantly lowered dopamine clearance (Dickinson et al., 1999).

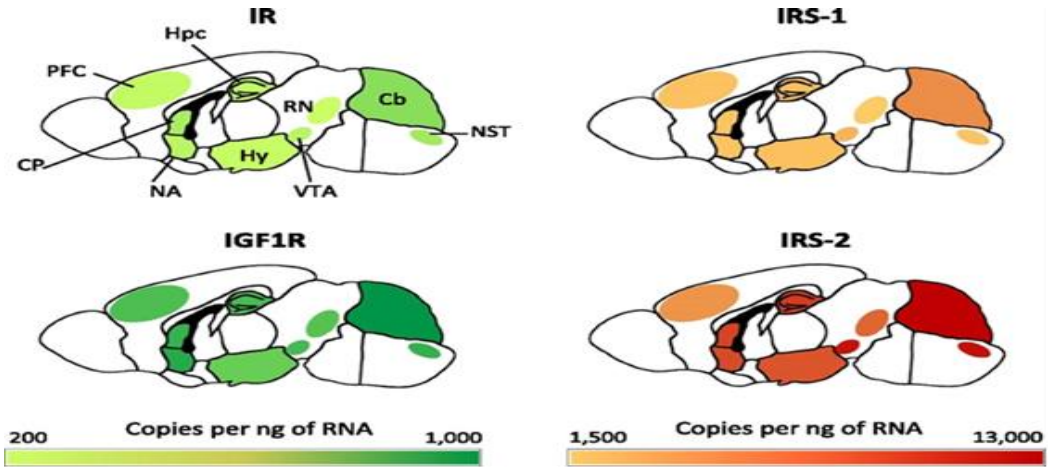


Fig 15: Expression of IR, IGF-IR, IRS-1 and IRS-2 in the brain determined by quantitative real-time PCR (Kleinridders et al., 2014). Expression of IR, IGF-1R, IRS-1, and IRS-2 in the brain. The expressions of IR, IGF-1R, IRS-1, and IRS-2 were determined by q real-time PCR of brain regions dissected from male C57BL/6 mice. Data are expressed as the copy number per nanograms of RNA transcribed. Cb, cerebellum; CP, caudate putamen; Hpc, hippocampus; Hy, hypothalamus; NA, nucleus accumbens; NST, nucleus tractus solitaries; PFC, prefrontal cortex; RN, raphe nucleus; VTA, ventral tegmental area.

The aim in this chapter was to study behavioural and neurochemical impacts of brain insulin level alteration, as well as how it impacts on D_2 signalling. We also investigated for the first time, by using a novel approach with diazoxide (a K_{ATP} channel activator inhibiting

insulin release) inducing a hypoinsulinemia model on rats. We investigated the consequential response to D-AMP-induced locomotor activity. Finally, we studied the electrophysiology of insulin on VTA dopamine neurons and PFC pyramidal neurons when administered either intravenously or through intracranial route in deeply anaesthetized rats.

II-2- Materials and methods

II-2-A-Subjects

Please refer to paragraph II-2-A for detail information on animal conditions and regulatory protocol on a study involving rodents.

II-2-B- Drugs

All drugs were purchased from Sigma (Sigma-Aldrich, UK), except for PPX in dose of 0.1 mg/kg given intraperitonially (i.p.) (Hellobio, UK), apomorphine hydrochloride (i.p.) (Tocris, UK), QNP in dose of 0.05 mg/kg (i.p.) (Tocris, UK). The drugs were dissolved into Krebs (release assays) and HEPES (uptake assays) buffers, as appropriate. The drugs were dissolved in distilled water or saline for studies involving behavioural and electrophysiological profiling.

II-2-C- *In vitro* ^{3}H-dopamine release assay

Please refer to paragraph I-2 for detailed experimental procedures. In this chapter, insulin 2µM and a powerful dopamine-releasing drug, D-AMP 10µM were investigated to determine their capacity to induce tritiated dopamine release on striatal brain slices. The interaction between the two drugs on postsynaptic for DA efflux was also investigated.

II-2-D-*In vitro* ^{3}H-dopamine uptake assay

In these experiments, two methods were used to study [DA] uptake activity.

II-2-D1-*In vitro* direct measurement of ^{3}[H]-dopamine uptake

Here, we studied ^{3}[H]-dopamine uptake according to paragraph I-2 protocol. Please, refer to the earlier mentioned paragraph for detailed experimental procedures. However, molecular interaction between insulin 2µM and a dopamine D_2/D_3 agonist, QNP 125nM was investigated as well as the effect of each drug on direct dopamine uptake process.

II-2-D2- *Ex vivo* kinetics of ^{3}[H]-dopamine uptake

In this set of experiments, three groups of rats (*n=5*), one pre-treated with saline (0.8 ml/kg) or challenged with insulin (single i.p. injection 20µg/kg, a low dose) supplemented by 5% glucose to prevent hypoglycemia (Qinna and Badwan, 2015) or diazoxide (DZ) 150 mg/kg to impair insulin secretion. Blood glucose levels were monitored with a glucometer (Accu-Check viva). After 1 hr of treatment, the animals were sacrificed by cervical dislocation and the brains region of interest (striata) were then quickly removed and placed on an ice-cold platform for further dissection (see paragraph I-2 for full protocols). The homogenized tissue was pre-incubated for 5 mins at 37^0C with ^{3}[H] DA and the kinetics of ^{3}[H]-dopamine uptake was assayed in a total of 300 µl of uptake buffer. We then varied the concentration of ^{3}[H] DA (5-80 nM) at each 5mins incubation period to determine the uptake rate per time. Protein level determined according to Bradford assay (Bradford, 1976). Apparent $K_{m,}$ and V_{max} values were calculated by nonlinear least-square curve fitting of data using the algorithm described by Schreiner et al., (2011) assuming Michaelis-Menten kinetics and, where appropriate, competitive inhibition. Models for one and two enzyme systems were

fit, and the significance test described by Munson and Rodbard, (1980) was employed to choose the best model to describe the data.

II-2-E- D-AMP and dopamine D_2/D_3 receptor agonist-induced behavioural studies

Rats were transferred individually from their home cage to a test cage and then allowed to habituate for 30 min before the start of the experiment. Adult male Sprague-Dawley rats were pretreated with saline (0.8ml/kg) or insulin 20µg/kg (5% glucose supplemented to prevent hypoglycaemia). The rats were later treated with single intraperitoneal injection 1mg/kg of d-amphetamine (D-AMP) or 0.1mg/kg of quinpirole (QNP). In another series of experiments, animals were pretreated with DZ 150 mg/kg, to prevent insulin secretion. Animals were then scored for behavioural locomotor parameters such as rearing (a dopamine-dependent motor manifestation), during 15-minute periods and up to a total of 60 minutes following the injection. Counting of yawning, pelvic grooming and pica eating behavioural traits were performed manually following QNP administration. Whenever possible, blood glucose levels were monitored with a glucometer (Accu-Check viva).

II-2-F-*In vivo* extracellular single-unit electrophysiology

Please refer to paragraph I-4 for detailed protocol. Recordings were done on both VTA DA neurons and pyramidal PFC neurons. However, in this study our recordings in the midbrain DA neurons were carried out in three ways: 1) we acutely administered insulin while recording firing activity 2). We also injected the VTA region with insulin. In these experiments, the tip of a 5 µl Hamilton syringe was lowered into the VTA (5 mm posterior to bregma and 1.5 mm lateral to the midline, 7.8 mm below the cortical surface). Insulin (1.5 µg in 1.5 µl) or saline was slowly injected over a 3 min.

3). We also carried out experiments where insulin was administered through the recording electrode, by filling the electrode with insulin (100 µg/ml) in saline to allow for diffusion into the distinct brain region with a concurrent single unit recording (Marks et al., 1988). This method of administration of drugs through the recording electrode has worked previously in our laboratory, with different types of drugs, including some drugs with relatively high molecular weight. However, in these experiments the amount of drug that can diffuse through the recording electrode is uncertain. We used this protocol to study the local effect of drug and to acquire electrophysiological information as well as recognition of cell populations at the site of local administration.

II-2-G- Data analysis

Please refer to paragraphs II-2-G for a detailed method of analysis on each protocol. However, in uptake kinetics experiment all values are expressed as mean ± standard error of the mean (SEM). The mean K_m and V_{max} values (expressed in units of nM and pmol/mg/min, respectively) were obtained from the individual K_m and V_{max} values derived from each experiment using nonlinear regression analysis and a single-site model (GraphPad Prism version 3.0; San Diego, CA). Data were analyzed by two-way analysis of variance (ANOVA), followed by appropriate post-hoc tests.

II-3- Results

II-3-A- Insulin did not induce ^{3}H-[DA] release on striatal slices

Administration of insulin at 2µM in the perfusion medium did not show any effect on basal ^{3}H-[DA] efflux from striatal slices (Fig. 16A). Also, insulin did not alter D-AMP-induced ^{3}H-[DA] release (Fig. 16B).

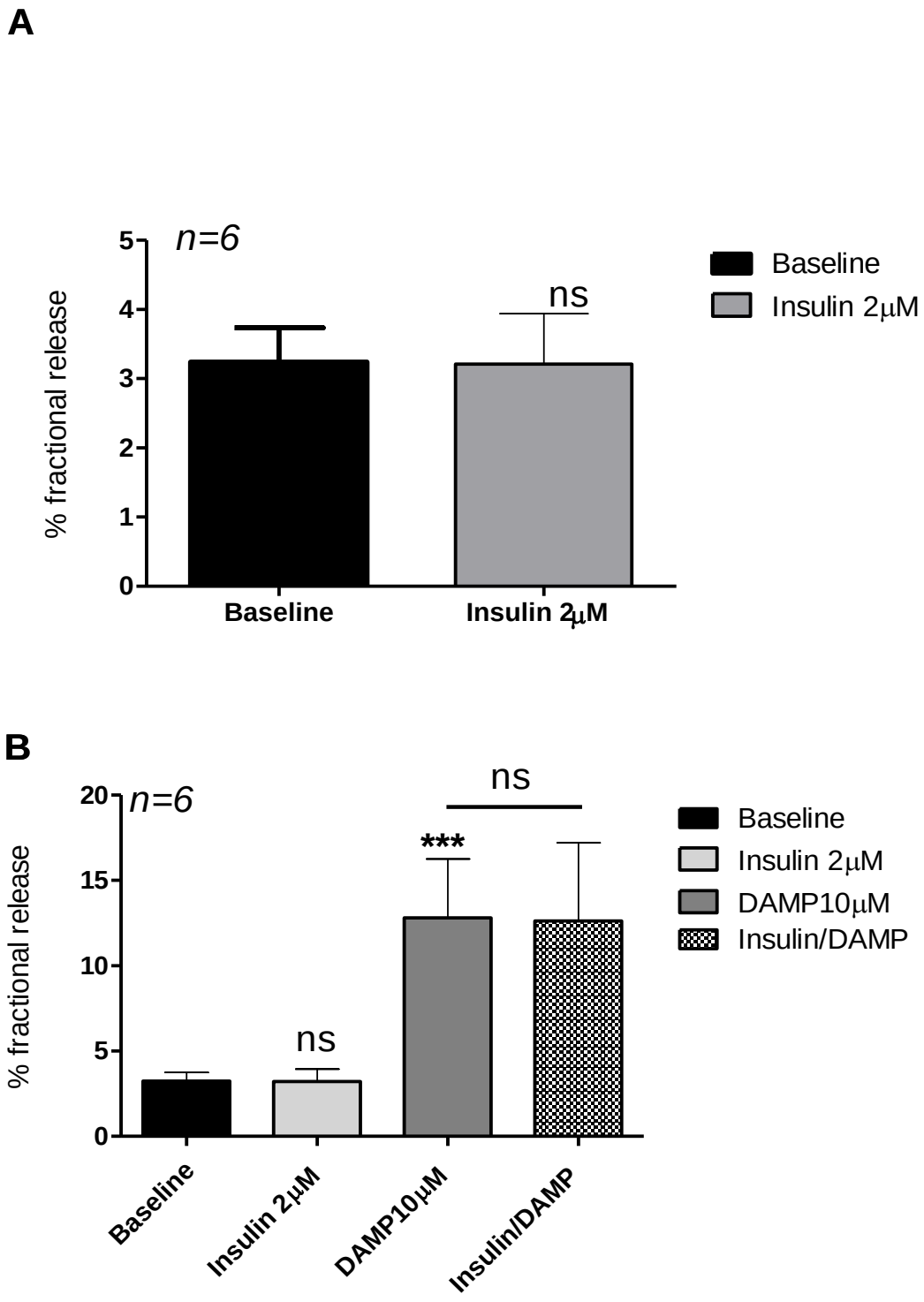


Figure 16: Insulin effect on the release of striatal [DA] efflux.

(**A**): Insulin 2µM did no induce a statistically significant change in ^{3}H-[DA] efflux from striatal brain slices. (**B**): Insulin neither potentiates nor attenuates DAMP-induced striatal release. P>0.05 *vs* baseline, unpaired student's t-test, P>0.05, ***P<0.0001 *vs.* respective conditions. *Neuman-Keuls,* after significant one-way ANOVA.

II-3-B- Insulin modulates striatal synaptosomal ^{3}H-[DA] uptake

II-3-B1- Insulin attenuates both baseline and QNP-induced ^{3}H-[DA] striatal uptake

The striatal synaptosomes incubated with insulin 2 µM exhibited reduced ^{3}H-[DA] uptake on both 5 mins and 30 mins pre-incubation period in the reaction medium. There was a significant (28%) inhibition after 30 min pre-incubation ($F=6.126$, $df=29$), which is more pronounced than after 5 min ($F=4.151$, $df=9$) (Fig. 17A and B). Interestingly, insulin also abolishes QNP-induced dopamine uptake ($F(4, 70) =13.39$) (Fig. 17C). Thus, dopamine transporter activity was simultaneously reduced in the presence of insulin.

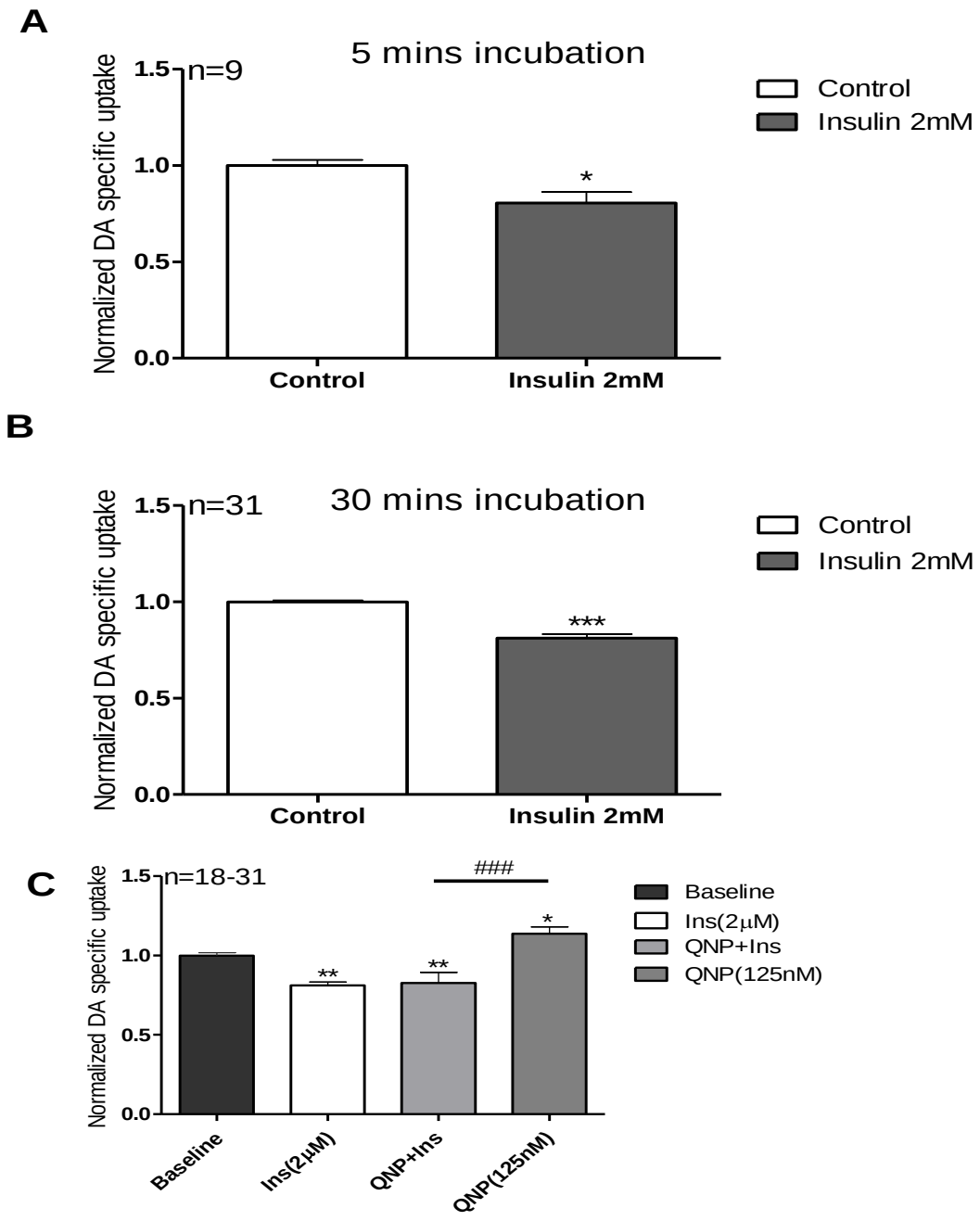


Figure 17: Effect of insulin with quinpirole interaction on [DA] uptake transporter activity

(**A&B**): Insulin 2µM significantly reduced [DA] uptake both in 5 mins and 30 mins incubation periods, unpaired student's t-test, * P<0.01, ***P<0.0001 *vs* baseline.

(C) In the presence of insulin, quinpirole-induced [DA] uptake was significantly reduced, compared to control experiments performed in parallel. This is an indication of molecular interaction with QNP at uptake level and reduced dopamine transporter activity. ###P<0.0001, *P<0.05, **P<0.001 *vs* baseline uptake, *Neuman-Keuls*, after significant ANOVA.

II-3-B2- Insulin modulates [DA] uptake kinetics on striatal synaptosomes

In the control group, Michaelis-Menten's plot analysis of [DA] uptake kinetic parameters on striatal synaptosomes revealed a direct proportionality between concentrations (up to 40 nM) and the uptake velocity, as well as adequate saturation at 80nM of [DA] (Fig. 18A). In the insulin-treated rats, Michaelis-Menten's plot analysis on striatal synaptosomes revealed that acute administration with insulin 2 µg/kg (supplemented with glucose 5%) in a single dose resulted in increase in V_{max} with no apparent change in K_M, which is a measure of affinity as compared with control group (Table 1). In another experiment with intraperitoneally administered DZ (K_{ATP} channel activator and an insulin release inhibitor), the V_{max} also increased considerably.

V_{max} values for DAT-mediated uptake after. Data are means±s.e.m R^2 indicates goodness of fit (*P<0.05 *vs.* control, one-way ANOVA followed by *Neuman-Keuls* post *hoc* test.

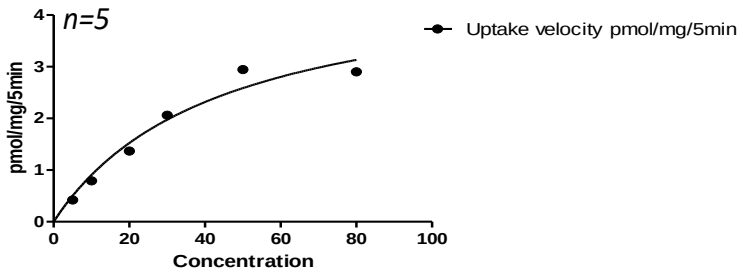


Fig 18: Michaelis-Menten plot for the determination of the Km and Vmax DAT activity on [DA] uptake in control rats.

Table 1: Insulin and diazoxide effects on kinetic parameters in DAT-mediated uptake on striatal slices

Kinetic parameters	Control	Insulin (20µg/kg)	Diazoxide (150mg/kg)
$K_{m\ (nM)}$	43.7±15.82	34.45±11.11	26.6±4.117
$V_{max\ (pmol/mg/5min)}$	4.834±0.8730	37.49**±5.509	38.4**±4.81
R^2	0.9588	0.9609	0.9992

II-3-C1- Presence of insulin and its inhibition by diazoxide modulate D-AMP-induced rearing activity

During the first 60 minutes of observation, following administration of the dopamine-releasing agent D-Amphetamine (DAMP, 1 mg/kg ip), animals that were pre-treated (15 min before the challenge) with insulin 20 µg/kg ip (supplemented with 5% ip. glucose administration to compensate for hypoglycaemia) did not show any more motor activity than the control group treated with D-AMP only, except during the first 15 mins of observation, where the insulin pre-treated group displayed more rearing activity (Fig. 19B). First, we investigated the glycaemic profile of DZ-treated rats for hyperglycaemic-inducing effect, through inhibition of insulin compared with control. The data showed a significant elevated (F=66.63, DFn=1, DFd=42) plasma glucose level than the control throughout the 3

hours of glycaemic observation (Fig. 19A). However, during the first 60 minutes of observation following administration of DZ 150mg/kg ip, an insulin inhibitor, animals that were pre-treated (15 min before the challenge) displayed significantly less locomotor activity ($F(1, 42) = 66.63$) throughout the observation period (Fig. 19C). In fig. 19D, insulin pre-treated group did not show any alteration in locomotor activity except during the first 15 mins of observation ($F(6, 120) = 9.5$). We demonstrated evidence that the impact of insulin on motor activity was not due to it hypoglycaemic-induced effect as we prevented such effect with supplemented 5% glucose to a normoglycemic state. We also observed that $n=value$, for the control group (5) done during the series of experiment is not different from the entire control group pulled together ($n=23$).

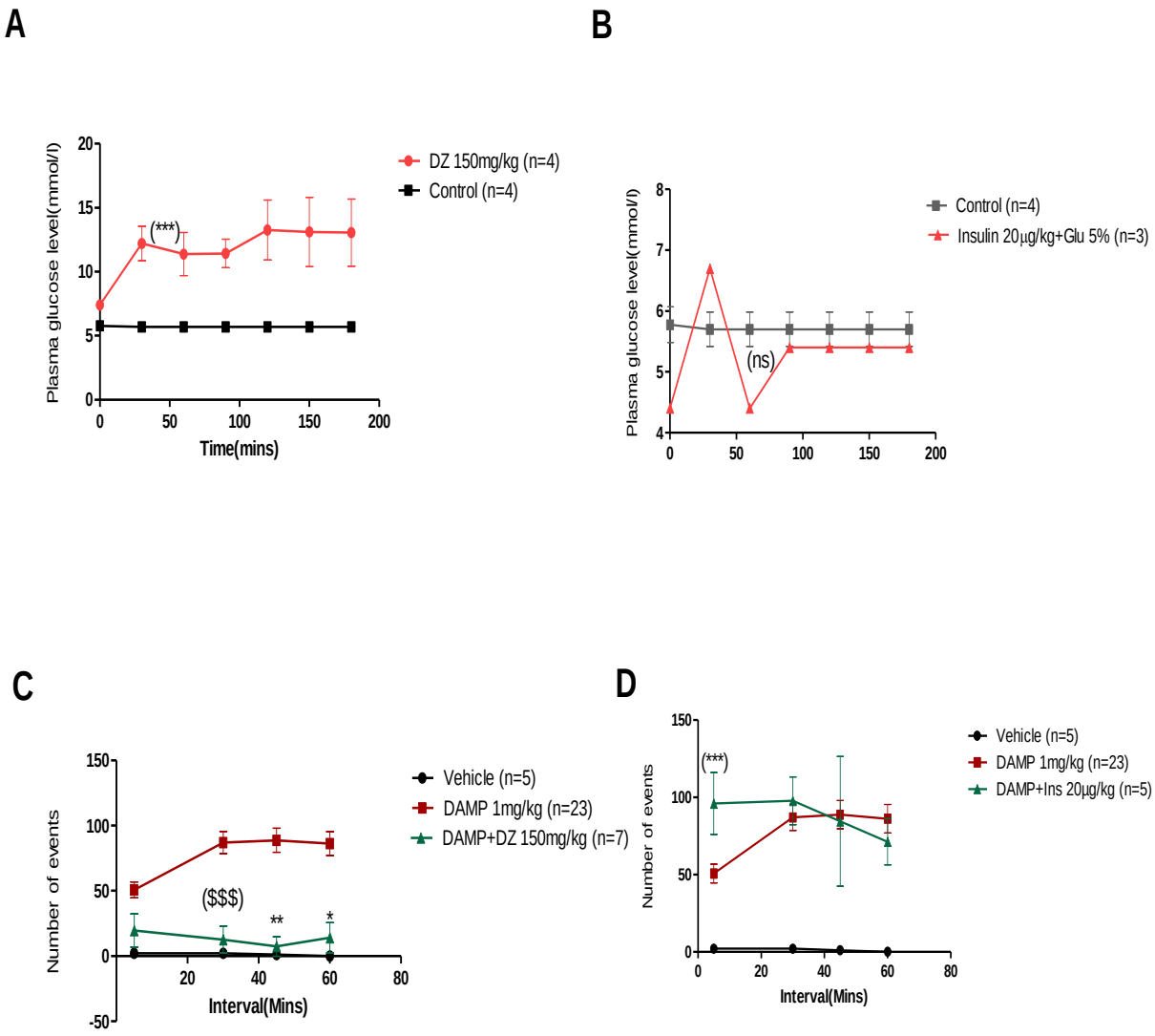


Figure 19: Impact of insulin and diazoxide on D-AMP-induced motor activity

(**A**): DZ treated rats exhibited consistent hyperglycaemic level likely due to insulin secretion inhibition throughout 3 hrs of observation, ***P<0.0001 *vs.* control at all times. **(B):** Insulin 20 µg/kg administered concurrently with glucose 5% (0.1 ml/kg) did not exhibit any significant alteration on glycaemic level. **(C**): In the presence of DZ, an inhibitor of insulin release, there was a significant reduction in locomotor activity, and pronounced statistical reduction during 30-45 and 45-60-mins periods. **(D)**: Insulin 20 µg/kg pre-treated group did not show a significant change in locomotor activity throughout the observation period, except during first 15 mins of observation. $$$P<0.0001, *P<0.5, **P<0.001, ***P<0.0001 *vs.* respective control. *Bonferroni post hoc* tests after significant two-way repeated measures ANOVA.

II-3-C2- Insulin and diazoxide inhibit both QNP and PPX-induced behavioural effects

As earlier indicated in the previous chapter, D_2/D_3 receptors induce classical behavioural traits such as yawning, pica eating, and pelvic grooming. A single dose of insulin 20 µg/kg pre-treatment supplemented with glucose, followed by QNP 0.1 mg/kg shows significant reduced yawning activity ($F(1, 32) = 6.41$) throughout the observation time compared with control (Fig. 20A). Similarly, significant reduced effects were also observed in pica eating ($F(1, 24) = 2.78$) and pelvic grooming ($F(1, 24) = 2$) but, only during the first 30 mins (0-15 and 15-30 min periods, Fig. 20B &C). Interestingly, with PPX, a preferential dopamine D_3 receptor agonist, 0.05 mg/kg, yawning ($F(1, 32) = 6.41$) and pelvic grooming ($F(1, 32) = 4.65$) effects were also significantly reduced in the insulin 20 µg/kg pre-treated groups compared to control (Fig. 21A, C). But apparent increase in pica eating than the control was demonstrated in rats pre-treated with insulin (Fig. 21B).

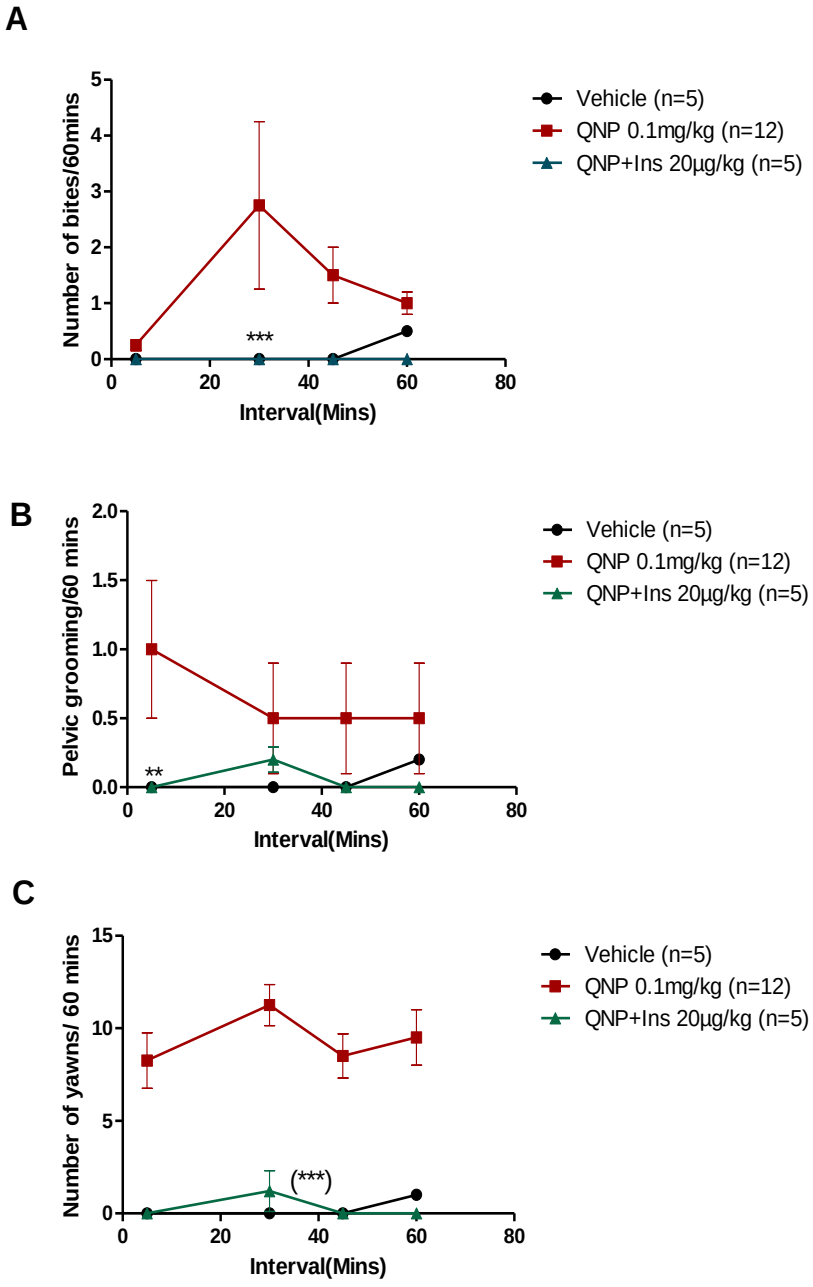


Figure 20: Impact of insulin on QNP-induced behavioural traits

(A, B): Insulin 20 µg/kg pre-treated group significantly reduced pica eating and pelvic grooming activities induced by QNP during the 30-45, and the 0-15 mins observation periods, respectively. (**C**): On the other hand, there is a statistically significant difference in QNP-induced yawning activity throughout the observation period when compared with the control *Bonferroni post-tests* after significant two-way repeated measures ANOVA. **P<0.001, *vs.* respective control, ***P<0.0001 vs control at all times.

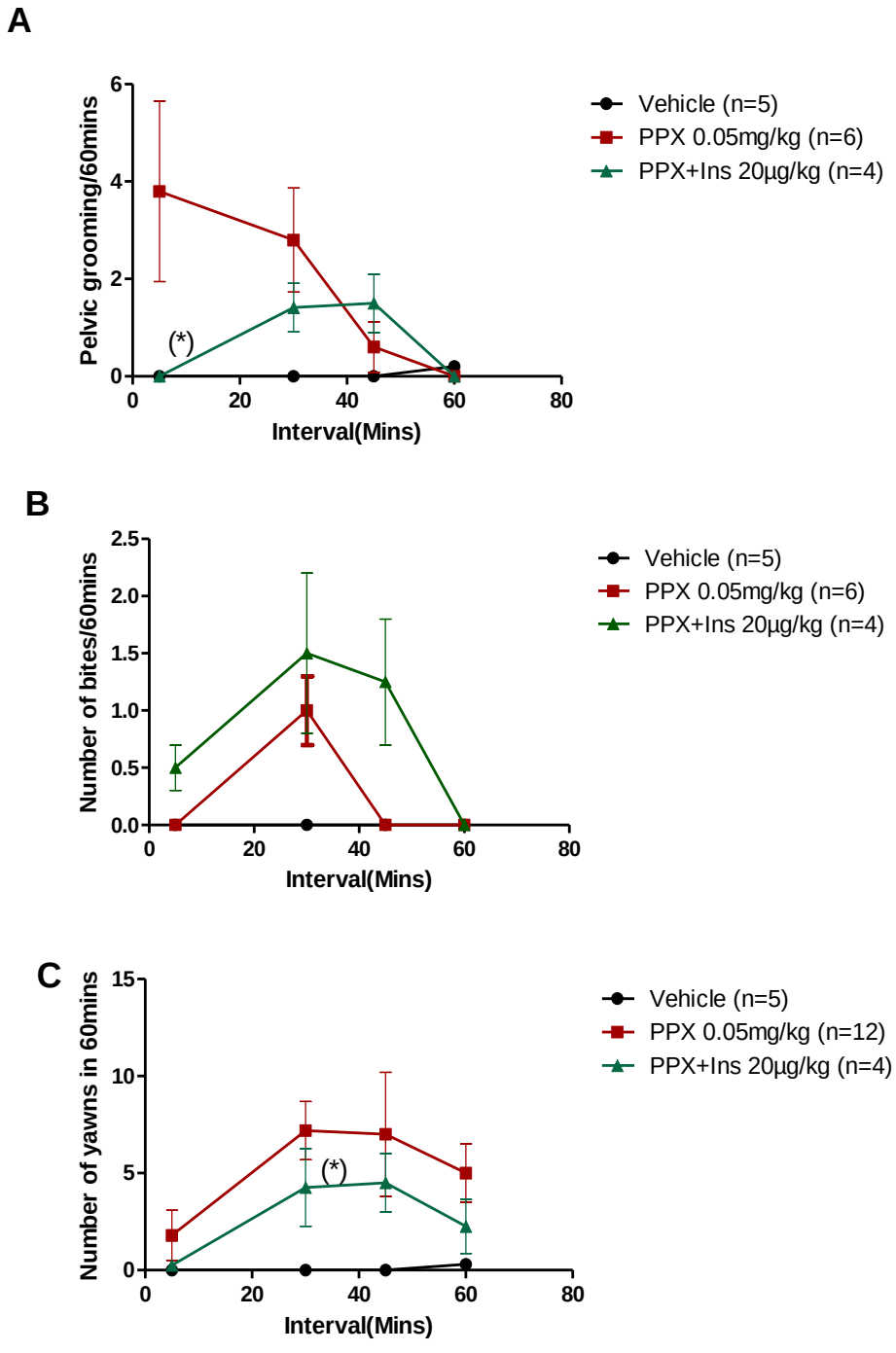


Figure 21: Impact of insulin on PPX-induced behavioural traits Insulin 20 µg/kg pre-treated group significantly reduced yawning and pelvic grooming activities (**A&C**) during the observation period. However, in **(B)** insulin pre-treated group exhibited more pica eating activity than the control group, albeit not a significant increase. Significant two-way repeated measures ANOVA. *P<0.05 *vs. control*

II-3-D1- Insulin modulates both pyramidal and DA neurons firing activities

First, in the PFC and VTA, we investigated the effect of DZ on the firing activity of neurons. DZ in these two brain regions did not alter the firing activities on both the pyramidal and VTA DA neurons. In the prefrontal cortex, intravenous administration of insulin 20 µg/kg does not affect the firing activity of PFC pyramidal neurons. However, following an additional dose of 20 µg/kg; the firing activity slightly but significantly increased (Fig. 22A). Three pyramidal neurons out of the seven tested had their firing rate increasing by more than 40% from baseline after a cumulative dose of 40 µg/kg. Similar finding has been reported by Zhou et al., (2017). On the other hand, in the VTA intravenous insulin had a slight decreasing effect on the firing rate of dopamine neurons (Fig. 22C). However, this was significant only at the high cumulative dose of 60 µg/kg.).

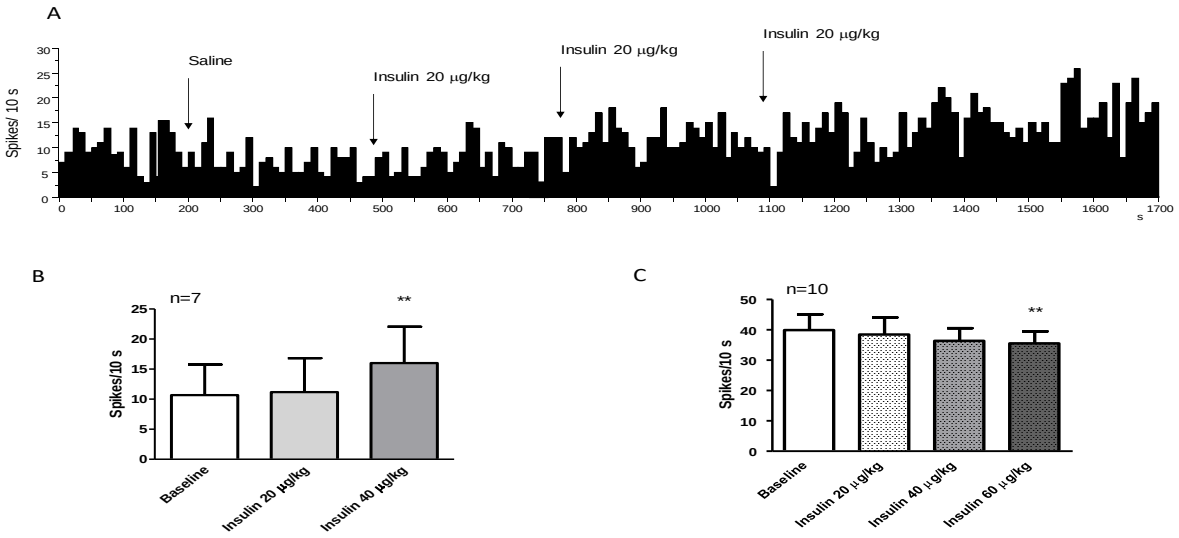


Figure 22: Electrophysiological effects of intravenously administered insulin on pyramidal prefrontal cortex neurons and VTA dopamine neurons

(A): Firing rate histogram of a pyramidal PFC neuron showing progressive activation upon administration of 3 subsequent doses of insulin (20 µg/kg, iv.) **(B):** Mean firing rates of PFC neurons before and after the administration of 2 doses of insulin (20 µg/kg, iv.). Neurons show a significant level of activation following the cumulative administration of insulin of 40 µg/kg (iv.). *P<0.03, vs baseline level, *Newman-Keuls* test after significant repeated measure one-way ANOVA. **(C):** In the VTA insulin given intravenously slightly but significantly reduced VTA dopamine neurons firing activity, but only at the highest cumulative dose tested (60 µg/kg, iv.). *P<0.03, vs baseline level, *Newman-Keuls* test after significant repeated measure one-way ANOVA.

II-3-D2- Insulin diffusion in brain progressively modulates VTA dopamine population firing rate

In these experiments, the recording electrodes were filled with insulin (final concentration 100 µg/ml) and lowered into the VTA for the recording of dopamine neurons. It is anticipated that during the recording the insulin solution is allowed to diffuse through the recording electrode into the VTA nearby the recorded neurons. In our laboratory, this method of administration of products directly to the neuron tested has proven to be efficient (Gronier, 2011a). Although the amount of product applied to the neurons cannot be predicted and diffusion efficiency can vary from one electrode to one another. Whenever possible, the dopamine neurons were recorded during two subsequent periods of 7 min and firing activity between period 1 and 2 was compared assuming that insulin will progressively diffuse through the electrode and that neurons will be exposed to insulin for longer in the second period of the recording. Firing activity of dopamine neurons recorded this way was not significantly different than the firing activity of a control population of neurons (though it tends to be lower, $p<0.2$). However, during the second period of the recording, the mean firing activity became lower than during the early period of the recording and lower than in the control (Fig. 23A &B).

II-3-D3.Intra-VTA administration of insulin

Previous data from our laboratory has shown that administration of saline (1-2 µl) within the VTA did not change the electrophysiological characteristics of the dopamine neurons (Gronier, 2008). This indicates that the insertion of the Hamilton syringe needle does not induce significant damage in this structure. We have confirmed this result on a small group of neurons (n=7) but used our main control population for comparison. Figure 24 shows that neurons recorded at least 40 min after the administration of insulin (1.5 µg) into the VTA region displayed a significantly lower baseline and lower burst activity than in the control

population. Interestingly the proportion of non-bursting neurons was particularly high in the treated group (12/16, 75% of neurons) compared to the control group (8/43, 18%).

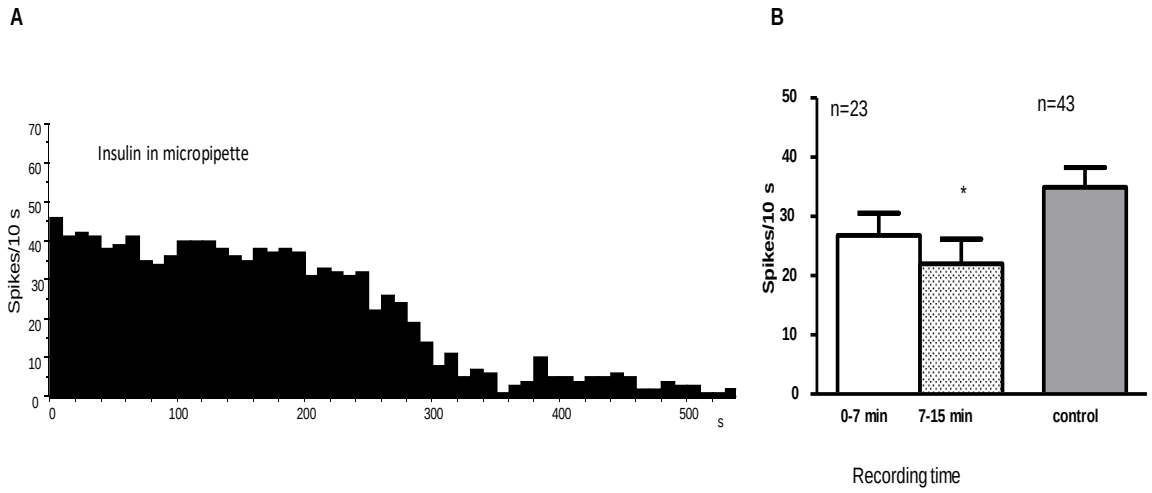


Figure 23: Population firing activity of intra VTA-infused insulin on DA neurons
(A): Firing rate histograms of a VTA dopamine neuron recorded with an electrode filled with insulin (100 µg/ml in saline). This particular neuron displayed a large decrease in firing activity during the recording period.

(B): The mean firing activity of VTA dopamine neurons recorded with an electrode filled with insulin decreased significantly during the later period of the recording (7-15 min), during that time the firing is also significantly lower than the mean firing activity of VTA dopamine neurons recorded in normal condition (with saline-filled electrodes). Note that in control condition the firing rate of dopamine neurons do not decrease with time. *P<0.02 compared to respective firing rate during the early period of the recording (paired Student's t-test) and compared to controls (unpaired t-test).

