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Modulation of Presynaptic Dopamine Synthesis and Storage Dynamics by D$_2$-Like Receptor Partial Agonist Antipsychotics in Rat Brain Striatum

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Abstract

Presynaptic dopaminergic regulation is important to maintain a homeostatic balance of dopamine stored levels and release. Changes in dopamine neurotransmission contribute to neurological and psychiatric disorders. Recent findings from our group (Ma et al., 2015; González-Sepúlveda et al.,-submitted) describe strong effects of several classes of dopaminergic drugs on dopamine synthesis, including L-DOPA (used in Parkinson), tetrabenazine (Huntington) and aripiprazole (schizophrenia). In this study, I confirm and extend those findings and compare the effects of D_2R partial agonist antipsychotics cariprazine and brexpiprazole, the psychostimulants amphetamine and methylphenidate, and several other selective and experimental compounds. Rat brain striatum was minced and incubated *ex-vivo* in the presence or absence of these drugs at different concentrations. Spontaneously, dopamine and serotonin accumulated over time reaching near-maximal storage levels. This experimental approach allowed me to evaluate their synthesis and storage dynamics under the influence of chosen pharmacological agents. My results could be useful to understand the mechanisms of action of antipsychotics, and they could facilitate further research with animal models and clinical trials using new dopaminergic agents.

Resumen

La regulación dopaminérgica presináptica es importante para mantener un equilibrio homeostático de los niveles almacenados y liberación de dopamina. Los cambios en la neurotransmisión de dopamina contribuyen a los trastornos neurológicos y psiquiátricos. Hallazgos recientes de nuestro grupo (Ma et al., 2015; González-Sepúlveda et al., presentado) describieron los fuertes efectos de varias clases de medicamentos dopaminérgicos en la síntesis de dopamina, incluida L-DOPA (utilizada en Parkinson), tetrabenazina (Huntington) y aripiprazol (esquizofrenia). En este estudio, confirmamos y ampliamos esos hallazgos y comparamos los efectos de los antipsicóticos agonistas parciales D_2R cariprazina y brexpiprazol, las psicoestimulantes anfetamina y metilfenidato varios otros compuestos selectivos y experimentales. El estriado cerebral de rata fue troceado e incubado *ex vivo* en presencia o ausencia de estos fármacos a diferentes concentraciones. Espontáneamente, la dopamina y la serotonina se acumularon con el tiempo alcanzando niveles de almacenamiento casi máximos. Este enfoque experimental nos permitió evaluar su dinámica de síntesis y almacenamiento bajo la influencia de los agentes farmacológicos elegidos. Nuestros resultados podrían ser útiles para comprender los mecanismos de acción de los antipsicóticos, y podrían facilitar la investigación futura con modelos animales y ensayos clínicos utilizando nuevos agentes dopaminérgicos.

Resum

La regulació dopaminèrgica presinàptica és important per mantenir un equilibri homeostàtic dels nivells emmagatzemats de dopamina i el seu alliberament. Els canvis en la neurotransmissió de dopamina contribueixen a trastorns neurològics i psiquiàtrics. Treballs recents del nostre grup (Ma et al., 2015; González-Sepúlveda et al.,-presentada) van descriure importants efectes de diverses classes de fàrmacs dopaminèrgics sobre la síntesi de dopamina, inclosa la L-DOPA (emprada en Parkinson), la tetrabenazina (Huntington) i aripiprazol (esquizofrènia). En aquest estudi, vam confirmar i ampliar aquestes troballes i vam comparar els efectes dels antipsicòtics agonistes parcials D_2R cariprazina i brexpiprazol, els pricostimulants amfetamina i metilfenidat i diversos altres compostos selectius i experimentals. L'estriat cerebral de rata va ser trocejat i incubat *ex-vivo* en presència o absència d'aquests fàrmacs a diferents concentracions. De manera espontània, la dopamina i la serotonina es van acumular al llarg del temps i van assolir nivells d'emmagatzematge gairebé màxims. Aquest enfocament experimental ens va permetre avaluar la seva síntesi i dinàmica d'emmagatzematge sota la influència d'agents farmacològics escollits. Els nostres resultats podrien ser útils per comprendre els mecanismes d'acció dels antipsicòtics, i podrien facilitar més investigacions amb models animals i assajos clínics mitjançant nous agents dopaminèrgics.

Abbreviations

5-HT	Serotonin
5-HIAA	5-Hydroxyindoleacetic acid
AC	Adenylyl cyclase
ADHD	Attention deficit hyperactivity disorder
AMPH	Amphetamine
ANOVA	Analysis of variance
ARI	Aripiprazole
BH_4	Tetrahydrobiopterin
BREX	Brexpiprazole
cAMP	Cyclic adenosine monophosphate
CARI	Cariprazine
COMT	Catechol-o-methyl transferase
D_2	Dopamine D_2 receptor
D_2R	Dopamine D_2-like autoreceptor
D_3	Dopamine D_3 receptor
DA	Dopamine
DAT	Dopamine transporter
DMSO	Dimethyl sulfoxide
DOPAC	3,4-Dihydroxyphenylacetic acid
GABA	γ-aminobutyric acid
GIRK	g-protein inwardly rectifying K^+ channels

GPCRs	G-protein-coupled receptors
HPLC-EC	High-performance liquid chromatography with electrochemical detector
HPLC-UV	High-performance liquid chromatography with ultraviolet detector
L-DOPA	L-3,4-dihydroxyphenylalanine
LUMA	Lumateperone
MAO	Monoamine oxidase
MPH	Methylphenidate
NMDA	N-methyl-D-aspartic acid
OKA	Okadaic acid
QUIN	Quinpirole
SUL	Sulpiride
TBZ	Tetrabenazine
TH	Tyrosine hydroxylase
TM	Transmembrane
TPH	Tryptophan hydroxylase
U.S. F.D.A.	United States Food and Drug Administration
VGCC	Voltage-gated Ca^{2+} channels
VMAT	Vesicular monoamine transporter

Index

Index of figures and tables

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I. INTRODUCTION

Introduction

Schizophrenia represents one of the leading causes of disability in the world. The disorder was first known as dementia praecox before it was renamed by Eugene Bleuler, who composed it from two Greek words: *skhízō* (to split) and *phrēn* (mind) between 1910 and 1911 to mean "the loss of the sense of reality" (Bleuler E., 1950). Even though schizophrenia has been diagnosed for more than a century and various antipsychotics are useful, we are still in progress to achieve an effective medical treatment covering different aspects of this illness. Now, schizophrenia alone is suffered by more than 21 million people worldwide. It is known, however, that the development of a new drug is a long and expensive process from research to market, often taking many years to move through each phase of preclinical and clinical development. In fact, total costs from discovery to market launch are in the range of $800 million for each new drug being developed, yet approximately 92% of new drugs fail between the preclinical and clinical development phases (Pampaloni & Masotti, 2009). This is primarily due to the lack of efficacy and various adverse drug reactions, and it has posed a serious challenge to new novel drug development (Olson, 2002). Thus, it is important to understand the pathological aspects of schizophrenia and the mechanisms of action of existing drugs in order to recognize the properties of different antipsychotics behind the success in the clinical treatments; these will be covered in my introduction.

Before I discuss the pathological aspects of schizophrenia, I should mention that this general knowledge of the structure and function of the nervous system is also of great importance. This will increase our understanding of how chemical compounds may treat a pathological condition. A nerve cell, also called a neuron, is the basic unit of the nervous system. A neuron is constituted by three major components: a **cell body** (soma), which acts as a controller to regulate

the functions of the cell; an **axon**, which conducts electrical impulses from the cell body to the axon terminals; and **dendrites**, which receive, process and integrate the incoming synaptic communications. The basic structure of a neuron is illustrated in fig. 1-1. The cell body and the dendrites form the postsynaptic component of the synapse, while the axon terminals form the presynaptic component. In this Ph.D. work, I have worked experimentally with brain minces containing axon terminals of a specific type of neurons synthesizing the neurotransmitter dopamine (DA).
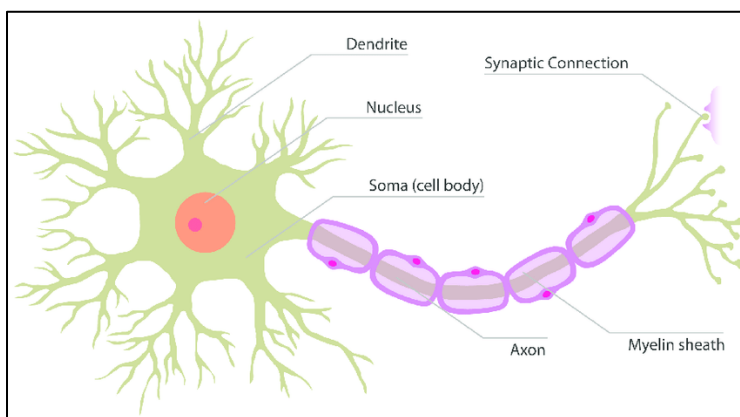
Fig. 1-1. The basic structure of a neuron (London & Fountas, Z., 2020).

Neurons can be sub-classified into different types based on their location, function or neurotransmitter types (e.g. DA, serotonin (5-HT), γ-aminobutyric acid (GABA), acetylcholine, norepinephrine, etc.). Neurons communicate with one another via these neurotransmitters. Since my work was focused mostly on the effect of compounds with pharmacology components on DA, I would like to elaborate more on dopaminergic neurons in the brain. In addition, I will also discuss some minor details of serotonergic neurons, as some of my results may also show the effect on 5-HT levels in comparison with DA concentration in the brain region called striatum. It should also

be borne in mind that all other neurotransmitters mentioned are present in other cells in the same tissue where DA and 5-HT are being analyzed.

1.   *DA*

DA is the predominant catecholamine in the brain, a monoamine neurotransmitter that has a catechol structure (benzene with two hydroxyl groups next to each other) and an amine side chain (fig. 1-2).
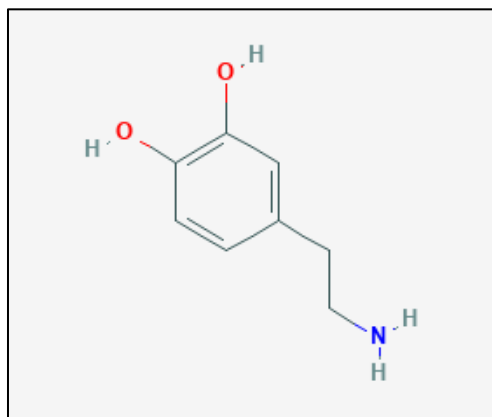
Fig. 1-2. Chemical structure of DA. The image is extracted from
https://pubchem.ncbi.nlm.nih.gov/compound/681#section=Structures

The discovery of DA in the brain dates back to more than 60 years ago, when Arvid Carlsson identified an intermediate compound or a precursor in the synthesis of adrenaline and noradrenaline in 1957. Following that discovery, the role of DA as a neurotransmitter was revealed in subsequent years after finding a distinct distribution pattern of the dopaminergic system from that of noradrenaline (Arvid Carlsson, 2001). For his significant contributions and works related to DA, Carlsson was awarded for Nobel Prize in Physiology or Medicine in 2000.

2. Dopaminergic System in the Brain

Anatomically, DA cells were designated as 'A' or aminergic-containing cells group. These cells were categorized from A8 through A14, succeeding the noradrenergic cells which were categorized from A1-A7. DA cell bodies are primarily located in the small brain regions of substantia nigra (DA cell group A8 and A9), ventral tegmental area (DA cell group A10), arcuate (DA cell group A12) and periventricular nucleus (DA cell group A14) of the hypothalamus. These regions are part of the mesencephalon, or midbrain area of the brain. The DA cell bodies project into other regions of the brain through three main pathways: (1) nigrostriatal pathway, (2) mesocortical pathway, and (3) mesolimbic pathway. The nigrostriatal pathway connects the DA cell bodies in the substantia nigra with dorsal striatum. This projection is involved in goal-directed behaviors and voluntary movements. Impairment within this region is implicated with neurological diseases such as Parkinson's, Huntington's and Tourette's syndromes. DA cell bodies in the ventral tegmental area form two projections. The first projection is the mesocortical pathway, which connects the ventral tegmental area with cortex and is involved in regulating motivation, emotion, and cognitive control. Impairment within this region may be implicated in the attention deficit hyperactivity disorder (ADHD) and the negative symptoms of schizophrenia. The second projection is the mesolimbic pathway, which connects the ventral tegmental area with several parts of the brain, including limbic structures, nucleus accumbens, olfactory tubercles, amygdala and hippocampus. This pathway is involved in incentive salience, reinforcement, learning and desire, and is thought to play an important role in addiction and the positive symptoms in schizophrenia (Berridge, 2007). Other dopaminergic projections include the tubero-infundibular pathway, which connects DA cell groups of A12 and A14 from the hypothalamus with the pituitary. This pathway

regulates pituitary hormones, especially prolactin secretion. Overall dopaminergic pathways in the brain are illustrated in fig. 1-3.
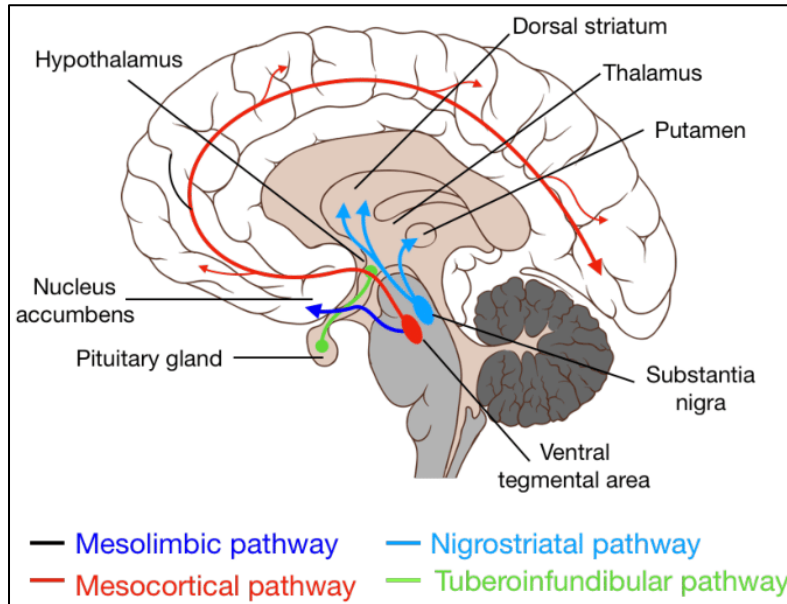
Fig. 1-3. Dopaminergic pathways in the brain (Nummenmaa et al., 2020).

3.   Striatum

Since the focus of my experimental design for this thesis is DA levels and dynamics in the rat brain striatum (discussed later in the pathophysiology of schizophrenia), I shall briefly explore the anatomy and neurophysiology of the striatum in the brain. The striatum is the largest structure and component of basal ganglia. It is located in the forebrain region. The striatum is divided into ventral and dorsal divisions based on its function and connection. The ventral striatum consists of the nucleus accumbens, while the dorsal striatum constitutes the caudate nucleus and the putamen; they are separate from each other by white matter in humans, but not in rats (consist mainly of nerve fibers with their myelin sheaths) (fig. 1-4). There are many strands of grey matter in the

striatum. White and grey matter overlay each other, creating the striatum's striped appearance. It was due to this characteristic that it was named striatum (the name comes from a Latin word for "striped"). The striatum receives glutamatergic and dopaminergic inputs from different sources. In fact, the striatum receives the densest DA innervation and contains the highest concentration of DA receptors in the brain (Gerfen, 2004). The dorsal striatum receives synaptic input projections from the prefrontal cortex and other frontal regions. The ventral striatum, on the other hand, receives extensive synaptic input projections from the ventral frontal regions (orbitofrontal, ventromedial and ventrolateral cortex). Both the dorsal and ventral striatum receive intense dopaminergic inputs from substantia nigra and ventral tegmental area, as discussed before, with regard to the dopaminergic pathways in the brain. The ventral striatum is associated with the limbic system and plays an important role in mediating cognition, movements, reward, reinforcement and motivational salience, while the dorsal striatum mediates the cognition involving motor functions, certain executive functions, stimulus-response learning and reward.
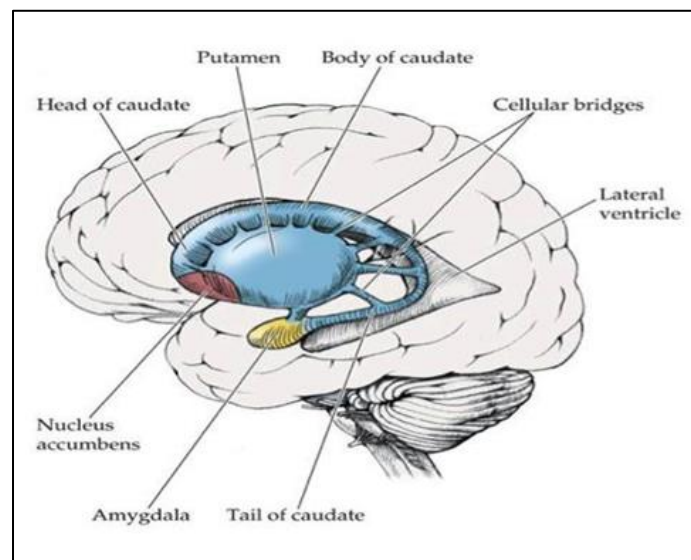
Fig. 1-4. The schematic shows the anatomical structure of striatum which constitutes of the caudate nucleus, putamen and nucleus accumbens. The image is extracted from http://www.learnneurosurgery.com/basal-ganglia.html.

The striatum contains both the projecting neurons and interneurons (neurons that innervate within the brain regions) (Bolam et al., 2000). Overall, there were two types of cells in the striatum as follows:

Medium spiny projection neurons: These are the principal neurons and comprise between 90 and 95% of the total neuronal population in the striatum. They contain GABA as their main neurotransmitter (GABAergic neurons) and are classified as inhibitory neurons. The medium spiny projection neurons can also be subdivided into two equal-size population of neurons based on their projection and their neurochemical contents, as follows:

- *Direct pathway:* project directly from the striatum to the output nuclei of the basal ganglia and express neuropeptide substances and D_1-like receptor in addition to GABA receptor.

- *Indirect pathway:* project from the striatum to the globus pallidus and express enkephalin and D_2-like receptor in addition to the GABA receptor.

Interneurons: These neurons comprise only between 5 and 10% of the total neuronal population in the striatum and are classified as excitatory neurons. They play an important role in regulating medium spiny projection neurons. An example of interneurons in the striatum is cholinergic interneurons, which release acetylcholine as a neurotransmitter.

4.   DA Biosynthesis

Three pathways have been described for the synthesis of DA (Delcambre et al., 2016). All three start from an amino acid L-tyrosine as a precursor. The first well-known and main pathway starts when L-tyrosine is hydroxylated into 3,4-dihydroxyl-l-phenylalanine (L-DOPA) by tyrosine hydroxylase (TH) with tetrahydrobiopterin (BH_4) act as a cofactor, O_2 and Fe^{2+}, resulting in the

formation of dihydrobiopterin and water (Dunkley et al., 2004). TH is known as a rate-limiting step in the synthesis of catecholamine. TH also exhibits a substrate inhibition phenomenon, which controls the synthesis and DA levels independently of the fluctuation of L-tyrosine concentration from meal intakes. Fe^{2+}, which is bound to TH, can be oxidized to Fe^{3+}. Fe^{3+} can bind to catechols (e.g. DA), and mediates a feedback-inhibition mechanism as summarized in fig. 1-5. The activity of TH can be regulated by two mechanisms: medium- to long-term regulations of gene expression or short-term regulation of enzyme activity through feedback inhibition by direct DA binding via competing for the BH_4 cofactor binding, allosterism regulation and phosphorylation (Dunkley et al., 2004). TH can be phosphorylated at serine (Ser): Ser^8, Ser^{19}, Ser^{31}, and Ser^{40} by a variety of protein kinases and phosphatases. Phosphorylation of TH alters the enzyme conformation which dissociates the catechols' binding, returning the enzyme to the fully active state.
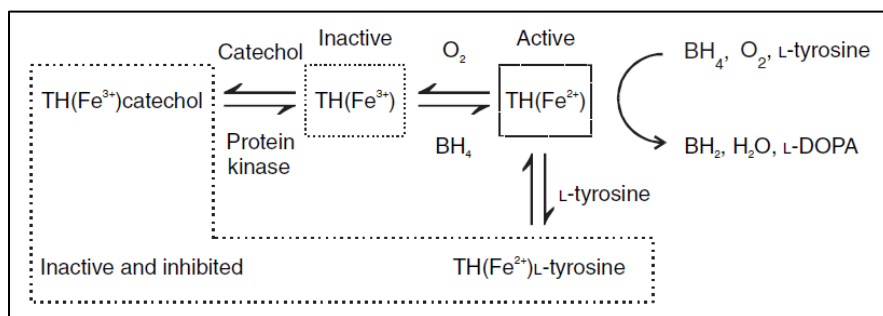
Fig. 1-5. Summary of TH' regulation (Dunkley et al., 2004).

The second pathway synthesizes L-DOPA from L-tyrosine via a hydroxylation process; however, the enzyme involved is tyrosinase, and this process can occur even in the absence of TH (Rios et al., 1999). L-DOPA is then decarboxylated by an aromatic amino acid decarboxylase to produce DA. The third pathway involves a different intermediate compound but also used L-tyrosine as a precursor. In this case, L-tyrosine is first to decarboxylate by the same DOPA decarboxylase

enzyme to yield tyramine before further hydroxylation by cytochrome-P450 Cyp2D enzyme to form DA. The overall steps for these three different biosynthetic pathways of DA are illustrated in fig. 1-6. In noradrenergic and adrenergic cells, DA can later be converted into other catecholamines, including noradrenaline and adrenaline.
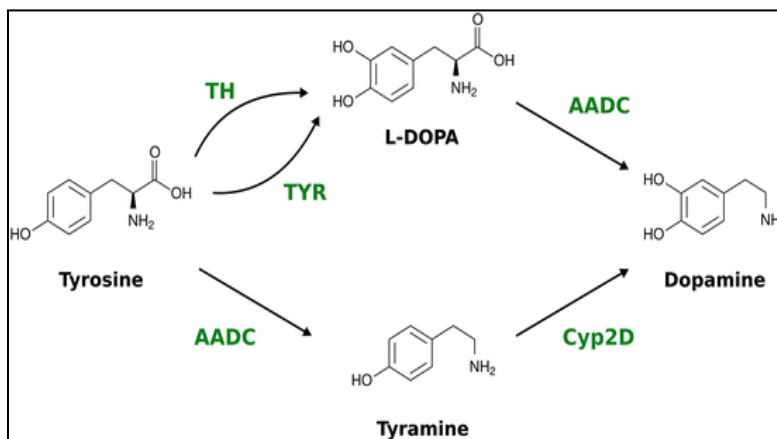
Fig. 1-6. Biosynthetic pathways of DA (Delcambre et al., 2016). Abbreviations: TH; tyrosine hydroxylase, TYR; tyrosinase, AADC; aromatic amino acid decarboxylase and Cyp2D; cytochrome-P450 Cyp2D enzyme.