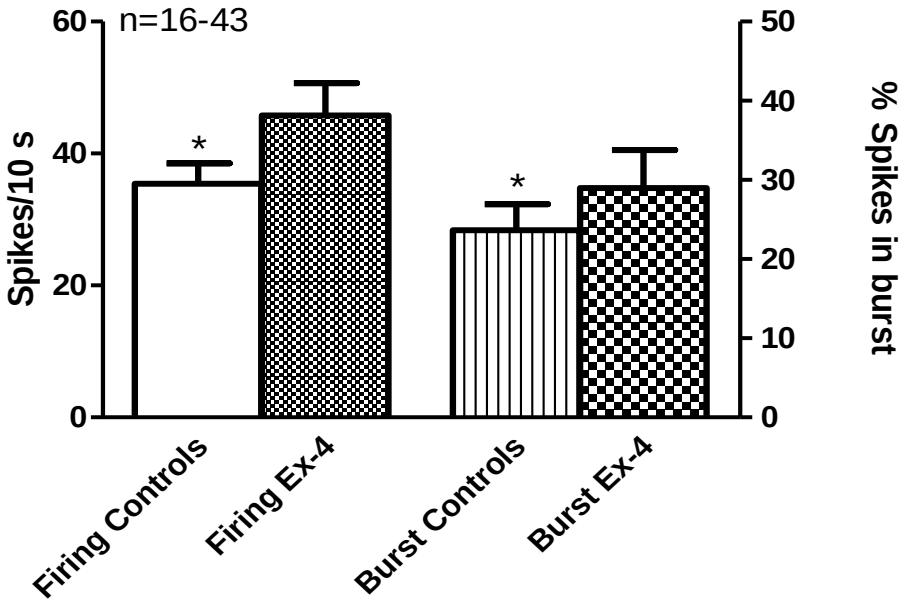


Figure 24: Individual electrophysiological characteristics of intra VTA insulin

Firing rate and burst firing of VTA dopamine neurons rats (n=4) that were administered insulin (1.5 µg) within the VTA at least 40 min before and in control animals (n=6). Both firing and burst activities were significantly lower than control values. *$p<0.03$, unpaired Student's t-test.

II-4-Discussion

Systemic peptides such as insulin, glucagon–like peptide (GLP-1), leptin and ghrelin can modify feeding habit via regulation of reward pathways (Figlewicz and Benoit, 2009). In this study, our neurochemical data reveals that insulin possibly does not have a pronounced effect on basal and D-AMP-stimulated striatal [DA] release (Fig. 16A, B). However, we reported that insulin may interact with dopamine uptake processes by causing a reduction in [DA] uptake via DAT clearance in our *in vitro* study on striatal synaptosomes (Fig. 17A, B, and C). Our ex vivo kinetic study indicates that the kinetic parameters of the DAT (V_{max} and Km) can be affected by insulin. A supplementation of insulin (administered 1 hr before animal sacrifice) seems to slightly reduce the K_M of the transporter. Therefore, it may increase its affinity for dopamine transport velocity when dopamine concentration is within its physiological range. However, a presumed deficiency of insulin in the brain caused by the blockade of its peripheral secretion (diazoxide acting on the pancreatic β-cells), seems to disturb more dramatically the activity of the transporter reducing considerably its affinity for dopamine. Therefore, the presence of insulin may be required in the synapse to stabilize the activity of the presynaptic transport of dopamine. It is not known whether this can be caused by a direct or indirect interaction between dopamine transporter and insulin receptor. As shown in the literature, insulin can represent a reward indicator, as well as function in the satiety signaling process. Previous proof has indicated that insulin can modulate DAT activity via an increased dopamine uptake (Schoffelmeer et al., 2011a; Williams et al., 2007a). However, there are contrasting studies that have shown that insulin role in the striatum is quite complicated. While, Schoffelmeer et al., (2011b) reported a decreased in electrically evoked release of ^{3}[H]DA from the striatum, another finding by Mebel et al., (2012) demonstrated that in the VTA there was a pronounced effect of insulin on dopamine uptake resulting in decrease dopamine efflux by fast-scan cyclic voltammetry method. Stouffer et al.,

(2015) however, discovered through cyclic voltammetry method that insulin magnifies striatal DA release in a nicotinic acetylcholine receptor-dependent manner by facilitating cholinergic excitability through insulin receptors. From all these controversial studies, one wonders from our data the reason for the paradoxical effect of insulin on ^{3}H[DA] release and uptake and possible desensitization of insulin receptors or downregulation in signaling pathways may be potentially implicated. Furthermore, insulin receptors experience fast endocytosis and breakdown after binding to peripheral tissue leading to neuronal desensitization of insulin receptors after exposure to supra-physiological concentration (Anthony et al., 2006; Mayer and Belsham, 2010; Schulingkamp et al., 2000). There are differences in protocols deployed from all the studies mentioned, as well as the methods used in our studies, and this could contribute to regional discrepancies in insulin activity. In some studies, it is hypothesized that insulin receptors stimulation induced an increase in surface expression and activity of DAT in the rat striatum, by facilitating dopamine reuptake via phosphatidylinositol 3-kinase (P13K)-dependent process (Carvelli et al., 2002; Garcia et al., 2005; Lute et al., 2008a). Phosphatidylinositol 3-kinases are a group of enzymes that regulate downstream intracellular signaling via binding to pleckstrin domains of protein kinase B (PKB) and phosphoinositide-dependent protein kinase (PDK) to cause modulation of phosphorylation of PDK (Shepherd et al., 1998). However, PI3K has assumed new roles in dopamine signaling process and implicated in multiple transduction of dopamine-induced control of gene expression, long-term striatal neuronal adaptation (Brami-Cherrier et al., 2002) as well as dopamine D_2 receptors regulation in addition to cyclic adenosine monophosphate (cAMP) pathways (Beaulieu et al., 2011). Our finding is consistent with these studies, having demonstrated an increased velocity above control following insulin administration. More data from our studies on radiometric uptake assay reveals that insulin receptors stimulation, modulate dopamine D_2 receptors which in turns contribute to the

regulation of DAT activity in the striatum (Fig. 17C). Mayfield and Zahniser, (2001) reported that dopamine D_2 receptor stimulation can facilitate surface expression and functional activity of DAT of Xenopus oocytes. Another interesting data has linked dopamine (D_1/D_2) receptors activation with cognitive enhancement (Mehta and Riedel, 2006), as well as suggesting a correlation between insulin administration and cognitive performance (Strachan, 2005a). Although, there is a disparity between data from animals and human studies, it is still plausible, that cognitive enhancing effect of insulin is partly due to its ability to modulate dopamine neurotransmission through the uptake process. Besides, when D_2 receptor agonist QNP was added to the reaction mix of striatal synaptosomes there was a significant increase in the velocity of [DA] uptake as in our study (Meiergerd et al., 1993b). In both studies, the effects were inhibited by dopamine D_2 blocker sulpiride, a study in agreement with D_2 receptor able to modulate DAT function and cell surface expression (Meiergerd et al., 1993b; Mayfield and Zahniser, 2001). We have demonstrated (for the first time) through *in vitro* radiometric assay an inhibitory effect of insulin on quinpirole-induced ^{3}[H]- [DA] uptake; a mechanism that is mediated by D_2 dopamine receptor. Whether this indicates that dopamine D_2 and insulin receptors can negatively (directly or indirectly) interact would be an interesting question to address. Data presented on behavioural studies further corroborate these neurochemical findings. However, we found that insulin pretreatment did not have a significant effect on d-amphetamine-induced locomotor activity when compared with the control. Furthermore, more locomotor activity was exhibited during the first 15 mins of observation in insulin pretreated group. Previous studies on the role of insulin in the behavioural manifestations of organic reinforcers and substances of abuse show that, insulin given in acute dose facilitates clearance of dopamine by enhancing the cell surface expression of DAT in the dopamine nerve endings (Figlewicz and Benoit, 2009; Lute et al., 2008a; Owens et al., 2005a; Williams et al., 2007b). Furthermore, it has been demonstrated that DAT

is the principal target for psychostimulant such as d-amphetamine (Rothman and Baumann, 2003), insulin receptors activation might probably play a key role in influencing cognitive and motivational functions of D-AMP (Giros et al., 1996). More homeostatic role of insulin in neurotransmission revealed that insulin receptors activation through activation of insulin receptors (IRS-2)/phosphatidylinositol 3-kinase (PI3K) pathway, maintains dopamine transporter function in the dopamine nerve endings, which is necessary for DAMP to facilitate dopamine neurotransmission (Khoshbouei et al., 2004; Lute et al., 2008a; Williams et al., 2007c). Therefore, our data indicate that insulin pre-treatment may worsen D-AMP effect at least in the first 15 minutes of its administration. Insulin also reduced DA uptake through attenuation of DAT activity to enhance the synaptic output of DA. In the presence of D-AMP, this effect was initially potentiated but later faded out. The insulin effect on D-AMP-induced motor activity is more of modulatory role rather than direct impact on uptake and release. A negative interaction with dopamine D_2 autoreceptor, as we have found, may slow down the negative control of dopamine release exerted by these receptors and in turn further increase DA release.

In another series of behavioural studies, we introduced diazoxide 150mg/kg (a drug preventing insulin release) for the first time to evaluate the effect of an absence of insulin hence; we made the rats acutely hypoinsulinemic. Our finding reveals a marked reduction of locomotor activity in DZ-pretreated group throughout the observation period. Previous studies have adopted the use of streptozocin to induce hypoinsulinemia or render the animal diabetic (Marshall, 1978; Owens et al., 2005a). In experimentally diabetic animals, there is a reduction of locomotor activity induced by D-AMP via insulin- mediated DAT activity in the striatum (Williams et al., 2007b). It has also been reported that insulin receptor knockout mice (NIRKO) exhibited insulin resistance causing alteration in dopamine turn over as well as behavioural disorders (Kleinridders et al., 2015b). Interestingly, other studies have

demonstrated that streptozocin-induced hypoinsulinemia reduced the dopamine-releasing effect of D-AMP via the blockage of PI3K signaling of InRs and attenuation of surface expression of dopamine transporter activity in the striatum (Lute et al., 2008b). It is possible also that change in DA clearance in DZ-treated rats are due, at least in part, to hyperglycemia resulting from insulin inhibition. However, according to our glycaemia data (Fig. 19A), it is unlikely that glucose in the brain could be in a concentration high enough to interact directly with the dopamine transporter. All together, these studies suggest that behavioural effects of amphetamine mediated by striatal DA release maybe insulin-dependent through DAT regulation. We further tested the effect of insulin status on D_2/D_3 dopamine agonists mediated behavioural traits such as; yawning, pica eating and pelvic grooming. Data from this study indicate that alteration in circulating insulin could significantly affect the behavioural activities of molecules acting on dopamine receptors. Our results also demonstrate that the activity of dopamine transporter and D_2/D_3 receptors is distinctively influenced by insulin status in rats. Quinpirole and PPX are D_2-like family receptor agonists, with different affinity for D_2 and D_3 receptor subtypes respectively (Kebabian et al., 1997). The main finding in our study is that insulin significantly reduced quinpirole and pramipexole-induced yawning, penile grooming and pica eating. Altszuler et al., (1977) earlier reported that DZ hyperglycemia is not mainly due to reduced insulin secretion, but may be directly linked to enhanced production of glucose and subsequent blockage of glucose uptake. Therefore, reduced behavioural sensitivity in DZ-treated rats compared with the insulin-treated group may be due to the differential level of glucose homeostasis that influences dopamine receptors density and signaling processes (Abbracchio et al., 1989; Lozovsky et al., 1981). We cannot exclude that the behavioural effect caused by lack of insulin is also due to an increase in glucose concentration. However, intracerebral glucose concentration is not regulated in the same way as peripheral glucose concentration and is much lower, suggesting

that the impact of a high concentration of glucose in the plasma will be possibly less significant in the brain. Albeit, an adequate concentration of glucose in the brain is of course absolutely essential for brain function. Also, from our data glycaemia is only increased by 2-3 times and it is not sure that it will be enough to cause important neurochemical alteration.

We also hypothesize that the near deficit in yawning, pica eating and pelvic grooming observed in the two groups might express reduced sensitivity in both receptor sub-types. Consequently, reduction in behavioural effects of D_2/D_3 receptor in DZ-treated rats might confirm the alteration in the dopaminergic transmission that takes place when insulin status is altered. We infer from this study, that changes in insulin and glucose status affect sensitivity to behavioural effects of dopaminergic drugs (Boucher et al., 2014; Caravaggio et al., 2015). The results also emphasize the vital role of insulin in modulating dopamine transmission, as well as understanding the comorbidity of eating disorders and addiction. Apparently, at the molecular level, insulin receptor signaling interferes adversely with dopamine receptor signaling and potentially amplifies action potential-DA dependent transmission in the mesolimbic DA pathway.

Our *in vivo* electrophysiological studies provide new information on the impact of insulin on the electrical activity of pyramidal neurons in the prefrontal cortex (PFC) as well as the dopamine neurons within the ventral tegmental area. First, we tested how intravenously injected insulin in anaesthetized rats modulates pyramidal neurons in the PFC. We demonstrate that insulin via i.v route slightly increased the excitatory effect of these neurons (Fig. 22A, B). Although the effect is very modest, some neurons appear to be sensitive to insulin. This might be as a result of insulin differential time-dependent process in crossing the blood-brain barrier (Banks and Kastin, 1998; Banks et al., 1997b). Hence, this physiological barrier prevents an optimum concentration of insulin from reaching the brain to activate insulin receptors. In support of this hypothesis, Csajbók and Tamás, (2016) propose that the

rate of pancreatic insulin transportation into the cerebrospinal fluid is much slower than the operating speed of neural networks. This leads to a slow supply of insulin to the brain by the pancreatic β-cell of the islet of Langerhans albeit; the neuronal insulin released locally in the brain provides more rapid means of neuromodulatory effect on the neural circuit. As a result, at any given time the plasma insulin concentration is usually higher than that in cerebrospinal fluid (Strubbe et al., 1988; Wallum et al., 1987). Zhou et al., (2017) recently demonstrated that locally administered insulin in the anterior piriform cortex (APC) increase the excitation of pyramidal neurons and resulted in a negative feedback inhibition of synaptic transmission performed on slice recordings. Data from experimental animals as well as epidemiological studies in humans suggest a biological correlate between insulin and cognitive performance (Strachan, 2005b). In addition, findings from (Reger et al., 2006; Shemesh et al., 2012), have revealed that intranasal insulin administration provides a therapeutic advantage in Alzheimer's patient by aiding memory performance. Since most of the excitatory inputs received by the pyramidal cells are glutamatergic, we can suggest that insulin effect on the pyramidal neurons potentially results in cognitive enhancement.

Since insulin is associated with cognitive and motivational processes which are linked with dopamine transmission, as well as insulin receptors activation (Labouèbe et al., 2013a); we tested this drug on VTA dopamine neurons' firing activity. Similar to PFC recordings, insulin on VTA dopamine neurons via intravenous injection did not show a significant change in the firing activity of the neurons (Fig. 22C), albeit, there was a modest decrease with high doses of insulin. High dose of insulin caused a reduction in VTA DA activity and through indirect activation of PFC pyramidal neuron this may enhance cognition. Our studies on insulin local administration through intra-VTA revealed a significant reduction in population firing activity when compared with the baseline. The data in this study suggested a mechanism by which insulin modulates dopamine transmission in the mesolimbic pathway.

Labouèbe et al., (2013) further reports that insulin induced a long-term depression (LTD) of excitatory postsynaptic currents released as a result of VTA dopamine neurons in mice's brain slices. This neuroadaptive mechanism (LTD) represents synaptic plasticity in the brain and is suggested to be associated with learning, memory retrieving and storage as well as information task. More importantly, in an *in vitro* study, long-lasting inhibition on VTA dopamine neurons is thought to be mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) excitatory synaptic transmission. Insulin-enhanced LTD has also been reported in the cerebellum (Man et al., 2000) and hippocampus (Wang and Linden, 2000). In addition to the significant reduction in firing activity following intra-VTA administration of insulin, we also report that the burst activity was significantly attenuated when compared with controlled animals. Findings from Labouèbe et al., (2013b) revealed that insulin in the VTA attenuates excitatory synaptic transmission and reduces burst activity of DA neurons. Together with all the studies, our findings and other articles highlighted show the vital connection between the functions of insulin and neurotransmission.

In conclusion, our behavioural data shows that insulin remarkably impairs dopamine-dependent behaviours induced by D_2/D_3 agonist. We infer a possible correlation between behavioural anomalies and suboptimal brain's insulin level, as also evident by the reduced locomotor effect to D-AMP following administration of DZ, an insulin release inhibiting agent. The fact that insulin weakens QNP and PPX-induced behavioural and neurochemical effects (dopamine uptake) suggest a potential interaction with D_2 /D_3 receptor signaling. A significant inhibitory effect on VTA dopamine neurons by insulin was observed when locally administered.

Chapter III- Electrophysiological and neurochemical effects of anti-diabetic sulphonylurea tolbutamide in the rat' ventral tegmental area and prefrontal cortex

III-1-Introduction

Tolbutamide is an orally administered sulphonylureas, used in the management of non-insulin-dependent diabetes mellitus (Fulton and Bell, 1957; O'donovan, 1959) and as an adjuvant therapeutic agent in insulin-dependent diabetes mellitus (Fabrykant and Ashe, 1959; Pontiroli et al., 1984). This class of drug acts on the sulphonylureas receptors through blockage of potassium adenosine triphosphate (ATP-regulated K^+-) channels to release insulin from pancreatic β-cell (Liss et al., 1999). In the presence of diazoxide, a K_{ATP} channels opener, the signalling process is reversed as the propagation of action potential initiation is shut down resulting in inhibition of insulin secretion (Fig. 25). Anatomically, Katp channels has on it sulphonylurea receptors, these receptors (SUR) are activated by TBT leading to blockage of Katp, this action is reversed by DZ. Also, some studies have shown the important modulatory effect of the channels on the excitability of substantia nigra neurons (Peltier and Vecchio, 1961; Röper and Ashcroft, 1995; Schiemann et al., 2012a) and dopaminergic midbrain neurons (Liss et al., 1999). Interestingly, Liss et al., (1999) further revealed that dopaminergic substantia nigra (SN) neurons express K-$_{ATP}$ channels subtypes with different sulphonylurea-sensitive receptors. These channels are configured in pore-forming subunits namely, Kir6.1 and Kir6.2 as well as a sizeable supplementary subunit, the sulfonylurea receptor (SUR1, SUR2A, and SUR2B) (Tinker et al., 2018). Thus, the Kir6.0 series is part of the inwardly rectifying member of potassium channels (K_{ATP}) while; the SUR is part of the adenosine triphosphate (ATP)-binding class of proteins.

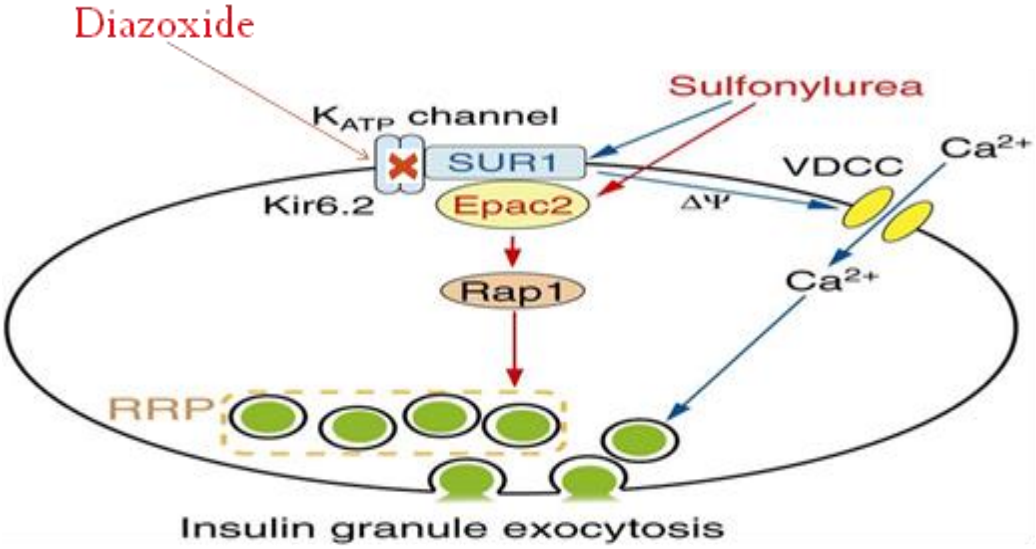


Fig. 25: Model of K_{ATP} channels modulation of insulin pancreatic beta-cell signalling (Babenko et al., 1998)

It was also reported that ATP responding K_{ATP} channels are termed as such, due to capacity to open when cellular ATP amount drops. This results in membrane hyperpolarization and hence relates cellular metabolism and neuronal excitability (Tinker et al., 2018). More *in vitro* studies demonstrate that activation of these channels differentially enhance degeneration of SN dopamine neurons (the hallmark of Parkinson disease), but not VTA dopamine neurons (Liss et al., 2005). The mechanism for this selective harmful effect on SN DA neurons was reported to be due to a process of K_{ATP} (Kir6.2) genetic mutation that led to a selective rescue of SN (apoptosis), but not in VTA DA neurons (Liss et al., 2005). In the pancreatic ß-cells, *in vitro* (Fridlyand et al., 2010a) and *in vivo* (Gomis and Valdeolmillos, 1998a) studies show that K_{ATP} channel opening enhances burst firing activity.

Nevertheless, there is very little documented data about the presence of these receptors, as well as their modulatory roles in the ventral tegmental area (VTA) and prefrontal cortex (PFC), two important brain areas involved in the control of our emotion. In the present study, we have investigated whether this drug can modulate neuronal activity in VTA and PFC using *in vivo* extracellular single-unit recordings, in anaesthetised rats. We also examined *in vitro,* the effects of this drug on dopamine transporter activity using striatum synaptosomes, as well as performed qualitative estimation of Kir6.2 channels distribution in specific brain regions such as PFC, striatum and the midbrain through the use of western blot technique.

III-2- Materials and methods

III-2-A-Subjects

Please refer to paragraph I-2-A for detail information on animal conditions and regulatory protocol on a study involving rodents.

III-2-B- *In vivo* extracellular single-unit electrophysiology

Please refer to chapter I for a description of electrophysiological experiments. All recordings in the ventral tegmental area (anteroposterior -4.5 to -5.5 mm to Bregma, lateral 0.3-1.2 mm, 7.2-9.5 mm below the cortical surface) were made in chloral hydrate (400 mg/kg) anaesthetised rats. Dopamine neurons were identified according to criteria summarised by (Ungless and Grace, 2012b). Only presumed dopaminergic neurons generally presenting a notch in the rising phase, a prominent negative compound and a time greater than 1 ms, from the start of the depolarization to the end of the repolarisation, were selected in our study. We also tested TBT on few identified non-dopamine neurons in the midbrain that did not fulfilled VTA dopamine neurons criteria (main criteria was action potentials (AP)

duration < 0.9 ms calculated from the spike propagation to the negative peak phase of the AP), as well as, when possible a lack of inhibition, or even an activation, of the neurons by systemic administration of low dose dopamine D_2 agonist (25 µg/kg i.v apomorphine) (Ungless and Grace, 2012a). Urethane (1.5g/kg) anaesthetized rats were used for recording pyramidal neurons in the prefrontal cortex (PFC, anteroposterior 2.5 to 3.5 mm to Bregma, lateral 0.3-1.7 mm, 1.5-5.5 mm below the cortical surface). Presumed pyramidal PFC neurons were identified according to previous electrophysiological criteria (Gronier, 2011b; Wang et al., 2011). A broad action potential (>1 ms), with a biphasic or triphasic large waveform action potential, starting with a positive inflection, a relatively slow firing rate (1-50 spikes/10 sec) and an irregular firing pattern, often with burst activities Outputs from the electrode were sent to a Neurolog AC pre-amplifier and amplifiers (Digitimer, UK). In some of the *in vivo* experiments involving tolbutamide, small volumes of a 5 % glucose solution were administered to prevent hypoglycaemia. However, this procedure was found to be unnecessary as we found that anaesthetic condition leads to a permanent state of hyperglycaemia, totally insensitive to insulin or insulin secretagogues like sulphonylurea (indicate if you have a figure showing that). Blood glucose levels were monitored with Accu-Check glucometer. Signals were filtered and sent to an audio amplifier, a Tektronix 2201 digital storage oscilloscope, and a computer running Spike 2, for data capture and analysis. Please refer to paragraph I-2- for detail information on animal conditions and regulatory protocol on a study involving rodents.

III-2-C- ^{3}[H] Dopamine uptake studies by striatal synaptosomes

Striata were isolated and used to prepare synaptosomes according to (Dunkley et al., 2008) with minor modifications as indicated in the use of not more than 5 strokes during homogenization of synaptosomes. Please, refer to chapter I for detail description of radiometric uptake protocols. Dopamine uptake was assayed using [^{3}H] dopamine, 10 nM)

and synaptosomes were incubated with tolbutamide 30 µM or diazoxide 100 µM in a total volume of 400 µl for 30 min at 37°C. Non-specific dopamine uptake was measured in the presence of 10 µM methylphenidate (dopamine uptake inhibitor). Dopamine uptake was stopped by addition of 3 ml ice-cold Krebs–Ringer buffer. The suspension was immediately filtered under vacuum through Whatman GF/B filters. Radioactivity was determined by liquid scintillation spectrometry. Specific [^{3}H] dopamine uptake was defined as the difference between dopamine accumulated at 37°C in the presence and absence of methylphenidate.

III-2-D- Western blot studies on Kir6.2 channel expression in specific brain regions

Naïve rats (*n=5*) were administered an overdose of chloral hydrate followed by rapid decapitation. The brain tissues were immediately dissected on an ice-cold platform and before being immersed in liquid nitrogen. Tissues were then stored at -80^{o}C until further analysis. The Western blot technique is employed for the detection of specific protein molecules from among a complex mixture of proteins. These mixtures may include all the proteins associated with a particular cell type or tissue. Western blots (WB) are used in evaluating protein expression or for assessing the size of a protein of interest (Kurien and Scofield, 2015; Mahmood and Yang, 2012a). In this chapter, the western blot technique was used to qualitatively evaluate the proportion of Kir6.2 protein expression in specific brain regions such as PFC, striatum and midbrain (containing the VTA). The first major step in a WB is sample preparation, where samples are mixed with a detergent, usually sodium dodecyl sulphate (SDS), which unfolds the proteins in the sample into linear strands and then coats them with a net negative charge (Kurien and Scofield, 2006; Mahmood and Yang, 2012b). The protein molecules are then separated according to their sizes via gel electrophoresis. After separation, the proteins on the gel are transferred onto a blotting membrane (e.g. nitrocellulose membrane). Following a successful protein transfer, the nitrocellulose membrane would carry all of the protein bands originally seen on the gels. The

membrane then goes through a treatment process called ‘blocking’ (carried out using non-fat dry milk or bovine serum albumin), which prevents the occurrence of any non-specific reactions. To detect a specific protein from the protein mixture on the membrane, the membrane is incubated with a primary antibody that specifically binds to the target protein. After primary antibody incubation, any unbound antibody is washed off with a buffer (e.g. using a tris buffered saline with tween i.e. TBST buffer). The membrane is incubated again but this time with a secondary antibody that recognizes and binds specifically to the primary antibody (Fig. 27). The secondary antibody used in this thesis was linked with a reporter enzyme (e.g. HRP i.e. horseradish peroxidase) that produces colour or light, permitting it to be detected, imaged and quantified (Kurien and Scofield, 2006; Mahmood and Yang, 2012b). Through these processes, therefore, the Western blot technique allows the detection and quantification of target proteins from among a complex mixture of proteins.

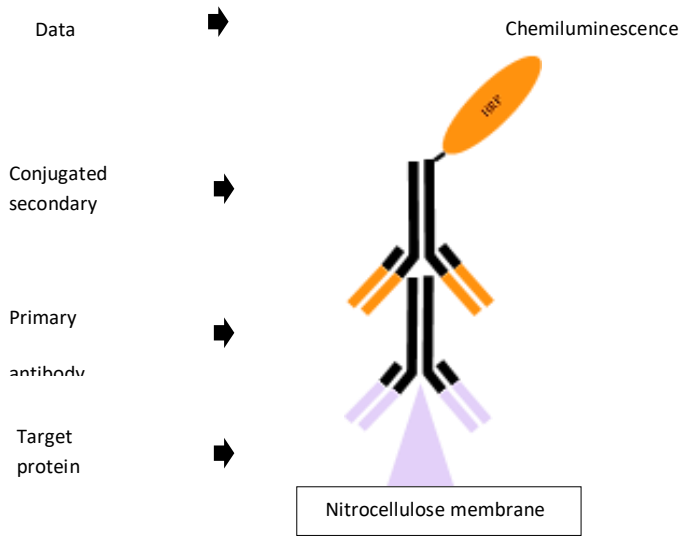


Figure 26: The primary and secondary antibody complex in Western blotting.

The primary antibody binds to the target protein and then a secondary antibody linked with a reporter gene (e.g. HRP – horseradish peroxidase) then binds to the primary antibody. This enzymatic complex can then be detected upon addition of chemiluminescence substrates.

Preparation of brain samples

A 1:1000 dilution of protease inhibitor and RIPA lysis buffer (Cocktail SIGMA) mixture was prepared. 500 µl of this mixture was added to each of the dissected brain regions of interests namely: frontal cortex, striatum and the midbrain. The tissue samples were then homogenised using Polytron PT318 (Kinematica, Switzerland) and then centrifuged for 10 mins at 13,000 g and 4°C using a benchtop temperature-regulated centrifuge (Sorvall Legend Micro 17R, Thermo Scientific, UK). The supernatant was extracted and 100 µl of the extract added to 100 µl of 2x electrophoresis buffer (5% β-2-mercaptoethanol, 20% glycerol, 0.5 M Tris-HCl of pH 6.8, 0.006% w/v bromophenol blue and 10% sodium dodecyl sulphate). The resultant mixture was heated at 90°C for about 3 mins and then stored at -80°C for later use. The remaining supernatants were used in estimating the concentrations of the total proteins using Bradford assay kit (Sigma Aldrich, UK), which allowed for equal protein concentrations (20 µg/µl) of the brain samples to be loaded. According to Lambert-Beer's law, the Bradford assay curve should produce a straight line curve. In practice, you may get a curve at high protein amounts. Disregard the curved data and also any data points that are clearly outliers. Then we use linear regression to calculate the slopes *b* of the lines, we make sure 0 µg standard was included and test for any signal produced by the buffer

Estimation of brain tissue protein concentration

A standard protein curve was generated from the Bradford assay by diluting 1.0 mg/ml of bovine serum albumin (BSA) in distilled water to yield a range of concentrations from 0 - 1 mg/ml at intervals of 0.1 mg/ml. The protein standards were made in duplicate. From the 1.0 mg/ml BSA stock, the standards were prepared as shown below:

- BSA stock (100 µl) 1.0 mg/ml
- 90 µl stock + 10 µl diluent 0.9 mg/ml

- 80 µl stock + 20 µl diluent 0.8 mg/ml
- 70 µl stock + 30 µl diluent 0.7 mg/ml
- 60 µl stock + 40 µl diluent 0.6 mg/ml
- 50 µl stock + 50 µl diluent 0.5 mg/ml
- 40 µl stock + 60 µl diluent 0.4 mg/ml
- 30 µl stock + 70 µl diluent 0.3 mg/ml
- 20 µl stock + 80 µl diluent 0.2 mg/ml
- 10 µl stock + 90 µl diluent 0.1 mg/ml
- 0 µl stock + 100 µl diluent 0.0 mg/ml

Along with the protein standards, tissue homogenates were also diluted 1 in 8 with distilled water and loaded into a 96-well plate (Multiwell plate, polystyrene 96-well, Fisher Scientific, UK). 200 µl of Bradford reagent (Sigma-Aldrich, UK) was then added to each well. The 96-well plate was mixed gently, covered with a foil and incubated at 37°C for 30 mins. Following incubation, the plate was allowed to cool for 10 mins, mixed gently and the foil removed before being placed in a microplate reader (uQuant^{TM}, BioTek, USA) set at 595 nm. Before the calculation of the concentration (µg/µl) of protein in each sample, the standard curves were checked for outliers.

SDS-PAGE electrophoresis

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the BIO-RAD Mini-Protean system. Glass plates (0.75 mm) along with its spacer plates (BIO-RAD, UK) were mounted in support cassettes. Before sealing the cassettes, the lower borders of both the glass plates and their spacers were checked to ensure correct alignment. The gel cassettes were then clamped against a casting frame, which allowed a level pouring of gels. To maximise the resolution of protein molecules, a discontinuous SDS-PAGE system

was employed. Both the stacking and the separation (resolving) gels were prepared with standard acrylamide gel reagents using concentrations estimated from the Chang Bioscience website (http://www.changbioscience.com/calculator/sdspage.html). A 10 ml of 12% separation gel (for 10 – 200 kDa proteins) was prepared as follows:

- 30% Polyacrylamide 4.0 ml
- 1.5 M Tris-base (pH 8.8) 2.5 ml
- 10% Ammonium persulphate 0.1 ml
- 10% SDS 0.1 ml
- TEMED (tetramethylethylenediamine) 0.004 ml
- Distilled water 3.3 ml

A 5 ml of 5% stacking gel was also prepared as follows:

- 30% Polyacrylamide 0.85 ml
- 1.0 M Tris-base (pH 6.8) 0.63 ml
- 10% Ammonium per sulphate 0.05 ml
- 10% SDS 0.05 ml
- TEMED 0.005 ml
- Distilled water 3.4 ml

The prepared separating gel solution was gently inverted two or three times and then dispensed into the space between the mounted glass plate and the spacer plate of the gel cassettes. To ensure a smooth surface, a small layer of isopropanol was placed at the top of

the separating gel and left for 30 mins to polymerise. After polymerisation, the isopropanol was decanted and the stacking gel solution was prepared and poured on top of the separating gel. A 15-well comb (3 mm x 10 mm, BIO-RAD, UK) was then inserted gently in the gel, refilled and left for an hour to polymerise. Subsequently, the wells were thoroughly rinsed with distilled water to remove excess polymerised acrylamide. The gels were then connected to the electrophoresis tank and then filled with 1x running buffer prepared from a 10x stock. A litre of the 10x running buffer stock was prepared as follows:

• Tris-base	30.3 g	25 mM
• Glycine	144 g	250 mM
• SDS	10 g	1%
• Distilled water	up to 1000 ml	

Equal concentrations (20 µg/µl) of each tissue sample were loaded into the wells. The first and the last wells contained 2 µl of all blue precision protein standard (BIO-RAD, UK) and 2 µl of 2x electrophoresis buffer (negative control), respectively. The standard protein aided in the later estimation of the molecular weight range of the proteins of interest. Before loading, all the samples were denatured at 90°C for 3 mins. The samples were initially run at 120 V for 15 mins to allow sample passage through the stacking gel followed by 100 V for 45 mins. The bromophenol blue tracking dye present in the electrophoresis (loading) buffer and/or the all blue standard protein marker allowed the visualization of protein movement in the gel.

Protein transfer

The separated proteins were transferred from the gels onto a Protran^{TM} nitrocellulose membrane (GE Healthcare, UK) via a transfer sandwich and an electroblotter. The transfer sandwich consisted of two pieces of white scotch-brite pads (200 mm x 160 mm, BIO-RAD,

UK), two pieces of 3 mm Whatman filter papers (Fisher Scientific, UK) and a piece of nitrocellulose membrane. The transfer sandwich was arranged as follows:

- Scotch-brite pad
- Whatman filter paper
- Gel
- Nitrocellulose membrane
- Whatman filter paper
- Scotchbrite pad

The black side of the transfer cassette was placed on the workbench and each element in the transfer sandwich was soaked in a transfer buffer, and then stacked on the cassette with air bubbles being dispelled at each stage by rolling sterile glass pipette over the sandwich. The cassette was then sealed and inserted into a Mini trans-blot tank (BIO-RAD, UK), while taking note of the colour codes (red for red). The transfer tank was filled with cold (4°C) transfer buffer. One litre of 1x transfer buffer (pH 9.2) was prepared as follows:

• Tris-base	5.82 g	48 mM
• Glycine	2.93 g	39 mM
• Methanol	200 ml	20%
• Distilled water	up to 1000 ml	

The transfer set up was then run at 100 V for 1 h. Following the transfer procedure, the nitrocellulose membranes were stained a Ponceau S dye to allow an estimation of transfer efficiency. The nitrocellulose membranes were immersed in the Ponceau S dye and left on a shaker for 5 mins. After staining, the nitrocellulose membranes were placed in a destaining

solution (5% glacial acetic acid) and left on the shaker for 5 mins. The destaining solution was changed 3-5 times until clear bands with almost no pink background were obtained. A 0.1% (x/v) Ponceau S dye in 1% (v/v) acetic acid was made as follows:

- Ponceau S dye 0.033 g
- Glacial acetic acid 0.3 ml
- Distilled water up to 30 ml

Dissolving 5 ml of the 100% glacial acetic acid in 95 ml distilled water also yielded a 100 ml of 5% glacial acetic acid.

Immunoblotting

Following Ponceau staining, a 10x TBS stock was (24 g Tris-base, 88 g NaCl, distilled water up to 1000 ml, pH 7.6) prepared. The membranes were blocked with 3% (w/v) non-fat dried milk in 1x TBST (0.1% Tween 20 in 1x TBST; blocking buffer) for 1 h at room temperature to prevent non-specific antibody binding. The 3% non-fat dried milk blocking buffer was observed to produce better quality bands with less background compared to 5% non-fat dried milk buffer. Primary antibodies were incubated overnight at 4°C in blocking buffer. Appropriate dilution ratio was used according to manufacturer's specifications (anti-potassium channel Kir6.2 antibody, 1:200, and predicted molecular weight, 44kDa). Following overnight exposure to the appropriate primary antibodies, the membranes were washed three times with 1x TBST for 5 mins each time, to wash off unbound antibody. The membranes were then incubated with the relevant secondary antibody in blocking buffer. In accordance with the enhanced chemiluminescence (ECL) system (Amersham Biosciences, UK), the secondary antibodies (bs-0295G-HRP 1:2000) used was linked to the detection

enzyme, horseradish peroxidase (HRP). After secondary antibody binding, the membranes were again washed three times with 1x TBST for 5 mins each. Following the last wash, the membranes were developed via the ECL plus detection kit (Amersham Biosciences, UK), which allows the emission of light produced in a multistep reaction in which peroxidase catalyses the oxidation of luminol. In this procedure, the nitrocellulose membranes were incubated with ECL reagent A (luminol), and reagent B (peroxide) for 5 mins (1:1 dilution). After the 5 mins exposure, excess ECL reagents were poured off and the membrane was sealed in a plastic wrap (Saran™ film). The image analysis was carried out by capturing the membrane images on the blot using a CCD-camera-based imager ChemiDoc MP (Bio-Rad).

Membrane stripping and reprobing

For all experiments, the nitrocellulose membranes were stripped following film development. Stripping allowed for the removal of the primary and secondary antibodies from the membranes. The membranes were then re-probed with the rat anti-β-actin monoclonal IgG_1 primary antibody. The β-actin protein was used as a control for the amount of protein loaded into each well. A mild stripping buffer (containing 15 g glycine, 1 g SDS, 10 ml Tween20, distilled water up to 1000 ml, pH 2.2) was prepared. The membranes were washed twice with stripping buffer for 10 mins each at room temperature. The membranes were then washed twice with 1x PBS for 10 mins each, followed by a 5 mins wash with TBST. A 10x PBS stock solution was prepared as follows:

- NaCl 80.0 g
- KCl 2.0 g
- KH_2PO_4 2.4 g
- Na_2HPO_4 14.4 g

- Distilled water up to 1 L
- pH 7.4

The membranes were then blocked for 1 h with 3% non-fat dried milk blocking buffer, after which the immunoblotting protocol was repeated with anti-β-actin primary antibody and an anti-rat secondary antibody.

III-2-E-Drugs

All drugs and antibodies were purchased from Abcam (Biotechnology company, UK), except, tolbutamide (Sigma-Aldrich, UK), primary antibody (anti-potassium channel Kir6.2, Sigma-Aldrich, UK in 1:200 dilution) and secondary antibody (bs-0295G-HRP, Bioss antibodies UK), The drugs were dissolved into Krebs (release assays) and HEPES (uptake) buffers, as appropriate. However, the drugs were dissolved in distilled water or saline for electrophysiological studies.

IV-2-F-Statistical analysis

All results are expressed as the mean +/- SEM. Changes were considered significant if $p<0.05$. This was assessed by using one or two-way ANOVA tests followed by Neuman-*Keuls* or Bonferroni posthoc tests (as appropriate), Student's t-tests and Chi-square, where appropriate. Kir6.2 channel protein quantifications were analyzed using ImageJ software following visual membrane images captured by CCD-camera-based imager ChemiDoc MP (Bio-Rad). The expression of each protein was normalized to the housekeeping protein β-actin. Data were expressed as a percentage of the control group (saline, mean±standard error of the mean).

III-3- Results

III-3-C1- Tolbutamide biphasically alters VTA dopamine neurons' firing activity

In the VTA, the insulin secretagogue tolbutamide (TBT, 20 and 40mg/kg, iv) induced biphasic effects on the basal activity of most of the dopamine neurons tested (Fig. 25A, C) large inhibition (up to 100%) during a relatively short period (1-3 min), caused by hyperpolarization, followed by a long-lasting statistical ($F(3, 37)=14.84$) significant excitatory effect (up to 40% of the baseline activity). Such an increase in neuronal excitability was slightly associated with alteration in the pattern of burst activity in some neurons (Fig. 27B). The proportion of neurons that responded to TBT and saline administration via activation or inhibition was analysed (Fig. 27D). We also noted that there is no significant decrease in glycaemia with anaesthetized animals after the administration of SU, as glycaemia has skyrocketed due to the presence of the anaesthetic.

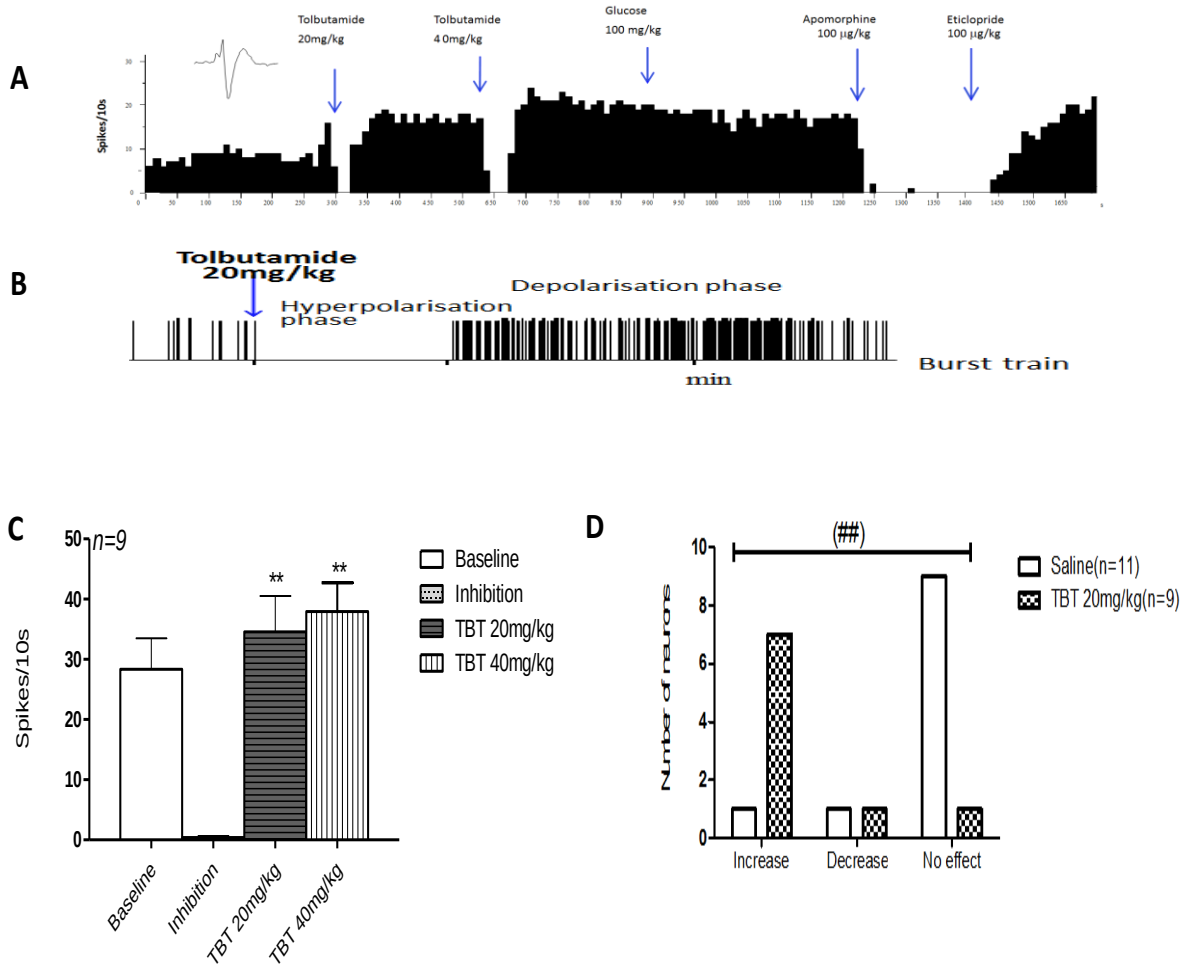


Figure 27: VTA dopamine electrophysiological recording during tolbutamide acute treatment

(**A**): TBT (20 and 40 mg/kg) biphasically altered the firing activity of DA neurons compared to baseline, first by brief inhibtion (1-3 mins) and followed by significant activation. (**B**): The burst activities were enhanced in most neurons tested with TBT. **(C):** TBT (20 and 40 mg/kg) dose-dependently increased the firing rate of DA neurons after the inhibitory phase. **(D):** Comparison between the two treatments, using Chi-square followed by *Fisher's exact* post hoc test. **P<0.01, **P<0.01 *vs.* baseline, *Newman–Keuls* after significant ANOVA. ##P=0.0045, *Fisher's exact* test after significant Chi-square analysis.