5. *DA Storage*

Two types of vesicular monoamine transporter (VMAT) are found in humans: VMAT-1, which largely expresses in neuroendocrine cells, and VMAT-2, which is majorly found both in monoaminergic cells in the brain and in the sympathetic nervous system (Erickson et al., 1996). Cytosolic DA, which is synthesized (as described earlier), is then uptaken, encapsulated and stored inside synaptic vesicles by VMAT-2 to protect them from degradation (German et al., 2015). This process is done against high concentrations gradient inside synaptic vesicles driven by transmembrane pH and electrochemical gradient generated by the vesicular H^+-ATPase in the granule membrane (fig. 1-7). This mechanism involves the exchange of one molecule of DA

transport into the synaptic vesicle with two H^+ ions out of the vesicle. Various pharmacological compounds inhibit VMAT-2 activity such as reserpine (the very first antipsychotic, but not used anymore), tetrabenazine (TBZ) and lobeline. Due to this action, TBZ is approved by the United States Food and Drug Administration (U.S. F.D.A.) for the treatment of chorea associated with Huntington's disease. Other compounds may also interfere with the physiological functions of VMAT-2: these include neurotoxin 1-methyl-4-phenylpyridinium (Darchen et al., 1988) and amphetamine (AMPH). AMPH interrupts VMAT-2 actions either via disrupting intravesicular pH gradients or by directly binding to the VMAT-2 and preventing the substrate from interacting with the transporter (Floor & Meng, 1996).
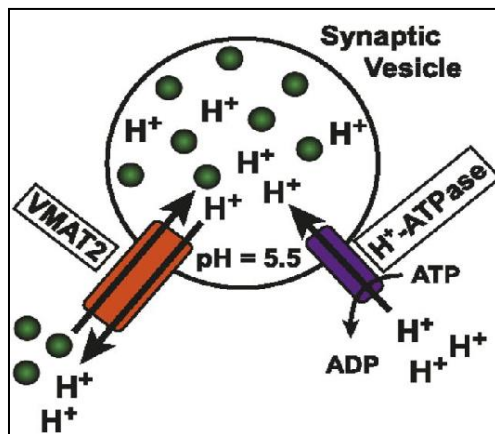
Fig. 1-7. DA (shown in green) is uptaken from the cytosol into the synaptic vesicles via VMAT-2. This is facilitated by H^+-ATPase (German et al., 2015).

6.   *DA Release*

The stored DA is released into the synaptic cleft via exocytosis in response to the presence of action potential stimulation at dopaminergic terminals. This action is mediated by ion-specific channels (e.g., K^+, Na^+, Ca^{2+}, etc.). For example, the Ca^{2+} channels increase the influx of Ca^{2+} ions,

thus increasing intracellular concentrations. These channels enable controlled membrane electrical potentials to propagate as action potentials through the neurite extensions towards the end of terminals. Such action potential stimulates the fusion of vesicles containing DA to the plasma membrane of dopaminergic terminals leading to a release of DA to the extracellular synaptic space. The amount of DA-mediated signaling is dependent on the amount of DA available in synaptic cleft. This DA that is available in extracellular synapse will then: (1) bind to target receptors, including DA receptor at postsynaptic (known as D_2L or D_2-long), which leads to the activation of various signal transductions; (2) binds to the DA receptor located at presynaptic (known as D_2S or D_2 short), which can modulate a negative-feedback inhibition on TH; (3) be recycled by reuptake into the presynaptic terminals, mainly via DA transporter (DAT); and (4) be metabolized by the action of several enzymes, e.g. catechol-o-methyl transferase (COMT).

7. *DAT*

Previously, I have discussed the mechanisms following the release of DA, where it can be recycled and reused mainly via presynaptically localized DAT to transport DA against its concentration gradients from the synaptic cleft back to the dopaminergic terminals. This process is dependent on extracellular Na^+ and Cl^- concentrations with the binding of two Na^+ ions and one Cl^- ion per DA molecule. This process will cause a conformational change from outward-facing to inward-facing and create a complex transport-associated current (German et al., 2015). The release of DA and ions into the cytoplasm will shift DAT back to its original conformation. The model of this process is illustrated in fig. 1-8 and the crystal structure of DAT is shown in fig-1-9. Various compounds have been recognized to target DAT for their pharmacological actions, such as psychostimulants;

these include cocaine, MPH and AMPH, which prolong the duration of DA action on its receptors. Impairment of DAT had been shown to be implicated with ADHD and depression, and has been correlated with drug abuse and their reinforcing properties.
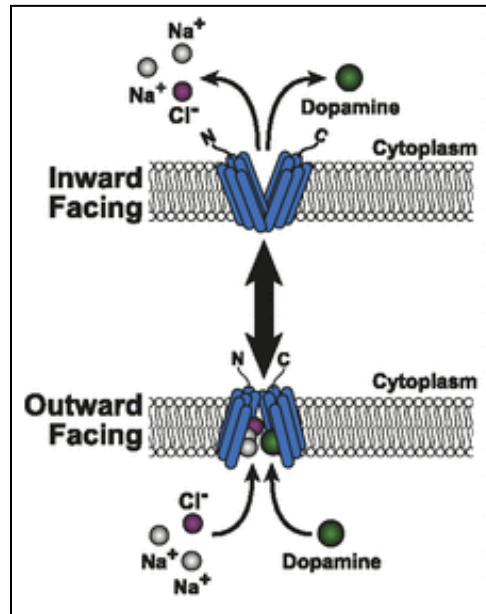
Fig. 1-8. The model of DAT shows the movement of DA from extracellular milieu to cytoplasm through a transition from an outward-facing to an inward-facing state (German et al., 2015).
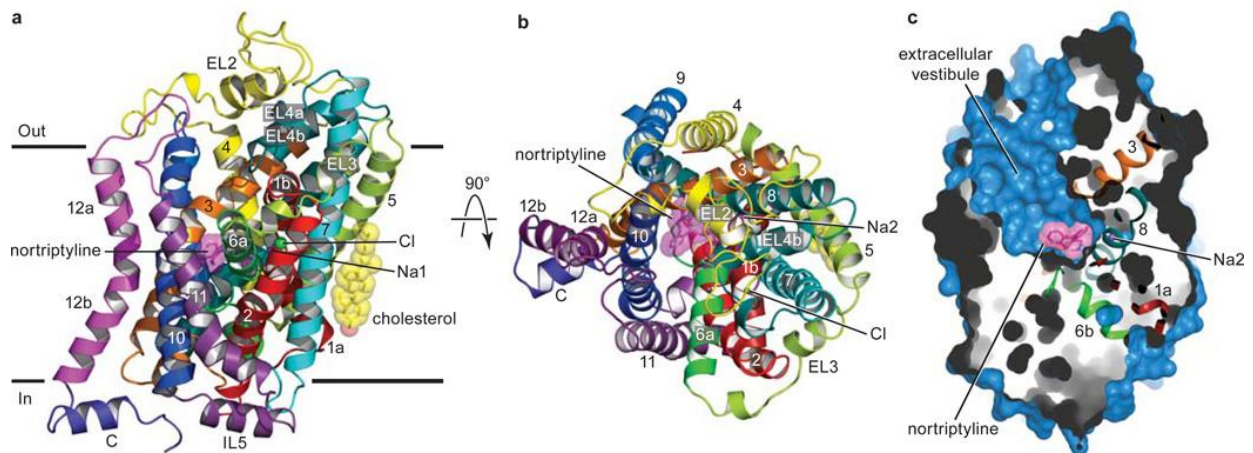
Fig. 1-9. (a) Crystal structure of DAT viewed parallel to the membrane with nortriptyline, sodium ions, a chloride ion, and a cholesterol molecule, shown in sphere representation in magenta, purple, green, and yellow, respectively. (b) DAT view from the extracellular face. (c) Surface representation showing that ligand and ion binding sites are accessible from the extracellular vestibule (Penmatsa et al., 2013).

8. *DA Metabolism*

The active metabolism of DA prevents its auto-oxidation, which leads to the formation of reactive oxygen species (Delcambre et al., 2016). In a neuron, DA is metabolized primarily by the action of monoamine oxidase (MAO), COMT and aldehyde dehydrogenase (ALDH). MAO can be found in two isoforms A and B: MAO A, found in catecholaminergic neurons, and MAO B, found mainly in astrocytes. MAO is located on the exterior of mitochondria inside the neuron, while COMT is located in postsynaptic neurons, glia, and also on the outer mitochondria membrane and endoplasmic reticulum. Previously, I have discussed that in neuronal cells DA is uptaken and stored inside the synaptic vesicles to prevent itself from being metabolized in the cytoplasm. MAO metabolize cytosolic DA into 3,4-dihydroxy-phenylacetaldehyde (DOPAL) before being oxidized into 3,4-dihydroxy-phenylacetic acid (DOPAC) by ALDH or reduced to form 3,4-dihydroxy-phenylethanol (DOPET). COMT, on the other hand, methylate the 3'-hydroxyl group of catechol ring on DA to yield a 3-methoxytyramine, and is further metabolized by MAO to form 3-methoxy-4-hydroxy-phenylacetaldehyde. Both DOPAC and 3-methoxy-4-hydroxy-phenylacetaldehyde can be subsequently metabolized to form homovanillic acid (HVA), both by COMT and ALDH. Details of the overall pathways of DA metabolism in the brain are clearly illustrated in fig. 1-10. Various compounds block the metabolism of DA and inhibit these enzyme activities for their pharmacological effects to increase DA availability and prolong the duration of DA action on its receptors. These include entacapone, which is a COMT inhibitor used for the treatment of Parkinson's disease, and pargyline, a MAO inhibitor used as an antidepressant.
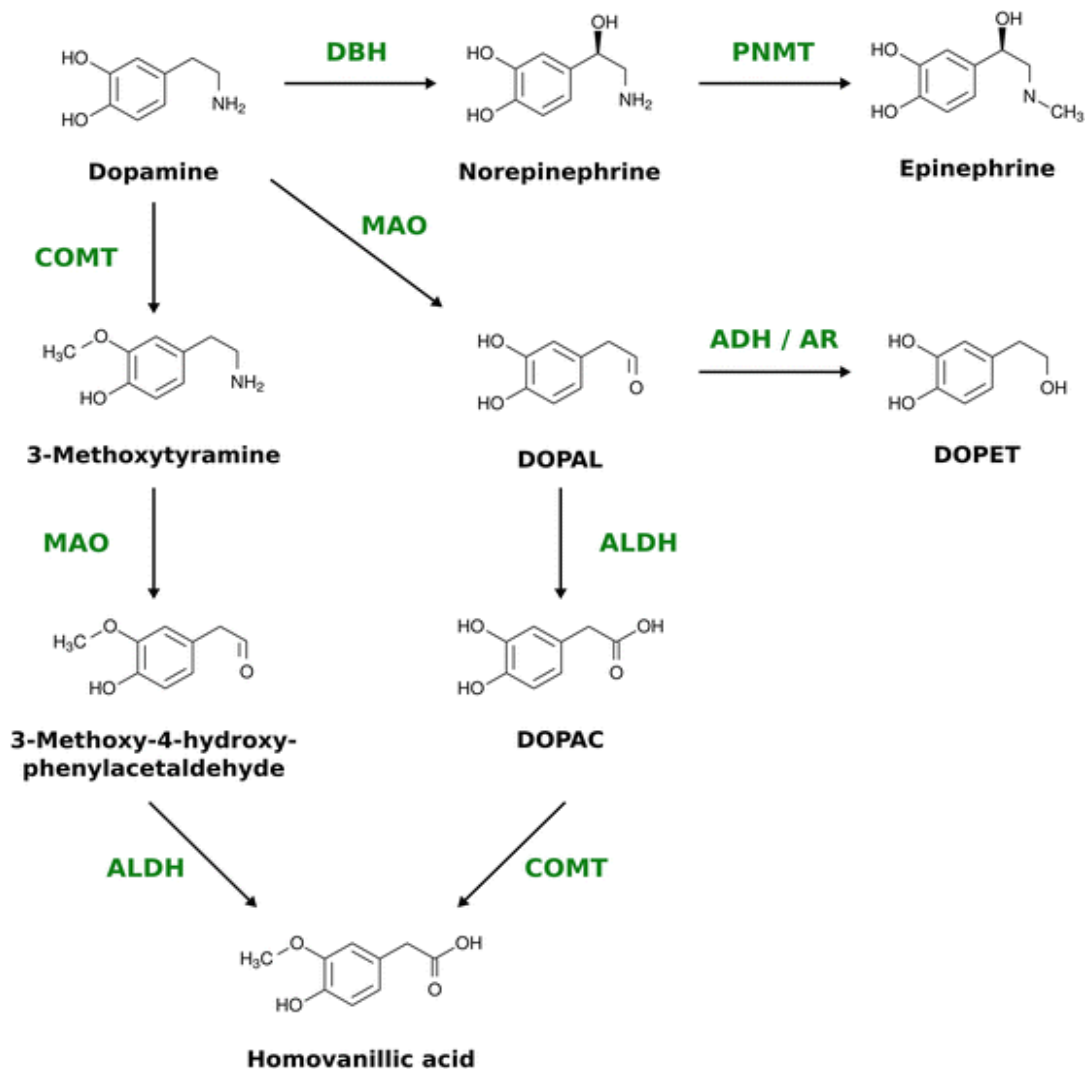
Fig. 1-10. The sequence of DA metabolisms in the brain (Delcambre et al., 2016). Abbreviations: MAO; monoamine oxidase, COMT; catechol-O-methyl transferase, ALDH; aldehyde dehydrogenase, AR; aldose reductase, DOPAL; 3,4-dihydroxy-phenylacetaldehyde, DOPAC; 3,4-dihydroxy-phenylacetic acid, DOPET; 3,4-dihydroxy-phenylethanol.