III-3-C2-Tolbutamide monophasically alters VTA non-DA neurons' firing activity

TBT (20 and 40 mg/kg, i.v) induced generally a monophasic excitatory effect on the non-DA VTA cell (Fig. 28B, C). Fig. 28A shows neurons with no alteration in firing rate.

Statistical analysis of neuronal responses (inhibition, excitation and no effect) between saline and acute TBT administration (Fig. 28D).

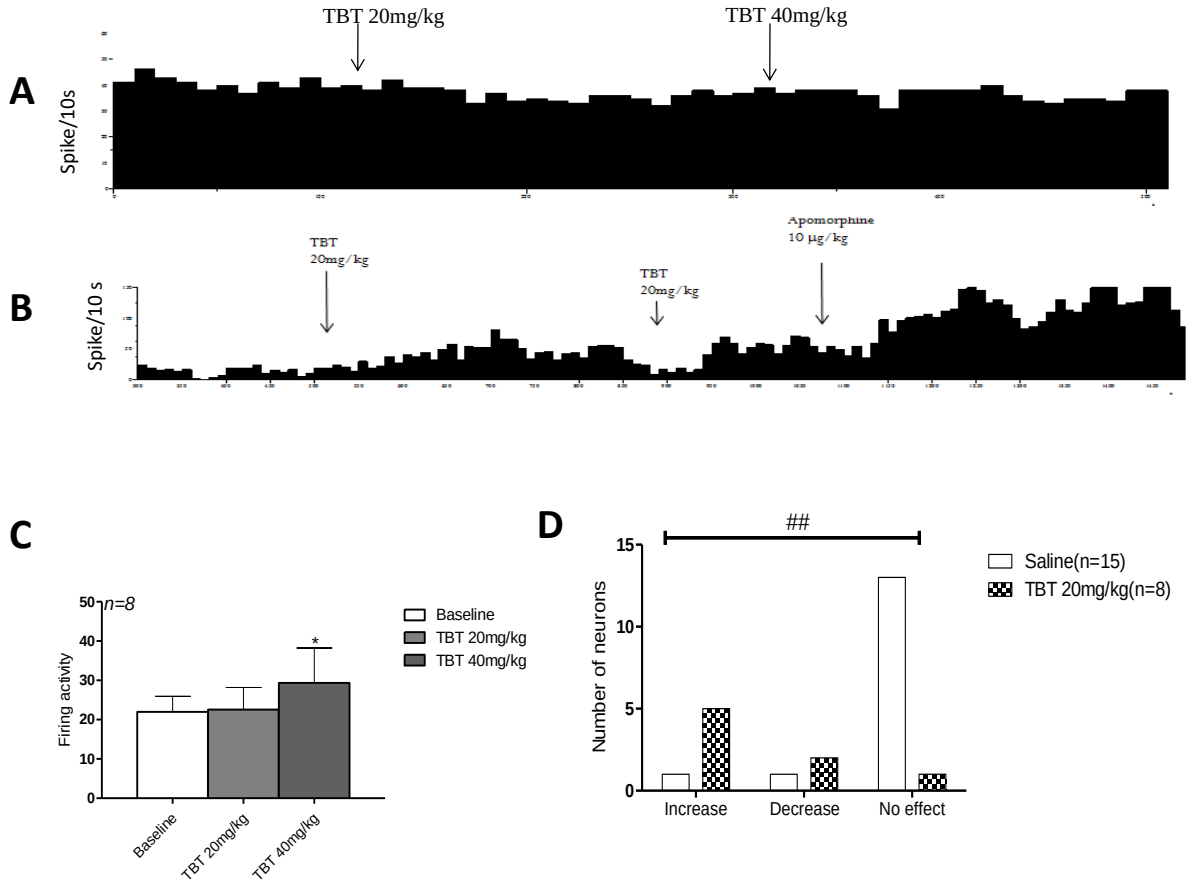


Figure 28: Midbrain non-DA electrophysiological recording during acute TBT administration

(**A**): TBT (20 and 40 mg/kg) did not alter the firing activity of this non-dopaminergic neurons (identified from established criteria) (**B, C**): Another non-DA cell exhibited a monophasic activation of the firing rate upon administration of TBT and apomorphine 10 µg/kg further increased its firing activity. This is a typical excitatory effect of apomorphine on non-dopaminergic neurons. (**D):** Proportion of TBT treated non-DA neurons responses (excitatory or inhibitory from more than 20% of basal firing activity. #P<0.001, *P<0.05 vs. baseline firing; ##P=0.0021, *Fisher's exact* test after significant Chi-square analysis.

III-3-C3-Tolbutamide increases PFC pyramidal neurons' firing activity

In the PFC, tolbutamide (TBT, 20 and 40 mg/kg, iv) acute administration dose-dependently and significantly increased the firing activity of most of the PFC neurons tested (about 75%) but, more usually, in a monophasic way (Fig. 29A, B), than in the biphasic pattern. Figure 29E, F show that the proportion of neurons showing increased firing activity following TBT is significantly higher than the proportion of neurons showing reduced and no effects. Effect of saline on PFC neurons displayed 76% of no response and about 15% of the neurons manifested an increase in firing activity (Fig. 29D, F). We also observed a biphasic neuronal firing in only about 10 % of the pyramidal neurons (Fig. 29C).

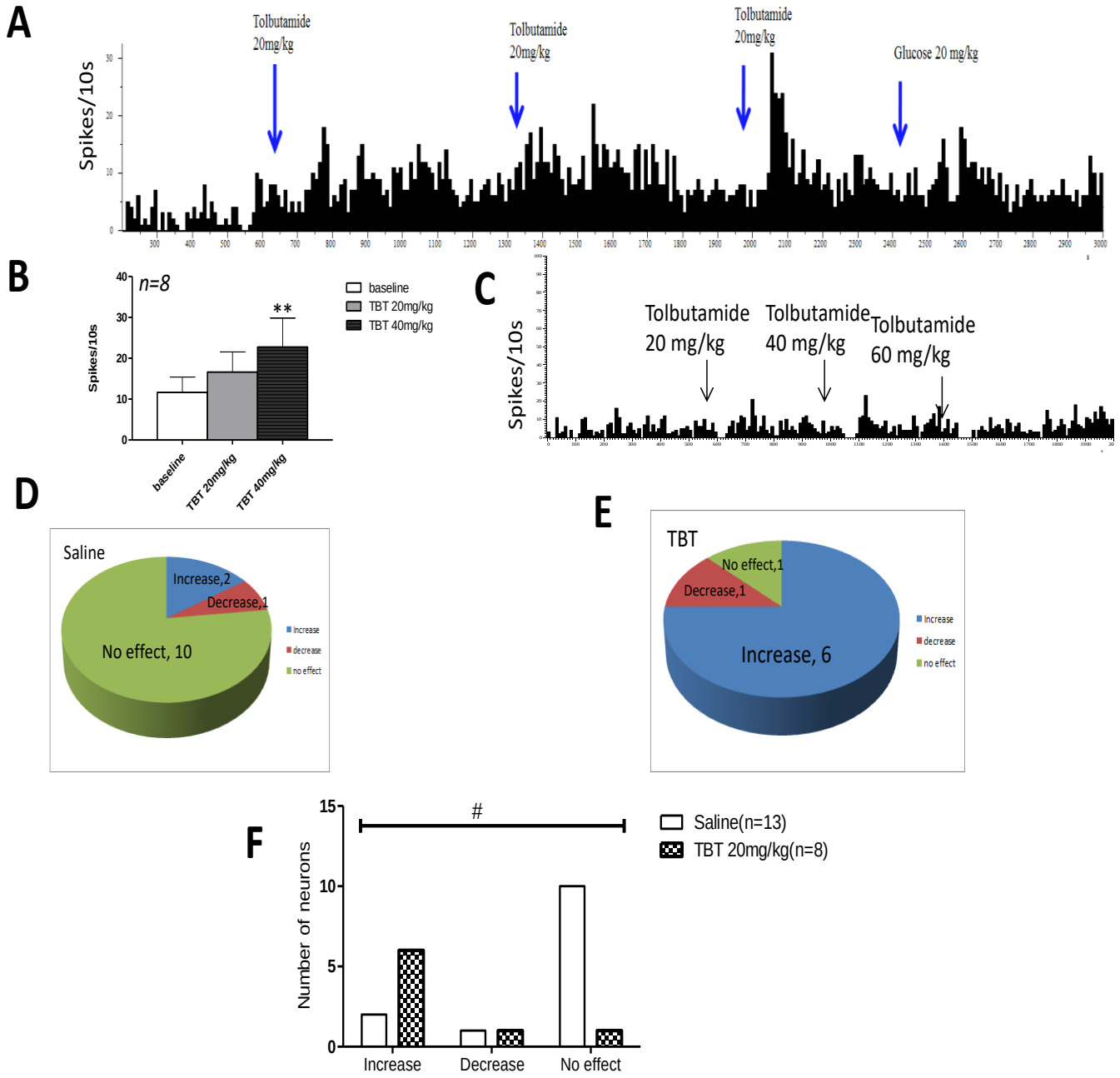


Figure 29: PFC electrophysiological recording during tolbutamide acute treatment

(**A, B**): TBT (20 and40 mg/kg) significantly increased firing activity on pyramidal neurons compared to the baseline firing rate **P<0.001 *vs*. baseline firing, followed by *Newman–Keuls* after significant ANOVA. (**C**): In few a neurons (about 10%), TBT administration induced biphasic effect similar to VTA DA neurons firing (see figure 27). (**D, E**): Proportion of electrophysiological responses (no response, increase, decrease) of PFC neurons to the administration of TBT or to administering saline. (**F**): The proportion of TBT treated pyramidal neurons responses (excitatory or inhibitory from more than 15% of basal firing activity.*P<0.05 vs. baseline firing, Chi-square analysis. #P=0.013

III-3-D1-TBT and DZ reduce synaptosomal^{3} [H] DA uptake to enhance the release

TBT (30µM) shows a significant reduction (t=2.655 df=10) in striatal ^{3}[H] DA uptake compared to the baseline control uptake (Fig. 30A). Similarly, in (Fig. 30B), we observed a paradoxical significant drastic reduction in ^{3}[H] DA uptake with 100µM DZ (t=3.361 df=16) an effect similar to TBT. The reduction with TBT was further significantly enhanced (F (2, 15) =92.56) in the presence of DZ (100µM), a K_{ATP} channels opener (Fig. 30C).

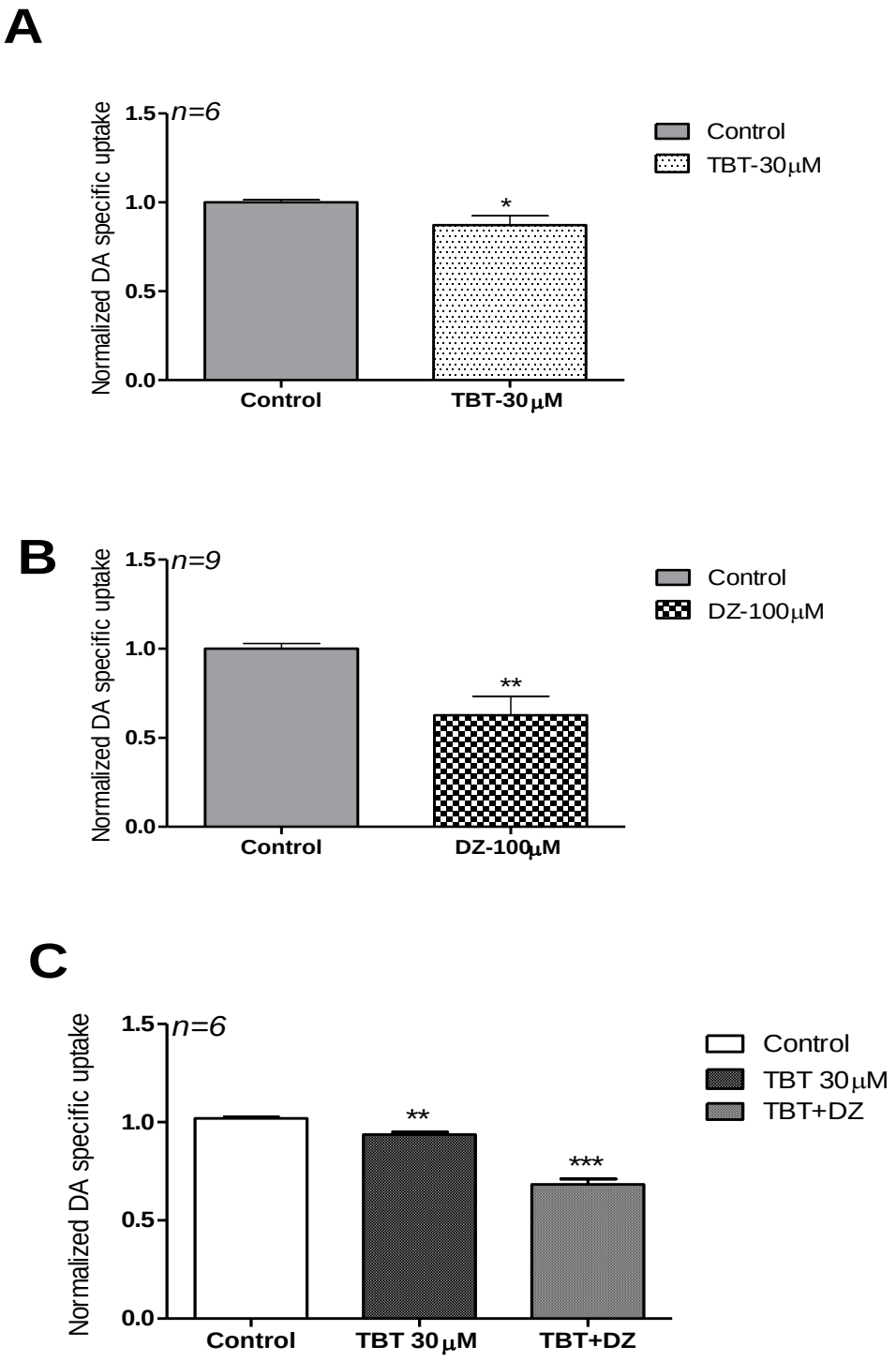


Figure 30: Striatal synaptosomal ^{3}[H] DA uptake of TBT and diazoxide

TBT (30µM) and DZ (100µM) showed a statistical significant reduction in ^{3}[H] DA uptake after 30 mins incubation period *P<0.05, and **P<0.01 respectively, Student's t-test (**A, B**).**C:** In the presence of diazoxide (DZ) 100µM, TBT inhibition on DA uptake was statistically enhanced. **P<0.01, ***P<0.0001 *vs.* control, ANOVA followed by Newman–Keuls after significant one-way ANOVA.

III-3-D2- High strength glucose dose-dependently reduces synaptosomal ^{3}[H]DA uptake

Effect of metabolic change (varying degree of glucose level) on striatal ^{3}[H]DA uptake was carried out. We aimed at testing the hypothesis that metabolic alteration may modulate dopaminergic transmission in the mesolimbic circuits (Narayanan et al., 2010). When the incubation medium for dopamine uptake was enriched to 40 mM, there was no alteration in striatal ^{3}[H] DA uptake when compared to the control (Fig. 31A). However, at 80mM of glucose concentration, striatal synaptosome ^{3}[H] DA uptake significantly ($F=1.021$, $DFn=11$, $DFd=8$) increased slowly in response to this dramatic change (Fig. 31B). The buffer that had a low concentration of glucose showed no changes in striatal ^{3}[H] DA uptake when compared with the control (Fig. 31C).

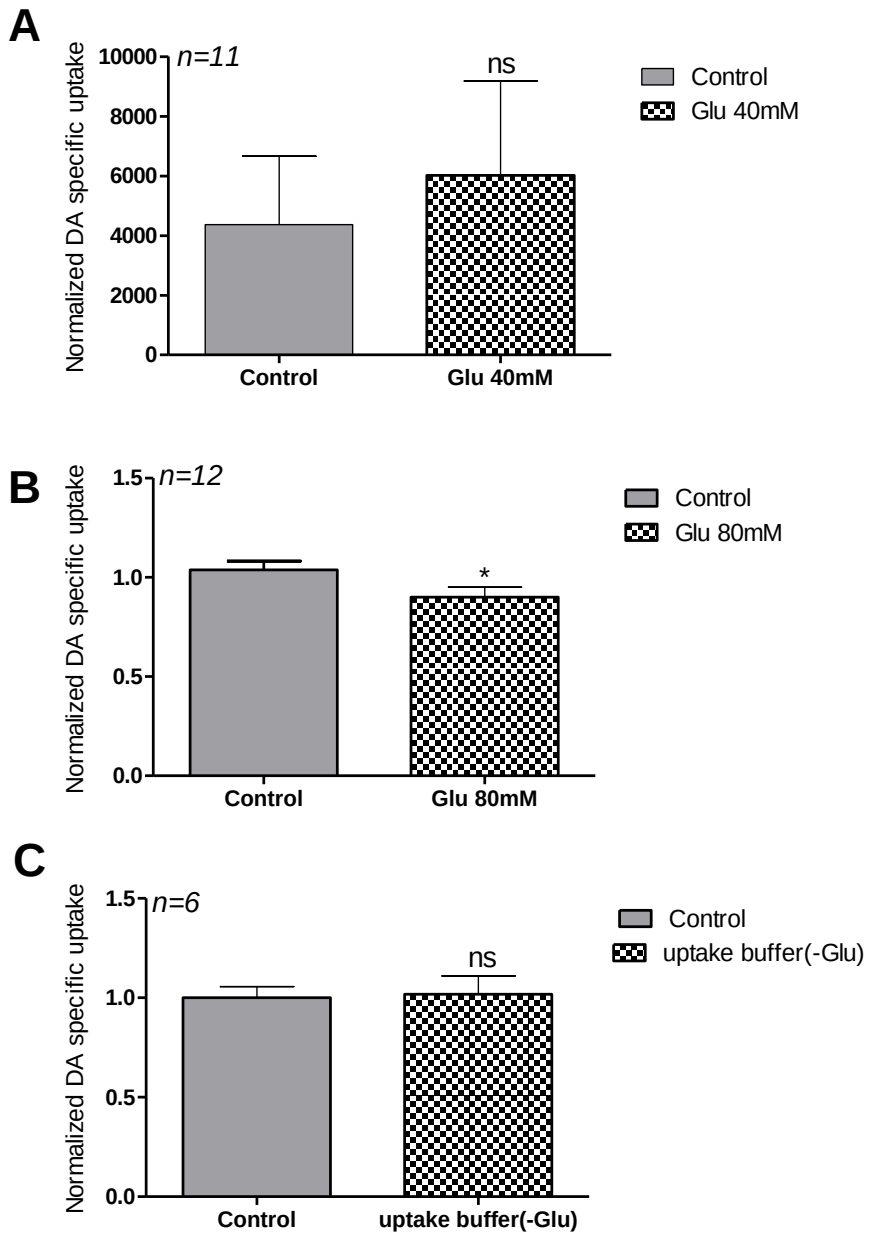


Figure 31: Striatal synaptosomal ^{3}[H] DA uptake of Glucose

(**A**): Glucose (40mM) did not show any alteration in ^{3}[H] DA uptake after 30mins incubation period P=0.6721, Student's t-test (**B):** Glucose (80mM) showed a statistically significant reduction in ^{3}[H] DA uptake after 30mins incubation period *P<0.05, Student's t-test. (**C)**: In the presence of low glucose concentration about 3-4mM there was no alteration in ^{3}[H] DA uptake at any point in time.

III-3-E- Protein expression of Kir6.2 channel on specific brain regions

This experiment aimed to investigate the presence of Kir6.2/K_{ATP} channels, and its proportion in the brain regions (PFC, striatum and midbrain) of interest. Since, our electrophysiology study has revealed that neurons containing Kir6.2/K_{ATP} channel in the PFC and VTA were activated by TBT, which may suggest the functions of the channels in mediating cognitive and reward processes. Our qualitative western blot results indicate that Kir6.2 channel was distributed in the three brain regions in seemly equal amount. Fig. 32A, B, C are the locations distribution of Kir6.2 channels in the PFC, striatum and VTA respectively. Fig. 32D illustrates representative blots from different brain regions namely, PFC, striatum and the VTA.

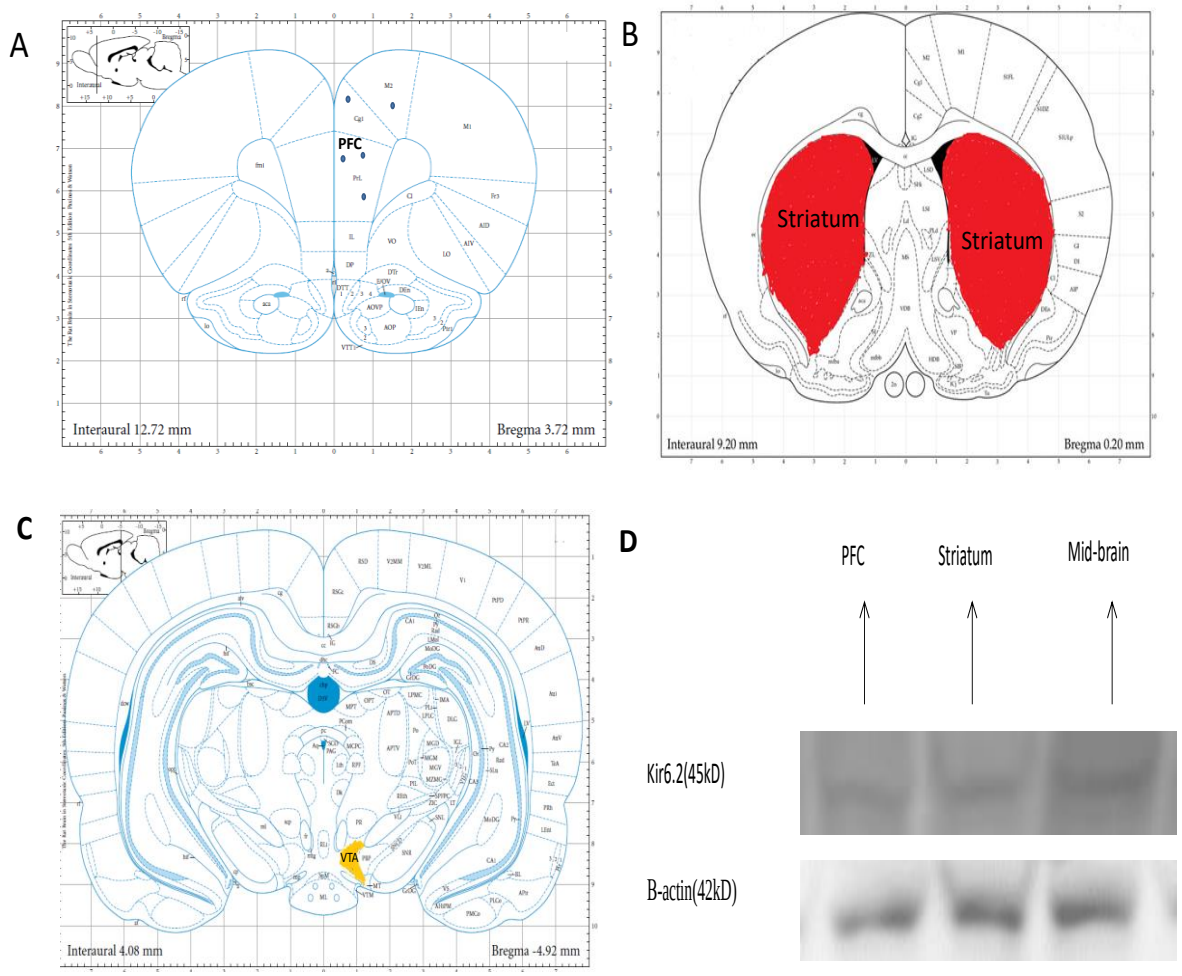


Figure 32: Kir6.2 channels distribution in the PFC, striatum and the midbrain

(A): Kir6.2 channels distribution in the PFC **(B):** Kir6.2 channels distribution in the striatum **(C):** Kir6.2 channels distribution in the midbrain containing the VTA **(D):** Western blots distribution of Kir6.2 channels in the three brain regions. There is limitation to western blot technique due to lack of spatial distribution of the protein.

III-4- Discussion

K_{ATP} channels are abundantly present in many sensitive tissues and act as a regulator of metabolically associated excitation of neurons, as well as control of energy homeostasis (Nichols, 2006). These channels are metabolic sensitive to ATP intracellular changes which are, at least in part modulated by glucose or lactic acid metabolism; thus, when the cells are in energy surplus the channels can be inactivated (Cota et al., 2007), which can promote depolarization and cell activation. Briefly, we evaluate the presence of Kir6.2 channel in all the three brain regions (PFC, striatum and midbrain). Our data demonstrated the presence of Kir6.2 channels in all the three regions, although, we did not quantify the level in each of the region (Fig. 32). To support our findings, previous studies have also demonstrated Kir6.2 channel expression in the cortex and hippocampus (Héron-Milhavet et al., 2004; Moriguchi et al., 2018), striatum (Patel et al., 2011; Shi et al., 2008) and in the SN (Schiemann et al., 2012b). Here, we provide a pilot finding of the expression of Kir6.2 in the midbrain. The VTA, part of the mesolimbic system, is concerned with reward as well as motivation hence, our data shows that manipulation of K_{ATP}/Kir6.2 channels may alter the electrical response of VTA DA neurons which can impact on reward (Ranaldi, 2014). Thus, our data showed that VTA dopamine neurons are sensitive to metabolic status. VTA neurons are important targets for metabolic hormones such as leptin, glucagon-like peptide-1, ghrelin and insulin. Insulin receptors are located on dopamine neurons in the ventral tegmental area (VTA) hence, any change in the level of this hormone affects the VTA neuron firing activity. In the presence of anesthetic with saline administration as control, we did not notice any alteration in the firing rate of the VTA DA neurons. However, in the presence of TBT, marked alteration beginning with inhibition and followed by excitation were noticed resulting from Katp/Kir6.2 channels blockage. The significance of this remains to be established. The results presented show that most VTA dopamine neurons responded to TBT acute administration

generally with inhibition followed by excitation of electrical activity (Fig. 27A). There is now evidence for a possible positive association between nigral dopaminergic neuronal loss, a pathological basis of Parkinson disease (Dauer and Przedborski, 2003; Shimohama et al., 2003), and K_{ATP} channels activation in particular from studies in 6-hydroxydopamine (6-OHDA) treated rats (Piri et al., 2017a). Electrophysiological studies on substantial nigra (SN) dopamine neurons revealed that K_{ATP} channels present in SN DA neurons may be involved in the regulation of burst activity as a genetic depletion of the Kir6.2/K_{ATP} channels in mice significantly reduced the burst activity of SNC neurons (Schiemann et al., 2012b). Recent papers suggest a potential use of Kir6.2/K_{ATP} channels blockers for the management of Parkinson disease (Zeng et al., 2008; Du et al., 2018; Han et al., 2018). According to some studies blocking the K-ATP channel can slow down neurodegeneration, by a mechanism that needs to be elucidated (Peltier and Vecchio, 1961). Interestingly, Kir6.2 inhibitors reversed the hyperpolarizing effect caused by reactive oxygen species (ROS) in particular hydrogen peroxide, which is a potent channel opener (Avshalumov et al., 2003). In simplified term, it is suggested that in a condition of stress the closing of this channel on the dopamine cells may play a role to prevent the cell to fall in an inactive state leading to cell death (Santos et al., 2019). Similarly, burst-stimulating discharge effect has also been reported by Fridlyand et al., 2010b; Gomis and Valdeolmillos, (1998b) on pancreatic β-cells. Accordingly, these channels add to the traditional mechanisms of burst regulation for dopamine neurons. We have for the first time demonstrated the effect of the K_{ATP} channels blockage on VTA dopamine neurons. We also report a biphasic effect starting as a near-complete inhibition during a relatively short period (1-3 min), associated with hyperpolarization, and followed by a long-lasting excitatory effect (up to 40% of the baseline activity). Here, we hypothesize that initial inhibitory effect caused by neuronal hyperpolarization was due to activation of GABA interneurons which are the main inhibitory neurons in the VTA. Activation of GABA spiny

neurons, possibly triggered by TBT, in the NAc may also relay powerful GABA modulatory innervations to the VTA. This was followed by stimulation of DA neurons with rapid depolarizing firing activity. Though being a hydrophobic molecule, TBT is an insulin secretagogue, K_{ATP} channels blocker and there is a direct connection between electrical discharge activity (Atwater et al., 1980) and exocytotic insulin release (Gilon et al., 1993). A body of literature has also demonstrated that TBT possesses a low brain distribution pharmacokinetic property, which has been attributed to P-glycoprotein-efflux transport system (Takanaga et al., 1998), as well as pH and membrane /potential –dependent transport efflux processes (Koyabu et al., 2004). Given this background, we utilized an escalated dosage of TBT at relatively high doses to allow for adequate concentration in the brain. Another recent finding from our laboratory on memantine, a drug used in the clinical management of moderate to severe Alzheimer's disease exhibited a dose-dependent activation of pyramidal neurons on PFC (unpublished). Our new report further demonstrated that activation of pyramidal neurons in the PFC is associated with cognitive enhancement. This possibly explains reason TBT alleviates cognitive impairment in a patient with a neurological disorder, an effect due to Kir6.2 blockage and pyramidal neurons in the PFC (Slingerland et al., 2008). This finding further suggests that TBT is possibly acting directly on the brain via Kir6.2/K_{ATP} channels inhibition and SUR1 activation.

Our findings on radiometric ^{3}[H] DA uptake assay revealed a significant reduction in dopamine uptake with TBT tested on striatal synaptosomes. An effect that potentially increases dopamine output in the postsynaptic terminals through the inhibition of DAT. The data described from this experiment shows that K_{ATP} channels can modulate striatal dopamine uptake, associated with DAT activity regulation under a non-pathological condition. Thus, it can be that TBT has some affinity for the dopamine transporter. Essentially, K_{ATP} channels in the brain are in the closed state under normal physiological condition and have been

implicated in ischemic brain injury, providing a neuroprotective function (al; Ben-Ari et al., 1990). These channels are also responsible for maintenance of glucose homeostasis via metabolic sensitivity in the hypothalamic region of the brain (Ashford et al., 1990; Routh, 2002) and modulation of vagal neuronal activity (Trapp et al., 1994b), as well as controlling the functional effect of respiratory neurones (Pierrefiche et al., 1996). The present investigations demonstrated the regulatory role of K_{ATP} channels in striatal synaptosomes that demands DAT as a principal regulatory transmembrane agent. Interestingly, there is general credence that activation of K_{ATP} channels in an ischemic event possesses neuroprotective effects by prompting depletion in energy utilization (Fujimura et al., 1997). It follows that opening of K_{ATP} channels causes hyperpolarization of the neurons and subsequently equilibrates the resting membrane potential in ischemic condition, particularly in an energy-deficient system when adenosine triphosphate (ATP) is reduced to adenosine diphosphate (ADP) (Sun et al., 2006; Yamada et al., 2001). However, recent evidence has contradicted the use of sulphonylureas in the management of cerebrovascular disorder as a compromising agent to achieve neuroprotection (Liu et al., 2016). The studies further explained that tolbutamide induces neuronal injury by stimulating oxygen-glucose deprivation (OGD) and persistent middle cerebral artery occlusion (MCAO). Whereas, the opening of these channels by diazoxide lowered the effects of both neuronal injuries (Liu et al., 2016). Liu et al., (2016) findings revealed that closing the Kir6.2/K_{ATP} channel by TBT enhanced the neuronal trauma caused by oxygen-glucose deficiency (OGD) and enduring middle cerebral artery occlusion (MCAO). Thus, they postulated that Kir6.2/KATP activation by DZ reduced OGD and MCAO in diabetic-induced mice through streptozotocin treatment. Another recent contrasting study has demonstrated the anti-Parkinson effect of K_{ATP} channels blocker in 6-hydroxydopamine –induced rat model of Parkinson (Piri et al., 2017b). Thus, may prevent the neuron to die as in Parkinson disorder in which there is a shortage of ATP

from mitochondria, which leads to the constant opening of the channel, the neuron may become silent and start dying more quickly. Interestingly, DZ a K_{ATP} channels activator exhibited a more pronounced striatal ^{3}[H] DA uptake reduction; an effect possibly expected to be reversed in this condition. This implies from our results that both drugs TBT and DZ may exhibit some specific similar effect on striatal synaptosomes by inducing a reduction of ^{3}[H] DA uptakes. This is in variance with a study that demonstrated an antagonistic effect of DZ on glucose-induced (Kr6.2/K_{ATP} channels blocking) dopamine release from pheochromocytoma-12 cells (PC-12) (Koshimura et al., 2003). In addition, paradoxical effect of DZ has been reported due to its non-specific action (Schikman , et al 1978). We also tested the effect of high concentration of glucose on DAT activity. We show that, at a glucose concentration of 80 mM which is an escalating dose, typical of a patient in a diabetic coma, the DAT activity was significantly inhibited which resulted in a reduction in ^{3}[H] DA uptake. Conversely, in the absence of glucose, the uptake process was aborted, indicating that glucose is primarily responsible for energy transport in the neuronal transmission. However, more recent studies have shown that other metabolic substrates such as lactate (Riske et al., 2017), pyruvate (Koivisto et al., 2016) as well as glutamate (Fendt and Verstreken, 2017) are also responsible for fueling of mitochondrial metabolism in the brain function. These findings enhanced the probability that the presence of glucose regulates neurotransmitter uptake in neuronal cells which represent similar activity in pancreatic β-cells of the islet of Langerhans (Taylor et al., 1999). Thus, glucose in high concentration (80mM) stimulated membrane depolarization, which signals the voltage-gated Ca^{2+} channels to trigger exocytotic dopamine release via DAT inhibition (Fridlyand et al., 2013; Han et al., 2018b; Komatsu et al., 2013). From all the neurochemical studies, our collective findings show that TBT and glucose can modulate K_{ATP} channels and bring about membrane depolarization via action on DAT to cause reduced striatal ^{3}[H] DA uptake. Since DAT activity has been known to be widely

dependent on the degree of ATP as well as sodium and potassium slopes, we hypothesize that TBT, DZ and glucose modulate K_{ATP} channels by inhibiting DAT activity which results into increase in extracellular dopamine levels.

In conclusion, TBT at high dose induced a biphasic effect on the dopamine neurons, an effect plausibly mediated by K_{ATP} channels blockage through sulphonylurea receptors activation. We also infer that period of inhibition (hyperpolarization) may be due to GABA interneurons or as possible negative feedback from activated accumbens neurons which send strong GABA inhibitory innervations to VTA. Interestingly, TBT activatory effect on pyramidal neurons of the PFC may indicate it could exert a positive effect on cognition and memory due to its ability to activate glutamatergic neurons on the PFC. Such an effect may not occur immediately in treated diabetic patients due to the relatively poor brain penetrability of the drug. The neurochemical studies show evidence of a significant reduction in specific dopamine uptake with TBT, DZ and glucose; these indicate a possible interaction of the drugs with dopamine neurotransmission (uptake or release) which may further be linked with reward processes (Sidló et al., 2008).

Chapter IV-Activation of glucagon-like receptor-1 modulates dopaminergic and glutamatergic transmission in rats' brain regions

IV-1- Introduction

Glucagon-like peptide-1 (GLP-1) is an endogenous insulin-producing gut hormone belonging to the incretin family (Calsolaro and Edison, 2015b). It is released by endocrine L-cells after feeding, stimulates insulin secretion and biosynthesis, prevents glucagon release as well as extends gastric fullness by reducing gastric emptying (Drucker, 2003a, 2003b). However, GLP-1 is not only essential metabolic hormones (Bae and Song, 2017), but can also modulate important brain function unrelated to feeding behaviour (Cabou and Burcelin, 2011). Different evidence suggests that these peptides can affect cognition (Abdelwahed et al., 2018; Gault et al., 2010a), motivation (Alhadeff and Grill, 2014; Alhadeff et al., 2014), addictive behaviour, as well as neuronal survival (Liu et al., 2015; Salcedo et al., 2012; Sharma et al., 2014). As a result of this neuromodulatory effect, GLP-1Rs have been identified in the cortex and hippocampus (Rebosio et al., 2018), hypothalamus (Liu et al., 2017), nucleus accumbens (Hernandez et al., 2019), as well as in VTA (Göke et al., 1995), where they perform devised neurophysiologic functions. Evidence has emerged that rodents that have their cortical and hippocampal GLP-1R deleted display impairment in a cognitive task, while amplification of GLP-1 in the hippocampus increased learning and working memory (Abbas et al., 2009; During et al., 2003a). In Abbas et al., (2009) findings, GLP-1 was administered intracerebroventricularly (i.c.v.), but not peripherally to elicit memory-enhancing effect in mice. Also, through genetic manipulation GLP-1R-deleted mice exhibited a phenotype characterised by learning deficiency and this was reversed subsequently to hippocampal GLP-1R gene transfer. Exendin-4 (Ex-4), a GLP-1, dipeptidyl peptidase-4

(DPP-IV)-resistant (Simonsen et al., 2006) and clinically used drug in the management of type 2 diabetes mellitus, as well as geniposide (GPO), a new putative GLP-1 agonist of plant origin (Rebosio et al., 2018), were the typical GLP-1agents studied in this chapter. GPO is an iridoid as well as found as secoiridoids (secondary metabolite), it has been extensively explored in the management of Alzheimer's and Parkinson disorders. Generally, plants iridoids of glycoside origin such as GPO, harpagoside and loganin have been shown to have pronounced neuroprotective effect on both Alzheimer's and Parkinson disorders (Dinda et al., 2019).

In the present study, we examined if the two GLP-1 agonists (Ex-4 and GPO) can affect some dopamine-dependent behaviours such as the ones we presented in chapter I. Following the behavioural investigations, we tested the effect of acute treatment of Ex-4 on specific protein expression such as, dopamine transporter (DAT) and GLP-1R, subsequent to D-AMP-induced motor activity with the aid of western blot technique. In another series of behavioural experiments, we also assessed, if chronically administered Ex-4 influences cognitive function in PCP treated rats, through the object recognition test (ORT) as well as the peptide acute effect on phencyclidine-induced hyperlocomotion activity. In addition, we investigated the role of neurochemical processes of radiometric release and uptake assays on ^{3}[H] dopamine when GLP-1R is activated. Finally, we have examined in rats whether the stable glucagon-like-peptide-1 receptor agonists Ex-4 or GPO can affect the electrical activity of dopamine neurons in the VTA and of pyramidal neurons in the PFC, using single-unit activity electrophysiology recording techniques.

IV-2- Materials and methods

IV-2-A-Subjects

Please refer to paragraph I-2-A for detail information on animal conditions and regulatory protocol on a study involving rodents.

IV-2-B- *In vitro* ^{3}[H]-dopamine and serotonin release assays

Please refer to paragraph I-2 for detailed experimental procedures. In addition, AMPA effect was studied on ^{3}H [DA] release following the same protocol in paragraph I-2. The effect of Ex-4 and GPO are studied on baseline [DA] release, as well as if these peptides influence D-AMP-induced ^{3}[H] DA efflux at the postsynaptic terminal of striatal brain slices.

IV-2-C-*In vitro* ^{3}[H]-dopamine uptake assay

Please refer to paragraphs I-2 for direct uptake assay protocol. In another series of experiments, 5-HT uptake was assessed in the PFC as well as uptake interaction of GLP-1 with a D_2/D_3 dopamine agonist in striatal synaptosome. For detailed experimental procedures, paragraph II-2-C2 discusses uptake kinetics for K_M/Vmax determination following intraperitoneal injection of the tested drugs. In this chapter, we studied Ex-4 and GPO effects on radiometric uptake parameters.

IV-2-D- Behavioural paradigm

Three major behavioural protocols were employed to ascertain the effect of Ex-4 and GPO on various behavioural traits. The experiments are object recognition test, PCP-induced hyperlocomotion activity and dopamine drug-induced behavioural traits assessments.

IV-2-D1- Novel object recognition (NOR) test

The experiment was carried out according to Broadbent et al., (2010) with little modifications in an open field circular box measuring 70 cm in diameter and 58 cm in height. The materials consisted of aluminium tin or plastic objects that varied in colour and size measuring, width=6.2–7.5 cm; height = 10–13 cm (Fig. 33). The objects were securely attached to the floor and separated 7 cm apart. The testing session was recorded manually under bright fluorescent lighting for effective illumination of the circular open field. Two groups of rats in each category of duration of treatment (*n=4*), control (0.8 ml/kg saline) and treated (ip Ex-4 5µg/kg) given in acute (24hr.), sub-chronic (5days) and chronic doses (2 weeks). They were acclimatized to the experiment room and the circular open field for two consecutive days before the testing (40 min in the experiment room and 5 min to explore the empty open field). In another series of experiments, rats were pre-treated with saline (0.8ml/kg) or Ex-4 (5µg/kg) and then treated with single i.p. injection 5 mg/kg of phencyclidine after the habituation period.

Figure 33: Images of materials used in object recognition model
The two identical objects represent the familiar objects placed about 7 cm apart during the training sessions, while one of the familiar objects is replaced during the test day by the novel object.

Object familiarization training

Rats were exposed for a period 4 days familiarization with the two identical objects (three exposures each day). Each animal was habituated to the testing room for 45 min and later placed in the empty open field for 1 min on each testing day. The rat is removed afterwards followed by centrally placing the two identical objects at 7 cm apart. The rat is then returned into the open field and allowed to explore for a period of 5 mins. As soon as all the rats in the group were familiarized with the two objects, the same protocol was repeated two more times on the same day (maximum interval of 60 min ensured between daily exposures). The two identical objects were repeatedly exposed to the rats for a period of 4 days.

Novel objects recognition (NOR) test

Memory testing started after 5 days of a washout for chronic study and immediately for an acute experiment. First, each rat was reacclimatized to the empty open field for a period of 1 min. The rat was subsequently removed and later placed in the open field to explore two objects (one new/novel object and a duplicate of the familiarized object) for a period of 5 mins. The exploration of the objects was scored manually when the rat's nose was about 1 cm away from the object and the whiskers were in motion according to Clark et al., (2000). It is noteworthy to know that exploration of objects was not considered an effective experimental scoring when the animal rears in an upright scrambling position on the object with the nose facing up. Proclivity for the novel object was demonstrated as the % time that the animal spent exploring the novel object compared to the familiar object. The preference for a novel object means that image of the familiar object still exists in animals' memory hence, the higher the % the more the memory for familiar object for discrimination.

IV-2-D2- PCP-induced hyperlocomotion studies

Rats were transferred individually from their home cages to the test open-field arena and then allowed to habituate for 30 min before the start of the experiment. First, we

tested the effect of acute Ex-4 (5 µg/kg) on basal motor activity of the rats, whether the drug on its own affects the normal basal horizontal movement of the subjects (assessed by number of times the rat walks a distance of 10 cm). Adult male Sprague-Dawley rats were treated with either Ex-4 (5µg/kg) or saline (0.8ml/kg). In another series of experiment, rats were pre-treated with either saline (0.8ml/kg) or Ex-4 (5µg/kg) and later treated with single intraperitoneal injection 10 mg/kg of PCP. The circular open field arena was divided and marked into 4 quadrants, and animals were then scored for behavioural locomotor activity such as running across (based on the number of the quadrant that was crossed over by the rat) and stereotypic head-shaking traits, during 15-minutes time periods and up to 60 minutes following the administration.

IV-2-D3- D-AMP and DA D_2/D_3 receptor agonist-induced behavioural studies

Please, refer to paragraph II-2-D for detailed behavioural protocols. In this study, Ex-4 (5µg/kg) and GPO (40mg/kg) were acutely (24hr) and sub-chronically (1 week) tested on D-AMP-induced locomotor activity, as well as dopamine D_2/D_3 agonist-induced yawning, pica eating and pelvic grooming in male adult Sprague-Dawley rats. In another series of experiment, we investigated the role of 30µg/kg of Ex-9 (a GLP-1 antagonist) on both D-AMP induced locomotors effect, as well as in the presence of Ex-4 agonist for possible pharmacological interaction at the receptor site.

IV-2-E- Western blot studies on DAT and GLP-1 protein expression

Following the last behavioural experiments (paragraph IV-2-D3), brain tissues were immediately dissected on an ice-cold platform and before being immersed in liquid nitrogen. Tissues were then stored at -80^{o}C until further analysis. Western blot technique was

deployed, according to paragraph III-2-D for the detection of specific protein molecules from among a complex mixture of proteins and how these proteins were altered by acute treatment with D-AMP 1 mg/kg or Ex-4 5 µg/kg+D-AMP 1 mg/kg. Appropriate dilution ratio was used according to manufacturer's specifications (DAT 1:1000, β-actin 1:1000 and GLP-1 1:500). Following overnight exposure to the appropriate primary antibodies, (the membranes were washed three times with 1x TBST for 5 mins each time, to wash off unbound antibody.

IV-2-E-*In vivo* extracellular single-unit electrophysiology recording

Please refer to paragraph I-4 for detailed protocol. Recordings were done on both VTA DA neurons and pyramidal PFC neurons. However, in this study Ex-4 5µg/kg and GPO 40mg/kg were investigated. At a point, we investigated the potential activatory or inhibitory effect of Ex-4, 5µg/kg combined with insulin 20µg/kg on the cell bodies of DA neurons following an intravenous administration. Next, we assessed the effect of Ex-4 when administered directly into the brain region of interest (VTA) through the use of Hamilton's syringe filled with the peptide and allowed to diffuse for up to an hour before extracellular recording and when Ex-4 is filled into the recording electrode, lowered into the brain for electrophysiological recording.

IV-2-F-Drugs

All drugs and antibodies were purchased from Abcam (Biotechnology company, UK), except for secondary antibody (bs-0295G-HRP, Bioss antibodies UK), geniposide (Carbosynth Ltd, UK) as well as phencyclidine (Tocris, UK). The drugs were dissolved into Krebs (release assays) and HEPES (uptake) buffers, as appropriate. However, the drugs were dissolved in distilled water or saline for behavioural and electrophysiological studies.

IV-2-G-Statistical analysis

Please refer to paragraph I-2-G for analysis involving radiometric release and uptake assays, electrophysiology as well as D-AMP-induced locomotor studies. However, for NOR study, data were expressed according to (Aggleton et al., 2010; Ennaceur and Delacour, 1988) studies, which stipulate that discrimination behaviour can be measured in two ways. The first discrimination index (D_I) measurement for acclimatization phase is calculated by the difference between exploration time for the novel and familiar objects i.e. [$D_1 = (T_N - T_F)$] where T_N=Time spent with novel object and T_F=Time spent with a familiar object. The second measurement of (D_I) states that, there is a ratio that exists between differences in time explored with novel and familiar object, to the sum of the total time spent with novel and familiar object i.e. [$D_{I'} = (T_N - T_F)/(T_N + T_F)$] of %. In both cases, the results were expressed as a magnitude of +1, 0 and -1. Where it is positive, this shows more exploration time with the novel, and negative exhibits more time spent with the familiar and zero means null preference. We utilized % DI for our statistic and further explain in term of magnitude for data interpretation. For behavioural experiment involving PCP-induced hyperlocomotion, the sums of each movement across a quadrant were counted manually and taken as a factor of 4. This means, numbers of movement across a quadrant (Nq) ÷4, equal to the total rat's locomotor activity occurring during 15 minutes interval period up to and 60 minutes of total experiment duration. This is plotted against the drug administered. Results are expressed as the mean±standard error of the mean (SEM) of counts for each rat's movement per group. Repeated 2-way ANOVA was also conducted when a significant difference was tested.

DAT and GLP-1R protein quantifications were analysed using ImageJ software following visual membrane images captured by CCD-camera-based imager ChemiDoc MP (Bio-Rad). The expression of each protein was normalized to the housekeeping protein β-

actin. Data were expressed as a percentage of the control group (saline, mean±standard error of the mean).

IV-3- Results

IV-3-A-GLP-1 effect on striatal ^{3}[H] DA and PFC ^{3}[H] 5HT efflux

IV-3-A1-Neither Ex-4 nor GPO alters baseline ^{3}[H] DA release

The application GPO (40µM) caused a significant reduction in dopamine efflux. Surprisingly, when Ex-4 (5µM) was applied, the effect on dopamine efflux remains statistically unchanged, yet with a slight reduction in [DA] efflux (Fig. 34A, B). This might suggest the seemly absence of GLP-1R on the dopamine terminals that resulted in a lack of activity on the baseline [DA] efflux.

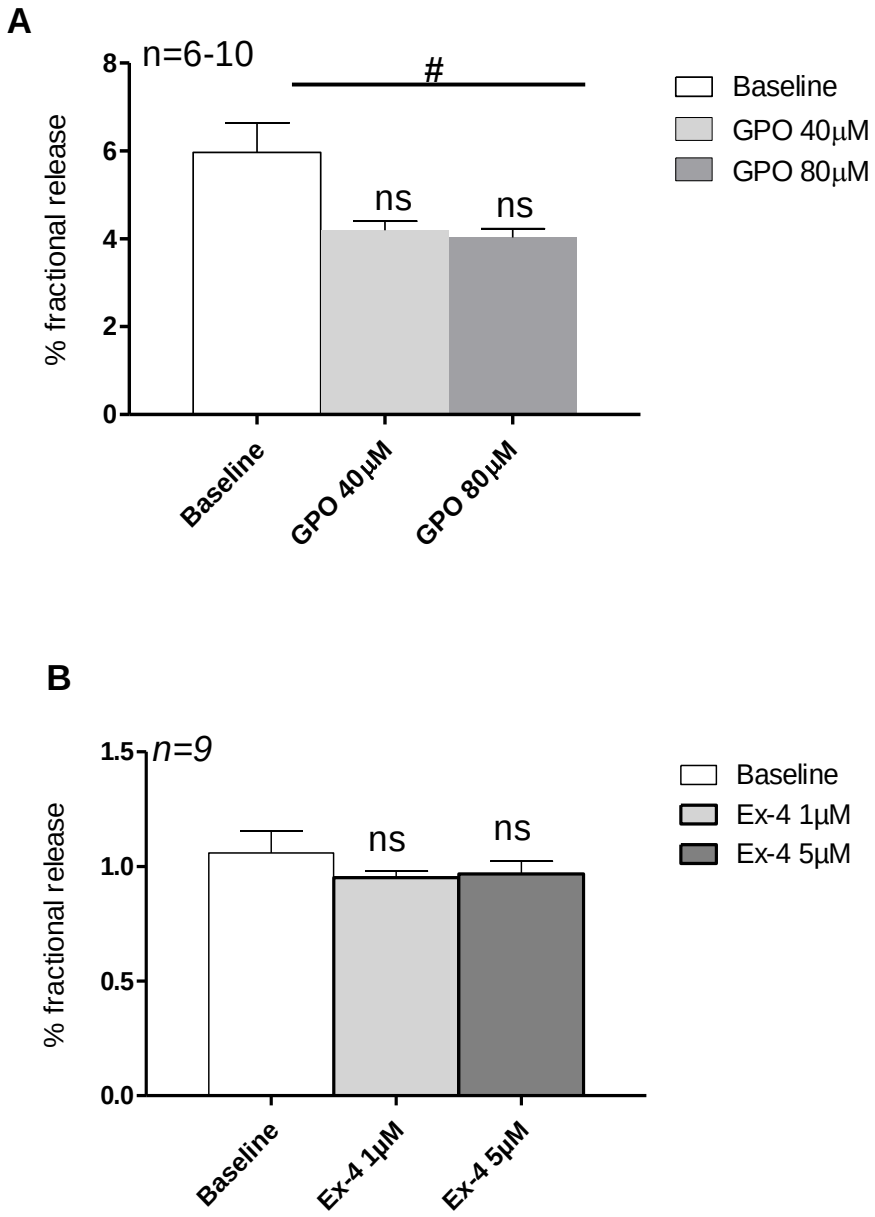


Figure 34: Dose-response relationship of GLP-1 drugs on striatal 3[H] DA efflux.
(**A**): GPO caused a non-dose response significant reduction in dopamine efflux when applied, #P<0.05, *Neuman-Keuls* after significant one-way ANOVA (**B**): Ex-4 showed a slight non-significant reduction of striatal ^{3}[H]DA efflux, even at an increased concentration.

IV-3-A2- Ex-4 and GPO moderately interact with D-AMP and KCL- induced DA efflux

In the striatum, a large amount of [DA] efflux was evoked in presence of 30 mM KCL, as well as with 10 µM D-AMP in the superfusion medium (Fig. 35A, B). The striatal slices pre-exposed to GPO 40 µM did not prevent the large DA releasing effects of D-AMP or KCL (Fig. 35C, E). Similarly, Ex-4 1µM tends to reduce slightly D-AMP-induced DA efflux, but this effect was not statistically significant (Fig. 35D) there were no effects of GPO on AMPA-induced [DA] efflux (Fig. 35F).

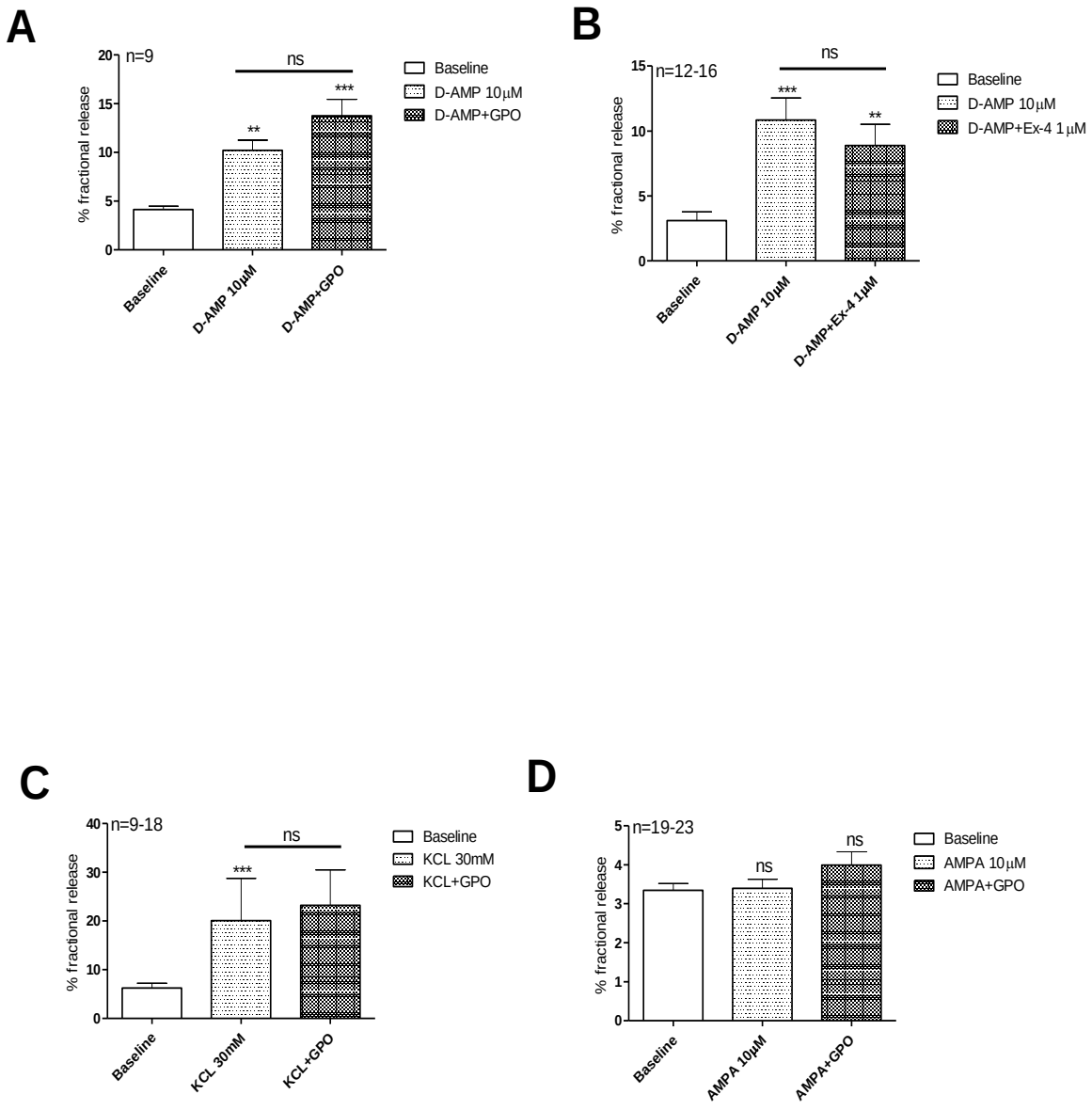


Figure 35: GLP-1 effect on D-AMP and KCL-induced 3[H] DA efflux

(A, C): Superfusion of GPO 40 µM in the presence of D-AMP and KCL did not affect the releasing magnitude of both drugs. **(B):** Ex-4 1 µM exhibited a slight inhibition of D-AMP-induced DA efflux, but not statistically significant. **(D):** GPO 40 µM was tested on AMPA 10µM, the GLP-1 had no effect on [DA] release ns: non-significant, **P<0.001, ***P<0.001 *vs.* respective baseline, ns, ***P<0.0001, Student's t-test P>0.05 *vs.* specified condition, *Neuman-Keuls* after significant ANOVA.

IV-3-A3- GPO attenuates KCL-induced ^{3}[H]-5HT on PFC slices

We tested the activity of serotonergic terminals on KCL-induced DA efflux using PFC slices. The addition of GPO to the superfusion medium significantly (t=2.405 df=15) reduced ^{3}[H] 5HT efflux (Fig. 36A). There was no apparent statistical interaction between KCL-induced ^{3}[H] 5HT efflux and GPO when co-superfused (Fig. 36B).

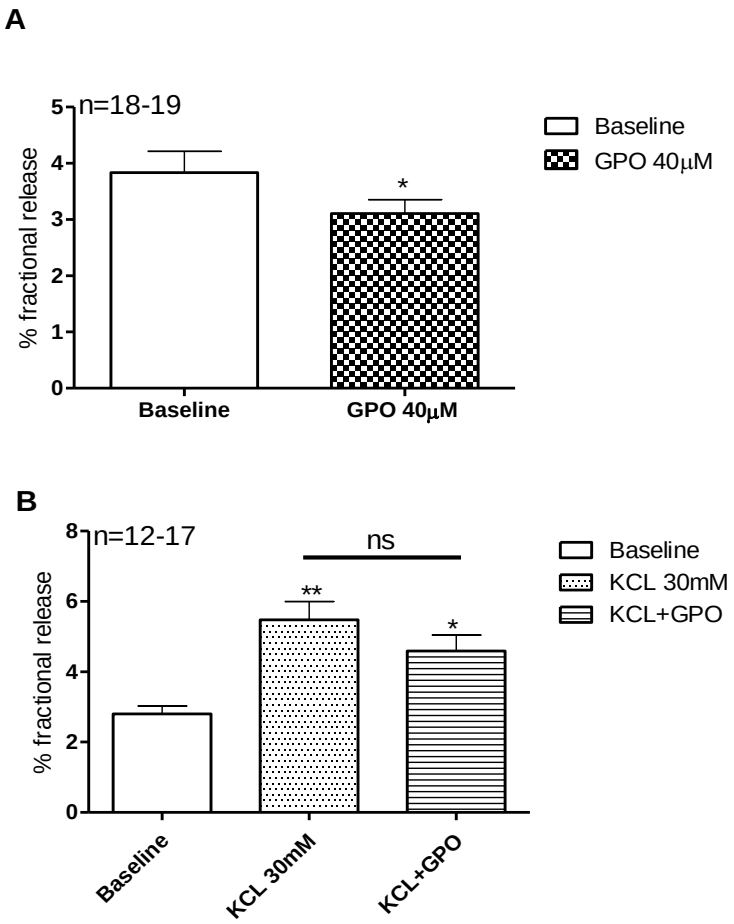


Figure 36: GLP-1 effect on serotonine terminals
(A): Superfusion of GPO on ^{3}[H]-5HT efflux. GPO exerts a significant reduction on baseline 5-HT release from PFC slices. *P<0.05, student's t-test **(B)** KCL 30 mM significantly and dramatically increases the release of ^{3}[H]-5HT (like for dopamine). This effect was not significantly affected in the presence of GPO 40 µM though, there was a tendency for a decrease.*P<0.05, **P<0.001 *vs.* respective baseline, ns, P>0.05 *vs.* specified condition, *Neuman-Keuls* after significant ANOVA.

IV-3-B- GLP-1 effect on striatal ^{3}[H] DA uptake processes

IV-3-B1-Ex-4 attenuates D_2/D_3 DA agonist-induced striatal^{3} [H] DA uptake

We investigate the dose-response relationship of Ex-4 (1 and 5 µM) and GPO (40 and 80 µM) on striatal ^{3}[H] DA uptake from striatal synaptosomes. Both drugs did not directly alter the uptake process (Fig. 37A, B). However, Ex-4 (1µM) significantly (F (2, 14) =27.61) inhibits QNP-induced striatal ^{3}[H] DA uptake (Fig. 37C). Similarly, Ex-4 treatment significantly reduced PPX-induced dopamine uptake in striatal synaptosomes (Fig. 37D). This significant neuromodulatory effect with dopamine D_2/D_3 receptor agonists may suggest the possibility of molecular and behavioural effects of GLP-1 on dopamine neurotransmission.

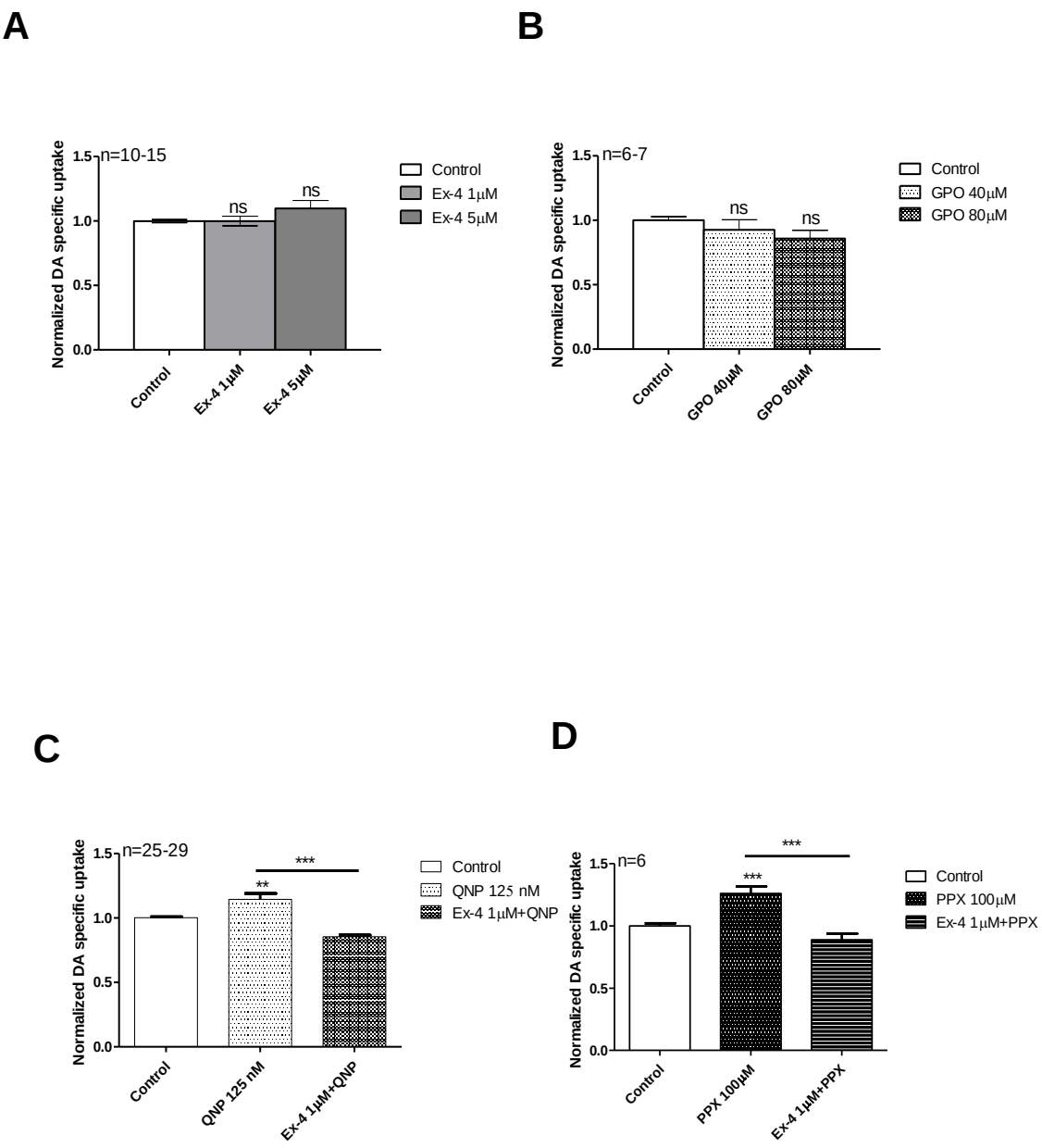


Figure 37: Effect of Ex-4 on quinpirole interaction on [DA] uptake transporter activity
(A, B): Neither Ex-4 (1 and 5 µM) nor GPO (40 and 80 µM) has any effect on striatal ^{3}[H] DA uptake **(C):** In the presence of 1 µM of Ex-4 quinpirole-induced [DA] uptake was significantly attenuated, an indication of neuromodulatory effect on dopamine transporter activity when co-administered with dopaminergic drug (**D**): Similar effect was exhibited with PPX a D_3-prefered dopamine agonist.**P<0.001, ***P<0.0001 *vs.* specific conditions, *Newman-Keuls,* after significant ANOVA.

IV-3-B2-GPO inhibits uptake of ^{3}[H] 5HT in the prefrontal cortex

We evaluate the impact of GPO on serotonin neurotransmission based on Cai et al., (2015) finding, that GPO possesses an antidepressant-like effect on a chronic unpredictable mild stress-induced rat. The mechanism of which is not fully understood. Here, through *in vitro* radiometric uptake assay of serotonin on the prefrontal cortex, GPO 40µM did not affect the uptake process (Fig. 38A). However, at a double dose of 80 µM of GPO ^{3}[H] 5HT uptake was slightly but significantly (t=2.244 df=16) reduced (Fig. 38B).

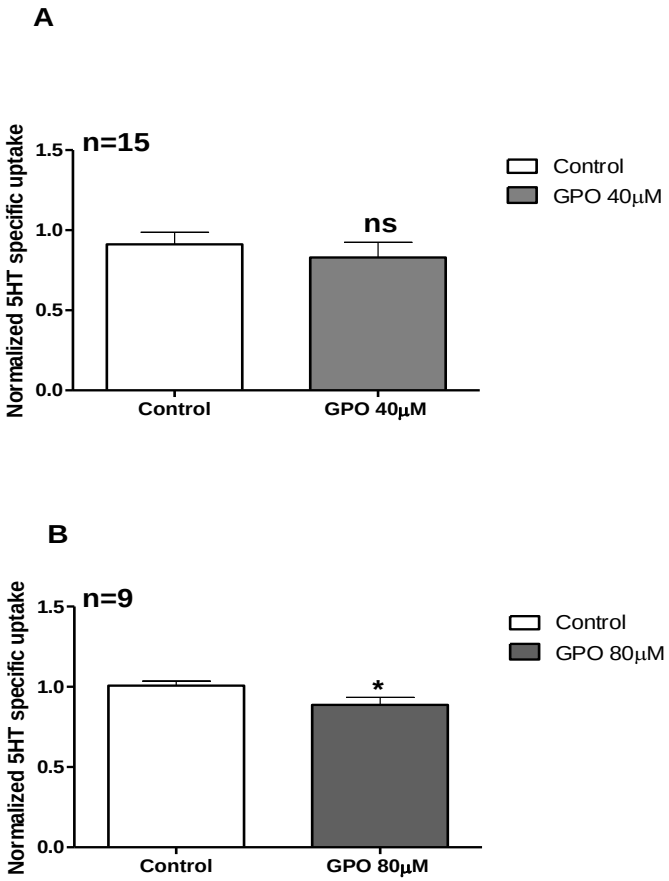


Figure 38: Effect of GPO on prefrontal cortex ^{3}[H] 5HT
(A): GPO 40 µM did not alter ^{3}[H] 5HT uptakes on the prefrontal cortex slices. **(B):** GPO at 80 µM significantly reduced the uptake process of ^{3}[H] 5HT to some degree. *P<0.04, Student's t test.

IV-3-B3- GLP-1 drugs alter uptake kinetics on striatal synaptosmes

Following a single acute intraperitonial administration of GPO (40 mg/kg) or Ex-4 (5µg/kg), derivation of uptake kinetic parameters (K_M and V_{max}) by Michaelis-menten's equation analysis were evaluated in striatal synaptosomes. Both Ex-4 and GPO significantly increased K_M values over the control. The increase in K_M is very similar for both agonists. This may further demonstrate a vital pharmacodynamic resemblance between Ex-4 and GPO, in terms of the effect on DA transmission. However, Vmax values in both groups remain unaltered when compared with the control. This was a preliminary finding and large sample size will be needed to ascertain the precision of the data. Data are means±s.e.m, R^2 indicates the goodness of fit. *P<0.05 *vs.* control, one-way ANOVA followed by *Neuman-Keuls* post *hoc* test.

Table 2: Ex-4 and GPO increased K_M for DAT-mediated uptake in striatal slices

Kinetic parameters	Control *n=4*	Ex-4 (5µg/kg) *n=4*	GPO (80mg/kg) *n=4*
$K_{m\ (nM)}$	43.47±15.82	83.07* ±19.6	103.2* ±9.117
$V_{max\ (µm/s)}$	4.834±0.8730	5.14±75	3.53±20.81
R^2	0.9588	0.9894	0.9989

IV-3-C-GLP-1 drugs modulate behavioural traits

IV-3-C1- Ex-4 intraperitoneal administration improves memory and cognitive tasks in rats

In this study, we tested the impact of Ex-4 5 µg/kg on cognition, in three main categories of treatment namely, acute (Ex-4 administered on the training day and test day), sub-chronic (5days administration of Ex-4) and chronic (2 weeks administration of Ex-4) exposures. Significant group differences were not observed on all of the individual test days. Generally, in all the groups and across the 3 categories of exposures exploration time for the familiar object decreases as the training interval progresses. The control and test groups in each category of exposure exhibited similar exploration time for the familiar objects during the 3 days training period (Fig. 39A, C, and E). For the discriminating index (DI') which is the measure of preference for the novel object expressed in percentage, acute exposure to Ex-4 did not improve preference for the novel object on the treated rats (Fig. 39B). However, sub-chronic and chronic exposures to Ex-4 induced significant ($t=2.223$ $df=12$) and ($t=6.538$ $df=6$) preferences for novel objects than the control groups (Fig. 39D, F). We also note with interest that, preference for novel object time-dependently increased from acute to chronic administration of Ex-4 (17%, 22.2% and 46.2%). In another series of cognitive study, we investigated the acute effect of Ex-4 5 µg/kg (i.p.) on a single dose of PCP-induced cognitive impairment. The PCP-treated group spent significantly more time ($F(2, 12) = 5.4$) exploring the objects during the 3-day familiarization phases than the Ex-4 pre-treated group (Fig. 40A). The PCP group has in average total time (21.7sec) and Ex-4 pre-treated has (8.8 secs) in each familiarization phase. However, Ex-4 pre-treated group showed a significantly longer time ($t=7.592$ $df=6$) on the novel object than the PCP treated group, which exhibited a complete cognitive impairment expressed in % negative value (Fig. 40B).

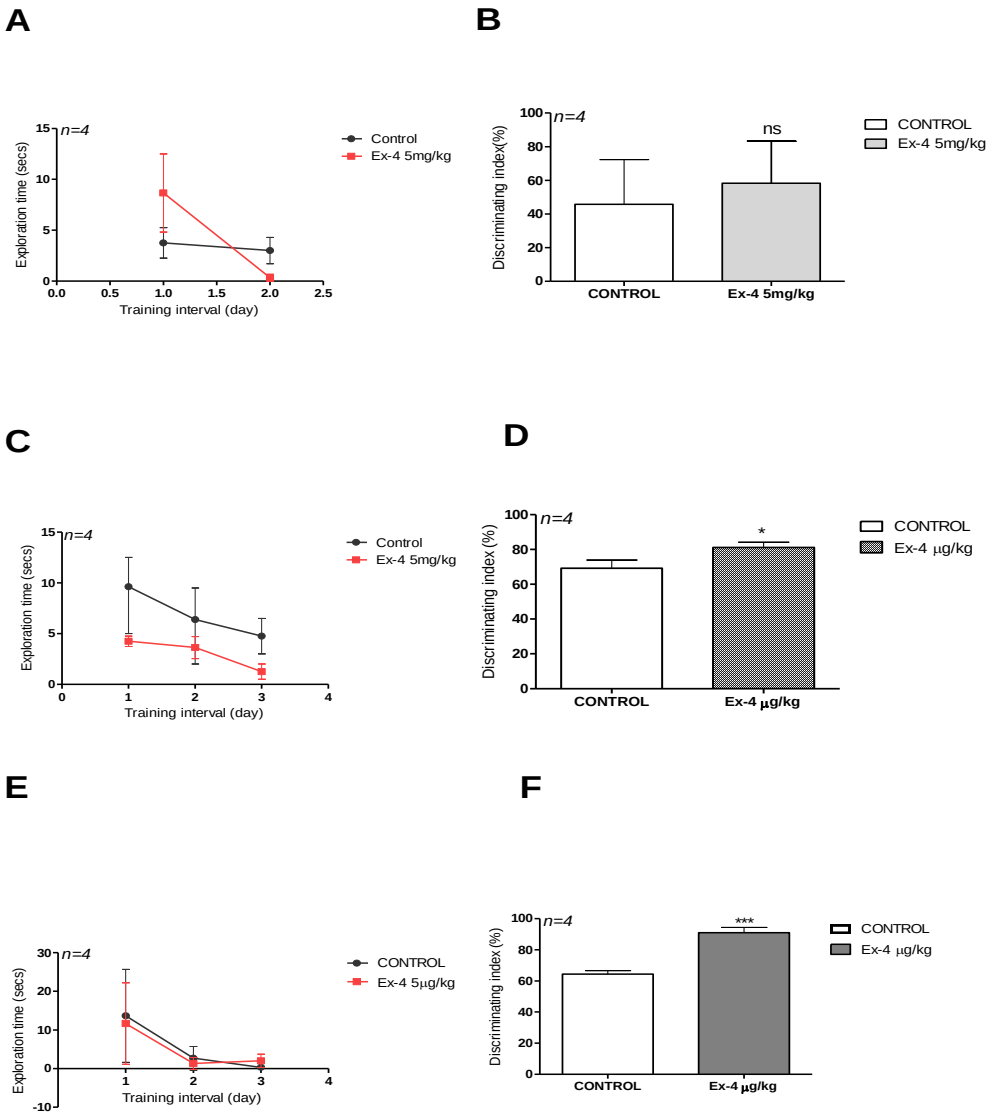


Figure 39: Effect of acute, sub-chronic and chronic Ex-4 treatment on NOR test
(A, C, E): Ex-4 treated groups in acute (**A, B**), sub-chronic (**C, D**) and chronic (**E, F**) exposures did not show any significant differences in exploration time of the familiar object, during the training exploration period, when compared with the saline control group. P>0.05 **(B):** In acute treatment, Ex-4 treated group did not show any significant alteration in preference for novel object P>0.05 **(D, F):** Ex-4 treated group in sub-chronic and chronic groups exhibited a significant increase in preference for the novel object than the control groups. *P<0.05, ***P<0.0001 *vs.* control, Student's t test.

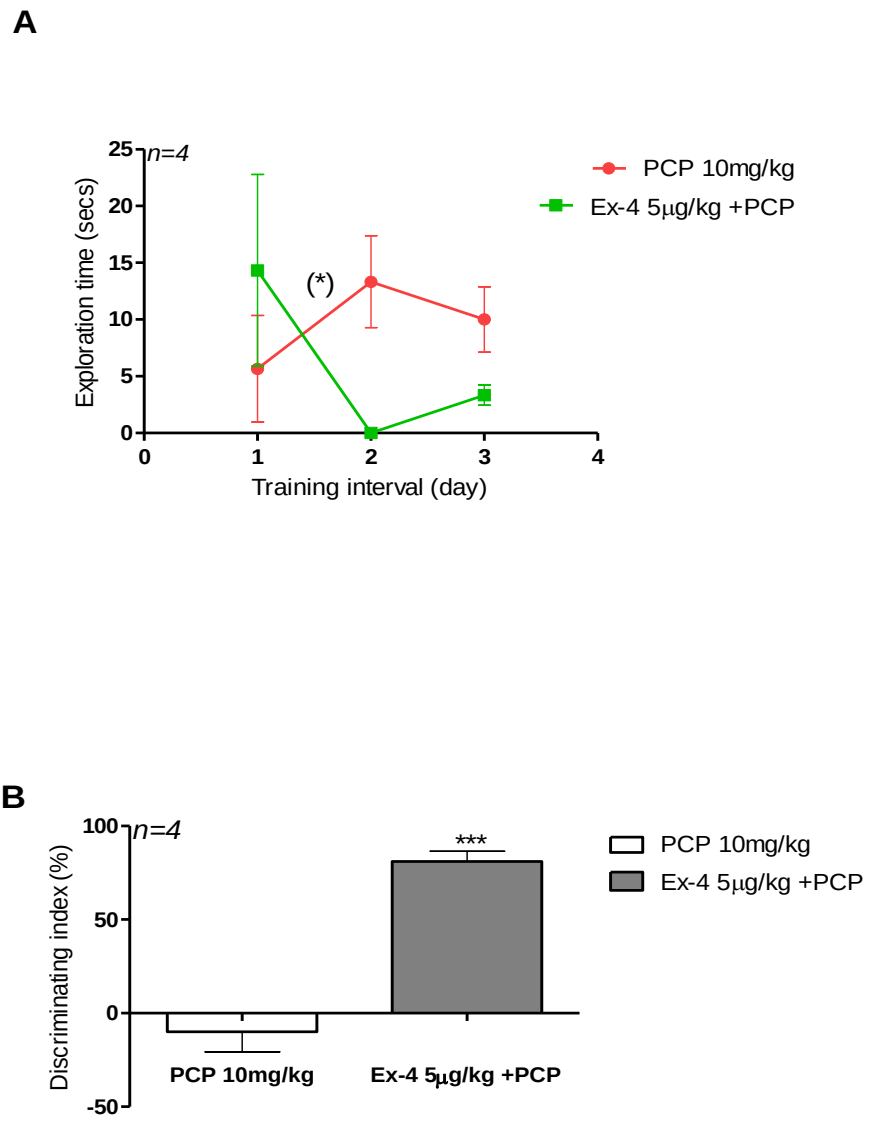


Figure 40: Effect of acute Ex-4 treatment on PCP-induced cognitive impairment
(A): The PCP treated group exhibited a significant increase in exploration time compared to the Ex-4 pre-treated group throughout the exploration period.*P<0.05 *vs.* Ex-4 group *Bonferroni*, after significant repeated measures two-way ANOVA. **(B):** Ex-4 treated group showed a significant increase above the PCP treated group during the preference test for the novel object. ***P<0.0001, Student's t test

IV-3-C2- Acute Ex-4 administration attenuates basal motor and PCP-induced hyperlocomotion

First, we tested the effect of Ex-4 5µg/kg on basal motor activity of the subjects (Fig. 41A). Briefly, the effect on motor activity was assessed after the single injection with Ex-4. The rats look calmer, but not drowsy, which did not last for a long period. Ex-4 caused a statistically significant ($F(1, 12) = 7.29$) reduction in basal motor activity of the rats. Then, we investigated the impact of Ex-4 on hyperlocomotive behaviours of PCP 10 mg/kg, when acutely administered intraperitoneally. The PCP-induced hyperlocomotion is usually used to model positive symptoms of schizophrenia in rats. During the first 60 mins of observation, after intraperitoneal administration of PCP 10 mg/kg, animals that were pre-treated with Ex-4 5µg/kg exhibited a significant reduction ($F(1, 16) = 39.26$) in locomotor activity than those pre-treated with 0.8 ml/kg saline (Fig. 41B). There were also apparent significant reductions ($F(2, 24) = 54.6$) in urination and headshaking stereotypic manifestation in Ex-4 pre-treated group when compared with the control (Fig. 41C).

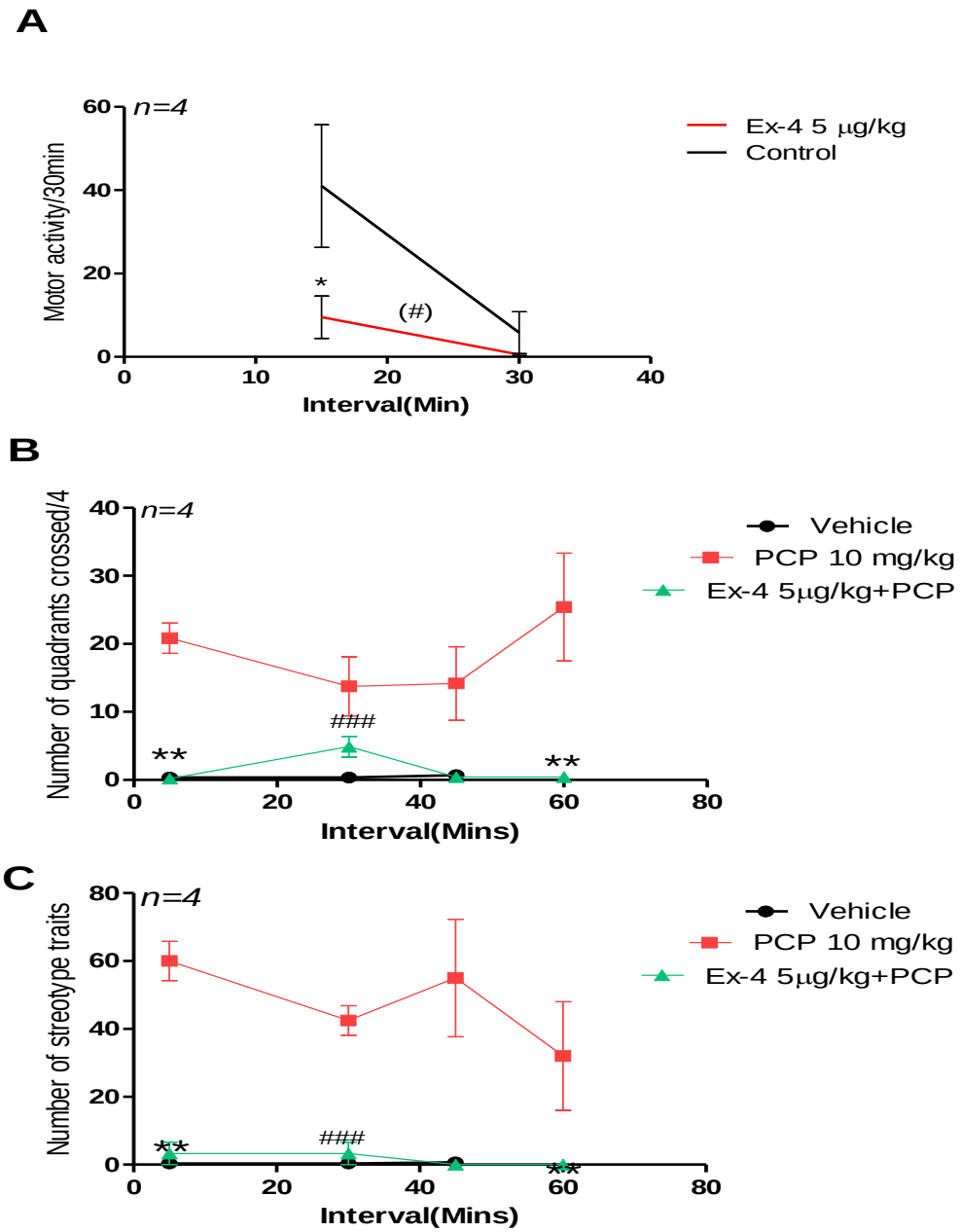


Figure 41: Impact of Ex-4 on basal and PCP-induced behavioural traits

(A): Acute intraperitoneal Ex-4 exhibited a significant reduction in basal motor activity (assessed by the number of times the rat walks a distance of 10 cm) in the first 15 min observation **(B):** Exendin-4 5 µg/kg pre-treated group significantly reduced PCP-induced hyperlocomotion activity in the first 15 mins and last 15 min of behavioural observations. **(C):** Ex-4 5 µg/kg significantly reduced the numbers of head shaking stereotyped activity on the first 45 mins during the observation period. #P<0.05, ###P<0.0001, *P<0.05, **P<0.001, ***P<0.0001 *vs.* control. *Bonferroni post hoc* tests after significant two way repeated measures ANOVA.

IV-3-C3- Acute Ex-4 and GPO differentially modulate D_2/D_3 DA agonist-induced behavioural traits

The various effects of dopamine agonists like PPX and QNP on different dopamine-dependent behaviours have been presented in chapter I. In PPX-induced behavioural traits, Ex-4 pre-treated rats exhibited significant reduced yawning ($F(1, 32) = 31.32$) and pelvic grooming ($F(1, 32) = 31.32$) activities throughout the observation time when compared with control (Fig. 42A, B), except for pica eating behaviour that showed no difference from the control (Fig. 42C). Similarly, Ex-4 pre-treated rats displayed a significant reductions ($F(1, 32) = 43.30$) in yawn, pelvic grooming ($F(1, 32) = 6.74$) and pica eating ($F(1, 16) = 2.97$) behavioural traits induced by QNP (Fig. 43A, B, and C).

In another series of experiment, GPO was tested on these behavioural characteristics induced by D_2/D_3 agonists. Yawning ($F(1, 32) = 6.84$) and pelvic grooming ($F(1, 32) = 23.09$) effects were significantly reduced in GPO pre-treated group, when compared with PPX only treated rats (Fig. 44A, B). But, in GPO pre-treated group there was a significant ($F(1, 32) = 7.72$) increase in pica eating activity compared to PPX treated group (Fig. 44C). In QNP-induced behavioural testing, GPO pre-treated group exhibited significant reduction ($F(1, 16) = 18.18$) on yawning effect (Fig. 45A), while there was no alteration on pica eating as well as pelvic grooming behavioural activities in the presence of GPO (Fig. 45B, C). However, Ex-4 has been reported to be associated with inducing mild pica response and reducing food intake due to nausea effect (Hernandez et al., 2018a).