9.   *G-protein coupled receptors*

After a release of DA from dopaminergic terminals, it binds mainly at DA receptors to exert its action. These DA receptors have been categorized as a member of class A (rhodopsin-like) G-protein coupled receptors (GPCRs). GPCRs constitute the largest family of transmembrane (TM)

protein, and regulate many cellular processes, including central nervous system activities (Jacoby et al., 2006). Due to this, GPCRs are a target of many pharmaceutical drug actions with 475 marketed drugs (~34% of all the U.S. F.D.A.-approved therapeutic agents) (Hauser et al., 2017). GPCRs are classified into three major classes (classes A, B and C) depending on the size of N-terminal, sequence homology and identity of seven TM-domains that participate in ligand binding. Among all three classes of GPCRs family, class A (rhodopsin-like receptor family) is the largest and counts for 85% of GPCRs genes; these include the receptor for hormones, neurotransmitters (including DA receptors), melatonin, and light receptors. These receptors have been classified into 19 subgroups from A1 to A19, with DA receptors under the A17 subfamily together with 5-HT, adrenergic and trace amine receptors.

9.1 Structure of GPCRs

GPCRs are integral proteins that possess seven TM-spanning domains or TM helices (Namkung et al., 2009). GPCRs are categorized by N-terminal, which can be glycosylated, followed by the seven TM α-helices (TM1 - TM7), which are connected by three intracellular (IL1-IL3) and extracellular loops (EL1-EL3), and ending with intracellular C-terminal. GPCRs also contain phosphorylation sites for cyclic adenosine monophosphate (cAMP)-dependent, protein kinase A, protein kinase C and GPCRs kinase (GRKs), generally in the second and third intracellular loops. The Schematic of the amino acid sequence of one of the GPCRs (D_2 receptor) is present in fig. 1-11. The crystal structure of the D_2-like receptors is shown in fig. 1-12.
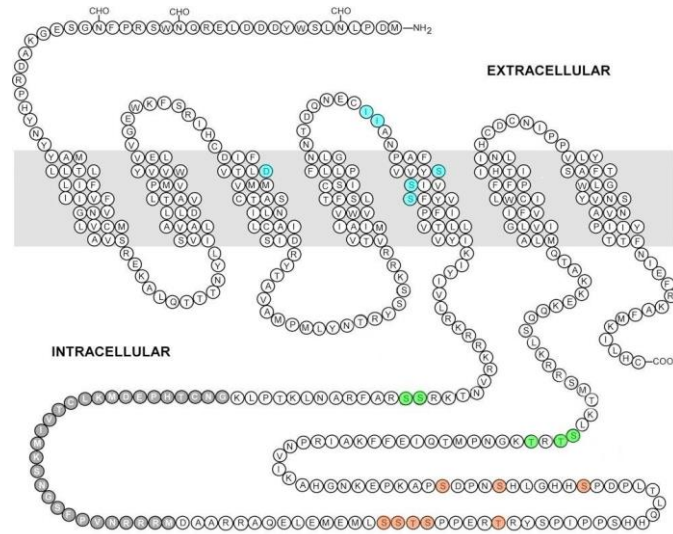
Fig. 1-11. **2D schematic of the amino acid sequence of the D₂ receptor**. Blue sites are involved in ligand binding, the green sites are for protein kinase C binding, while orange sites are for GRKs. The 29 amino acids present at the D₂L variant are indicated with the grey residues (elaborated in more detail in the classification of DA receptors later) (Namkung et al., 2009).
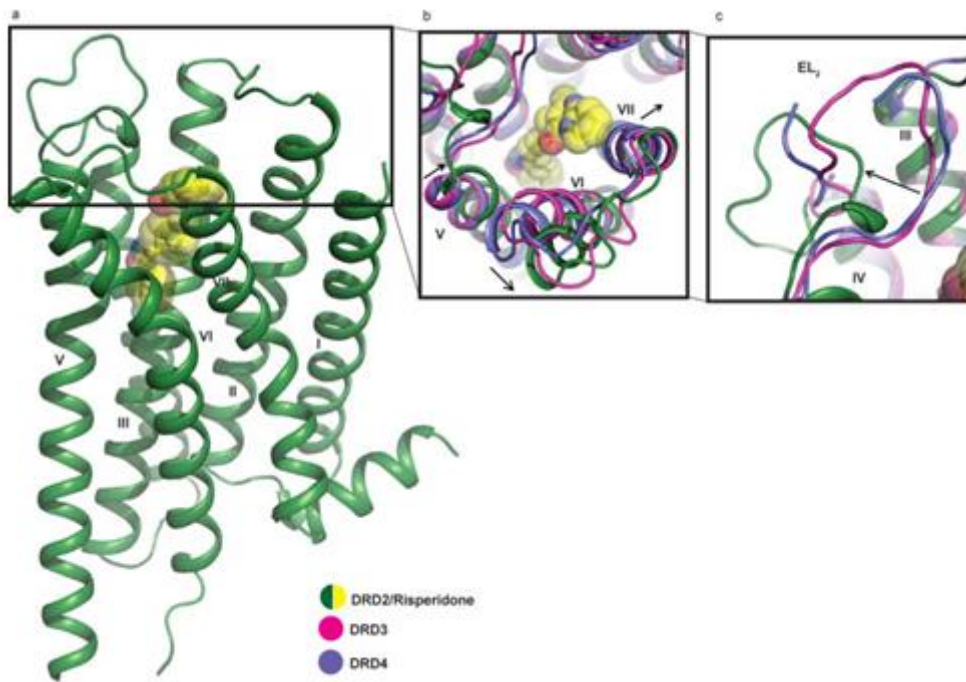
Fig. 1-12. Crystal structure of D_2 (shown in green) in comparison with D_3 (shown in magenta) and D_4 (shown in blue) receptors. (a) Structure of D_2 receptor with risperidone. (b and c) view from extracellular (adapted from Wang et al., 2018).