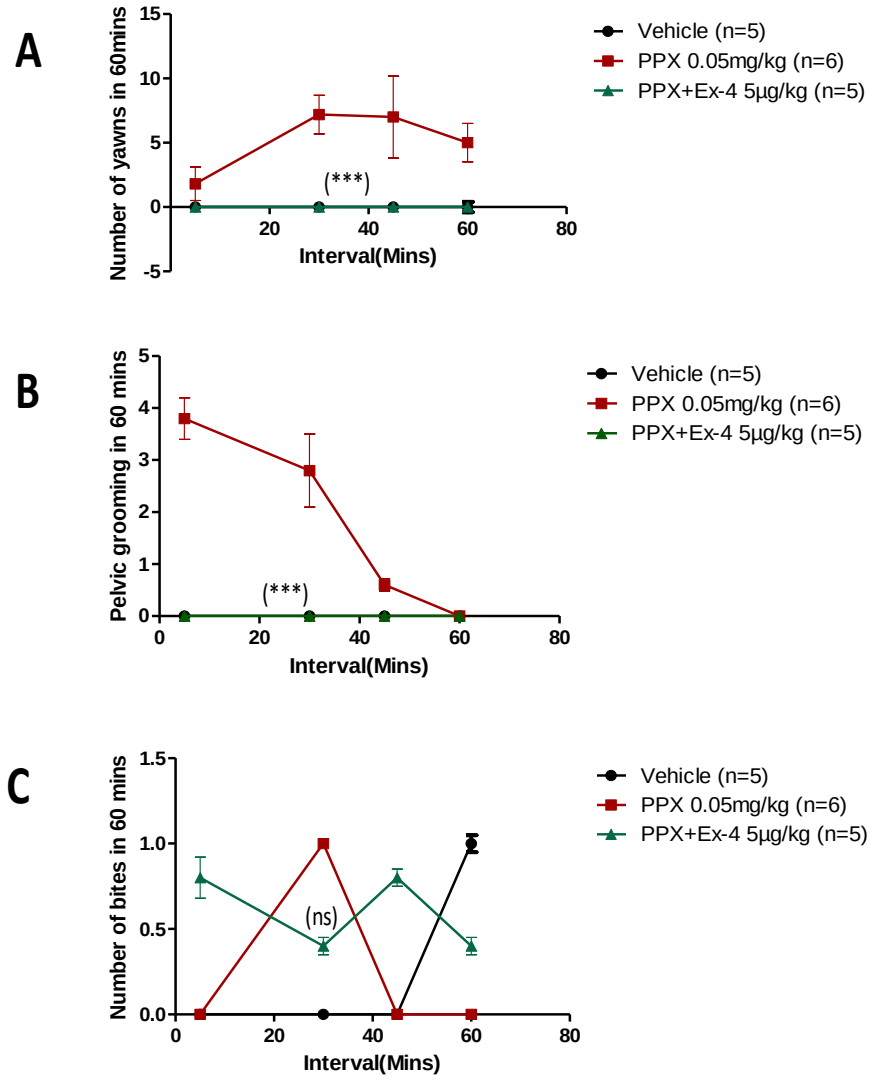


Figure 42: Ex-4 inhibits PPX-induced yawning and pelvic grooming effects

(A, B): Exendin-4 5 µg/kg pre-treated group significantly reduced PPX-induced yawning and pelvic grooming effect during the 60 mins observation (**C**): Ex-4 at the same dose administered (5 µg/kg) shows no apparent effect on pica eating activity ***P<0.0001 *vs.* PPX control. Significant two-way repeated measures ANOVA.

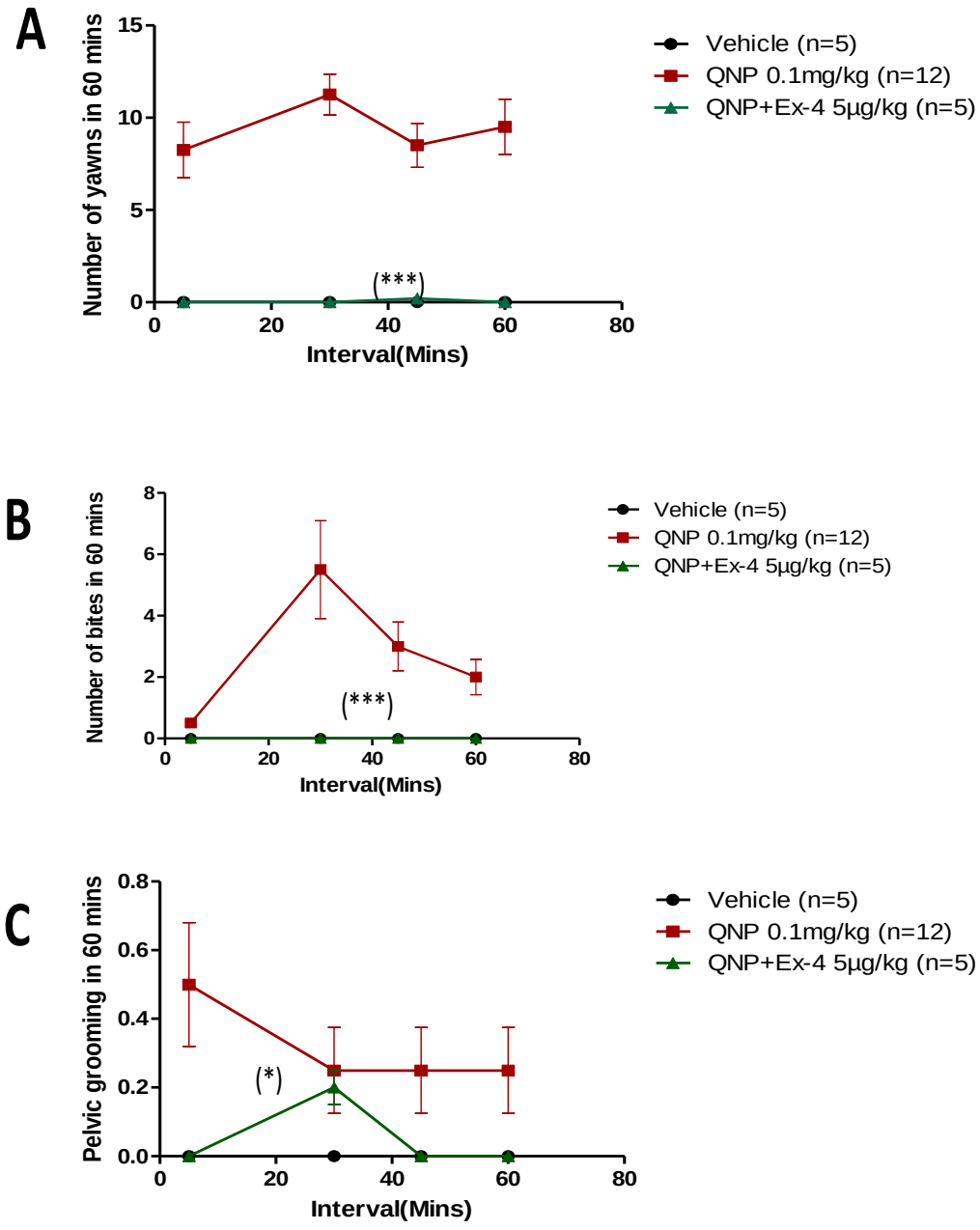


Figure 43: Ex-4 attenuates QNP-induced yawning and pelvic grooming effects

(A, B, C): Exendin-4 5 µg/kg pre-treated group significantly reduced QNP-induced yawning, pica eating and pelvic grooming effect during the 60 mins observation *P<0.05, **P<0.001, ***P<0.0001 *vs.* QNP control. *Bonferroni* post hoc tests after significant two way repeated measures ANOVA.

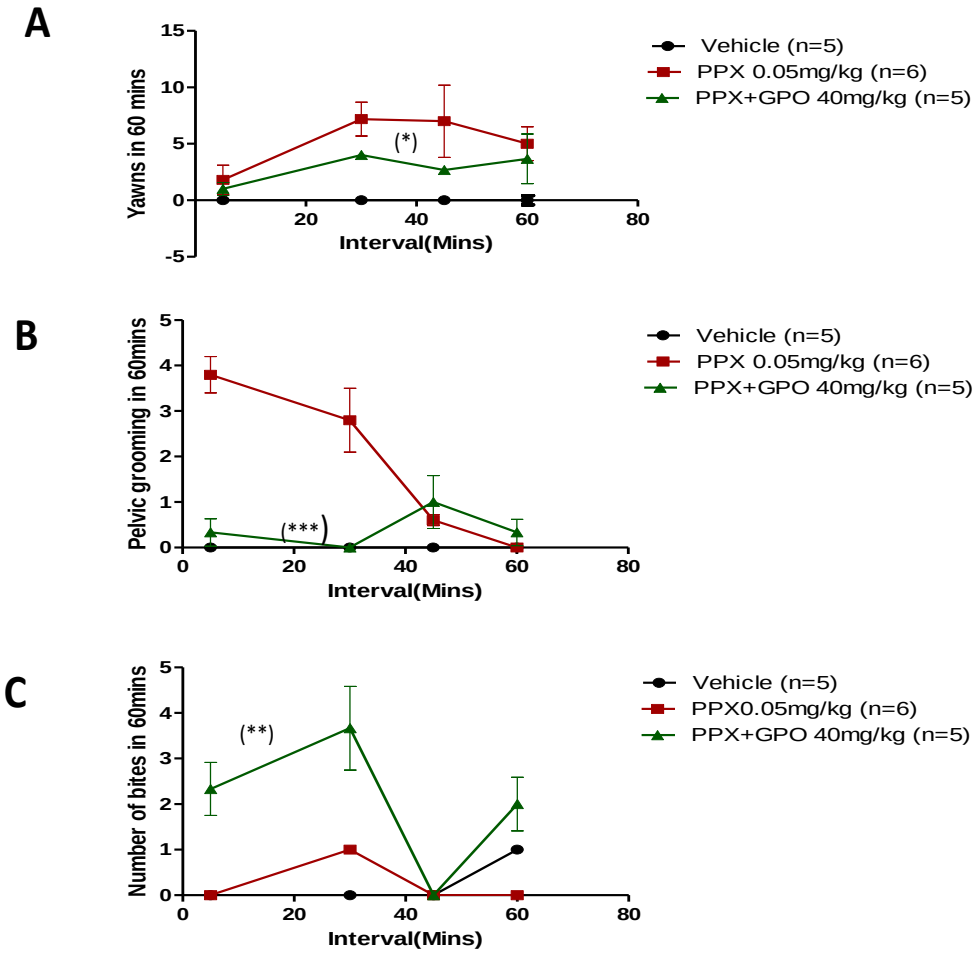


Figure 44: GPO reduces PPX-induced yawning and pelvic grooming effects

(A, B): GPO 40 mg//kg pre-treated group significantly reduced PPX-induced yawning and pelvic grooming effects during the 60 mins observation (**C**): GPO at the same dose administered (40 mg/kg), significantly increased pica eating activity above the PPX control group. We show another evidence of probable inducement of pica response by GLP-1, albeit it is an inconsistent trait across the group. *P<0.05, ***P<0.0001 *vs.* PPX control, **P<0.001 *vs.* Ex-4+PPX treated group. *Bonferroni* post hoc tests after significant two way repeated measures ANOVA.

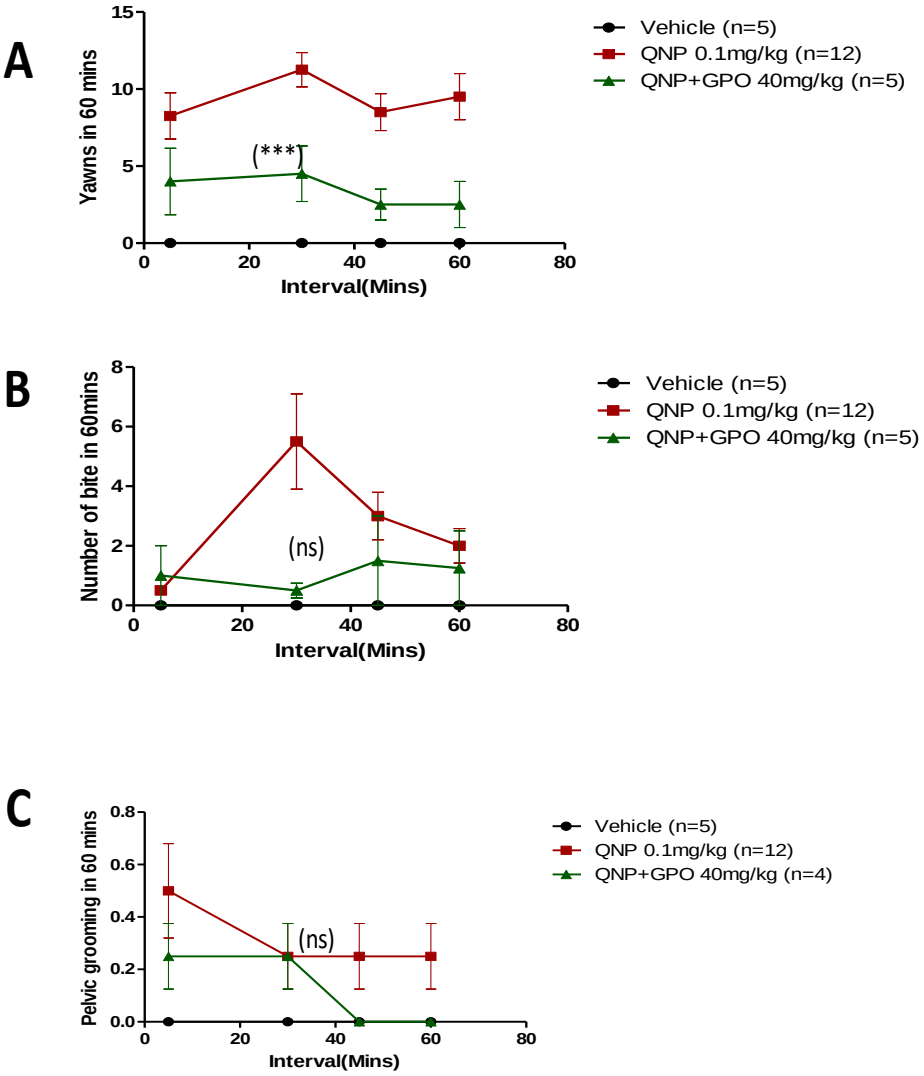


Figure 45: GPO attenuates QNP-induced yawning effect

(A): GPO 40 mg//kg pretreated group significantly reduced QNP-induced yawning effect during the 60 mins observation (**B, C**): GPO, at the same dose administered (40 mg/kg), did not affect both pelvic grooming and pica eating effects, ns: not significant, ***P<0.0001 *vs.* respective control at all times. *Bonferroni* post hoc tests after significant two way repeated measures ANOVA.

IV-3-C4- Acute Ex-4 and GPO administrations differentially modulate D-AMP-induced locomotor activity

During the first 60 minutes of observation, following administration of the dopamine-releasing agent D-Amphetamine (DAMP, 1 mg/kg ip), animals that were pre-treated (15 min before the challenge) with Ex-4 5µg/kg. i.p. show a significant reductions in motor activity both pooled control group ($F(1, 47) = 20.69$) and control group experiment at the same time with treated group ($F(1, 47) = 36.47$) when compared to the non-pre-treated rats (Fig. 46A, B). Similarly, GPO-pre-treated animals exhibited the same significant ($F(1, 48) = 14.31$) inhibitory effect on D-AMP-induced locomotor activity, but at lower magnitude (Fig. 46C, D). We also access if the GLP-1 antagonist Ex-9 could reverse the effect of Ex-4. Interestingly, in the presence of Ex-9 a GLP-1 receptor antagonist, there was no significant difference between the Ex-9 pre-treated group and D-AMP treated control (Fig. 47A). We took a further step to compare motor activity between D-AMP control group and rats already pre-treated with GLP-1R blocker (Ex-9, 2µg/kg) followed by Ex-4 5 µg/kg and then D-AMP. The pre-treated group (Ex-9 +Ex-4) exhibited a significant reduction in motor activity when compared with D-AMP control which shows that the drug did not block the effect of Ex-4 at this dose (data not shown). However, at an increased dose of 100 µg/kg Ex-9, motor activity of the rats was partially restored and Ex-4 activity seems partially to be blocked (Fig. 47B). It is important to note that in all the behavioural experiments, controls were investigated exactly at the same time as treated for accuracy purpose, but when compared with a larger population of control such as $n=23$ for DA-MP, we got the same results.

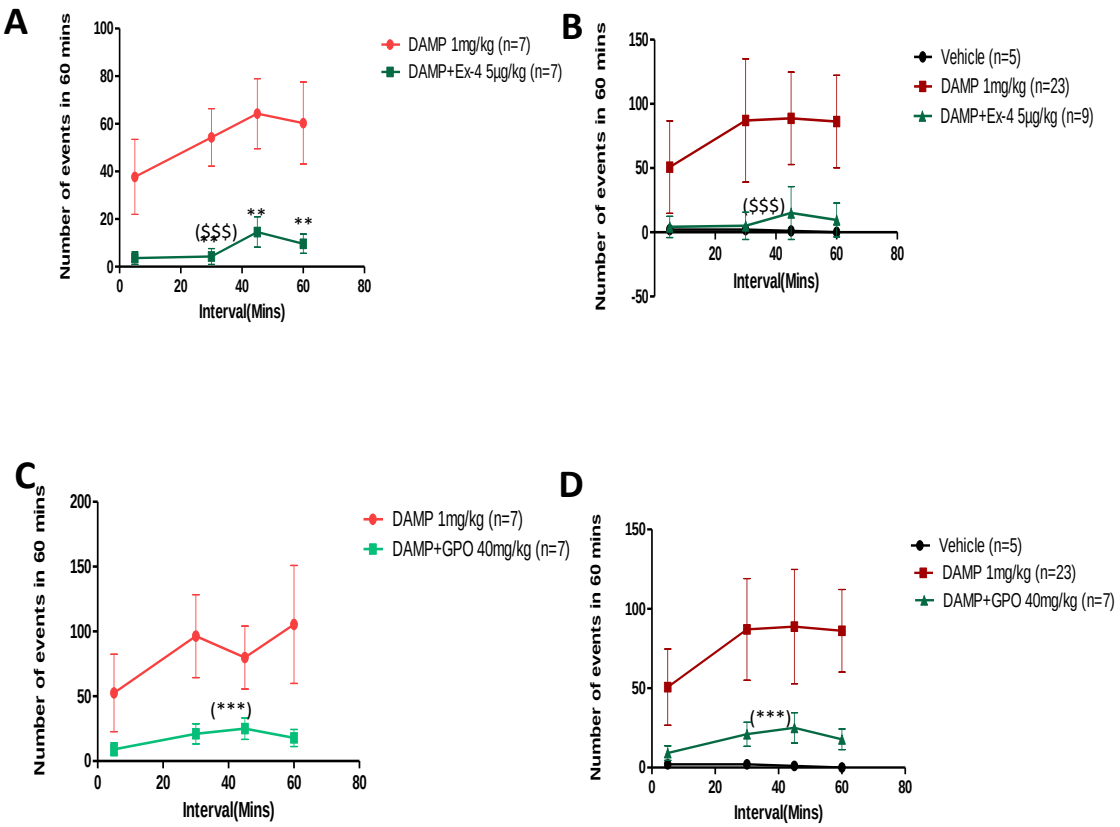


Figure 46: Impact GLP-1 on D-AMP-induced motor activity

(A): Ex-4 (5 µg/kg) pre-treated group exhibited a significant reduction in locomotor activity and in particular at 30, 45 and 60 mins of observation compared to control animals run in parallel**. (B):** This is a similar figure than (**A**), but showing how the treated group deviates from the pool of control subject of 23 animals. **(C):** GPO pre-treated group also exhibited a global significant reduction from D-AMP treated control run in parallel**.** (**D**): GPO-treated group deviation from a pool of total control subjects used in the experiment. The large error indicates pronounced variability within the wide control group of 23 rats. $$$P<0.0001, **P<0.001, ***P<0.0001 *vs.* respective control at all times. *Bonferroni* post-tests after significant two-way ANOVA.

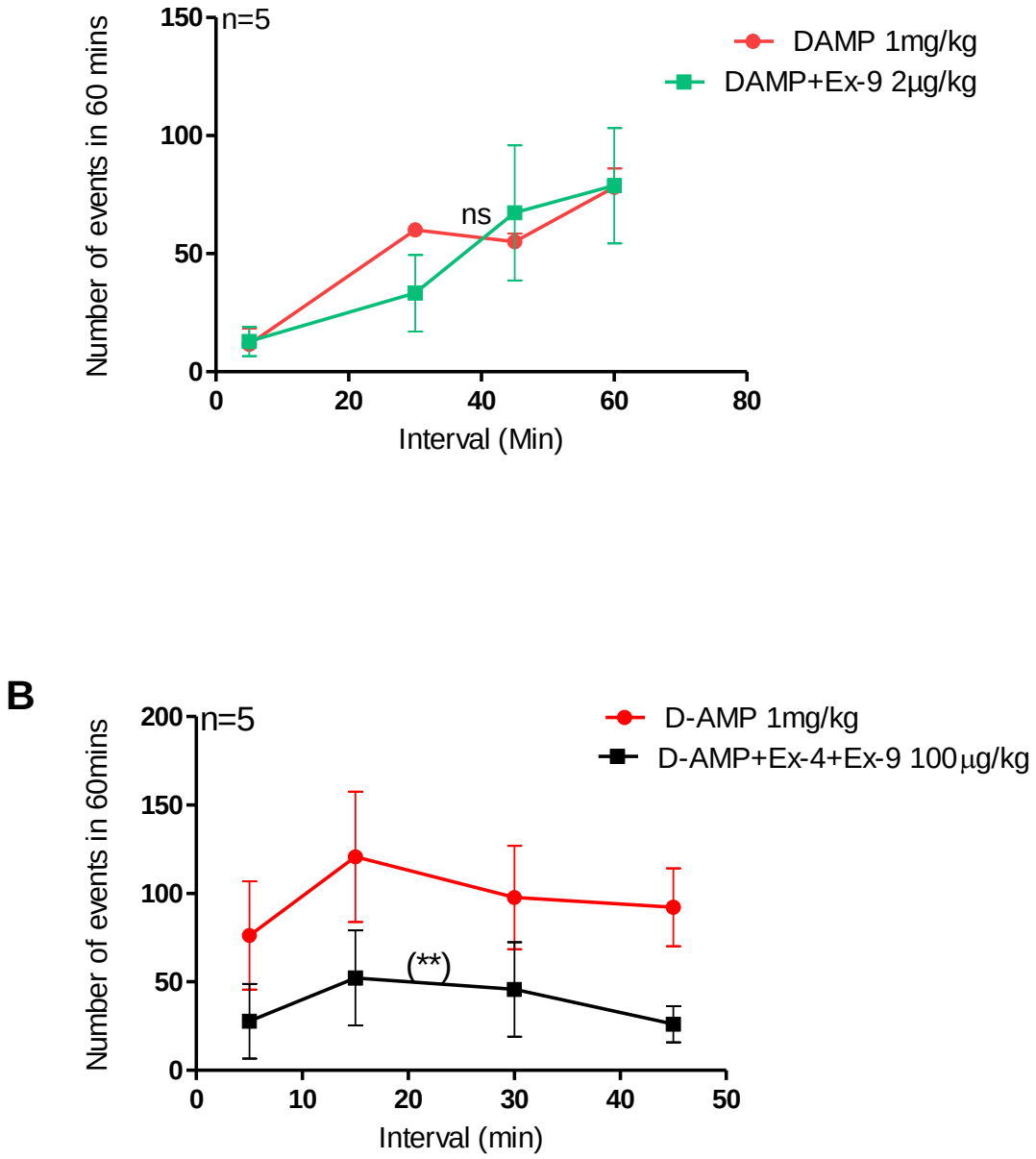
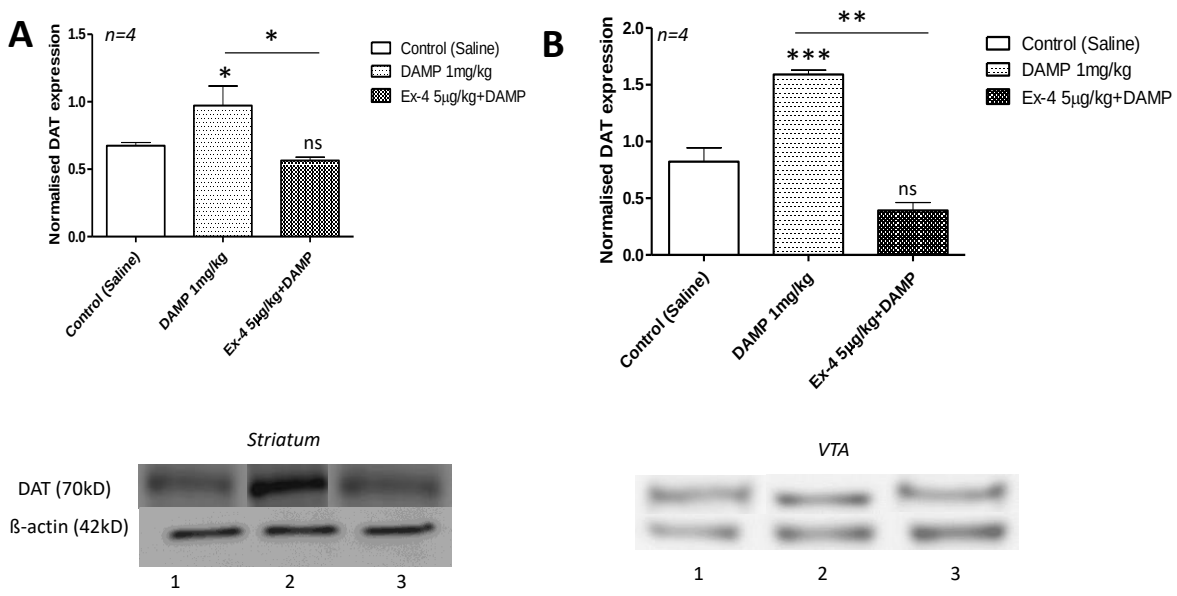


Figure 47: Impact of Ex-9 on D-AMP-induced motor activity

(A): Ex-9 2µg/kg, a GLP-1 antagonist pre-treated group showed no significant alteration in motor activity when compared to D-AMP control group. **(B):** The treated group in which the antagonist (Ex-9, 100 µg/kg) was first administered, followed by Ex-4 (15 mins interval) and later challenged by D-AMP, showed that Ex-9 to some extent did not block the effect of Ex-4 agonist compared with D-AMP control group. Thus, Ex-9 at 100 µg/kg partially attenuated the effect of Ex-4. **P<0.0001 *vs.* control. *Bonferroni* post-tests after significant two-way ANOVA.

IV-3-D-Acute Ex-4 administration regulates DAT and GLP-R protein expression

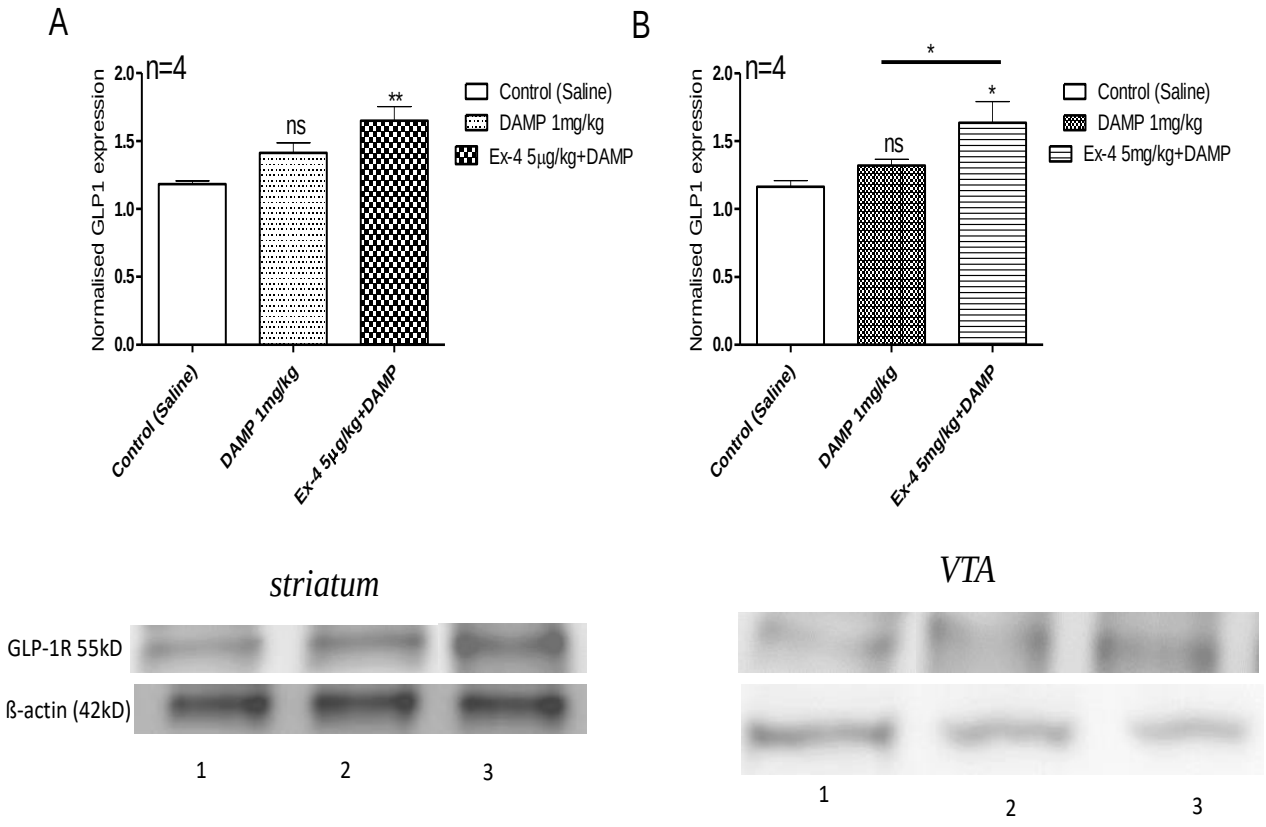
Following behavioural assessments of Ex-4 acute treatment on D-AMP-induced motor activity; the extent of DAT and GLP-1 protein expression in each treatment group was studied. We observed significant increases of DAT protein expressions in the striatum as well as in the mid brain (containing the VTA) with D-AMP treated group. However, in the groups pre-treated with EX-4 following D-AMP administrations, there were significant reductions in the level of DAT expressions both in the striatum ($F(2, 11) = 6.954$) as well as the midbrain ($F(2, 11) = 17.35$) (Fig. 48A, B). In another series of western blotting studies, we evaluated the impact of Ex-4 pre-treatment on GLP-1R protein expression. Here, we observed an increased in GLP-1R expression in group pre-treated with Ex-4 when compared with the control in the striatum, after 1 hr. of Ex-4 administration. Similarly, in the midbrain Ex-4 pre-treated group expressed significantly ($F(2, 11) = 9.595$) more GLP-1R protein when compared with both control (saline) and D-AMP treated drug (Fig. 49A, B).



Note: 1=control (saline), 2=D-AMP, 3= Ex-4+D-AMP

Figure 48: Ex-4 acute administration increases striatal and midbrain DAT protein expression.

Following behavioural experiments with D-AMP, DAT protein expression levels were quantified in different regions. **(A):** In the striatum, D-AMP (1 mg/kg) exposed group significantly increased DAT protein expression when compared to both control (saline) and Ex-4+D-AMP groups. **(B):** In the midbrain, D-AMP (1 mg/kg) exposed group significantly increased DAT protein expression in comparison with control (saline) and Ex-4+ D-AMP groups. The more incremental effect was observed in the midbrain. The bottom is displayed representative immunoblots with protein sizes in kilodaltons (kDa). *P<0.05, ***P<0.0001, *vs.* control (saline), *P<0.05, **P<0.001 *vs.* Ex-4+D-AMP group, Newman–Keuls test after significant ANOVA.



Note: 1=control (saline), 2=D-AMP, 3= Ex-4+D-AMP

Figure 49: Ex-4 pre-treated rats increase striatal and midbrain GLP-1R protein expression.

Following behavioural experiments with D-AMP administration, GLP-1R protein expression levels were quantified in different regions. **(A):** In the striatum, Ex-4 pre-treated group in combination with D-AMP, significantly increased GLP-1R protein expression when compared with the vehicle, while a single administration of D-AMP has no significant effects. **(B):** In the midbrain, Ex-4 pre-treated group significantly increased GLP-1R protein expression in comparison with control (saline) and with D-AMP-only groups. Below are displayed representative immunoblots with protein sizes in kilodaltons (kD). *P<0.05, **P<0.001, *vs.* control (saline), *P<0.05*vs.*D-AMP group, *Newman–Keuls test* after significant ANOVA.

V-3-E-Impact of acute systemically administered GLP-1 agonists on the firing rate of midbrain dopamine neurons and pyramidal PFC neurons

IV-3-E1- GLP-1 drugs modulate the firing rate of midbrain dopamine neurons.

In these electrophysiological studies, we assessed the effect of acute systemic administration of Ex-4 as well as GPO on the firing rate of DA neurons and pyramidal PFC neurons in anaesthetized rats. In the midbrain, we study the dose-response relationship of Ex-4 and the firing activity of VTA DA dopamine neurons. We observed no effect on the firing activity of VTA DA neurons. When the dose was increased to 20 µg/kg Ex-4, the firing activity remains unchanged (Fig. 50A, B). However, following co-administration with insulin 20 µg/kg, as indicated in the previous chapter, the firing rate of dopamine neurons became slightly and progressively reduced, when compared to saline treated animal in the same condition, indicating a possible synergetic effect between the 2 hormones on dopamine neurotransmission (Fig. 50A &C). Similarly, GPO 40 and 80 mg/kg tested on VTA dopamine neurons did not alter the firing activity of the DA neurons (Fig. 51A, B). We further tested the effect of two strengths of GPO (40 and 80 mg/kg) on non-dopamine VTA neurons. Our observation was homogenous to the effect of GPO on VTA DA neurons, as there was no alteration in the firing activity of non-dopamine neurons (Fig. 51C). The distribution of the responses of VTA neurons to the different treatments were compared using Chi-square test (no change; increase defined as 20% over baseline; decrease defined as 20% below baseline, following administration). The proportion of neurons responding to GPO (Fig. 52A) and Ex-4 (Fig. 52B) administrations were different from the responses from saline administration (Fig. 52C).

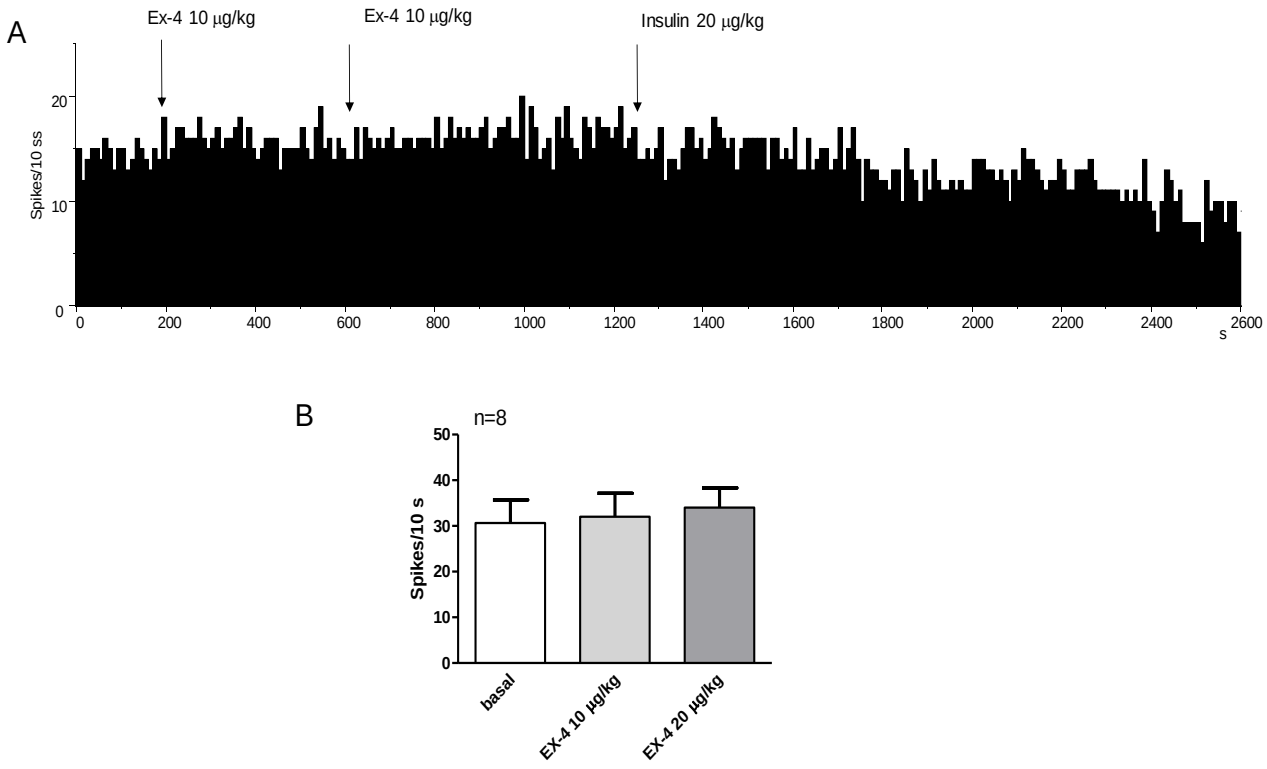


Figure 50: Electrophysiological effects of intravenously administered Ex-4 on VTA dopamine neurons

(A): Firing rate histograms of a VTA dopamine neuron following 2 subsequent *iv.* administration of Exendin-4 (cumulative dose 20 µg/kg, iv.) followed by an administration of Insulin (20µg/kg, iv.).

(B): Mean firing activity of VTA dopamine neurons recorded during and after 2 administration of Exendin-4 (cumulative dose 20 µg/kg, iv.). Ex-4 administration did not alter the firing rate of VTA dopamine. However, when co-administered with insulin 20 µg/kg (iv.) a decrease in the firing was observed in some neurons.

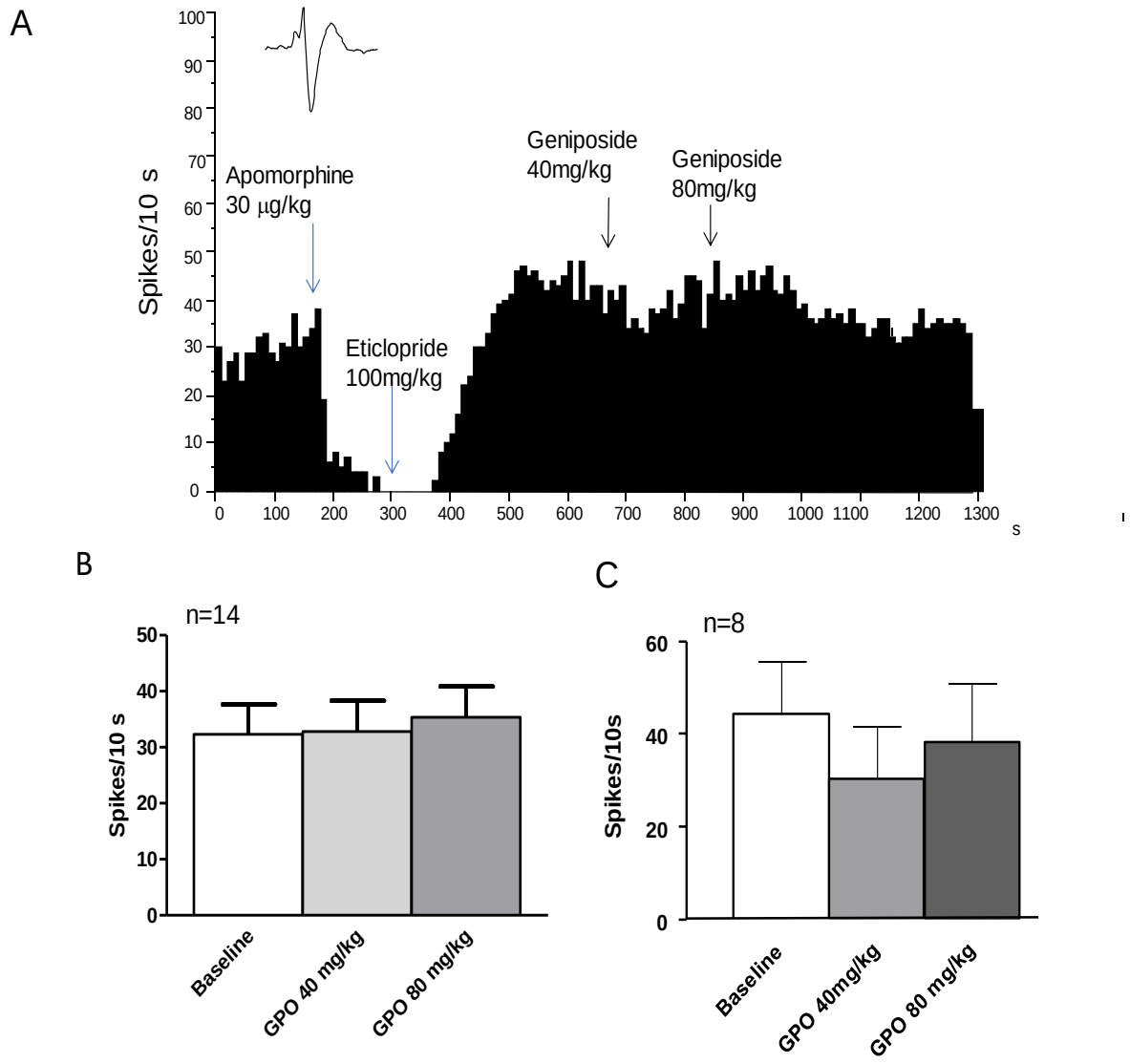


Figure 51: Electrophysiological effects of intravenously administered GPO on non-dopamine VTA neurons

(A): Firing rate histogram of a confirmed VTA dopamine neuron (response to apomorphine/eticlopride) showing no response to administration of 40 and 80 mg/kg of geniposide (GPO, iv.)

(**B, C**): Administration of 2 subsequent doses of geniposide did not alter the mean firing rate of VTA dopamine (B) and non-dopamine neurons, ns: non-significant, P>0.05.

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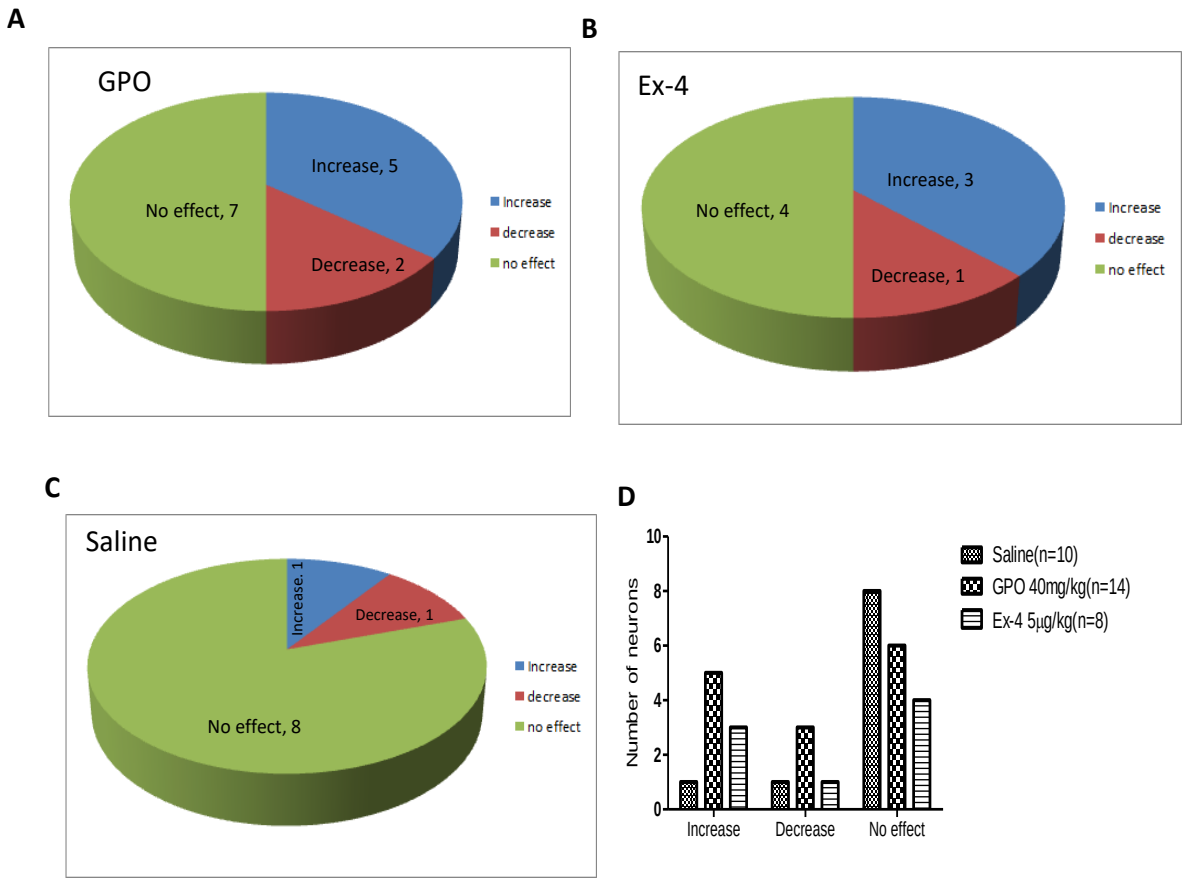

Figure 52: Comparing the firing rate distributions of saline, GPO and Ex-4 on VTA DA neurons

(A): Proportion of neurons showing changes in firing activity with intravenously administered GPO, about 35% of neurons responded to an increase in firing activity **(B):** Proportion of neurons showing changes in firing activity with intravenously administered Ex-4, about 43% of the neurons displayed an increase in firing activity. **(C):** Change in firing activity with saline administered intravenously, showing 80% of the neuron's response to no effect. **(D):** There was no statistical significant difference in the population of neurons distribution within the three treatment groups, P>0.05 Chi square test

IV-3-E2- Ex-4 diffusion in the brain modulates electrophysiological characteristics and sensitivity of VTA dopamine neurons

In the first series of experiments, the recording electrodes were filled with exendin-4 (final concentration 100 µg/ml in saline) and lowered into the VTA for the

recording of dopamine neurons. As mentioned in the previous chapter, it is anticipated that during the recording Ex-4 can diffuse through the recording electrode into the VTA nearby the recorded neurons. In our laboratory, this method of administration of products directly to the neuron tested has proven to be efficient (Gronier, 2011a). Although, the amount of product applied to the neurons cannot be predicted and diffusion efficiency can vary from one electrode to one another. Whenever possible the dopamine neurons were recorded during two subsequent periods of 7 min and firing activity between period 1 and 2 was compared assuming that Ex-4 will progressively diffuse through the electrode and that neurons will be exposed to insulin for longer in the second period of the recording. Firing activity of dopamine neurons recorded this way was not significantly different from the firing activity of a control population of neurons (though it tends to be 20% higher, $p<0.2$). During the second period of the recording, the mean firing activity increased in the majority of the neurons tested, but this increase did not reach statistical significance ($p<0.15$; Fig. 53A &B). The mean firing activity of these neurons (late recording period) was also 39% higher than in the control population, but this was not statistically significant ($p<0.2$). Believe that this may be due to the small sample size of dopamine neurons in the tested group.

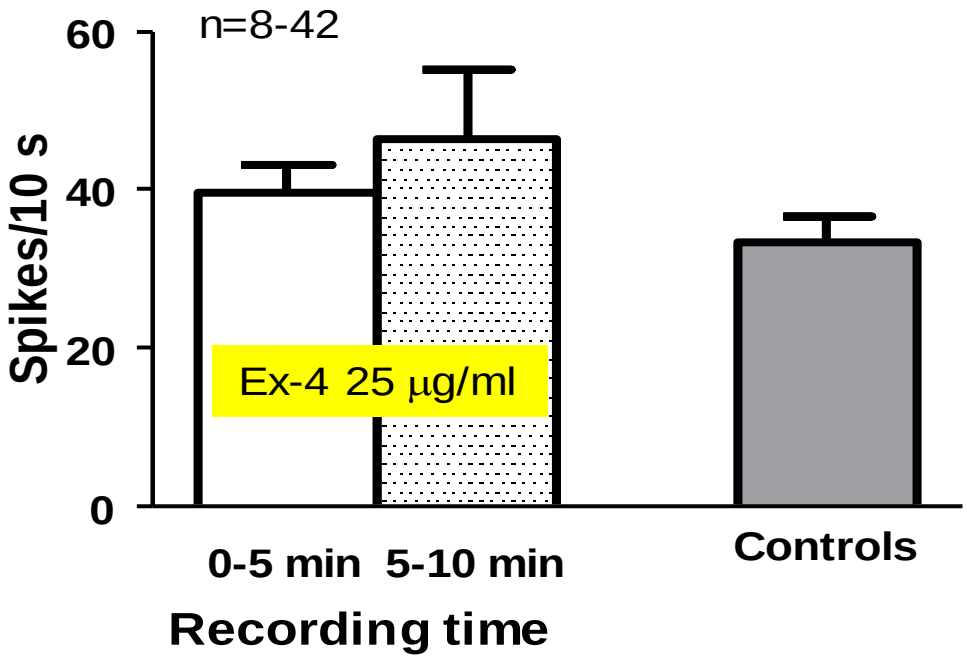

Figure 53: The mean firing activity of VTA dopamine neurons

The recording was carried out with an electrode filled with Exendin-4 (left). There was an increase during the later period of the recording (7-15 min), during that time the firing is also significantly higher than the mean firing activity of VTA dopamine neurons recorded in normal condition (with saline-filled electrodes). Note that in control condition the firing rate of dopamine neurons do not decrease with time and the controls were obtained from previous experiments.

IV-3-E3- Intra-VTA administration of Ex-4

As discussed, previous data from our laboratory has shown that the administration of saline (1-2 µl) within the VTA did not change the electrophysiological characteristics of the dopamine neurons (Gronier, 2008). This shows that the insertion of a Hamilton syringe needle does not induce significant

damage in this structure. Like in the previous study we have used our main control population (no VTA administration of saline) for comparison. Figure 54 shows the distribution of neurons recorded at least 40 min after the administration of insulin Ex-4 (1.5 µg) into the VTA compared to controls. Treated neurons displayed generally higher baseline and burst activity than in the control population, and results were nearly significant for firing activity ($p<0.08$, unpaired Student's t-test), but not for burst activity ($p<0.3$). Nevertheless, the proportion of neurons firing in the burst was particularly high in the treated group (16/19, 85% of neurons).

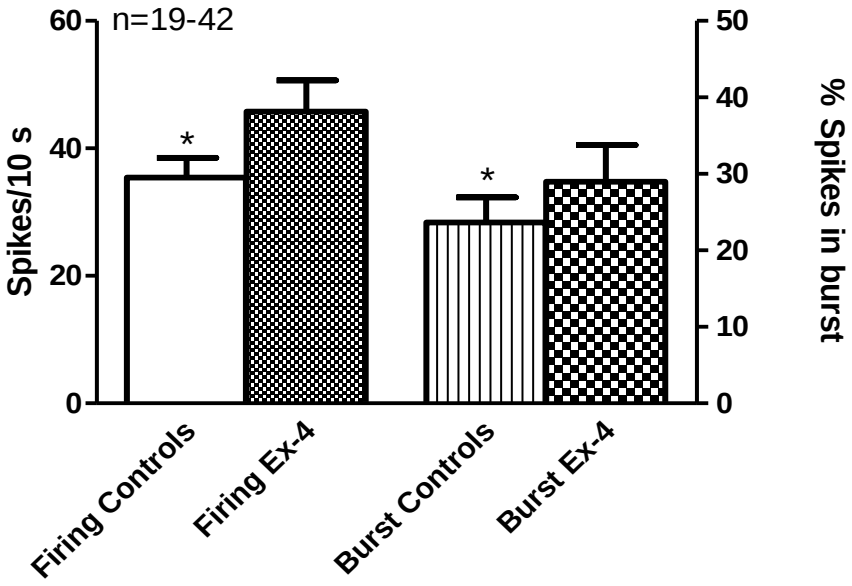

Figure 54: Individual electrophysiological characteristics (firing rate and burst firing) of VTA dopamine neurons rats

There were increases in both the firing rate and the burst firing of the VTA dopamine when Ex-4 (1.5 mg) doses were administered within the VTA at least 40 min before and in control animals (n=6).

IV-3-E4- Intra-VTA administration of GLP-1 changes the sensitivity of dopamine neurons towards the dopamine D_3 agonist pramipexole

We investigate the effect of a wide range of pramipexole (a Dopamine D_3 preferential receptor agonist, see previous the chapter) doses intravenously administered, with progressive 20 µg/kg increments and up to a cumulative dose of 100 µg/kg, in naïve and in VTA-treated animals (same condition as before: 1.5 µg Ex-4 injection within the VTA). As mentioned in chapter 2, naïve animals displayed a significant progressive reduction in firing rate upon each administration of PPX leading to a complete (or nearly) complete shutdown of the firing activity, an effect reversed by the administration of the potent D_2/D_3 receptor antagonist eticlopride (100-200 µg/kg). Neurons previously exposed to Ex-4 displayed a shift in their sensitivity to the D_3 agonist, as seen in Figure 55. Analysis indicates a significant dose and treatment effect. Equivalent results were obtained when % of firing decreases from baseline were considered (not shown).

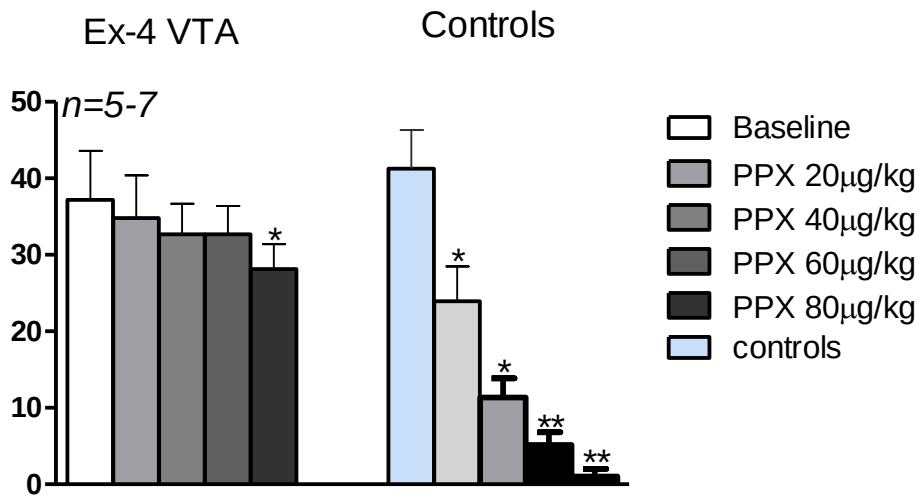

Figure 55: Intra-VTA exposure to exendin-4 induces partial desensitisation of dopamine neurons to the preferential dopamine D_3 receptor agonist pramipexole.
Compared to control animals, dopamine neurons from intra VTA-treated animals displayed significantly lower firing rate reductions following progressive pramipexole challenges, administered in a dose response manner and with progressive 20 µg/kg increments. *p<0.05; **p<0.01 vs. respective control, repeated measure ANOVA

IV-3-E5- GLP-1 drugs enhance the firing rate of pyramidal prefrontal cortex neurons

Here, we tested the effect of GLP-1 on the firing rate of pyramidal neurons located in the PFC. Intravenous administration of Ex-4 dose-dependently (10 and 20 µg/kg) increased the firing rate of pyramidal neurons in a significant pattern (Fig. 57A, B). This provides us with potential evidence of correlation with behavioural paradigm on cognition and memory effect of Ex-4 in rats, as earlier demonstrated. However, in one neuron (about 11.6%), Ex-4 induced reduction in the firing activity of the pyramidal cells during the experiment (Fig. 56C). Similarly, GPO at a dose of 40 mg/kg did not alter the firing activity of the neurons. But, at 80 mg/kg a significant excitatory effect was observed (Fig. 57A, B). Considering the total population of PFC pyramidal cells studied, about 33% of the neurones showed a reduction in firing activity (Fig. 57C).

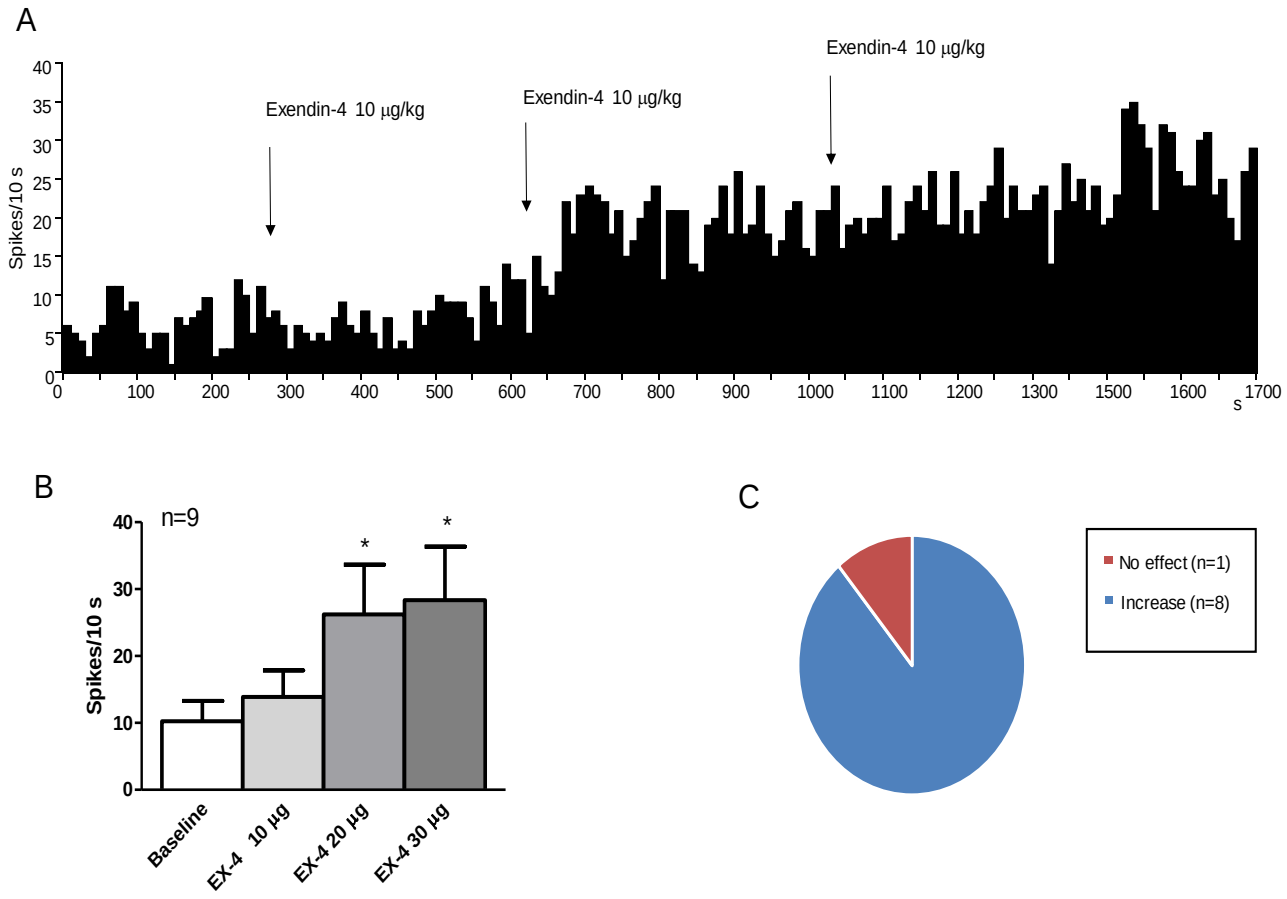

Figure 56: Ex-4 administered intravenously increase the PFC pyramidal cell firing rate
(A, B): Ex-4 (iv.) significantly increased the firing rate of pyramidal neurons in a dose-dependent pattern showing firing rate histograms of an individual neuron in A, and mean firing rates in B.
(C): Only one neuron out of the 9 PFC pyramidal neurons tested, exhibit no increase in firing rate following by Ex-4 (30 µg/kg). *P<0.05, *vs*. baseline, *Newman–Keuls* test after significant ANOVA.

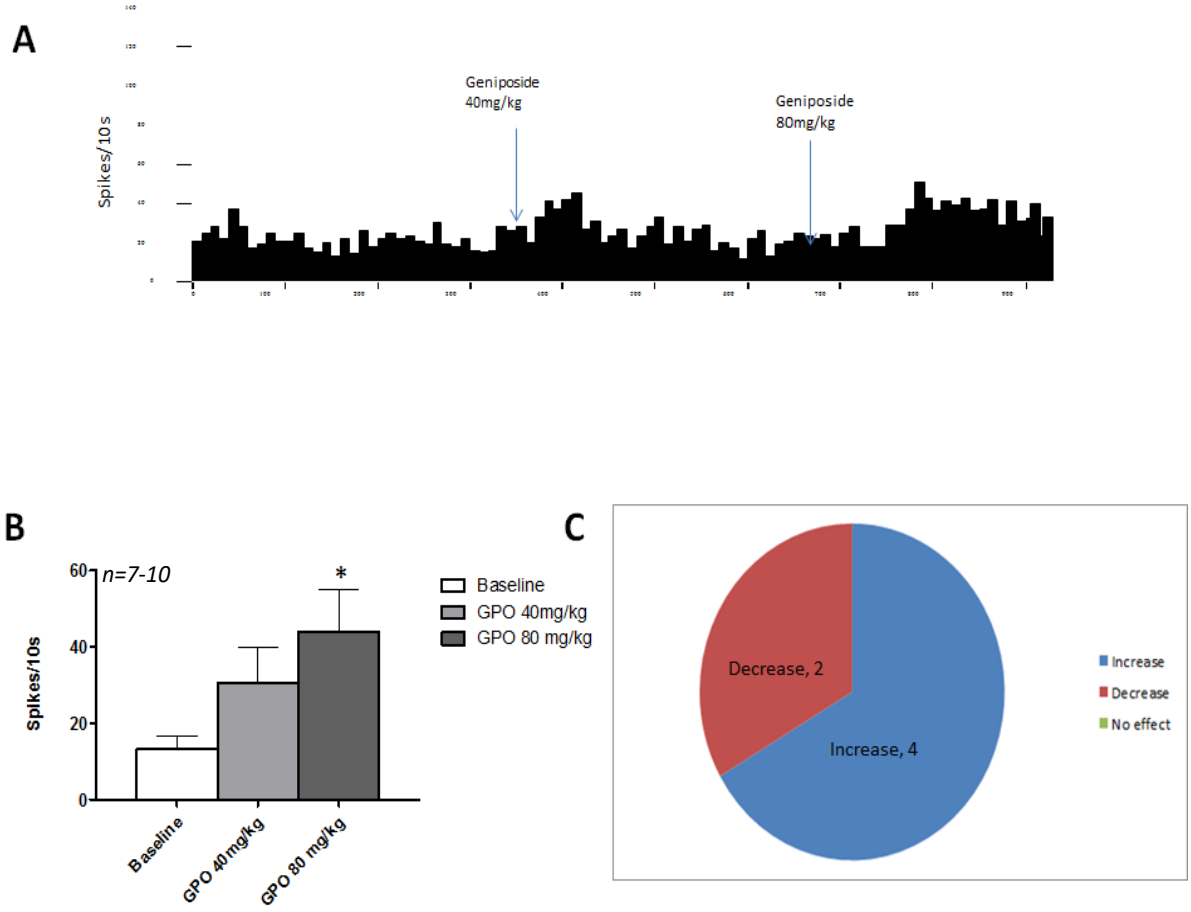

Figure 57: GPO administered intravenously increase pyramidal cell firing rate
(A, B): GPO at 80 mg/kg (iv.) significantly increased the firing rate of pyramidal neurons while the 40 mg/kg did not affect. (**C**): In the PFC pyramidal 2 neurons out of the 6 neurons exhibited a reduction in the firing rate induced by GPO, while the remaining neurons showed activation. *P<0.05 *vs.* baseline, *Newman–Keuls test* after significant ANOVA

IV-4-Discussion

In this chapter, we have demonstrated that the gastrointestinal role of GLP-1 receptor activation goes beyond feeding and glucostatic regulations, but also involves central nervous system action. Thus, we present for the first time that D-AMP-induced motor response and D_2/D_3 dopamine agonist-induced behavioural traits (yawning, pica eating and pelvic grooming), as well as PCP-induced cognitive impaired condition, are attenuated in rat intraperitoneally pretreated with GPO or Ex-4. We further unravel that dopamine transporter modulation may be involved in these processes. The previous study has reported a reduction in basal motor activity when GLP-1 analogue is intracerebroventricularly administered into the hypothalamus (Turton et al., 1996). As part of our pilot study, we initially tested the effect of single acute administration of Ex-4 on basal locomotor activity and observed significant attenuation in motor activity with rats treated with Ex-4 5 µg/kg when compared with the control (Fig. 41A). Several studies have shown that Ex-4 easily transverses the BBB when given peripherally and may be partly responsible for the reduction in basal ambulatory activity by stimulating GLP-1Rs in the medial subnucleus of the NTS (Kanoski et al., 2011a; Kastin and Akerstrom, 2003a). While we observe reduced motor activity at 5µg/kg Ex-4, Erreger et al., (2012a) reported a reduction in motor activity at doses 6 times (30 µg/kg) higher than in our study. Nevertheless, the tendency of Ex-4 and GPO to reduce the motor effect of D-AMP are partly modulated through GLP-1Rs located within the mesocorticolimbic DA pathway (Kastin and Akerstrom, 2003b). However, peripheral administration of GLP-1 may easily access expressed GLP-1Rs in the NTS, and can also elicit its effect through vagal interaction. Thus, NTS, in part via GLPergic fibres, innervates the mesolimbic dopaminenergic system, peripheral GLP-1 may be able to modulate reward-mediated brain regions such as VTA and NAc (Alhadeff et al., 2012b; Alvarez et al., 1996; Rinaman, 2010). Some studies have also demonstrated that craving induced by highly

palatable foods was largely reduced when Ex-4 was locally administered in the main brain reward areas (Alhadeff et al., 2012b). Especially, in this chapter, we demonstrate through western blot the possibility that GLP-1Rs that are abundant in striatal areas, particularly in the ventral striatum, are involved in weakening the motor stimulating activity to D-AMP (Chartoff et al., 2005; Dossat et al., 2011; Heusner et al., 2003). Albeit, GPO shows a lesser degree of attenuation of D-AMP-induced motor response compared with Ex-4 albeit, both still exhibited a significant level of reduction (Fig. 46A, C). We hypothesize that GLP-1R activation attenuate D-AMP-induced motor activity by interfering with DA D_2/D_3 autoreceptor sensitivity, since we did not observe any significant modulatory effect on the release and basal DA uptake assays. This is a plausible mechanism, because Ex-4 weakened QNP and PPX-induced DA large uptake. Another possible mode of action is the possibility of change in the sensitivity or activity of post synaptic receptor (whether D2/3 or even D1) involved in the effects of DAMP that can be involved. This may occur in DA-rich region like in striatum. We also tested the effect of 2 µg/kg Ex-9, a GLP-1Rs antagonist on D-AMP-induced locomotor response. However, the effect was similar to D-AMP treated group contrary to our expectation of increased locomotor activity (Fig. 47A). This might be due to low dose administered that was not enough to block the effect of endogenous GLP-1R. Next, we attempted blocking the effect of intraperitonially dosed Ex-4 by pretreatment with Ex-9 2 µg/kg (GLP-1R antagonist) followed by Ex-4 5µg/kg treatment before D-AMP challenge. Our findings revealed that Ex-9 at 2 µg/ kg did not effectively block the agonistic effect of Ex-4 but, at 100 µg/kg there was a blockage of the GLP-1R to a lesser extent (Fig. 47B). It might be suggestive that intraperitoneal administered Ex-9, poorly transverses the BBB and blocks peripheral GLP-1R through efflux of Ex-9 from the brain to the periphery (Kanoski et al., 2011b). It is plausible that GLP-1Rs activation impede with the pharmacological effect of D-AMP to enhance the release or synthesis of DA or facilitate the activity of VTA DA via an

interaction with the dopamine transporter (Heusner et al., 2003; Koob and Volkow, 2010). An interesting hypothesis will be that GLP-1Rs activation may result in modification of DAT or dopamine receptor activity, which leads to attenuation of a rewarding trait of D-AMP (Owens et al., 2005b; Thompson et al., 2010).

The capacity of psychostimulants to induce dopamine release are familiarly connected with the boosting characteristic of habit-forming drugs and these variables are thought to be an integral part of craving or addiction process (Everitt and Wolf, 2002; Wise and Rompre, 1989). By adopting *in vitro* radiometric ^{3}[H] DA release assay protocol, we further elucidate the potential mechanism by which GLP-1 drugs attenuate D-AMP-induced motor activity using striatal slices. Here, we report that neither Ex-4 1µM and Ex-4 5µM nor GPO 40µM and GPO 80µM has a significant effect on basal ^{3}[H] DA release from the striatal slices (Fig. 34A, B). The null effect of GLP-1 drugs on basal dopamine release is in agreement with an in vivo microdialysis dopamine measurement experiment performed on mice by Egecioglu et al., (2013). They further reported that at a selected dose of 2.4 µg/kg Ex-4, there was no significant effect on accumbal DA release when compared with the saline treated group. We have also demonstrated an increase in DA efflux induced by D-AMP 10 µM on striatal slices, which may be key to locomotor and addiction effects of psychostimulant (Wise and Rompre, 1989). Interestingly, in our radiometric release model we found that D-AMP-induced striatal dopamine release was slightly reduced by pre-treatment with Ex-4 although, not statistically significant (Fig. 35A, B). Since we didn't find the same result in vitro and in vivo, models this indicates that Ex-4 may act indirectly to modulate D-AMP induced release and not directly on the dopamine terminal. These results show that activation of GLP-1Rs on the striatal DA terminals has an inhibitory effect on striatal DA efflux. These findings are in agreement with previous studies that reported reduction of alcohol, cocaine and D-AMP-induced DA release in *in vivo* microdialysis experiment on mice following treatment with Ex-

4 (Egecioglu et al., 2013e, 2013e; Erreger et al., 2012b; Sørensen et al., 2015). The slightly weaker effect of GPO on D-AMP-induced locomotor activity might be due to its lower efficacy on GLP-1Rs activation, having reported the drug as a putative GLP-1 agonist (Gong et al., 2014b).

As part of our investigation to unravel the mechanism of action of GLP-1 drugs attenuating effect on D-AMP-induced locomotor activity, we assessed the role of DAT and GLP-1R protein expressions in the striatum as well as in the midbrain within various groups of treatment. Here, we report that Ex-4 pretreated group significantly reduced DAT expression induced by D-AMP treated control group, both in the striatum and VTA (Fig. 48A, B). However, this technique is limited by inability to express spatial distribution of the proteins from various anatomical point of view, as well as lack of probable antibodies specificity leading to off-target binding. DAT has been generally considered as a principal regulator of DA signaling process, by driving uptake of extracellular DA back into the presynaptic neurons (Vaughan and Foster, 2013a). In the clinical management of attention deficit hyperactivity disorder (ADHD), DAT is the major target for the pharmacological actions of psychostimulants such as, methylphenidate, methamphetamine (METH) and D-AMP (Han and Gu, 2006a). DAT is thought to be the chief terminating transmembrane for dopamine transmission hence, controls and maintains the strength as well as extent dopaminergic signaling (Giros and Caron, 1993). D-AMP is a potent substrate for DAT at which it competes with dopamine to enter the presynaptic dopamine terminals. Within D-AMP the terminal, DAMP blocks the vesicular monoamine transporter (VMT) to cause massive dopamine depletion. Then excessive intracellular dopamine will be in part released by a reverse transport system (Fischer and Cho, 1976; Rothman and Baumann, 2003; Han and Gu, 2006b). Previous neurochemical investigations revealed an elevation in DAT protein surface expression when a striatal synaptosome was acutely treated by D-AMP through the

process of biotinylation to mark surface DAT (Johnson et al., 2005). The studies further demonstrated that elevation of surface DAT expression by D-AMP was as a result of exocytosis emanating from continuous activation with D-AMP to facilitate DAT internalization. Further study by Furman et al., (2009) has also supported the evidence that DA, as well as D-AMP treatment on the striatal synaptosome, expeditiously elevate DAT trafficking to the cell surface with the aid of real- time internal reflection microscopy. Our findings on D-AMP-induced DAT overexpression is thus consistent with the earlier mentioned studies. It is possible that D-AMP can have access to the intracellular medium of the synapse and causes more depletion of the dopamine storage thus, leading to more DA to be reversely transported outside the synapse. We have for the first time, examined the neuromodulatory role of acute Ex-4 pretreatment on D-AMP-induced motor activity as it affects DAT degree of protein expression. A considerable body of literature has directly demonstrated that DAT (Fumagalli et al., 1998), change in temperature (Albers and Sonsalla, 1995) as well as energy metabolism alteration (Burrows et al., 2000) are required for the dopaminergic neurotoxity induced by methamphetamine (METH). Fumagalli et al., (1998) further revealed that DAT is principally required for METH-induced dopaminergic neurotoxicity, as confirmed by the pronounced change in dopaminergic cell integrity in wild-type mice and a complete deficit of any alteration in DAT −/− mice. Also, Salahpour et al., (2008), through the use of transgenic mice model and western blotting technique revealed that D-AMP at a dose range of 0.5-2 mg/kg induced upregulation of DAT leading to a striking increase in hypersensitivity to locomotor and craving effect of D-AMP. The observations in these findings may also support the hypothesis that symptomatic manifestations of ADHD is probably more of persistent loss rather than a gain of DAT function in humans. We have consistently demonstrated that Ex-4 pretreated groups both in the striatum as well as in the midbrain show a pronounced reduction in DAT protein

expression compared with D-AMP control group thus, it prevents D-AMP-induced upregulation of DAT. In agreement with this finding, data from our laboratory have shown that blockage of transporters by methylphenidate prevents D-AMP effect (Di Miceli and Gronier, 2015).

Furthermore, in this present study Ex-4 has been shown to reduce variables that may indicate a rewarding process such as, motor activity enhancement and striatal dopamine release. Then activation of GLP-1R in the reward centre of the brain by GLP-1 drug should have an indirect effect on DAT regulation and may prevent, at least in part, psychostimulant (D-AMP)-induced neurotoxicity. Here, we report a significant increase in GLP-1R protein expression in Ex-4 pretreated group when compared with D-AMP treated group in the VTA brain sample (Fig. 49B). Interestingly, in the striatum, the increase in GLP-1R expression induced by Ex-4 pretreated group was significant when compared with the naive control, but not with D-AMP treated group (Fig. 49A). Earlier studies have identified the neuroanatomical evidence for a possible function of GLP-1 in reward, particularly the expression of GLP-1R mRNA and GLP-1 immunoreactivity in specific brain regions such as NAc and VTA, and also substantia nigra, hippocampus and hypothalamus (Merchenthaler et al., 1999b). Since GLP-1Rs are expressed centrally, it is plausible to report that activation of these receptors will attenuate drug-dependence and craving behaviours. Several emerging literature has reported the role of GLP-1Rs in food and drug reward processes (Hayes and Schmidt, 2016a; Schick et al., 2003). Thus, peptides that impact on feeding behaviour are relevant in drug reward and could be an important marker for targeting pharmacologic therapy in the management of addiction (Erreger et al., 2012b; Kenny, 2011b). Another study by Schmidt et al., (2016) revealed that stimulation of VTA GLP-1Rs remarkably attenuates the boosting reward magnitude of cocaine in the mesolimbic DA pathway. Interestingly, in our study, we found desensitization of dopamine D_2/D_3 receptor in VTA following

application of Ex-4. Since feeding behaviour and drug craving or dependence share an interrelated brain circuitry mechanism, we infer GLP-1R stimulation may be a potential modulator of addictive behaviour associated with DA pathway and uncontrollable psychostimulant use.

Next, we discuss our findings on the effect of GLP-1 drugs on well-defined behaviours such as yawning, pica eating, and pelvic grooming induced by D_2/D_3 receptors dopamine drugs. We also studied the possible mechanism of action by deploying *ex vivo* radiometric ^{3}[H]DA uptake assay in striatal synaptosomes to corroborate the *in vivo* behavioural data. In chapter I, we demonstrated the behavioural effects (yawning, pica eating and pelvic grooming) of intraperitoneal acute administration of D_2/D_3 dopamine receptor agonists namely, PPX and QNP. The current investigations were aimed at assessing the effects of Ex-4 and GPO on putative D_2/D_3 agonist-induced behavioural traits such as yawning, pica eating and pelvic grooming by D_3-preferring agonist PPX and QNP. Here, we report that Ex-4 5 µg/kg significantly attenuated both QNP and PPX-induced behavioural traits (Fig. 42A, B and 43A, C) except, pica eating activity that was not changed (Fig. 42C). Similarly, GPO 40mg/kg pre-treated group exhibited significant reductions in all the behavioural activities induced by PPX (Fig. 44A, B, C) but there was an exception for behaviour involving pica eating and pelvic grooming traits induced by QNP (Fig. 45B, C). Nevertheless, the yawning trait was remarkably reduced in GPO pre-treated group for QNP (Fig. 45A). The organic basis of yawning is attributed to be linked with alteration in physiological conditions of the body such as a reduction in lungs' oxygen volume as well as an increase in brain temperature, in response to environmental stimulus in the paraventricular nucleus (PVN) of the hypothalamus (Chan and Tseng, 2017). On the other hand, pelvic grooming arises from a series of a complex integrated neuronal and vascular episode that results in a build-up of blood in the phallus to attain rigidity (Andersson and Wagner, 1995).

While these two behavioural traits are positively correlated and majorly mediated by D_2/D_3 receptor agonist (Collins et al., 2008). Pica eating, an index of nausea induction does not share this mechanism (Takeda et al., 1993b). Paradoxically, while dopamine agonist has been widely implicated in inducing of all the behavioural traits under consideration, GLP-1 equally induces pica eating effect (Skibicka, 2013). This might be responsible for null effects exhibited by Ex-4 and GPO nearly in all the pica eating assessments. Previously, in this chapter, we have established the effects of GLP-1R activation on D-AMP -induced behavioural traits. A few numbers of works of literature has provided evidence for the presence of D_1 receptors on GABAergic neurons, projecting directly to the midbrain (VTA/substantia nigra) in what is term "direct dopamine pathway" (Groenewegen et al., 1999). The D_1 receptor found in these regions performs an important role in mediating behavioural activities induced by DA receptors activating drugs (Le Foll et al., 2009). Based on the few available data so far, Ex-4 has been suggested to potentially exert its effect through mediation of D_1 receptor signaling pathways and by affecting psychostimulant-induced striatal DA release (Sørensen et al., 2015). From our behavioural findings of the GLP-1 drug's effect effect on dopamine D_2/D_3 receptor agonist, it raises the curiosity that besides the documented data of Ex-4 effect on D_1 receptor mediation in psychostimulant induced motor activity, attenuating effect of GLP-1 observed may also be connected to D_2/D_3 receptor signalling. Besides, some experiments from our laboratory, not presented here, have shown that dopamine D1 receptor activation has rather a complex role on basal and drug-stimulated motor activity. Indeed, we found that the selective dopamine D_1 receptor agonist, A-68930 reduces basal as well as D-AMP-induced motor activity.

We further investigated the hypothesis that predicts an interaction with D_2/D_3 receptor signalling by GLP-1 with PPX and QNP-induced behavioural traits. Through *in vitro* uptake technique, using striatal synaptosomes, we were able to probe for a possible correlation

between our behavioural findings and neurochemical studies. Our data shows that neither Ex-4 nor GPO dose-dependently altered basal ^{3}[H] DA uptake activities (Fig. 37A, B). Interestingly, the two GLP-1 drugs under investigation differentially weaken PPX and as well as QNP-induced ^{3}[H] DA uptake activities when incubated with striatal synaptosomes in specific conditions (Fig. 37C, D). Also, we assessed the impact of GLP-1 drugs on the kinetic parameters (K_M/V_{max}) in the treated rat before isolation of the striatum. Surprisingly, we observed an increase in K_M value in both GLP-1 drugs, and no significant apparent change in V_{max} when compared with the naïve group. This translates to a possible modulatory effect of GLP-1 on DAT activity. This data demonstrates that GLP-1R stimulation potentially alters dopamine uptake, and modulates D_2/D_3 receptor signaling process to possibly cause an inhibitory effect of well-defined behavioural traits. Whether this modulation is involved in the inhibitory effect of behavioural traits induced by dopamine agonists is an interesting question. We have previously demonstrated in chapter I that D_2/D_3 receptors agonists (PPX D_3 preferred and QNP D_2 preferred) presynaptically regulate extracellular DA output in the limbic brain areas, an effect that has been ascribed to alteration in DA release (Joseph et al., 2002). There is more evidence that these agonists also interfere with dopamine transporter and uptake processes in the NAc as well as VTA, resulting in an enhanced rate of DA clearance and large transport velocity (Zapata and Shippenberg, 2002; Zapata et al., 2007a). This neuronal effect has been identified to be dependent upon the duration of agonist exposure to the brain region under investigation. In addition, it was reported that rapid fast increase in DA uptake induced by D_2/D_3 agonist is associated with enhanced cell surface as well as large DA transporter exocytosis (Zapata et al., 2007b). In converse, D_3 antagonist assessed under *in vitro* studies showed a reduced dopamine uptake when incubated with the striatal synaptosome (McGinnis et al., 2016). Our current findings further agree with these previous studies on D_2/D_3 receptor agonist effect on DA uptake and transport velocity

enhancement. On GLP-1 agonist interaction, studies have investigated GLP-1 agonists effect on DA transporter and revealed an increase in ^{3}[H] DA uptake in synaptosome prepared from the lateral septum of the brain region (Reddy et al., 2016a). Unfortunately, there are very few studies available that have provided a lucid neurochemical explanation for GLP-1R signalling effect on DAT and DA uptake activities. From our data, we can infer that GLP-1R signalling modulates DAT and alters DA uptake velocity. While, in the presence of D_2/D_3 receptor agonist, the activation of GLP-1R modulates and weakens D_2/D_3 induced DA uptake process. Whether this activity is partly responsible for perceived inhibition of behavioural traits such as yawning, pica eating and pelvic grooming exhibited by GLP-1 drugs, is a very interesting question to address. Interestingly, the same negative interaction between D_2/D_3 receptors and GLP-1 receptors seems to exist also in the VTA. We found then an intra-VTA administration of Ex-4 induced large desensitization of the inhibitory D_2/D_3 autoreceptors. The functional consequence of this effect remains to be elucidated. However, it may reflect a general role of GLP-1 R in the brain reducing dopamine receptor signalling, possibly via possible complex local interaction.

Besides ubiquitous presence of GLP-1Rs expression in the limbic system (Dickson et al., 2012c; Egecioglu et al., 2013d; Shirazi et al., 2013), we sought to investigate the possible impact of these peptides on the neurotransmitters present in cortical region (PFC) of the brain as well as drugs that impair memory and cognitive function in the brain. Recent data has demonstrated the presence of GLP-1Rs in the hippocampus, as well as cortical neurons of rodent brain regions critical for memory, cognitive and learning functions (Mossello et al., 2011a). Furthermore, it potentially provides molecular explanation for neuroprotective effects impelled by Ex-4 exhibited in various cognitive and memory disorders connected with Alzheimer's disease (Li et al., 2010), type II diabetes mellitus (T2DM) (Chen et al., 2012b) as well as Parkinson's disease (Harkavyi et al., 2008; Kim et al., 2009a). From these behavioural

studies, we report that both sub-chronic and chronic Ex-4 treated groups exhibited a more cognitive enhancement effect than the control group (Fig. 39D, F). It is important to note that, both groups (Ex-4 and saline) exhibited a positive preference for novel objects albeit, Ex-4 treated group demonstrated more profound cognitive effect. However, a single acute dose of Ex-4 5 µg/kg showed no statistical difference in cognitive task with the control (Fig. 39B). A study from Lovshin and Drucker, (2009) revealed that 5µg/kg Ex-4 administered twice daily improved glucostatic control, metabolic function and cognitive task in a rodent. In another cognitive task test demonstrated with water maze protocol, Ex-4 exhibited an enhanced learning performance in naive rats at the same dose when compared with saline-treated control (During et al., 2003b; Gault et al., 2010b). It was also reported that insulin resistance pathological condition, associated with the dysfunctional activity of GLP-1R is also connected with cognitive decline (Mossello et al., 2011b). Thus, enhancement of cognitive task after Ex-4 administration could be further evidence for a secondary effect of the drug on metabolic regulation and unmediated effect through direct activation of GLP-1Rs in the brain.

Next, we sought to test the hypothesis of cognitive and memory enhancement impact of GLP-1 on experimental rats' model of schizophrenia by acute single dose PCP-induced cognitive deficit and hyperlocomotion (Neill et al., 2016). Here, we report that acute 10 mg/kg PCP increased the exploration of familiar objects (a reflection of cognitive deficit) compared to Ex-4 5 µg/kg pre-treated group. However, in preference for novel objects recognition (NOR) testing, PCP induced a pronounced NOR deficit, while Ex-4 pre-treated group exhibited a large degree of preference for novel object, an indication of significant enhancement in cognitive function. We also assessed the impact of acute Ex-4 on PCP-induced hyperlocomotion activity. Our findings revealed significant inhibition in the locomotor activity of Ex-4 pre-treated group. Unlike D-AMP-induced motor activity, studies

have shown that dopamine is not essential for the hyperlocomotor activity of NMDAR blockers (Chartoff et al., 2005). The studies further suggested that glutamate, rather than DA is necessary for the motor and molecular responses of NMDAR blockers albeit, DA and glutamate can operate mutually. Other findings by Swanson and Schoepp, (2002) demonstrated that NMDAR antagonists increased locomotor activity in a rodent with depleted catecholamines and that this effect is distinguished from DA. There are substantial evidences that acute NMDAR antagonist treatment (Gilmour et al., 2012; Meltzer et al., 2013), as well as sub-chronic administration (Dawson et al., 2014; Nomura et al., 2016) at therapeutically used doses, induce synaptic structural dysfunction in specific brain regions such as, PFC and hippocampus in both man and animal subjects (Elsworth et al., 2011; Yuen et al., 2012a). These anomalies are profoundly associated with a glutamatergic aberration in the PFC (Yuen et al., 2012b), and GABAergic abnormality in the hippocampus (Nomura et al., 2016). More support for this evidence reported that an increased degree of NMDAR hypofunction within the brain is linked to learning and memory impairments, in ageing patients leading to psychotic and Alzheimer's tendencies (Newcomer et al., 2000). Beyond NMDAR signaling implication in cognitive and memory functions, cerebral insulin signalling dysfunction also results in extensive impairment of synaptic hippocampal neurotransmission and cognitive task (Trudeau et al., 2004). Thus, it is evocative that inadequate insulin production (T1DM) or insulin insensitivity (T2DM) induces cognitive deficit and deficiencies of long-term potentiation (LTP) in the hippocampal (Gault et al., 2010b) and PFC (Erberk Ozen and Rezaki, 2007; Frith and Dolan, 1996) regions of the brain. Because of these neurochemical and behavioural data, it is suggestive that glutamatergic neurotransmission signalling, altered by PCP may be essential to account for hyperlocomotor and cognitive-deficit responses of the drug (Adams and Moghaddam, 1998). Interestingly, some studies have found that GLP-1R activation in the NAc suppresses reward and craving activities by

enhancing presynaptic glutamatergic AMPA/Kainate transmission (McIntyre et al., 2013; Mietlicki-Baase et al., 2013a; Liu et al., 2017). Therefore in our study, Ex-4 action on PCP may–be partly through augmenting or compensating a glutamatergic -deficient neuronal circuit.