9.2 Orthosteric and allosteric ligand binding sites at GPCRs

Ligands' binding sites at GPCRs are classified as orthosteric and allosteric binding sites. The orthosteric site is the active ligand binding site at GPCRs. The key component of ligand binding at the orthosteric site depends on the ligands' affinity towards the receptor. A higher affinity of the ligand would allow a low dosage to activate the binding sites. The other protein surface at GPCRs, which can change the conformation of the orthosteric protein binding site, is known as the allosteric binding site (Jeffrey Conn et al., 2009) (fig. 1-13). An allosteric modulator acts via shifting the free energy landscape. An allosteric modulator binding towards GPCRs disturbs the protein surface atoms, which propagate like a wave to reach the orthosteric binding sites. Thus, the design of allosteric modulator depends on the protein conformational ensemble and the preferred propagation states (Tsai, 2012). Interestingly, this action can potentiate (positive allosteric modulator) or decrease (negative allosteric modulator) ligand responses at the orthosteric site (fig. 1-14). Therefore, positive and negative allosteric modulators can change the affinity and efficacy of the ligand on the receptors. The other type of allosteric modulator is a silent allosteric modulator. This modulator does not alter the action of an orthosteric ligand but blocks another allosteric modulator to bind. Overall, allosteric ligands act at GPCRs to: (1) induce a conformation change on a target receptor, which increases or decreases the binding affinity and/or efficacy of the receptor agonist; (2) stabilize the conformational changes from the agonist binding and increase or decrease the probability of the receptor-active states; and (3) prevent or facilitate (change) the desensitization of the receptor due to continuous activation or intense exposure of an agonist.
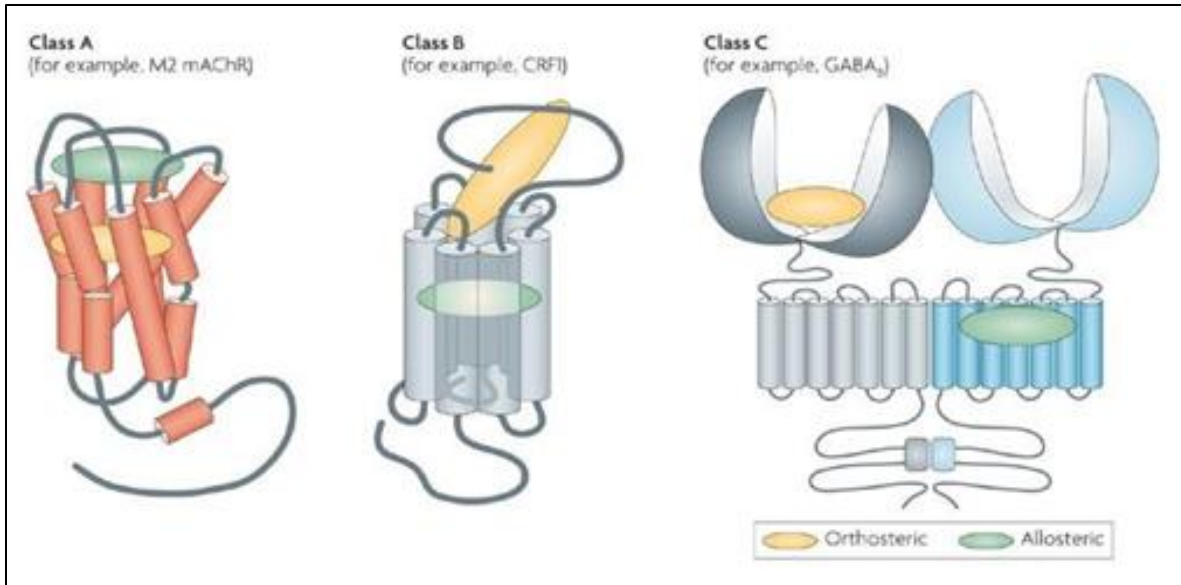
Fig. 1-13. Schematic diagram of GPCRs highlighting two common endogenous ligand binding sites, the orthosteric and allosteric sites for each GPCRs family members (Jeffrey Conn et al., 2009).
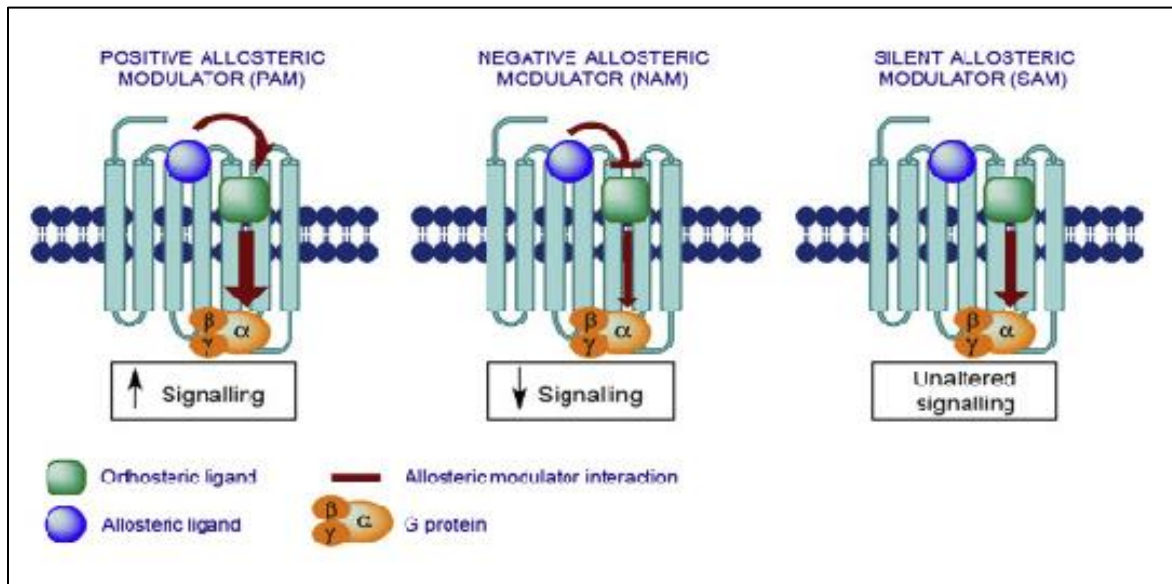
Fig. 1-14. Schematic diagram illustrates three types of allosteric modulator actions on GPCRs and their effects to the orthosteric ligand (López-Rodríguez et al., 2020).

9.3 GPCRs properties and signaling action

G-proteins are a heterotrimer complex that consists of α, β and γ subunits. Without stimulation or as in the inactive state, all three subunits are bound together with the GDP (guanosine diphosphate) on a G_α subunit. Binding of GPCRs with extracellular ligands (e.g. agonist) results in a conformation change on GPCRs structure that is transmitted to the G_α subunit of the heterotrimeric G-protein. This activation facilitates an exchange of GTP (guanosine triphosphate) in place of a GDP on G_α subunit. The activated G_α-GTP complex triggers the dissociation of the G_α subunit from the $G_{\beta\gamma}$ dimers from the receptor. Such dissociation also leads to losses of affinity of the ligand. The dissociation of both the G_α subunit monomer and the $G_{\beta\gamma}$ dimers then becomes free to interact with other intracellular proteins upon their downstream effectors to initiate intracellular signaling responses (e.g. adenylyl cyclase (AC), which I will describe later). After the signal propagation, the GTP of G_α-GTP complex is hydrolyzed to GDP and the G_α becomes inactive (G_α-GDP), which leads to its re-association with the $G_{\beta\gamma}$ dimers to form the inactive heterotrimeric complex. The overall cycle of the GPCRs active/inactive states is illustrated in fig. 1-15. A conformation equilibrium between active and inactive biophysical states leading to the classification of a ligand from an agonist, antagonist and inverse agonist will be described later.
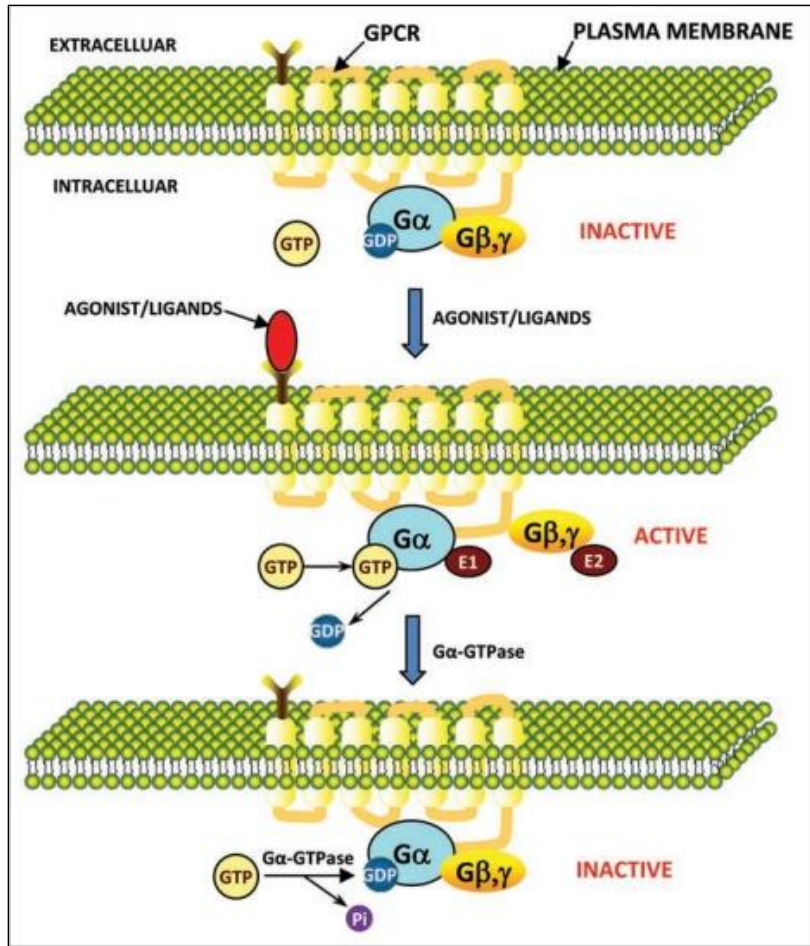
Fig. 1-15. Model of G-protein activation/deactivation cycle (Tuteja, 2009).

There are three common types of G-protein α subunit, G_s, $G_{i/o}$ and G_q. As shown in fig. 1-14, the activated G-protein will bind to the effector to activate the intracellular signal transduction. The effector for the G-protein α subunit, G_s and $G_{i/o}$ effectors are cAMP, which acts as a second messenger while the G_q activates phospholipase C which then catalyzes the cleavage of phosphatidylinositol 4,5-biphosphate into the second messenger inositol (1,4,5) triphosphate and diacylglycerol. The mechanism by which the G-protein α subunit mediates signal transduction on cAMP leads to the classification of DA receptors; I will discuss it in detail later. The g-protein $G_{\beta\gamma}$

21

dimers, on the other hand, act on various ion channels such as G-protein inwardly rectifying K^+ channels (GIRKs) and voltage-gated Ca^{2+} channels (VGCC).

9.4 Ligand classifications based on their biological responses on GPCRs

I have mentioned earlier (fig. 1-15) the conformation equilibrium between the active and inactive biophysical state of the receptor leading to the classification of a ligand from an agonist, antagonist and inverse agonist. Here, I would like to discuss this classification, as it is important to know the differences between each of these ligands and their effect, since this thesis works with a different type of ligands, including an agonist, partial agonist and antagonist on D_2 receptor. A ligand is a substance or molecule which binds to other sites on a target protein (in this case a receptor) to produce a desired biological response (effect). Referring to this response or effect, this ligand can be classified into different classes, as per below:

Full agonist: Ligand that stabilizes the receptor in an active conformation state. A full agonist may bind and stimulates the receptor to produce the maximal biological responses (a term known as the maximal effect).

Partial agonist: In comparison with a full agonist, a partial agonist is a ligand that binds and stimulates the receptor but can only produce part of the maximal biological responses.

Antagonist: Ligand that binds the receptor but does not produce any effect or biological responses. A full antagonist may block other ligands (agonist or partial agonist) to bind on the same receptor types, thus antagonizing the biological responses produce by these ligands. The presence of a competing antagonist causes a rightward displacement of the agonist concentration-response curves.

Inverse agonist: In comparison with the full agonist, which stabilizes the receptor in an active conformation state, an inverse agonist stabilizes the receptor in an inactive conformation state. The difference with an antagonist is that this ligand decreases the spontaneous coupling of the receptor to the G-protein and suppresses the constitutive activity.

The overall action of these ligands on the receptor is illustrated in fig. 1-16, and the differences in their biological responses are represented in fig. 1-17.
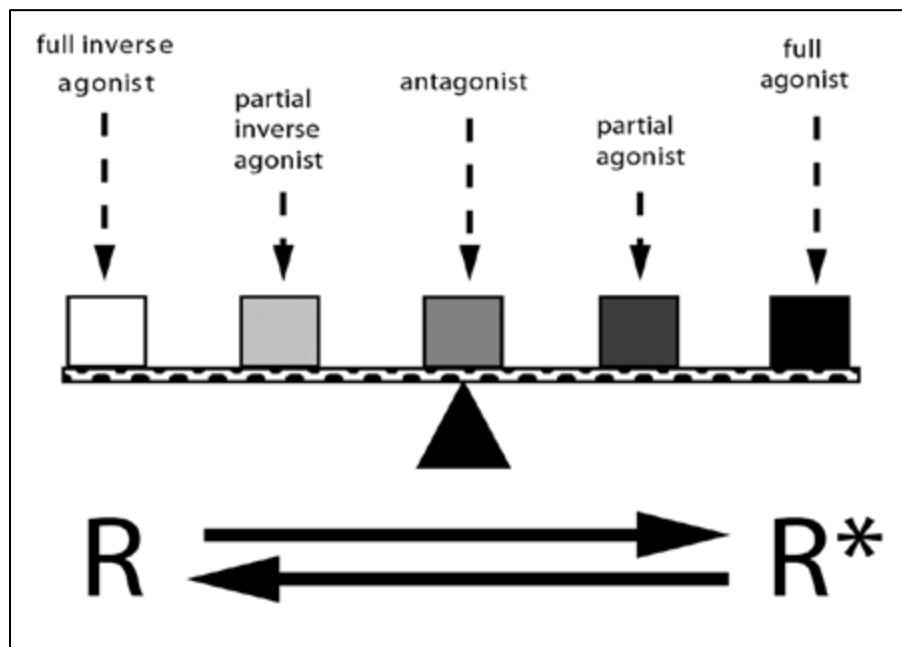
Fig. 1-16. The action of an agonist, partial agonist, antagonist and inverse agonist can be interpreted as changing the conformation equilibrium balance between the active and inactive biophysical state of the receptor (Seifert et al., 2003).
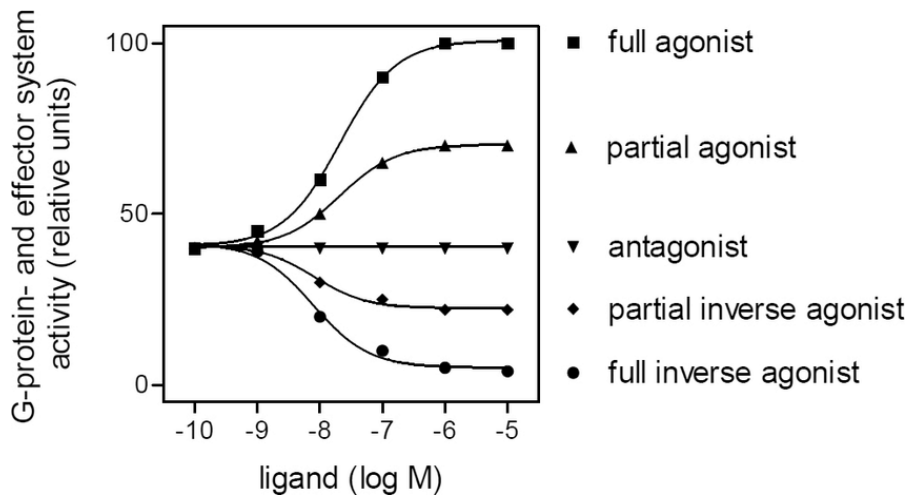
Fig. 1-17. The concentration-response curve represents the effect of ligands of each classification on the receptor. The biological response of a ligand typically follows a sigmoidal function with an agonist increases, while an inverse agonist decreases the activity below the basal values. A partial agonist, on the other hand, produces submaximal responses. An antagonist has no activity but is able to block the receptor from an agonist, partial agonist or inverse agonist (Seifert et al., 2003).