Finally, we assessed the electrophysiological impact of Ex-4 and GPO on the VTA dopamine neurons as well as PFC pyramidal neurons. Our electrophysiological data shows that GLP-1R activation can change the excitability of prefrontal cortex neurons, an effect that can be associated with the putative pro-cognitive action of GLP analogues (Mansur et al., 2018b). These electrophysiological findings suggestively correlate with behavioural data of Ex-4 effect on cognition, memory and PCP-induced hyperlocomotion activity. Previous studies using whole-cell voltage-clamp electrophysiological paradigm revealed that GLP-1 elicited a stimulating effect on hypocretin neurons, located in the lateral hypothalamic region, through rapid depolarization of membrane potential and enhanced spike frequency (Acuna-Goycolea and van den Pol, 2004c). The paper further illustrates that GLP-1R activation facilitated glutamate release at the axonal terminals partly by activation of presynaptic GLP-1Rs and that this effect was reversed in the presence of exendin (9-39), a GLP-1R antagonist. Another *in vitro* electrophysiological studies by Mietlicki-Baase et al., (2014), revealed that presynaptic GLP-1Rs in the NAc core was activated by Ex-4 to enhance the activity of medium spiny neurons (MSN) through the glutamatergic transmission. Importantly, the glutamatergic transmission sends a signal to the PFC, NAc, amygdala as well as the hippocampus (Kelley et al., 2005). Primarily, GLP-1Rs located in the NAc core physiologically control feeding behaviour in which the activation produces GLP-1 to facilitate presynaptic glutamatergic signalling and modulation (Alhadeff et al., 2012b; Dossat et al., 2011). From all the findings, we can infer that activation of presynaptic GLP-1R potentially modulates glutamatergic PFC pyramidal neurons which are of prime relevance in

cognition, memory and drug-craving anomaly (Caballero et al., 2016; Dauvermann et al., 2017a; Isherwood et al., 2018; Kelley et al., 2005). Thus, we provide an *in vivo* electrophysiological evidence to support these findings of Ex-4 activatory effect on the firing rate of PFC pyramidal neurons. Interestingly, on the VTA dopamine neurons, GLP-1 receptor activation through intravenous administration of Ex-4 did not affect the DA neurons in the midbrain. However, a moderate inhibitory action of insulin on firing activity was potentiated by Ex-4 when co-administered intravenously. This synergistic effect with insulin might be due to secondary neuronal inhibition synchronized in a pool of DA neurons in the VTA. To circumvent the problem of ineffective penetrability into the brain, we administered Ex-4 directly into the VTA. Although, there was no significant effect on intravenously administered Ex-4 on the VTA DA neuronal activity however, we found a pronounced activatory effect on VTA DA neuronal activity when administered through intra-VTA. Ex-4 may exert its central effect indirectly by activating a peripheral component (e.g. vagus nerve) that can alter CNS activity. It has been demonstrated that some of the effect of gut peptides, including GLP-1 were suppressed following vagotomy (Plamboeck et al. 2013). However, BBB penetration is also possible in particularly at the level of the area postrema in the brain stem. Interestingly the area postrema is strongly connected to the nucleus of the solitary tract which contains a group of GLP neurons that innervate the VTA and also higher structures 183). Our finding reveals relatively high firing DA neurons than the control and desensitisation of D_2/D_3 receptors following intra-VTA administration of Ex-4 (3 µg). The desensitization effect observed with dopamine D_2/D_3 may be general in the brain that GLP-1R interacts negatively with authoreceptors. A few numbers of *in vitro* electrophysiological studies have demonstrated that GLP-1R activation in the VTA involves glutamatergic transmission which enhances excitatory postsynaptic current (EPSC) in the VTA DA neurons (Mietlicki-Baase et al., 2013b). Also, the studies show that intra-VTA administered Ex-4

enhances tyrosine hydroxylase in the VTA (Mietlicki-Baase et al., 2013b), demonstrating increased dopamine production. This group further revealed that Ex-4 did not change the phasic DA efflux in striatal brain slices using fast-scan cyclic voltammetry, which further suggests that GLP-1Rs are possibly not found on the presynaptic DAergic terminals, but rather on the cell bodies of DA neurons (Mietlicki-Baase et al., 2014b; Schmidt et al., 2016). Our release radiometric studies of GLP-1 drugs on ^{3}[H] DA are in agreement with these findings. We have earlier demonstrated through behavioural paradigm that GLP-1 drugs significantly attenuated D-AMP-induced reward, as metered by locomotor stimulation. The lack of effect of intravenously administered Ex-4 on VTA DA neurons is possibly due to the poor penetrability of the peptide in traversing the BBB when intravenously administered in anaesthetized rats as well as suboptimal dose inadequate to elicit an effect. It is noteworthy, that GLP-1 agonist may have low brain penetrability, yet it also activates a few neurons too, but a large proportion of GLP-1 in the brain comes certainly from GLP-1 neurons. Indeed, there is specific GLP-1 innervation for the NTS to the VTA. Therefore some of the effects we see in the VTA may be rather mediated via this innervation. Albeit, some studies have shown that GLP-1R activation may lead to inhibition of excitatory synaptic VTA DA neurons through an *in vitro* electrophysiology paradigm using a striatal slice of whole-cell patch-clamp recordings (Wang et al., 2015b). This effect potentially leads to reduced expression of VTA/NAc dopamine signaling and regulating drug and food reward (Volkow et al., 2011). More studies have revealed that, while food intake can be partly regulated by dopamine homeostatic signaling, the drug-craving signalling mechanism is controlled by the mesolimbic dopamine system (Kenny, 2011c). We can thus infer that dopamine neurons exhibit a reduction in excitatory synaptic ability and enhanced inhibitory synaptic inputs when Ex-4 is co-administered with insulin. In agreement with these studies, our data

demonstrate that administration of GLP-1 peptides for VTA electrophysiological recording is potentially implicated in reward behaviour.

Finally, in this chapter, we have demonstrated that exendin-4 alters dopamine-dependent behaviour with a remarkable inhibitory effect on D_2/D_3 DA agonist-induced yawning, pelvic grooming and pica eating. Evidence was also provided for GLP-1 inhibitory effect on D-AMP-induced motor activity, a DAT mediated mechanism from GLP-1R activation. In addition, we reported the precognitive effect of GLP-1on PCP-induced cognitive impairment as well as an excitatory effect on PFC pyramidal neurons. Also, we revealed the activatory effect of GLP-1 in the VTA and its desensitization effect on DA D_2/D_3 authoreceptors. We propose that GLP-1 interacts with DAT, an important transmembrane for D-AMP–induced reward activity, by attenuating its surface expression and cause less of it to be available for D-AMP to act on. This is to be transported inside the neuron to exert its depleting effect on dopamine vesicles besides DA D2/D3 autoreceptor sensitivity. These riveting data indicate that GLP-1R activation is essential for control of reward episode from drugs as well as cognitive and memory enhancing tendencies.

Chapter V- Ad libitum administration of sucrose during adolescence alters dopamine neurotransmission and dopamine-dependent behaviour modulation by glucagon-like peptide

V-1- Introduction

Adolescence constitutes a transformational phenomenon that exists after prepubescence and before adulthood in all animals' lifespan. In human, neuroimaging researches have shown that specific brain regions such as PFC progress to maturity after the age of 20 years old (Sowell et al., 2001; Wahlstrom et al., 2010; Mills et al., 2014). However, different studies from functional magnetic resonance imaging (fMRI) on reward system during adolescence reported striatal hyper-responsiveness leading to a high reward-craving effect (Blum et al., 2000; Galvan, 2010). Conversely, it requires more repeated rewarding inducement to reach similar effect as in adulthood, due to hypo-responsiveness caused by a deficiency in the motivational task (Bjork et al., 2004; Blum et al., 2000). It is noteworthy that, brain development activity during adolescence is significantly impacted on by sex hormones (Sisk and Foster, 2004), which elicit modulatory effect on memory, cognition, reward as well as motivational changes (Martini and Melcangi, 1991; Zhou et al., 2002; Peper et al., 2011). In response to rapid brain developmental effect in adolescent, there is a high degree of sensitivity to neural abuse as well as manifestations of signs of various psychiatric disorders such as feeding, emotional thought and behavioural disorders (Paus et al., 2008; Spear, 2000). Unfettered consumption of extreme palatable sugary foods and fatty diets, which are affordable and easily accessible, play a principal part in the event of corpulence (Malik et al., 2013a). While there are well documented outcomes of overconsumption of sugar resulting in high risk of diabetes mellitus (Macdonald, 2016b), obesity (Delli Bovi et al., 2017), metabolic syndromes (Stanhope, 2016), cardiovascular

diseases (DiNicolantonio and OKeefe, 2017), acne (Melnik, 2015), cancers (Miles et al., 2018) and chronic kidney disease (DiNicolantonio et al., 2016), little is known about how sugar influences memory and cognition, motivation and impulsivity, reward-seeking behaviour, depression as well as other mental health conditions. Adults studies conducted on the individual that consumed high quantities of soft drinks of about 500 mL per day have been linked with a significant possibility of developing depression, suicidal tendency and some psychological disorders, then those individual adults that did not consume soft drink as control subjects (Shi et al., 2010). Additional studies revealed that probability for the occurrence of depressive traits was much higher in adults drinking a large quantity of soft drink (Yu et al., 2015). It is quite disturbing that sugar intake is more among adolescents than in adults (Bremer and Lustig, 2012). Thus, animal models have demonstrated that rats which excessively ingested sugar during adolescence were not motivated to response for saccharin, due to possible depressive behaviour. However, they are highly vulnerable to reward-related disorders as well as cocaine self-administration (Vendruscolo et al., 2010). More studies using rat models have revealed a neurobiological alteration in craving such as, a significant reduction in DA D_2 receptors in the striatum and escalated D_1 receptors binding in the NAc, in rats that were exposed to 25% glucose for several weeks (Colantuoni et al., 2001a).

The present investigation asked the question whether prolonged continuous consumption of sucrose solution (5%) by adolescent rats impacts on cognition and memory of the subjects and if sucrose consumption was sufficient to trigger alterations in the dopaminergic system. In particular, we tested the electrophysiological characteristics of the neurons such as DA firing and burst activities. We also studied the modulation of autoreceptor effect by PPX, a DA D_3R preferred agonist. We further assessed the effect of dopamine-producing drugs (D-AMP, PPX) on dopamine dependent-behaviours, if these behaviours are altered by sucrose consumption. Finally, we examined whether the

behavioural traits could be modulated by glucagon-like peptide, a gut metabolic hormone which is known to interfere with food and drug rewarding systems (Jerlhag, 2018).

V-2- Materials and methods

V-2-A-Subjects

Please refer to paragraph I-2-A for detail information on animal conditions and regulatory protocol on a study involving rodents. In this study, 4 groups *(n=8)* of male adolescent rats (PND 20-25) were administered sucrose 5% and water *ad libitum*, or water for 2 weeks, with or without the stable glucagon-like peptide agonist analogue exendin-4 (0.01 mg/kg/day, ip).

V-2-B- Sucrose, chow consumptions and weight measurement

Upon reception, animals were acclimatized to their housing conditions for at least 4 days before access to 5% sucrose solution. Fresh sucrose was made daily and was accessed by the rats continuously within 24 hrs for 15 days, with an option of water (two bottles choice). Sucrose and water consumptions were recorded by weighing the bottles before and after the 24 hrs periods. Rats were randomly assigned to one of the following groups, as indicated in figure 58:

- Rats receiving vehicle alone throughout the experimental period-**(Control)**
- Rats receiving 5% sucrose with an option of a vehicle (water) during the dark phase, and 5% sucrose alone throughout the light phase for 15 consecutive days and later received D-AMP and PPX treatment before electrophysiological recording.-**(Sucrose+D-AMP)**

- Rats receiving 5% sucrose with an option of a vehicle (water) during the dark phase and 5% sucrose throughout the light phase as well as exendin-4 (0.005 mg/kg/day, ip) for 15 consecutive days. The group also received D-AMP and PPX treatments before electrophysiological recording**.-(Sucrose+D-AMP+Ex-4)**

The weights of the rats and chow consumed were measured accordingly on every 3 days during the 15 days continuous sucrose access by comparing the weights of the animals before and at the termination of the treatment. The rats were isolated during night and reunited in the morning. It is mostly during the dark phase that they eat and drink. We calculated the % chow consumed by measuring the quantity of chow consumed by each rat per day over the actual rat weight in %). Therefore, the weight ratio between the end and the beginning of the chronic treatment was measured for each animal and was a direct reflection of body weight change.

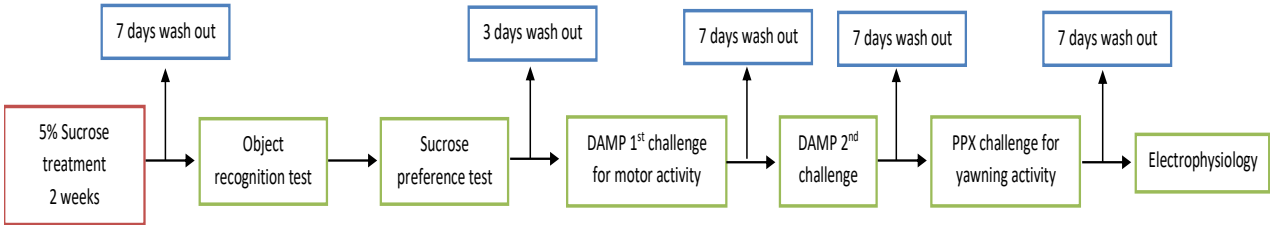

Figure 58: Experimental timeline for the entire investigation

Each of the group was treated with 5% sucrose for 15 days, except for the naïve and the D-AMP only group. For an unbiased electrophysiological recording, we also added an additional group which was sucrose-only treated rats without exposure to D-AMP and PPX treatment before electrophysiological recording.

V-2-C- Evaluation of glycaemic parameters

Briefly after the end of 15 days sucrose treatment, oral glucose tolerance test (OGTT) was assessed over a period of 2 hrs in the control (vehicle) as well as 5% sucrose treated group. This was carried out by making a slight prick on the lateral tail vein of the

animals to obtain a blood drop and the glycemic levels were consequently measured using the Accu-Chek® blood glucose system (Aviva). Previous studies have reported a glucose range levels between 5.6 and 7.9mMol/L in naïve rats (Florence et al., 2014; Wang et al., 2010). However, in experimentally-induced diabetes mellitus, the glycemic level may escalate to a mean value of 12.4 and 21.8 mMol/L in alloxan and streptozotocin-induced DM respectively (Howland and Zebrowski, 1976).

V-2-D- Sucrose preference test (SPT)

Subsequent to 7 days washout period, animals were subjected to SPT according to previously published techniques (Mateus-Pinheiro et al., 2014; Tang et al., 2015; Xu et al., 2012). In essence, the animals were tested for sucrose preference for a 5 days using a two-bottle option test. Animals had *ad libitum* access to food and water throughout the experiment. On the first day, rats were housed singly and accustomed to drinking from two water bottles. On the following three days, rats were trained on the sucrose preference test, in which one out of the two water bottles was replaced by a bottle containing a 5% sucrose solution (w/v). Rats were allowed to drink freely from both bottles during the 12-hour nocturnal phase (7.30 PM until 7.30 AM). During the light phase, both bottles were replaced by bottles containing water only. The bottles were weighed and refilled each day at the same time in the morning. The positions of the bottles were switched daily to avoid position preferences, which has been observed in rodents (Gaudez and Cail, 2016; O'Connell, 1971; Sinclair, 1976; Velimirov et al., 2011). Sucrose preference was determined after the last trial, on the fifth day, by the quotient of sucrose consumption over 12 hours to the total liquid intake, consisting of both water and sucrose intakes. That is, the percentage of preference was calculated as follows: sucrose intake/total fluid intake × 100. A decrease in sucrose preference is classically used to determine anhedonia. A sucrose preference score lower than

65% was considered a depressive-like phenotype according to previously published protocols (Conti et al., 2017; Gueye et al., 2018a; Qin et al., 2019; Yun et al., 2016).

V-2-E- Novel object recognition (NOR) test

After 1 week washout period, the animals were tested for NOR task. Please refer to paragraph IV-2-D1 for detailed protocols on NOR test (Chater 4).

V-2-F- D-AMP and DA D_2/D_3 receptor agonist-induced behavioural studies

Please, refer to paragraph II-2-D for detailed behavioural protocols. In this study, each group was tested on D-AMP-induced motor activity, with the second challenge of D-AMP after a week from the first D-AMP exposure, except in the control and sucrose-only group. Similarly, PPX was tested on each group of the animal subject to assess the response to DA D_2/D_3-induced yawning effect (experiments not done in the control and sucrose-only group). Collectively, we investigated if the consumption of sucrose modulates DA associated behavioural traits.

V-2-G-*In vivo* extracellular single-unit electrophysiology recording

Please refer to paragraph I-4 for detailed protocol. Recordings were done on VTA dopamine neurons. In this electrophysiological studies, we assessed the impact of intravenously administered PPX (a D_3 preferred DA agonist) on auto-receptor in the midbrain dopamine neurons. We compared the DA neurons firing rate and burst activity within the 3

groups. We investigated whether continuous sucrose consumption during adolescence can alter dopaminergic neuronal activity in the VTA.

V-2-H-Statistical analysis

Please refer to paragraph IV-2-G for detailed statistical analyses deployed in these studies. However, weight change analysis was deduced by finding the weight difference every 3days. The % chow consumption was calculated by measuring the quantity of chow consumed by each rat per day over the actual rat weight in %, while the percentage of preference was calculated as follows: sucrose intake/total fluid intake × 100.

V-3- Results

V-3-A-Effects of sucrose consumption on growth, feeding and glycaemic levels

The impact of continuous sucrose consumption during adolescence on the growth variables was investigated. We observe that at the end of 15 days continuous sucrose consumption, rats treated with vehicle and sucrose consumed similar quantities of chow. While the group treated with Ex-4 5 µg/kg along with continuous 5% sucrose demonstrated a significant reduction in chow consumed, particularly in the last days of treatment (Fig. 59A). Similarly, Ex-4 treated rats showed a pronounced weight reduction when compared with the control and sucrose treated groups. However, the sucrose (alone) treated rats manifested an increase in weight gain than all the other groups (Fig. 59B). Rats exposed to daily access of 5% sucrose generally demonstrated increases in blood glucose levels compared to the control group (Fig. 59C).

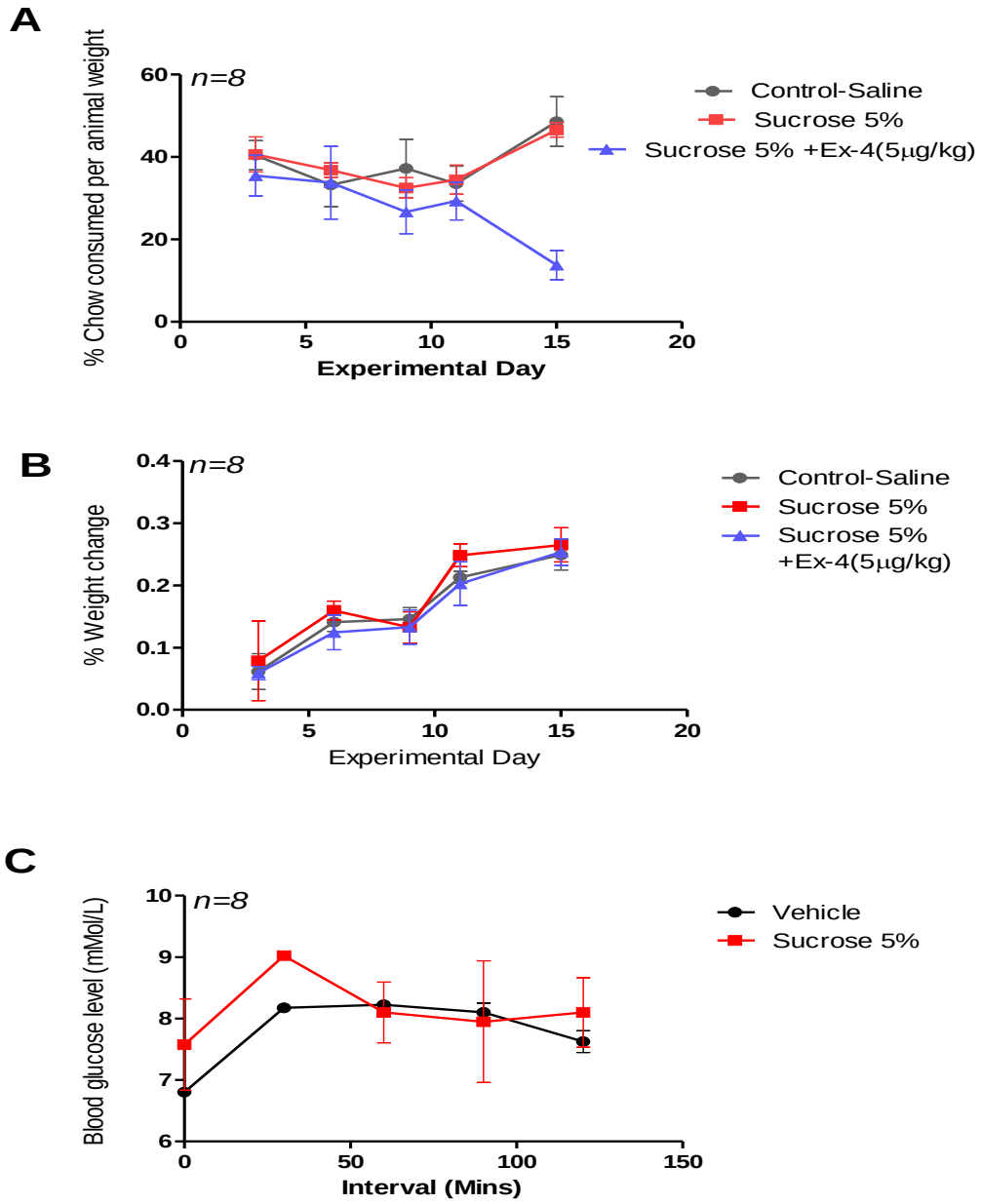

Figure 59: Sucrose consumption does not alter weight and feeding pattern
(**A**): There was no significant difference in the chow consumed between the sucrose (alone) treated and the control. However, during the last 3 days of the treatment, the Ex-4 treated rats displayed a significant reduction in the quantity of chow consumed when compared with the control. *P<0.05 *vs.* control, Bonferroni after significant repeated measures two-way ANOVA. (**B**): Sucrose consumption did not significantly affect the weight changes in all the groups. However, Ex-4 treated group showed the lowest AUC value (1.9, followed by the control (2) and the sucrose alone treated rats (2.2). This may indicate the anorexic property of Ex-4 that eventually culminates to low chow consumption, reflecting on the weight change. (**C**): Random plasma glucose levels were measured immediately at the end of 15days sucrose administration. The sucrose treated rats slightly became hyperglycaemic at the end of 30 mins treatment than the control albeit, no significant discrepancy in blood sugar level at the end of 2 hrs observation following the end of 15 days sucrose treatment.

V-3-B-Effects of sucrose consumption on depressive-like phenotypes in adulthood

Three days after the termination of the washout period, the rats were investigated for sucrose preference test which is an index for depressive-like behaviour. We observed, that rats treated with sucrose alone as well as those treated with sucrose plus Ex-4, showed a strong preference for sucrose (about 100% preference); indicating that sucrose preference was not prevented by Ex-4 ip. Administration, although there was a statistical significant difference ($F=8.6$, $DFn=2$, $DFd=36$) within the group (Fig. 60A). Interestingly, controls show a slightly lower preference index than the treated, but this was not found to be significant (Fig. 60B). We equally observed a pronounced increase effect in total fluid consumption both in sucrose alone and sucrose plus Ex-4 treated groups (Fig. 60C).

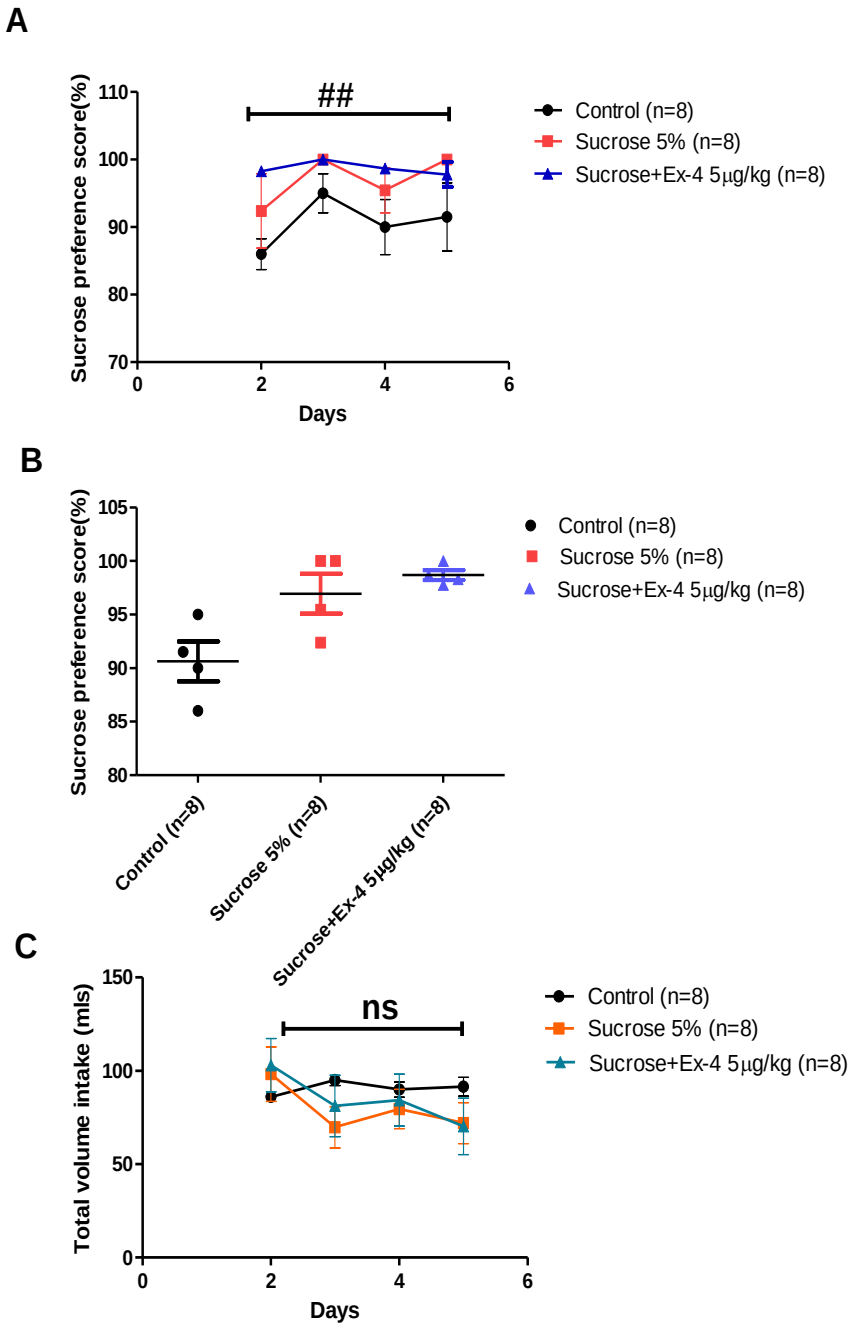

Figure 60: Adolescent exposure to chronic consumption does not induce depressive-like phenotype

(**A, B**): In all the 3 groups of adolescent rats exposed to continuous sucrose consumption, there was an apparent preference for sucrose in adulthood (above 90%), with no depressive-like phenotype exhibited. A two-way repeated measure ANOVA show significant difference among the groups **(A),** but no significant difference at any time. ##P<0.001 within the group. (**C**): Adolescents exposed to both sucrose as well as sucrose plus Ex-4 consumed the same volume of fluid when compared with the control group **ns:** not significant.

V-3-C- Sucrose consumption induces cognitive impairment

In this study, we investigated the impact of continuous sucrose consumption on cognitive activity, as well as whether Ex-4 intraperitoneally administered concurrently for 2 weeks may affect sucrose-induced cognitive impairment. During the exploration period, sucrose-treated (alone) group spent significantly less time exploring the objects during the 3 day familiarization phases than sucrose +Ex-4-treated rats, while the control (vehicle) group spent the most time during exploration period. There was no significant difference within the group (Fig. 61A). We found that during the familiarization phase the control groups significantly differ from sucrose groups. At test period, the discriminating index, which is the ratio of the differences in time explored with novel and familiar object, to the sum of the total time spent with novel and familiar object i.e. [$D_{I'} = (T_N - T_F)/(T_N + T_F)$] of %), differs moderately although, not statistically significant between control and the sucrose groups, but significant difference (F=5.694, df=2) exists within the group (Fig. 61B). There was a varied disparity in DI' among the groups, while the control exhibited 85% in DI', the sucrose and sucrose +Ex-4 treated displayed -8.3 and -20% for mean DI' respectively. In this instance, the control group spent the most time exploring the novel object than the sucrose access rats. In addition, the total amount of time spent exploring the novel object during the experimental phase was deduced as control=13 sec, sucrose=11 sec and sucrose+Ex-4=6 sec. Interestingly, preference for the familiar object during the test period was reported as control group having the least time spent, while the sucrose (only) group spent significant (F=1.073, df=2) longest time with the familiar object Preference for familiar was introduced to further appreciate the level memory deficit associated with higher preference for familiar object over novel object (Fig. 61C).

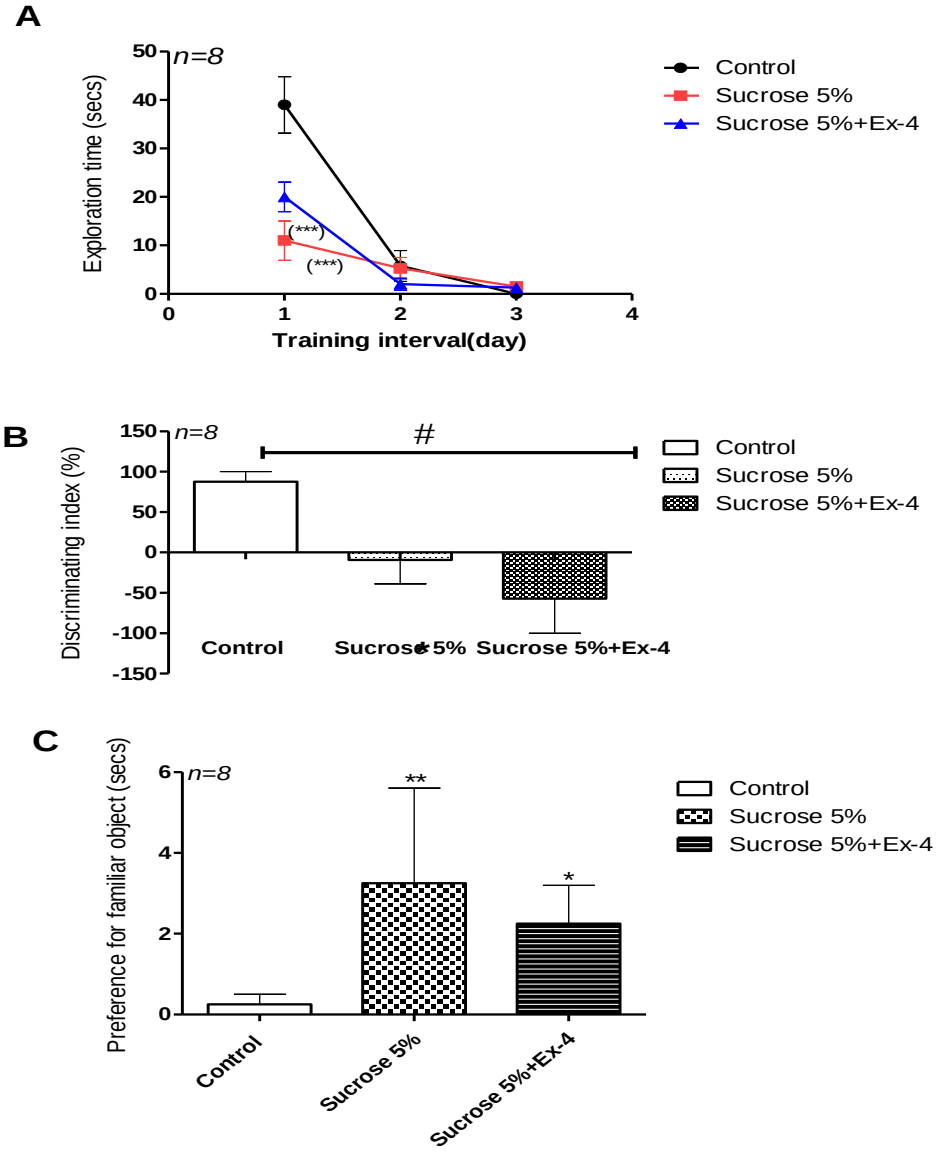

Figure 61: Effect of concomitant Ex-4 treatment on continuous sucrose-induced cognitive impairment

(**A**): During the exploration period, the sucrose treated (only) group, as well as sucrose+Ex-4 treated group exhibited a significant reduction in familiarization time when compared with the control group(**B**): Ex-4 treated group object did have a significant impact on sucrose consumption on preference for novel (DI'). Indeed, sucrose induced a long term cognitive impairment and this was not reversed by Ex-4 concurrent administration. (**C**): Interestingly, sucrose (only) treated group displayed the longest time spent on the familiar object during the test phase than the control, similar results were observed in sucrose+Ex-4 treated rats #P<0.05, *P<0.05,**P<0.001, ***P<0.0001 *vs.* control. *Bonferroni* post hoc tests after significant two way repeated measures ANOVA.

V-3-D- Ex-4 concomitant treatment with sucrose alters DA drugs induced behavioural traits.

After 1 week of washout, rats were challenged with D-AMP 1 mg/kg to assess response to motor activity. Rats that were treated with sucrose-only displayed a significant (F=65.37, DFn=2, DFd=84) motor response than the naïve rats to the administration of D-AMP (1 mg/kg), as shown by a larger increase in rearing activity (Fig. 62A). There is also a significant difference within the group. Following 1 week of D-AMP 1 mg/kg treatment, we acutely challenged the rats with additional D-AMP 1mg/kg to assess if there may be behavioural proportionality from the first treatment and the second challenge. Interestingly, a more significant increase (F=94.85, DFn=3, DFd=84) in motor response within the group was observed in D-AMP challenge II within the group (Fig. 62B). In another series of experiment, we tested the effect of D_3 preferred agonist PPX 0.1 mg/kg) on yawning activity (Fig. 63A). There was a significant difference within the group and sucrose-treated rats also displayed a significantly (F=36.29, DFn=3, DFd=36) larger response than naïve animals to the D_3 preferential agonist PPX (0.1 mg/kg, ip) on yawning activity. Yawning effect was also markedly reduced in rats that were co-administered exendin-4 with sucrose than in sucrose treated alone.

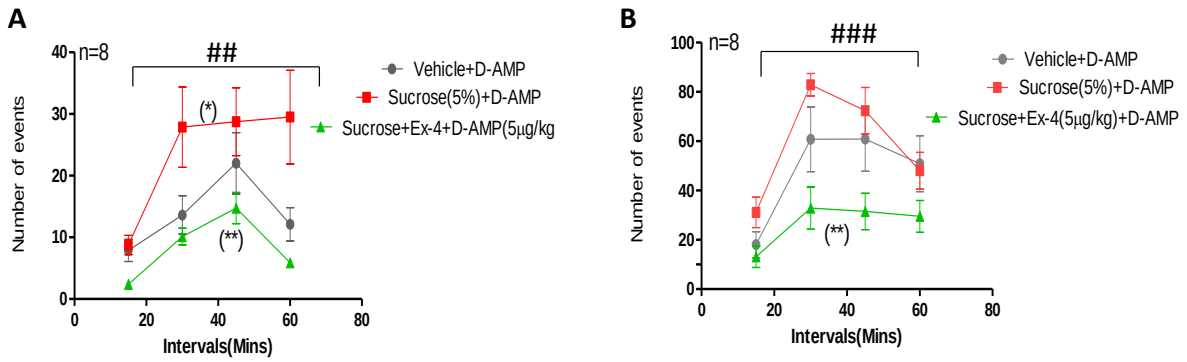

Figure 62: Ex-4 treatment alters D-AMP-induced motor activity in sucrose-treated rats after double challenge

(A): Sucrose-only treated group exhibited a higher statistical significant motor activity than the control and the sucrose/exendin-4 treated rats when challenged with D-AMP, except during the 0-15 min interval. **(B):** In the second D-AMP challenge, the sucrose+Ex-4 group exhibited significant motor reduction (assessed by measuring rearing-like activity) when compared with the control and the sucrose-only treated rats. However, the entire group still displayed relative increase in motor activity than in the first D-AMP challenge ##P<0.05, ###P<0.0001, *P<0.05, **P<0.001, *vs*. control, *Bonferroni* post hoc tests after significant two-way ANOVA.

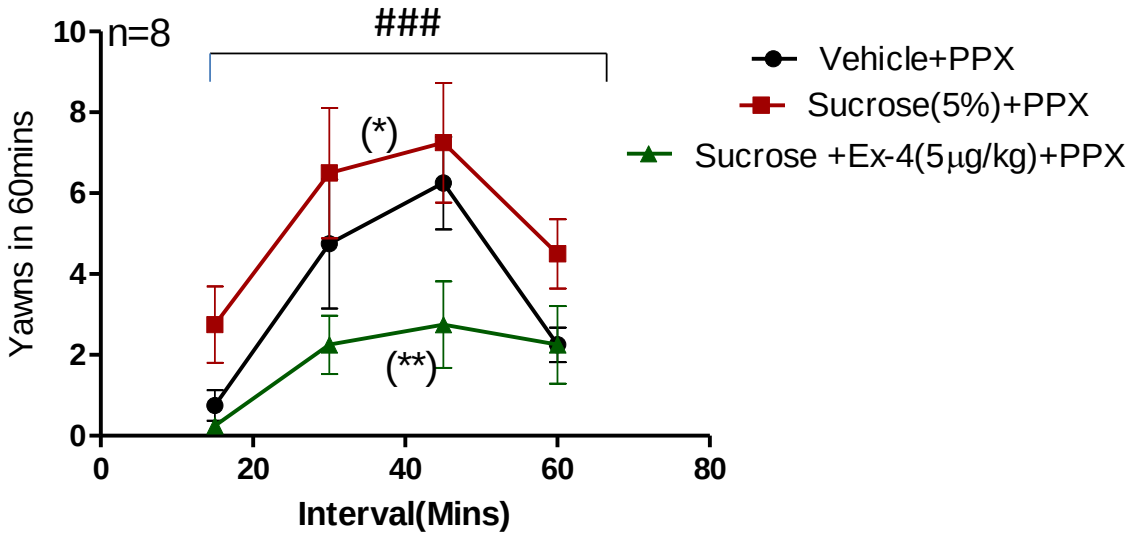

Figure 63: Ex-4 treatment alters DA drug-induced behavioural traits in sucrose-treated rats

Sucrose-only rats exhibited a significant increase in yawning activity following PPX administration than the control. Furthermore, the yawning effect was significantly reduced in sucrose+Ex-4 group ###P<0.0001, *P<0.05, **P<0.001 *vs.* control, *Bonferroni* post hoc tests after significant two-way ANOVA. All animals have been exposed to D-AMP once.

V-3-E1- Rats treated with sucrose altered neuronal sensitivity to acute PPX administration

In this experiment, we investigated the effects chronic treatments on the ability of the preferential dopamine D_3 receptor agonist PPX (cumulative doses 20-100 µg/kg, iv) to reduce the firing activity of VTA dopamine neurons (to test dopamine D_2/D_3 autoreceptor sensitivity). The naïve rats displayed a complete inhibition to PPX at the 4^{th} dose administered and the firing rate was reversed by administration of the D_2/D_3 antagonist eticlopride 0.2 mg/kg to initial baseline firing (Fig. 64A, B) as shown also in chapter I. Animals treated with sucrose only displayed a partial decrease in their responses to PPX (Fig. 64C, D) compared to controls. Surprisingly, the desensitization effect was more prominent in VTA dopamine neurons from rats that were co-administered sucrose with exendin-4 (Fig. 64E, F). Fig. 65 shows a comparison among the different groups (naïve, sucrose-only, sucrose+Ex-4 treated). Apo (Apomophine) in doses of 10 µg/kg and Apo max (maximum dose of apomorphine) of 20 µg/kg was injected shortly before the administration of eticlopride to test the response of the neurons to DA D_1/D_2 agonist.

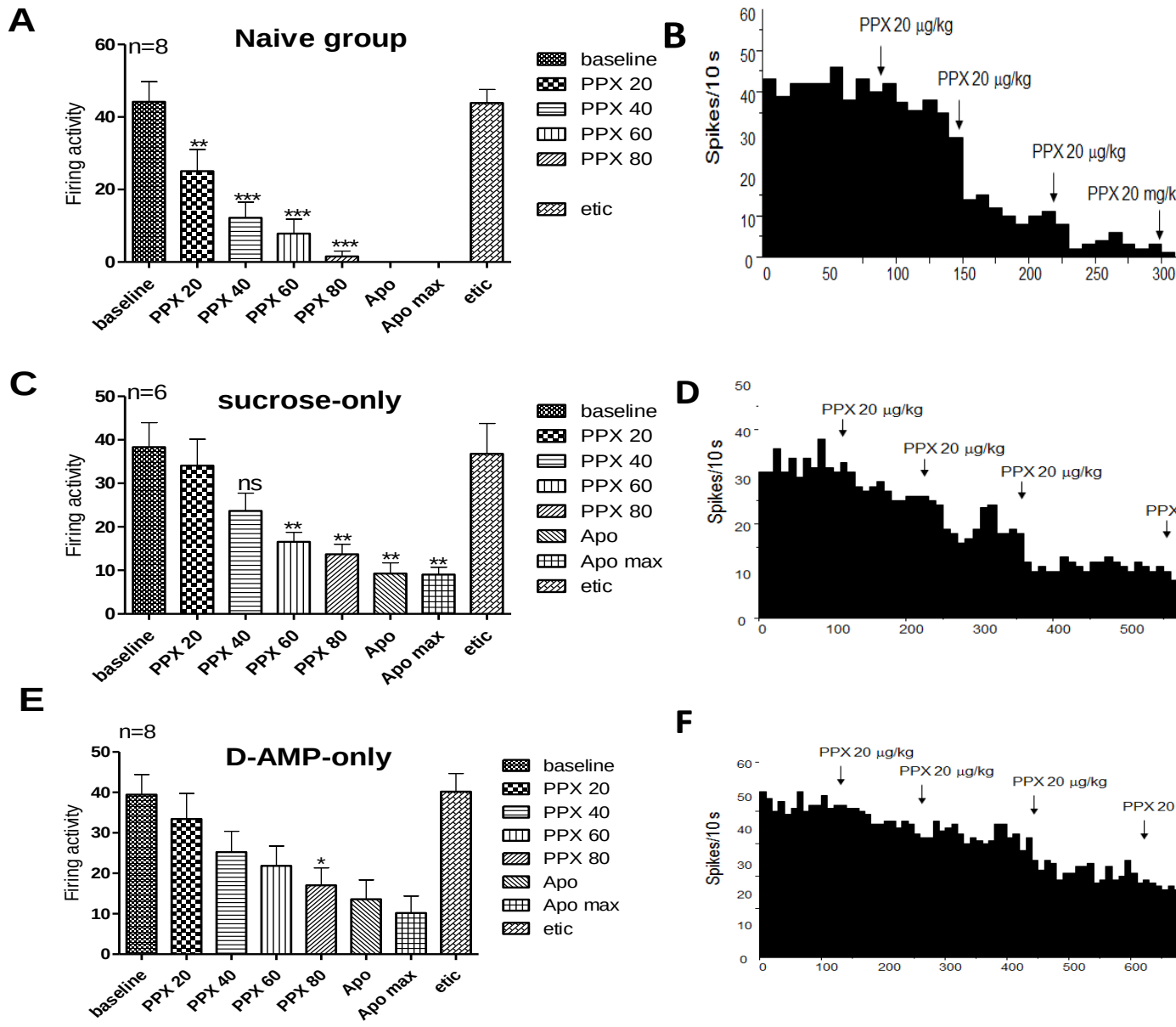

Figure 64: Sucrose consumption reduces VTA dopamine neurons sensitivity to the dopamine $D_{2/3}$ receptor agonist pramipexole (PPX), an effect potentiated by ex-4 co-administration.