9.5 Basic pharmacological terminology

For an optimal understanding of the differences between the pharmacological responses and the properties of these ligands, it is necessary to know the basic pharmacodynamic terminology, as follows:

Affinity: Since these ligands bind to the receptor to produce desired biological responses, the nature of such a binding can be quantified by characterizing how tightly the ligand and the receptor interact. The higher the affinity of the ligand, the higher their chemical force and the attraction of this ligand towards the receptor at any given time.

Efficacy: The word efficacy is used when referring to the ability of the ligand to produce an effect or response. The maximal effect is quote as E_{max} value. Differences in drug efficacy are compared by the E_{max} value obtained at high concentrations.

Potency: Potency describes the amount of a ligand to produce maximal effects. A highly potent ligand is able to produce a maximal response at low concentrations. The potency of a ligand is evaluated by comparing EC_{50} or IC_{50} value, as described below:

EC_{50}: effective concentration of a ligand to produce half of the maximal effects.

IC_{50}: inhibitory concentration of a ligand to inhibit half of the specific biological functions. The relationship between ligand affinity, efficacy and potency can be further understood with the dose-response curves illustrated in fig. 1-18.
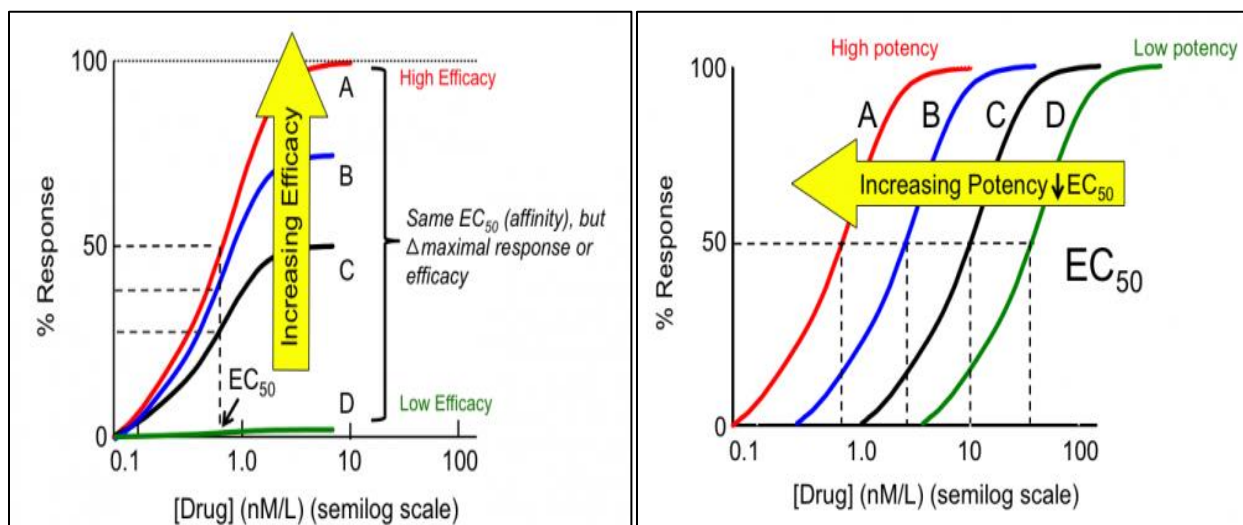
Fig. 1-18. Comparison between ligand affinity, efficacy and potency can be clearly illustrated based on the displacement or properties in their dose-response curves. The image is extracted from http://tmedweb.tulane.edu/pharmwiki/doku.php/basic_principles_of_pharm

10.   DA receptors

10.1 Classification of DA receptors

The first identification of DA receptors was based on their action on the second messenger of AC. Based on this action, DA receptors were classified into two types: D_1 and D_2 receptors. The D_1 receptor stimulated, while the D_2 receptor had either no effect on AC activity or inhibited it (Kebabian & Calne, 1979). With further advancement in medical genetic cloning techniques, DA receptors were then separated into five distinct receptor subtypes (D_1 to D_5) in the late 1980s. Based on their pharmacological and biochemical properties, these five receptor subtypes were categorized into two major groups or subfamilies: the D_1-like family, which contains both the D_1 and D_5 receptor subtypes; and the other D_2, D_3 and D_4 receptor subtypes were classified into the D_2-like family. As mentioned earlier, these classifications were based on their action on AC activity. The D_1-like family receptors are coupled to the stimulatory G_s family of G-protein to stimulate cAMP production by stimulating AC, while the D_2-like family receptors are coupled to the inhibitory $G_{i/o}$ family of G protein to inhibit cAMP production by inhibition of the AC (Osinga et al., 2017) (fig. 1-19). Location-wise, the D_1-like family receptors were exclusively found at the postsynaptic terminals, and the D_2-like family receptors were expressed in both post- and presynaptic on dopaminergic neurons. Genetically, both the D_1- and the D_2- like family receptors are different based on the availability of introns in their coding sequence, with the D_1-like family receptors not containing introns, while the D_2-like family receptors contain several introns in their sequence. This structure later leads to the generation of two major D_2-like family receptor variants, D_2S (D_2-short) and D_2L (D_2-long) with the addition of 29 amino acids at the later variant, as shown

in fig. 1-10 (Usiello et al., 2000). The D_2S variant is mostly expressed presynaptically and is involves in autoreceptor function, while the D_2L seems to be expressed at the postsynaptic dendrites. For the scope of this thesis, I will focus and elaborate on the D_2-like family receptors, since they play an important role in the treatment of schizophrenia, as all approved antipsychotics act on D_2-like receptors.
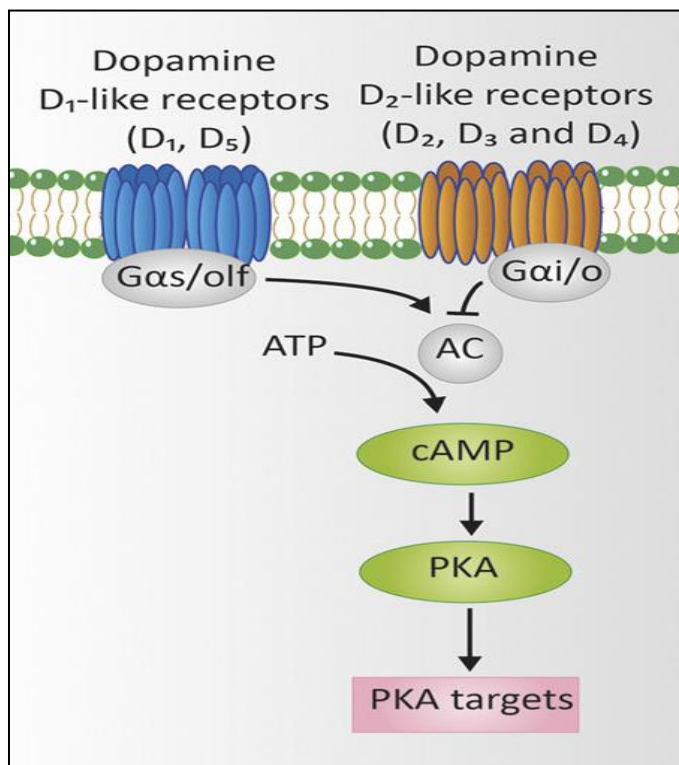
Fig. 1-19. Classification of DA receptors bases on their signaling action on AC (adapted from (Osinga et al., 2017).

10.2 Distributions and localizations

I have shown and discussed in detail the dopaminergic system in the brain (fig. 1-2). Here, I would like to concentrate on the distribution of D_2-like receptors from the dopaminergic system in the brain. The D_2 receptor is found at the highest level in the striatum, nucleus accumbens and the

olfactory tubercle. However, the D_2 receptor is also expressed at a high level in other parts of the dopaminergic system, including substantia nigra, ventral tegmental area, hypothalamus, hippocampus, amygdala, septum and cortex in the brain. Compared to the D_2 receptor, the D_3 receptor has a limited pattern of distribution, with the highest level of expression being found in the limbic region, such as in the shell of nucleus accumbens, olfactory tubercle and the islands of Calleja (Murray et al., 1994). The D_3 receptor is also found in the ventral tegmental area, substantia nigra and the medial olfactory area. The D_4 receptor, on the other hand, has the lowest level of expression in the brain, as only a few areas have been documented, including cortex, amygdala, hippocampus and hypothalamus (Rondou et al., 2010). The overall distribution of D_2-like receptors in the brain can be clearly illustrated in fig. 1-20.
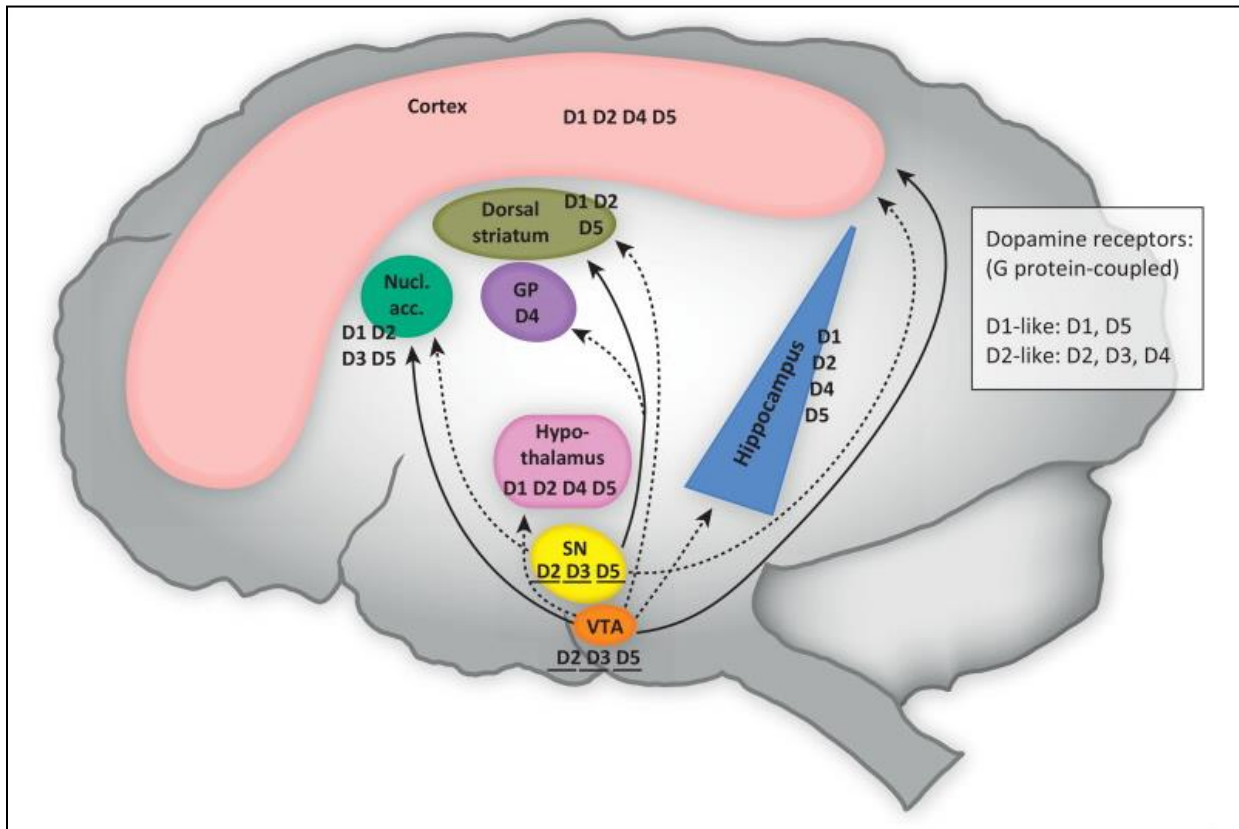
Fig. 1-20. Distribution of DA receptor subtypes in the brain (Brichta et al., 2013).

28

10.3 Functions

Previously, I have mentioned the location of the D_2-like family receptors (D_2S variant) which was found both post- and pre- synaptically on dopaminergic neurons. This D_2 receptor located at presynaptic DA terminals was involved in autoreceptor function. Since most parts of this thesis are based on the effects of partial agonist antipsychotics and the main effect analyzed is on this D_2-like autoreceptor (D_2R), it is important to know the regulation of this receptor and its functional action at the presynaptic terminals. There are several mechanisms involved in the regulation of D_2R at the presynaptic terminals. The major action of D_2R is regulating DA release from axon terminals (Benoit-Marand et al., 2001), This regulation involves inhibition of VGCC (Herlitze et al., 1996), which are responsible for the Ca^{2+} entry triggering DA release and hyperpolarization via voltage-dependent K^+ channels ($K_v1.2$) (Martel et al., 2011) or the activation of GIRK. This action is mediated through G-protein $\beta\gamma$ inhibition, as mentioned earlier. As the amount of DA-mediated signaling at post-synaptic receptors is dependent on the amount of DA available in the synaptic cleft, the clearance of DA at extracellular milieu is primarily determined by the reuptake mechanism, mainly by the DAT. Thus, the second mechanism of action of D_2R in regulating DA neurotransmission is altering the reuptake of DA via DAT (Cass & Gerhardt, 1994; Wu et al., 2002). The third mechanism, in turn, works by decreasing DA synthesis (W. Kehr et al., 1972). This regulation involves down-regulation on TH activity via an inhibition of AC, leading to the reduction of cAMP-protein kinase A, and provides a feedback mechanism by which DA can regulate its own inhibition at the presynaptic terminals. The overall mechanisms of D_2R regulations of DA transmission at the presynaptic terminals are illustrated in fig. 1-21. In addition to these three regulations of D_2R at the presynaptic terminals, another study also shows that D_2R

activation may also regulate and increase the reuptake of DA into the synaptic vesicles via VMAT-2 (Truong et al., 2004).

In *in-vivo*, these effects will lead to the changes of extracellular DA levels (Rouge-Pont et al., 2002; Schmitz et al., 2002) and decrease the activity and reinforcement properties of drugs of abuse, including ethanol (Phillips et al., 1998) and opiates (Maldonado et al., 1997). Furthermore, the absence of D_2R has been shown to elevate DA synthesis and release together, with the changes in behavior, leading to increased motivation for rewards, hyperlocomotion and supersensitivity, induced by cocaine (Bello et al., 2011). These indicate the important role of D_2R in regulating dopaminergic neurotransmission at the presynaptic terminals.
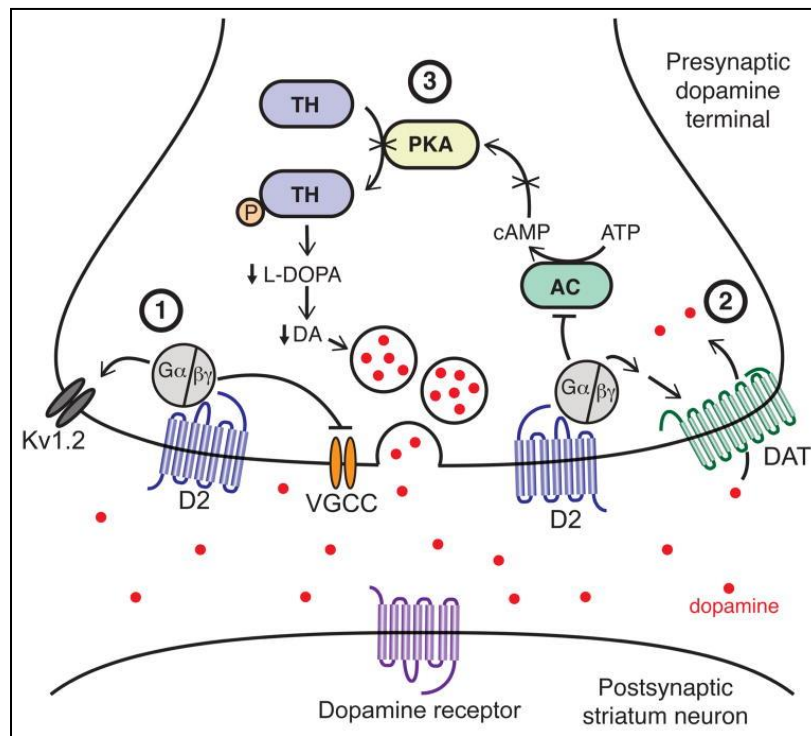
Fig. 1-21. Schematic of the D_2R signaling and regulation of DA transmission at presynaptic dopaminergic terminals (Ford, 2014).

11.   *Serotonergic systems in the brain*

In addition to DA, another neurotransmitter that also plays an important role in schizophrenia symptoms is 5-HT (see section 13.2 in the pathophysiology of schizophrenia). Therefore, I would like to discuss some of the general knowledge of serotonergic systems in the brain. The discovery of 5-HT in the brain dates back to earlier than that of DA, when Erspamer and Vialli found a compound later named enteramine in enterochromaffin cells in rabbit intestines during the 1930s (Erspamer & Asero, 1952). Later, in 1948, a vasoconstrictor substance that was the same molecule as enteramine was discovered and named 5-HT (Rapport et al., 1948). 5-HT is an indoleamine (a family of molecules that has a common structure), which consists of an indole compound (a six-member of benzene ring fused to a five-member of pyrrole ring) that contain an amine group (fig. 1-22).
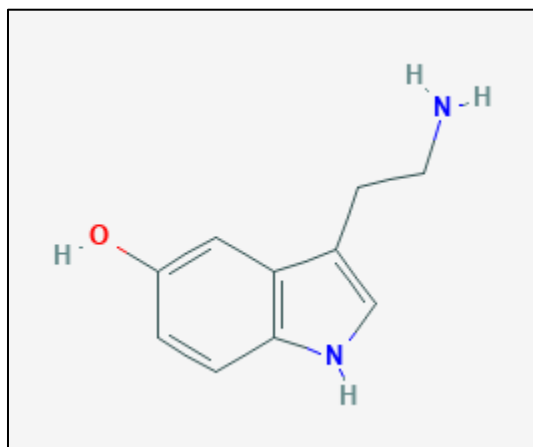
Fig. 1-22. Chemical structure of 5-HT. The image is extracted from
https://pubchem.ncbi.nlm.nih.gov/compound/Serotonin

Serotonergic systems in the brain constitute a defined neuronal population with the cell bodies located in the raphe nuclei of the brain stem (Dahlström & Fuxe, 1964). From the raphe nuclei, the 5-HT neuron projects to almost all brain regions, including the cortex, amygdala, hippocampus and hypothalamus. Serotonergic systems in the brain are illustrated in fig. 1-23.
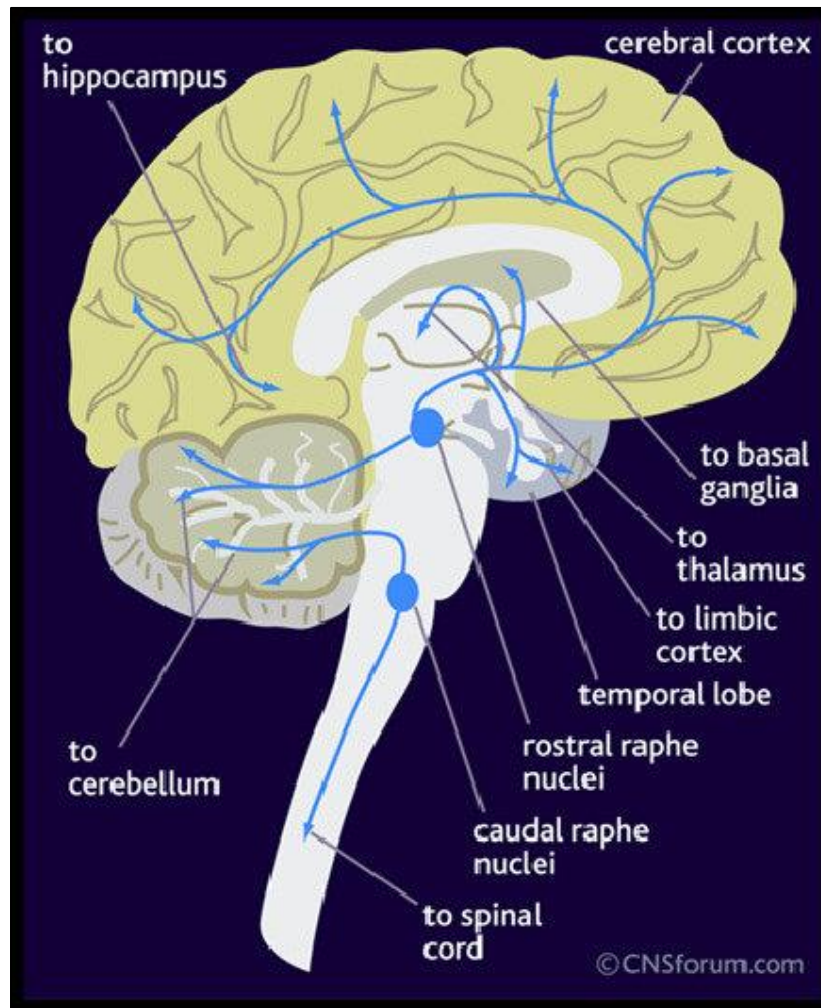
Fig. 1-23. Serotonergic pathways in the brain (Tarlacı, 2011).