(A, B): In naïve rats, the VTA dopamine (DA) neurons exhibited a complete inhibition to a cumulative administration of PPX (up to 80 µg/kg, iv). The cells were reactivated by the selective dopamine D_2 antagonist eticlopride (0.1 mg /kg) to their original firing rate.

(C, D): In sucrose-only treated rats, the VTA DA cells exhibited a significant desensitization to PPX acute administration. The inhibition of firing was only statistically significant at the high doses of 60 and 80 µg/kg PPX.

(E, F): In sucrose+Ex-4 treated rats, the desensitization was remarkably more pronounced.

ns: non-significant, *P<0.05, **P<0.001, ***P<0.0001 *vs.* baseline firing. *Newman-Keuls* post hoc test after one-way ANOVA.

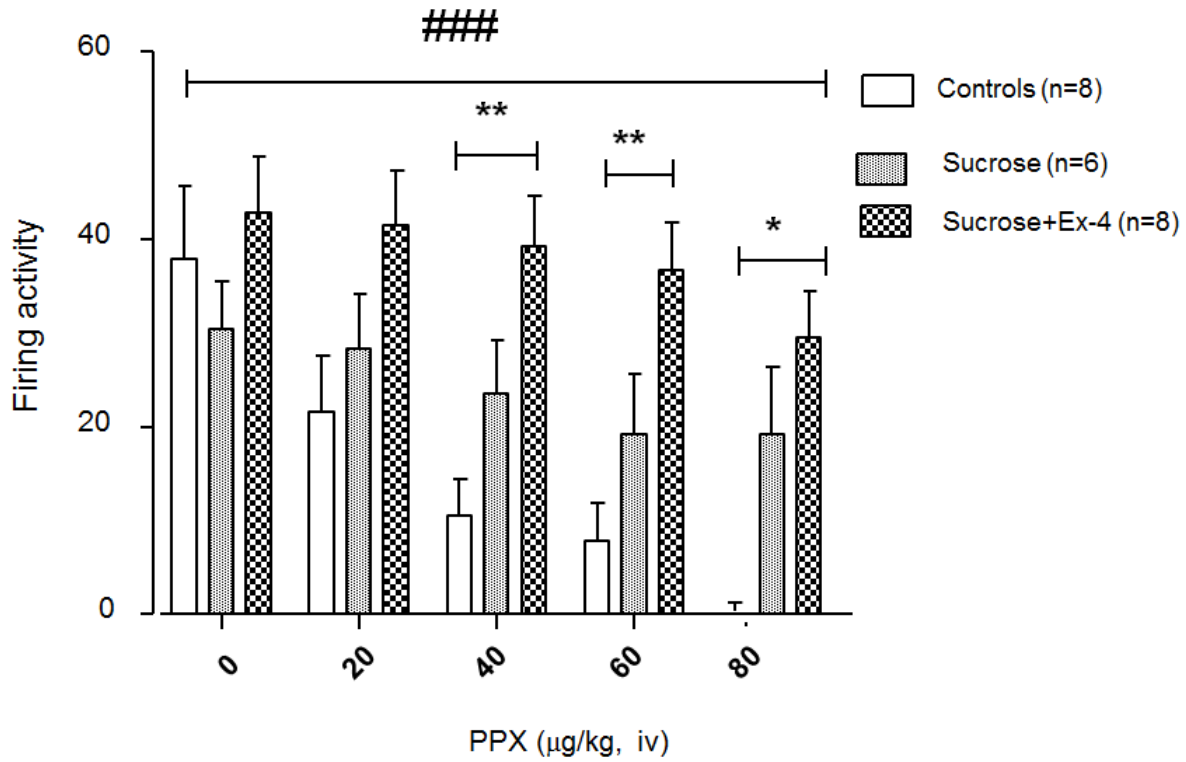

Figure 65: Statistical comparison among the groups (naïve, sucrose-only and sucrose+Ex-4)

Control rats compared with sucrose-only and sucrose+Ex-4 treated rats exhibited significant difference in sensitivity to PPX within the groups. ###P<0.0001 *vs.* all conditions, *P<0.05, **P<0.001 *vs.* naïve rats. *Bonferroni* post-tests after significant two-way ANOVA within the 3 groups.

V-4- Discussion

In this study, we continuously exposed adolescent rats (PND 20-25) to a 5% sucrose solution for 15 days and subsequently investigated their cognitive behaviours, impact on D_2/D_3 dopamine agonist drugs, as well as the effect of electrophysiological characteristics on VTA DA neurons. We also examined if the co-administration of exendin-4 to sucrose can modulate the effects of sucrose. First, sucrose consumption was assessed on growth parameters. We found that 2 weeks sucrose feeding on the rats induced a slight weight increase compared to both controls and sucrose+Ex-4 treated rats (Fig. 59B). This is in agreement with previous findings by Bes-Rastrollo et al., (2016); Bissonnette et al., 2017; Malik et al., 2013; Vermunt et al., (2003) in which sucrose sweetened food enhanced weight gain in adolescent rats as well as young human subjects. Interestingly, rats that were treated with sucrose+Ex-4 displayed relatively lowest weight change and consumed fewer chows (Fig. 59A). This is suggested to be as a result of the ability of GLP-1R agonist to quell feeding via satiety induction which may lead to weight loss (Bradley et al., 2010; Yang et al., 2014a). Additional studies, including our findings (see chapter IV), have implicated hypothalamic homeostatic and dopaminergic reward pathway in playing a vital role in the reduction of food intake elicited by peripheral Ex-4 administration (Brunetti et al., 2008; Dalvi et al., 2012; Lu et al., 2018). The studies further explained that Ex-4 potentially decreases energy intake and facilitates energy dissipation although, a debate arises on which of the processes takes the lead when it comes to weight loss. We also observed that chow consumption was significantly low with sucrose+Ex-4-treated rats than in both control and sucrose-only treated rats. A direct correlation between weight change and food consumption has been reported in previous studies (Chow and Hall, 2008; Yang et al., 2014b), our findings undoubtedly agree with these reports. Notably, while sucrose+Ex-4 treated rats were able to exhibit a significant reduction in chow consumption and weight loss, Ex-4 did not seem to

affect the volume of sucrose intake throughout the experiment (Fig. 60C).We also assessed the impact of sucrose consumption on the glycaemic level when compared with the control. Previous findings have shown that high intake of sugar apparently causes some health risks, due to excessive ingestion of energy and may not necessarily disrupt metabolic or glycaemic homeostasis (Johnston et al., 2013; Macdonald, 2016c). Here, there was no significant glycaemic alteration at the end of 15 days continuous sucrose treatment, although, the rats were slightly hyperglycemic the effect quickly faded out within 60 mins of observation (Fig. 59C). Next, we investigated the impact of chronic sucrose consumption during adolescence whether it may induce depressive-like phenotype, an index for anhedonia in adulthood. Our finding revealed that continuous 5% sucrose consumption during adolescence does not lead to depressive-like phenotype at adulthood (Fig. 60A, B). Generally, the sum of the fluid intake increased on the first day of the test and later became constant on the remaining 4 days across the groups. However, sucrose-only treated rats, as well as sucrose+Ex-4, the treated group exhibited escalated increase in daily fluid intake and reached about 100mls per day initially at PND 44. There have been seemly conflicting findings on whether unlimited access to sucrose consumption during adolescence in rats can induce depressive-like phenotype or not. We have previously reported from our laboratory that rats exposed to 5% sucrose did not induce any long-term anhedonia traits, whether early adolescence or adulthood treated (Di Miceli et al., 2019b). In support of our findings, another study by Gueye et al., (2018) reported that continuous sucrose consumption by adult Wistar rats had no impact on sucrose preference and anxiety-like behaviour. In essence, all these findings indicate that unlimited sucrose consumption even at adulthood may likely not induce a long-lasting impression on an emotional trait. It has also been reported that rats with a history of sucrose consumption during adolescence demonstrated a decrease in hedonic activity as well as reduced reward efficacy to 1% saccharin preference (Naneix et al., 2016; Vendruscolo et al., 2010). In the

following experiment, we assessed the impact of sucrose consumption on cognitive processes and whether GLP-1 administered concomitantly with sucrose intake might positively affect a potential deficit or has no direct correlation. We discovered that sucrose+Ex-4 treated rats spent relatively same time exploring the familiar objects when compared with a sucrose-only treated group (Fig. 61C). While the control rats spent the shortest time in exploration of the familiar object. Furthermore, in the control rats, the total exploration time spent during training was the longest with familiar objects (Fig. 61A). It has been reported that total time spent in exploration of a familiar object is not an adequate parameter to make a meaningful inference on cognitive interference (Morellini et al., 2010). Yet, our finding is indicative of the fact that unlimited sucrose intake adversely affects exploratory behaviour possibly by inducing fear and anxiety which eventually culminate into loss of attention to explore, as well as more interested in the familiar object, an indication of cognitive disturbance (Haagensen et al., 2014). In particular, NOR is based on the hypothesis that inherent novelty-seeking of rodents steers them to spend extra time investigating a novel object against the familiar one (Mathiasen and DiCamillo, 2018). In agreement with previous accounts (Beilharz et al., 2014; Heyward et al., 2012; Ross et al., 2009), sucrose-only treated rats exhibited a pronounced cognitive decline when compared with the control rats (Fig. 61B). Surprisingly, in sucrose+Ex-4 treated rats the cognitive deficit was not corrected. More studies have shown that daily controlled sucrose exposure during adolescence induces a long-term consequence on the role of the prefrontal cortex, which presents in form of cognitive impairment and memory deficit (Reichelt et al., 2015a). Hence, it is plausible that high sucrose diet in adolescence potentially induces long-term adverse effects on specific brain regions concerned with cognitive mediated behaviour involving the PFC. We also observed with interest that, Ex-4 could not reverse the cognitive deficit elicited by sucrose-treated rats. This may be due to a suboptimal concentration of peripherally administered Ex-4 able to transverse the BBB

central effect. Thus, we have demonstrated in the previous chapter that acutely administered Ex-4, as well as its chronic treatment, aids cognitive functions. Another study using Morris water maze test on STZ-induced T2DM plus high sucrose intake, demonstrated that GLP-1 ameliorates hyperglycemia as well as improved learning and memory (cognitive abilities) deficits in T2DM rats (Cai et al., 2017). Our data reveal that Ex-4 works on cognition only, in a condition of hyperglycemia, by correcting the hyperglycemia in inducing insulin release. Chronic sucrose treatment seems to induce a kind of long-lasting deficit in cognition, not corrected by exendin-4. Thus, we saw in previous chapter sub-chronic and chronic effects of Ex-4 on cognition in non-treated rats, here it is a different condition in treated rats (sucrose exposed) and after a washout.

Next, we report our findings on the impact of continuous sucrose intake on D-AMP-induced motor activity as an index for impulsivity and reward-related traits. We observed that sucrose-only treated rats exhibited a remarkable hypersensitivity to D-AMP-induced motor activity when compared with both the control and sucrose+Ex-4 groups (Fig. 62A). The behavioural sensitization effect was further escalated on the second challenge with D-AMP after 7 days of the previous acute treatment (Fig. 62B). A well-liked and persevere speculation that has lasted for about 3 decades, is that sugar intake promotes hyperactive or vigorous behaviour, especially in children (Dykman and Dykman, 1998; Hoover and Milich, 1994; Wolraich et al., 1995a; Kim and Chang, 2011a). This perspective was supported by previous investigations, demonstrating a positive relationship between sugar intake and devastating-truculent behaviours arising from restlessness in children (Prinz et al., 1980). Early research also showed that there was a marked improvement in the behaviour of adolescent prison inmates as well as hyperactive teenagers when sugar was removed from their diet (Crook, 1974; Schoenthaler, 1983). Further prevalence human study in adolescents demonstrated that odd ratios (the connection between exposure and result) for mental

disorders such as anxiety, hyperactivity and attitudinal anomalies were elevated in subjects on excessive sugar consumption (Lien et al., 2006). However, the connection between sugar and hyperactivity observed in some survey investigations is in variance with vast experimental researches which have discovered no causal relationship effect between sucrose intake and behavioural anomalies (Benton, 2008; Kim and Chang, 2011b; Wender and Solanto, 1991; Wolraich et al., 1995b). For example, in the study with Wolraich et al., (1995b), they found that sugar intake does not impact on the behaviour or cognitive execution of children and in Wender and Solanto, (1991) investigation, it was observed that ADHD children were significantly more aggressive than the control, when both were exposed to sugar ingestion. Nevertheless, a growing evidence from animal models shows that rodents fed with excessive sucrose exhibit cognitive disorders (Beilharz et al., 2015; Gueye et al., 2018c; Reichelt et al., 2015b) and may change response to drug-induced behaviour (Colantuoni et al., 2001b, 2002), while having not much effect on basal behavioural activity. Gosnell, (2005) has earlier discovered that sucrose consumption in rats facilitates behavioural sensitization elicited by cocaine, as well as enhancement of sensitization after repeated cocaine administration. Our data demonstrated that sucrose-only treated rats displayed hypersensitivity to D-AMP treatment as well as D_3 preferred DA agonist (PPX) yawning effect. We also found out, that the double challenge of D-AMP corresponds to double behavioural responses (Figure 62A, B). In other words, repeated administration of D-AMP (2 doses) resulted in about twice behavioural responses in all the groups. This may suggest that repeated D-AMP exposure can result in continuous disturbance of dopaminergic homeostasis, which may further impede cognitive and behavioural activities consistent with the psychostimulant addict. (Tse et al., 2011). Our finding is in line with Gosnell, (2005) and Avena and Hoebel, (2003) studies who also demonstrated that sugar dependency leads to behavioural cross-sensitization to D-AMP administration. The pharmacological interaction

involved in this events is likely to be initiated by the tendency of excessive sucrose intake to activate brain regions implicated in reward processing as well as energy homeostasis (Kenny, 2011b, 2011d). Precisely, the stimulation of brain reward system by highly palatable sugary foods is considered to reverse energy regulation and facilitates uncontrollable eating behaviour that leads to obesity (Kuźbicka and Rachoń, 2013; Volkow and Wise, 2005; Chen et al., 2019). In addition, excessive sucrose intake has been shown to activate opioid (Mysels and Sullivan, 2010) as well as triggers DA release (Avena et al., 2008; McCutcheon et al., 2012; Pritchett and Hajnal, 2011; Skibicka et al., 2012) in the NAc, accompanied by indirect signaling effects on the corticolimbic regions of the brain (Grimm et al., 2002; Murray et al., 2016; Pomonis et al., 2000; Shariff et al., 2016). In this case, DA and opioid antagonists can specifically reverse the reinforcing effects of excessive sucrose intake (Levine et al., 2003). From our data, sucrose+Ex-4 rats displayed the lowest motor activity response to D-AMP challenge as well as minimal yawning effect to PPX (Fig. 63). Although Ex-4 did have any effect on the intake of sucrose during the chronic treatment, we observed with interest the remarkable lower effect exhibited by this group to D-AMP and PPX-induced DA mediated behavioural traits. A rich body of literature has previously provided evidence for the inhibitory role of GLP-1R activation in food craving and feeding incentive, implicating hypothalamic homeostatic regulation to produce anorexigenic effects (Dickson et al., 2012d; Montelius et al., 2014; Pritchett and Hajnal, 2011, 2012). We have also mentioned in chapter IV that GLP-1R activation may inhibit drug-seeking behaviour demonstrated in the form of increased motor activity and impulsivity effect to D-AMP treatment (Hernandez et al., 2018b). Bringing all the available data together, the new emerging role of GLP-1R activation in food and drug-reward behaviour such as, psychostimulant addiction is further underpinned by the implication of mesolimbic DA reward pathway, playing a critical role in the process (Hayes and Schmidt, 2016b; Skibicka, 2013; Dickson et al., 2012c).

In view of this background, we investigated the electrophysiological characteristics (firing, burst and population activity) of DA neurons in the VTA for sucrose-only, naïve as well as sucrose+Ex-4 treated groups. We then tested the effects of these chronic treatments on the ability of the preferential dopamine D_3 receptor agonist PPX (cumulative doses 20-100 µg/kg, iv) to reduce the firing activity of VTA dopamine neurons (to test dopamine autoreceptor sensitivity). We found out the different groups exhibited non-significantly different electrophysiological characteristics (firing, burst and population activity) throughout the recording period. However, sucrose-only treated group tends to exhibit lower firing activities (38.3±5.2), compared with the naïve (43±5.0) rats (figure not shown). Specifically, VTA DA neurons in naïve rats displayed complete sensitivity to PPX autoreceptor activity. While sucrose treated exhibited desensitization to PPX administration. Addition of Ex-4 pronounced the degree of desensitization. Interestingly, previous behavioural studies have demonstrated that craving effect of sucrose ingestion induced an increasing ratio performance, which was dose-dependently terminated by raclopride a DA D_2/D_3R blocker (Cheeta et al., 1995). Ratio performance technique is used as a means of evaluating the power of a reinforcing occurrence by an addictive substance, without reference to the degree of response (Hodos and Kalman, 1963). This is an arrangement in which each reinforcer is presented on the completion of a particular number of responses and the number of responses required increases after each reinforcement. In addition, more neurochemical and behavioural studies have supported this hypothesis with implication of DA D_1/D_2R being involved in sucrose induced craving, as well as dependency, rather than its tonic and metabolic activity (Hajnal and Norgren, 2001; Hajnal et al., 2004; Hsiao and Smith, 1995; Yu et al., 2000). More recent reports have indicated the role of high sugar intake on the neuronal activity of specific brain regions such as, the hippocampus (Lemos et al., 2016; Mitra et al., 2016), nucleus accumbens shell (NAcSh) (Villavicencio et al., 2018) as well as the prefrontal cortex

(Petykó et al., 2009). However, there is still a paucity of data on the impact of continuous intake of sucrose on electrophysiological parameters of DA neurons in the VTA, a region that is largely implicated in food and drug-reward. In some studies on the cortico-hippocampal slices using extracellular electrophysiological recordings, chronic sucrose (35%) consumption did not alter the general hippocampal metabolic configuration, nonetheless exhibits a critical hippocampal impairment through long-term potentiation (LTP) that is associated with behavioural (memory and cognitive) deficits (Lemos et al., 2016; Wang et al., 2014). A few numbers of studies has further demonstrated that high sucrose ingestion in rats causes upregulation of DAT (Bello et al., 2003) and enhances mesoaccumbens dopamine release (Hajnal et al., 2004), providing more evidence for rewarding effect of sucrose and increased behavioural hypersensitivity to DA drugs treatment in such manner similar to drugs of abuse (Volkow et al., 2011a). Thus, continuous sucrose intake enhances both accumbal DA release as well as upregulates DAT which in turn acts on DA D_2/D_3R to cause an alteration in firing rate. Future consideration must investigate the time-course effect of sucrose consumption and concentration variations. Finally, we tested the effect of sucrose treatment on the tendency of preferential DA D_3R agonist PPX (cumulative doses 20-100 µg/kg, iv) to reduce the firing activity of VTA dopamine neurons. In this study, we aimed to test for DA autoreceptor sensitivity in each of the group. We discovered that sucrose treatment induced a partial but significant decrease in the sensitivity of dopamine autoreceptors (Fig. 64C, D, G), which was surprisingly exacerbated by Ex-4 co-administration, indicating that Ex-4 may exert differential pre and postsynaptic neuronal effect (Fig. 64E, F). We previously reported in chapter I of this thesis that, PPX D_2/D_3 autoreceptor in the VTA resulting in a decrease in the firing rate of DA neurons in naïve rats (Fig. 64A, B). However, the partial but pronounced decrease in the sensitivity of DA autoreceptors in sucrose-only treated rats to PPX might be attributed to a decreased DA

D_2/D_3R sensitivity induced by chronic intake of sucrose. This possibly induced downregulation of the of DA receptors leading to desensitization to acute PPX administration (Bello et al., 2003). In addition, DA D_2/D_3R are associated with behavioural plasticity and loss of cognitive control and lack of goal-directed attention (Yawata et al., 2012). On the other hand, Ex-4 (5µg/kg) co-administered with sucrose, amplified desensitization of PPX to DA D_2/D_3R autoreceptors. This last series of study involving the investigation of PPX sensitivity to autoreceptors in sucrose+Ex-4 rats demonstrates that VTA DA neurons in the presence of Ex-4 activates the GLP-1R, and may lead to further downregulation of DA signalling in the mesolimbic pathway. Our finding agrees with Wang et al., (2015) that demonstrated through *in vitro* electrophysiological technique, the abolishing effect of GLP-1R activation on mesolimbic DA pathway involving VTA/NAc signalling process to regulate homeostatic and hedonic signals. Few reports on the function of Ex-4 in controlling synaptic activity reveal that activation of GLP-1R enhances the neuronal firing of orexin and hypocretin in the hypothalamus via modulation of vital membrane characteristics such as neurotransmitter release and uptake as well as channel conductivity of Na^+ /Ca^{2+} ions (Acuna-Goycolea and Pol, 2004). This desensitization process may be a general effect common to any structure containing GLP-1R and DA D_2/D_3 receptors. Whether it is on the cell body of dopamine neurons or on the postsynaptic neurons in dopamine rich area, such as striatal neurons, the mechanism involved is unknown. It may also be an interaction at the molecular level on intracellular signalling, do GLP-1 receptors and dopamine coexist on the same neuronal membrane, or is it an indirect effect. Thus, it is plausible to suggest that Ex-4 elicits a paradoxical effect of neuronal desensitization on the presynaptic DA neurons to attenuate the behavioural effect of DA releasing drugs.

In conclusion, the present findings consolidate the negative impact of continuous sucrose consumption on memory and cognitive tasks. We further infer that prolonged ad

libitum access to sucrose in adolescent rats may alter brain circuits related to dopamine neurotransmission. It also increases the behavioural traits to dopamine agonists and is possibly associated with hypersensitivity of some postsynaptic dopamine receptors. These effects were partially prevented by exendin-4, which may elicit some protective and therapeutic effects on dopamine receptor function, but with further desensitization effect on the VTA DA authoreceptors. On the other hand, we noticed that sucrose treatment induced a partial but significant decrease in the sensitivity of dopamine autoreceptors which was surprisingly exacerbated by exendin-4 co-administration, indicating that exendin-4 may exert differential effects on pre- and post-synaptic dopamine receptors, provided DA receptor and GLP-1R are present. It will be of particular interest to find out how long these differential effects will last.

Chapter VI – Concluding remarks and perspectives.

The overall goals of this study were to explore the gut-brain interaction phenomenon, as it relates to gut peptides effect on neurotransmission, behavioural traits as well as the impact on altered nutrition in animal studies. This study has contributed to our understanding of GBA involvement in various central activities such as reward, motivation, and cognitive tasks (Carabotti et al., 2015).

Finding from chapter I, serves as a template for subsequent studies in the thesis. We found from this study that D-amphetamine (DAMP) triggers a huge dopamine efflux in the striatum (Fig. 6B), when superfused on striatal slices (as expected), and caused an increase in motor activity in particular rearing associated activity (Fig. 9A), an effect certainly due to increased dopaminergic transmission in the mesolimbic DA system (Zetterström et al., 1983). We were able to also establish that D-AMP can induce sensitization in the form of escalated motor activity triggered by the challenge administration of the drug after repeated-dose (Fig. 10A). This effect is likely associated with activation of the cortico-limbic system, with initial propagation in the NAc (Chinen et al., 2006). Although, there are still unanswered questions of how long can this neuroadaptive process last? And if this time lag is accompanied with some neurochemical alteration that could be quantified? Indeed, it was accompanied by electrophysiological alteration. Another crucial investigation carried out was to investigate

potential behavioural correlate of varying degree of motor activity (Fig. 10B), with DA neurons electrical firing activity in the VTA (Fig. 14). We observed from our results, that a slight correlate exists between behavioural phenotypes and the rate of DA neuron firing. This indicates that rats with low or moderate motor activity response to D-AMP possibly possess a reduced rate of DA neuronal firing in the VTA. We show that a causal link exists between the behavioural traits that are DA-dependent and DA firing rate (Hamidovic et al., 2009). However, we used very limited samples and there is an absolute need for a more expanded investigation by increasing the sample size and adopt more consistent criteria for the classification of degree of motor activity. The indispensable functional role of DA D_2/D_3 receptors in the modulation of D-AMP-induced motor activity was established. Dopamine D_2R blockage through eticlopride, drastically reduced D-AMP induced motor activity (Fig. 11A), while NGB 2904 a selective DA D_3 antagonist, augments the motor activity (Fig. 11B). On the other hand, things appear more complicated concerning DA D_1 receptors involvement as preliminary data shows that dopamine D_1 agonist, such as SCH-23390 unexpectedly block this locomotor effect. All the results taken together, revealed an indirect modulatory activity of DAT, through DA D_2/D_3 receptor signaling (Beaulieu et al., 2007; Bolan et al., 2007; Boyd and Mailman, 2012). The more expansive study is required in term of DAT quantification in both acute and chronically administered DA D_2 blockers. We and other reported that acute administration of dopamine D_3 preferential agonist PPX reveals a dose-dependent inhibition on the neuronal firing of VTA dopamine neurons of naïve rats (Piercey, 1998; Chernoloz et al., 2009c) (Fig. 13A, B). However, in once weekly single intraperitoneal D-AMP 1 mg/kg injection for a period of two weeks, the DA neurons exhibited significant desensitization after low dose PPX administration (Fig. 13C, D). Thus, disturbance in D_2/D_3 autoreceptors functions with repeated D-AMP (once weekly for two weeks) led to abnormal dopamine activity in the VTA which can be part of the basis for the drug-craving behavioural

anomaly (Gorter et al., 2016; Keebaugh et al., 2017). Whether this partial desensitization is indeed related to the increased motor response to DAMP remains to be demonstrated.

The radiometric *in vitro* results from chapter II show that insulin can modulate striatal [DA] uptake (Fig. 17A, B), likely through an indirect effect on the DAT. This further underpins the role of insulin in dopaminergic transmission (Stouffer et al., 2015; Patel et al., 2018b). Particularly, the DA signaling processes involved is possibly mediated through interaction with DA D_2 receptors (Fig. 17C). Our studies were based on the premise that insulin receptors (InRs) are distributed throughout the brain with high density in the striatum and cerebral cortex (Schulingkamp et al., 2000). In spite of this vast distribution, peripherally administered insulin under anaesthetized rat using *in vivo* electrophysiology paradigm seems not to alter the firing activities of both DA VTA neurons, though an excitatory effect was observed with a high dose on PFC pyramidal neurons (Fig. 22B, C). However, slight progressive decreases in DA neurons firing were observed when insulin was administered through an electrode to allow for gradual diffusion into the VTA (Fig. 23A, B), as well as when co-administered with the stable GLP-1 agonist Ex-4 in intravenous administration (Fig. 22D, E). We posit that insulin crossing from the plasma through the BBB into the brain may not be inadequate concentration to alter the electrophysiological activity of dopamine neurons in a short time frame. The situation may be slightly different in the PFC, brain penetration of insulin, which involved selective transporter at the levels of the endothelial cells can be different depending on the brain region (Strubbe et al., 1988; Wallum et al., 1987; Zhou et al., 2017). For different complex reasons, locally produced insulin may be more efficient than administered insulin in regulating brain function, likely in a glucose-regulated manner (Molnár et al., 2014; Begg, 2015; Gray and Barrett, 2018). We further performed an intra-VTA administration of insulin, which produced significant attenuating effects in both firing and burst activities of VTA DA neurons (Fig. 24). These effects show that there was an

interaction with the local insulin receptors for a direct response (Zhou et al., 2017). Furthermore, using a novel approach to impair insulin secretion with intraperitoneal administration of DZ 150 mg/kg, we report that in the absence of insulin, D-AMP-induced motor activity response was significantly inhibited and the effect was restored when insulin was administered (Fig. 19C, D). Our finding on the modulatory effect of insulin on D-AMP-induced motor activity was in line with the classical streptozotocin-induced diabetes model (Sevak et al., 2008). We propose that DZ model be tested within a wider range of sample size and different strain of animals with varied concentration to ensure repeatability, precision and reproducibility of data. Modulatory effect of insulin on dopamine neurotransmission was further confirmed with DA D_2/D_3 receptor agonist-mediated behavioural responses such as yawning, pica eating and pelvic grooming, induced by PPX and QNP. We demonstrated that insulin modulates these responses by inhibition of the behavioural traits (Fig. 20, 21), an effect also confirmed by radiometric [DA] uptake inhibition of QNP (Fig. 17C). The fact that insulin weakens QNP and PPX-induced behavioural and neurochemical effects (dopamine uptake) indicate a potential interaction with D_2/D_3 receptor signaling. Clinical consideration of insulin dysregulation is of importance in disease management, as brain insulin dysregulation has been linked with Parkinson (Athauda and Foltynie, 2016; Folch et al., 2018), Alzheimer (Cholerton et al., 2013; Neth and Craft, 2017) and other neurodegenerative disorders (Craft and Watson, 2004). In addition, this chapter demonstrates a possible association between insulin level and psychostimulant induced craving indexed as an enhanced motor activity.

Results in chapter III on the K_{ATP} blocker tolbutamide (TBT), indicates a modulatory role of the Kir6.2/K_{ATP} on dopamine neurotransmission and neuronal excitability that can be of therapeutic interest. This provides evidence for likely modulatory action of Kir6.2/SUR1 in reward/motivation as well as cognition processes (Héron-Milhavet et al., 2004; Moriguchi

et al., 2018). First, we qualitatively established the presence of Kir6.2/K_{ATP} channel on the PFC, striatum and the mid-brain containing the VTA (Fig. 32). We suggest more studies on this investigation to ascertain the level and proportion of distribution of the channel in different brain regions, as well as a possible alteration in protein expression of the channels after TBT administration. A very interesting finding is that a high dose of TBT (20 mg/kg) induced a biphasic effect on the dopamine neurons (Fig. 27A, B, C). We also observed that period of inhibition (hyperpolarization) may be due to GABA interneurons or as possible negative feedback from activated accumbens neurons which send strong GABA inhibitory innervations to VTA and in the SN (Schiemann et al., 2012b) (Fig.28A). We are yet to fully confirm this theory and it will be possible in the future to try the use of specific GABA agonist and blockers to support this hypothesis (a similar mechanism is known to occur with the effect of nicotine on VTA dopamine neuronal activity). Conversely, TBT activatory effect on pyramidal neurons of the PFC is monophasic (Fig. 29A, B) and may indicate it could exert a positive effect on cognition and memory due to its ability to activate glutamatergic neurons on the PFC (Yuen et al., 2009; Dauvermann et al., 2017b). There was a pharmacokinetic problem of penetrability of TBT into the brain tissue. An attempt was made to circumvent this problem by the use of a large dose of TBT and co-administration with sodium deoxycholate. A more viable delivery mechanism of TBT into the brain might be developed in the future for an effective central response. The neurochemical studies show evidence of a significant reduction in specific dopamine uptake with TBT and glucose (Fig. 30A, 31B), these indicate a possible interaction of the drugs with dopamine neurotransmission (Sidló et al., 2008). This chapter shows that although, TBT is an insulin secretagogue, yet its effect on the brain is mediated by antagonistic action on Kir6.2 channels.

Finding in chapter IV is crucial to understanding the role of GLP-1 beyond feeding and glucostatic regulations, but also as a vital mediator in reward, motivation and cognition.

Our results show that GLP-1 alters dopamine-dependent behaviour with a significant inhibitory effect on D_2/D_3 DA agonist-induced yawning, pelvic grooming and pica eating (Fig. 42, 43, 44, 45). We also provide evidence for GLP-1 inhibitory effect on D-AMP-induced motor activity (Fig. 46A, B, C, D). Indeed, we found that DAT protein was significantly up regulated with D-AMP-treated rats and remarkably downregulated in Ex-4 pre-treated rats (Fig. 48A, B). This could be attributed to some degree of plasticity of DAT, resulting from synaptic alterations which have been found in some studies (Vaughan and Foster, 2013b; German et al., 2015). The western blot analysis was conducted within an hour of experimental treatment of the animal; it will be of interest to see how long such dysregulation of DAT is sustained after the D-AMP treatment discontinuation. Further research is needed to unravel the impact of DAT protein expression following acute GLP-1 administration, whether DAT is regulated by complex processes such as phosphorylation (Foster et al., 2012; Gorentla et al., 2009), protein–protein interactions (Egaña et al., 2009; Eriksen et al., 2010) and changes in intracellular localization (Lee et al., 2007; Vaughan and Foster, 2013b). We observed a direct correlation between GLP-1 acute treatment and upregulation of GLP-1R protein expression, particularly with Ex-4+D-AMP treated rats (Fig. 49A, B). This is suggestive of GLP-1R activation partly involves in reward and motivation associated with the drug of abuse (Reddy et al., 2016b; Suchankova et al., 2015). Indeed, we report the precognitive effect of GLP-1 on PCP-induced cognitive impairment (Fig. 40B), as well as a clear excitatory effect of Ex-4 on PFC pyramidal neurons (Fig. 56A, B). These data further indicate that GLP-1R activation may have a positive impact on cognition, learning as well as memory, which might be indirectly associated to its neuroprotective effect to resist oxidative stress on all neurons that express GLP-1R (Oka et al., 1999; During et al., 2003a; Gault et al., 2010c; Tweedie et al., 2013; Hölscher, 2014b; Li et al., 2016; Gault and Hölscher, 2018). It will be of interest to know the extent of this cognitive effect in chronic

treatment and if specific blocker of GLP-1R could reverse such action. We also suggest carrying out a dopaminergic lesion with 8-OHDPAT and then proceed to GLP-1R measurement via WB analysis to precisely validate the presence of GLP-1R on the striatal terminal. We have initially presumed that the effect might not be presynaptic as there was no effect with radiometric [DA] release on the striatal slices (Fig. 34A, B). Notwithstanding, we found an interaction between Ex-4 and quinpirole or pramipexole-induced dopamine uptake (Fig. 37C, D) indicating that GLP-1R may be present on DA terminals and there, GLP-1R signaling may negatively interact with DA D_2/D_3 receptors. This was in a way also confirmed in our study on intra VTA administration of Ex-4 which appears to impair D_2/D_3 autoreceptor function on firing rate of DA cells (Fig. 54). Also, a study has demonstrated that Ex-4 reduced nicotine-induced motor response as well as accumbal DA release through the use of *in vivo* microdialysis (Egecioglu et al., 2013a). Since our *in vitro* (release assay) study did not agree with this, it is suggestive to adopt another neurochemical assay to ascertain the actual mechanism of GLP-1effect on the attenuation of psychostimulant-induced motor response. GLP-1 effect on electrophysiological paradigm, when given through intravenous administration, elicits excitability of prefrontal cortex neurons, an effect that can be due to the cognitive-enhancing property of GLP-1 analogue as demonstrated in this chapter (Fig. 56A, B). A direct administration of GLP-1 into the VTA seems to induce an increase in firing and burst activities of DA neurons than the control (Fig. 54). But, this effect needs to be confirmed with a larger population of neurons. Even more, interestingly, the intra-VTA administration of the GLP-1R agonist also showed pronounced desensitization of D_2/D_3 receptors upon PPX administration (Fig. 55). This confirms the negative relationship between D_2/D_3 receptors and GLP-1 receptor function, which may exist at every level of the dopaminergic system (cell bodies, neuron terminals, post-synaptically). Whether GLP-1R and

dopamine D_2/D_3 receptors coexist on the same membrane and interact negatively with each other will be a very interesting question to explore.

The final chapter (chapter V) discusses how GLP-1 modulates the impact of diet alteration in form of continuous sucrose consumption on behavioural and electrophysiological parameters. Our data shows that there is a long-term negative impact of continuous sucrose consumption on memory and cognitive task (Fig. 61B, C), and also on behavioural response to psychostimulants (Fig. 62A, B). Indeed, this effect was not reversed by acute administration of GLP-1 although, co-administered for two weeks with sucrose. Previous studies have shown the involvement of PFC and hippocampus in adolescent rats exposed to daily sucrose intake (Reichelt et al., 2015b). We also observed that prolonged ad libitum access to sucrose in adolescent rats may alter brain circuits related to dopaminergic neurotransmission (Bello et al., 2003; Hajnal et al., 2004; Alsiö et al., 2011; Marella et al., 2012) (Fig. 64C, D). It also increases the behavioural traits to dopamine agonists (Fig. 63), and is possibly associated with changes in sensitivity of some postsynaptic dopamine receptors. Indeed, these effects were partially prevented by GLP-1, which may elicit some protective effects on dopamine receptor function (Kim et al., 2009b; Li et al., 2012; Darsalia et al., 2014). However, we also observed that sucrose treatment (on its own) induced an incomplete but significant decrease in the sensitivity of dopamine autoreceptors which was surprisingly exacerbated by exendin-4 co-administration (Fig. 64F, H) indicating; again, that exendin-4 may exert similar desensitizing effects on pre- and post-synaptic dopamine receptors. It will be of particular interest to find out how long these differential effects will last, as well as possible neurochemical and molecular parameters that may be involved in these changes. Putting the results together, our findings suggest that consumption of sugar by adolescents may impair neurocognitive functions affecting decision-making and memory, response to addictive drugs, potentially rendering them at risk for developing mental health

disorders such as ADHD. More studies are required to validate this finding whether the effect is long-term or short-term induced.

In the future, GLP-1R agonist might potentially find a new use in the management of neuropsychiatric, neurodegenerative and addiction disorders. As demonstrated in this thesis, exendin-4 alters dopamine-dependent behaviour with a remarkable inhibitory effect on D_2/D_3 DA agonist-induced yawning, pelvic grooming and pica eating. Evidence was also provided for GLP-1 inhibitory effect on D-AMP-induced motor activity, a DAT mediated mechanism from GLP-1R activation. These might suggest a potential use in the management of addiction, reward and other dopamine-dependent disorders. In addition, we reported the precognitive effect of GLP-1on PCP-induced cognitive impairment as well as an excitatory effect on PFC pyramidal neurons. Also, these effects might be potentially explored in neurodegenerative disorders such as, Alzheimer disease associated with memory loss and cognitive dysfunction. TBT has been investigated and explored in the early clinical practice days in the management of Parkinson disease. In this thesis, a potential new role has emerged due to its biphasic effect on VTA. Data presented on sucrose consumption is vital to help understand how our diet influences drugs use in CNS. In this thesis, limitation encountered in various techniques deployed has little impact on the data presented. In radiometric release assay, leakages of tritiated neurotransmitter as well as other drugs loaded on the chamber could result in suboptimal concentration of the drug to elicit an efficient efflux. Thus, another neurochemical techniques such as, microdialysis and static release assays may provide a more precise neurochemical data. In radiometric uptake assay, a careful consideration of how to preserve the integrity of the synaptosome is of concern. We circumvented this challenge by reducing the numbers of strokes utilized during homogenisation, a vital step to achieving an experimentally viable synaptosome. The western blot technique would have also be synergized with the aid of immunohistocytochemical

elucidation, which provides a vital information on spatial distribution of protein. This information is however absent with western blot protocol. All the behavioural assays were conducted using visual manual scoring. This approach potentially introduces some errors due to manual spotting of behavioural trait. The use of cam camera coupled with software that can record either online or offline for data analysis will be of help to minimize this error. Anesthesia and invasive surgical procedures greatly alter the physiology of the CNS environment, directly affecting neurotransmission and motility. To fully realize the advantages of in vivo brain electrophysiology, neural recording and stimulation must be conducted in conscious animal models. Advancing with the tools for single-unit recordings in awake animal models demands new and innovative neural microelectrode technology. However, unlike in anesthetized animal, signal quality in awake animal might be a challenge. It is also worth noting that anaesthetics used throughout our study did not have any neuromodulatory effect on the tested drugs.

Publications

Research articles

From the present laboratory

Matteiu Di micelli, **Omoloye Adesina** Benjamin Gronier (2019). Chronic methylphenidate treatment during adolescence has long-term effects on monoaminergic functions. *Journal of Psychopharmacology 33 (1): 109-121*

From previous laboratory

Abdullahi A Muritala, **Omoloye AA,** Adeyinka A. Aderinola (2019). Evaluation of anticonvulsant activities of the ethanoic leaf extract of *Uvaria afzelli* (Annonaecae) in mice. *IOSR (in press)*

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Omoloye AA, Ladoja F, Muritala AA. (2015). Toxicological profile of hydro-ethanolic extract of the leaf of *Solenostemon monostachyus* (P.Beauv.)(Lamiaceae) in rats. *Advancement in Medicinal Plant Research* 3(3):126-136

Omoloye AA, Quasim KO, Onnolome JS, Muritala AA. (2015). Endocrine and metabolic effects of hydroethanolic extract of *Solenostemon monostachyus* on haloperidol induced hyperprolactinemia. *Journal of Pharmacy and Pharmacology 3:391-399*

Olaitan O. J., Sulola E.O., Adejumo E.O., **Omoloye AA** (2015). Cytotoxic effects and genotoxic screening of pharmaceutical effluents using onion bulbs (Allium cepa L.). *Journal of Advances in Biology &Biotechnology*.2 (1): 51-58

Research posters

Adesina Omoloye, Benjamin Gronier (2019). Ad libitum administration of sucrose during adolescence causes changes in dopamine neurotransmission and dopamine dependent behaviour which are partially reversed by glucagon-like peptides**.** *Eur Neuropsychopharmacology accepted for ECNP conference, 2019 Coppenhagen, Denmark*

Benjamin Gronier, **Adesina Omoloye**, Sabrina Weisenburger, Egon Koch (2019). Electrophysiological effects of menthacarin a proprietary combination of essential oils, on visceral nociception. *Neurogastroenterology conference, Berlin, Germany*

Adesina Omoloye, Lara-Malenka Sakiroff**,** Benjamin Gronier (2018). Insulin modulates neural functions via dopamine signalling in reward-related behaviours. *Br J.Pharmacol.2019;176:2977–3081*
https://bpspubs.onlinelibrary.wiley.com/doi/epdf/10.1111/bph.14681

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Adesina Omoloye, Benjamin Gronier (2018). Electrophysiological and neurochemical effects of sulphonylurea tolbutamide, in the rat ventral tegmental area and prefrontal cortex. *FASEB J.* 32, 1_supplement: 685.2https://www.fasebj.org/toc/fasebj/32/1_supplement. *Experimental biology, 2018, ASPET Conference, San Diego, California, US.*

Benjamin Gronier, **Adesina Omoloye** (2018). Insulin, insulin secretagogue and glucagon-like peptide differentially modulate neuronal activity in the prefrontal cortex and dopaminergic systems. *Eur Neuropsychopharmacology* 29:S228-S229 DOI:10.1016/j.euroneuro.2018.11.370

Di Miceli, **A. Omoloye,** B. Gronier (2017). Possible long term consequences of a chronic oral treatment with methylphenidate on monoamine neurotransmission.

Adesina Omoloye, Timothy Fajemirokun (2015). Medication errors assessment in paediatric outpatient clinic of a primary healthcare centre in Lagos, Nigeria. *West African Postgraduate College of Pharmacy annual conference.* (Oral presentation)

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