11.1 Biosynthesis, storage and metabolism of 5-HT

5-HT is synthesized in two steps from the amino acid L-tryptophan as a precursor (Gray & Roth, 2007). The first step involves hydroxylation of L-tryptophan into 5-hydroxy-l-tryptophan (5-HTP) via tryptophan hydroxylase (TPH) with BH_4 acting as a cofactor. Similar to TH, TPH is also known as the rate-limiting enzyme. The second and final step of the biosynthesis involves converting 5-HTP into 5-hydroxytryptamine or synonym as the 5-HT via AADC. The overall steps for the biosynthetic pathways of 5-HT are illustrated in fig. 1-24. 5-HT can be metabolized by the enzyme MAO to produce 5-hydroxyindole acetic acid (5-HIAA). Similarly to DA, following the synthesis, 5-HT is then uptaken, encapsulated and stored inside the synaptic vesicles by the same VMAT-2 to protect them from degradation. The same mechanism is involved as for the uptake of DA, and this process is done against a high concentration gradient inside the synaptic vesicles. 5-HT is stored at the presynaptic terminals until the action potential of the neurons triggers the release into the synaptic cleft, where it can be reuptaken by 5-HT transporter or bind to 5-HT receptors at the postsynaptic terminals.
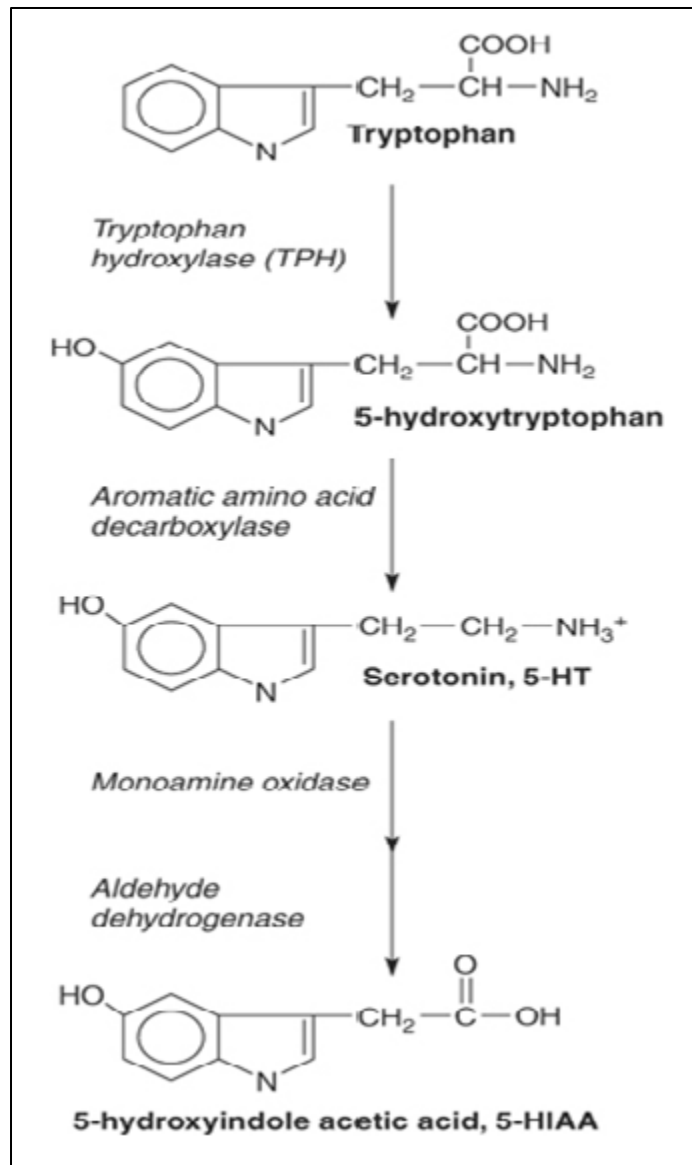
Fig. 1-24. Biosynthetic pathways of 5-HT (Gray & Roth, 2007).

11.2 5-HT receptors

After 5-HT is released from the serotonergic presynaptic terminals, it later binds mainly at 5-HT receptors to exert its action. There are at least 14 different 5-HT receptor subtypes, which have been grouped into seven classes (5-HT$_{1-7}$) based on their structural and operational characteristics. Similarly to the DA receptors, all 5-HT receptors subtypes are GPCRs, except for 5-HT$_3$, which is

a ligand-gated ion channel. I have discussed that the D_2 receptor was expressed both at the post- and pre- synaptic terminals; all 5-HT receptor families were expressed only at postsynaptic terminals, except for the 5-HT$_1$ family, which also existed at the presynaptic terminals. The 5-HT$_{1A}$ receptor subtype is expressed in the somatodendritic region and exerts a general inhibitory influence of neuronal firing, while both the 5-HT$_{1B}$ and the 5-HT$_{1D}$ receptors subtypes are expressed at the serotonergic nerve terminals, thus mediating presynaptic autoinhibition regulations via negative feedback mechanisms (Hoyer et al., 2002). The density of 5-HT receptor subtypes varies between different brain regions. The 5-HT$_{2A}$ receptor subtype is more highly expressed in the cortex than any other brain region (Hall et al., 2000). The 5-HT$_{1A}$ receptor subtype is abundantly found in the limbic system (hippocampus, posterior entorhinal cortex and subcallosal area), and the 5-HT$_{1B}$ receptor subtype is highly expressed in the ventral striatum (Varnäs et al., 2004). The overall distribution of 5-HT receptor subtypes in the brain is clearly illustrated in fig. 1-25.
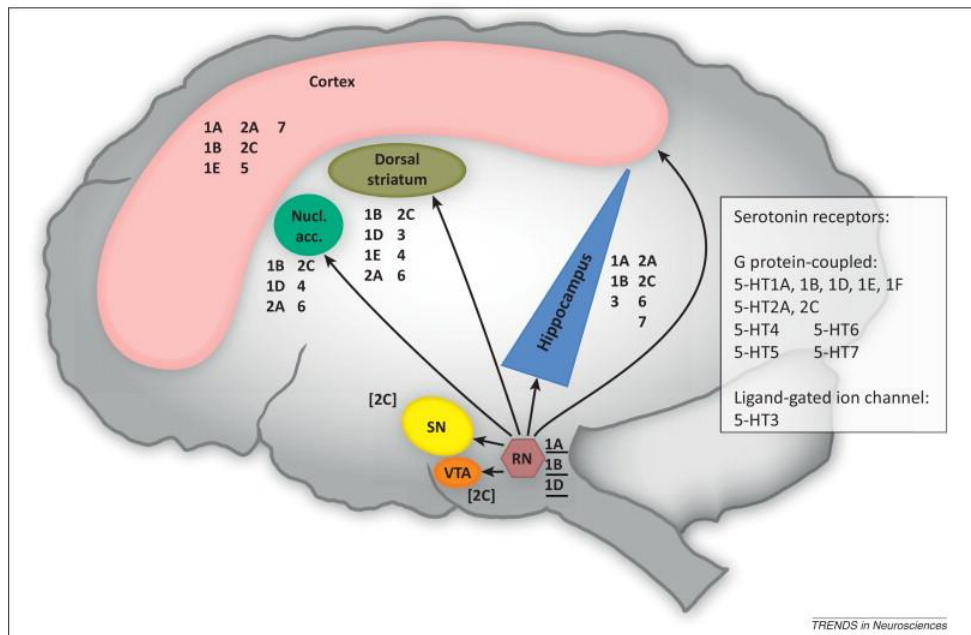
Fig. 1-25. Distribution of 5-HT receptor subtypes in the brain (Brichta et al., 2013).

12.   *DA-related neurological disorders*

DA plays an important role in brain functions, including cognitive, motor, motivation, reward and reinforcement. Dysfunctional dopaminergic neurons have been implicated in many neurological disorders. Throughout this thesis, several compounds that target presynaptic dopaminergic terminals as their pharmaceutical actions are used for understanding the mechanisms involved in regulating DA synthesis These compounds have been approved for the treatment of some of the dopaminergic related diseases. Therefore, I would like to give an overview of the pathophysiology of these illnesses and the mechanisms of action of the drugs used for treatments. These illnesses include Parkinson's disease, Huntington's disease and ADHD. In addition to treating these disorders, some drugs (psychostimulants) also contribute to reinforcement properties, and I will discuss them before continuing to the second part of the introduction of this thesis, which is related to schizophrenia.

12.1 *Parkinson's disease*

Parkinson's disease is one of the most common neurodegenerative disorders causing motor impairment. It arises from the death of cells in the basal ganglia. This affects most of the DA neurons, resulting in a depletion of DA levels in the striatum. The earliest changes can be observed in the medulla oblongata and olfactory bulb (Braak stages 1 and 2) where patients are found to be pre-symptomatic (Braak et al., 2006). In the later stages (Braak stages 3 and 4), the changes are spread into the substantia nigra areas of the midbrain and basal forebrain. During this phase, the illness probably becomes symptomatic and clinically manifest. It later goes to the final stages of 5

to 6, when the lesions appear in the neocortex. Classical symptoms and signs of parkinsonism are associated with movement disorders: these include hypokinesia (poverty of movement, e.g. loss of facial expression), bradykinesia (slowness of movement), rigidity and rest tremor. Other symptoms include depression and pain. The most common characteristic of Parkinson's disease is the accumulation of Lewy bodies in DA neurons in substantia nigra (Davie, 2008). Lewy bodies are abnormal aggregations of protein that develop inside the nerve cells; their distribution varies depending on the pathological stages mentioned above. Histologically, Lewy bodies appear as spherical masses that displace other cell components (fig. 1-26). They consist of α-synuclein with a primary structural component like a dense core made up of neurofilament proteins and proteins responsible for proteolysis (ubiquitin).
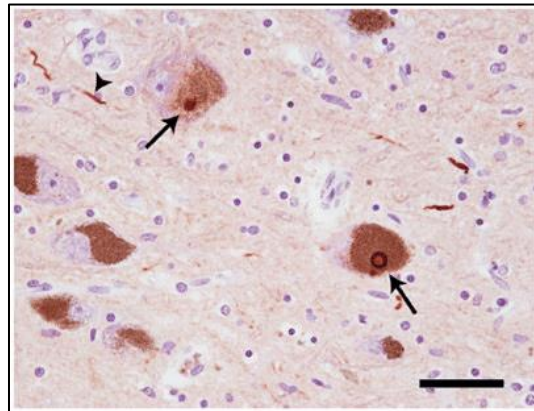
Fig. 1-26. Characteristics of Lewy bodies showed with the arrows from Parkinson´s disease patients (Ingelsson, 2016).

At the moment, there is no cure for Parkinson's disease, and the treatments are aimed to ameliorate the symptoms. Understanding that symptoms are related to the depletion of DA, it was assumed that treatments should increase DA levels. Several pharmacological compounds have been

approved for the treatment of Parkinson's disease and are based on its mechanism of action on DA. These include:

> ➢ **Increasing DA synthesis**

Treatment with L-DOPA, the intermediate compound for the synthesis of DA has been used for more than 50 years. In fact, L-DOPA has become the gold standard for the treatment of Parkinson's disease. However, long-term treatment with L-dopa is complicated with dyskinesia.

> ➢ **Mimic DA**

Other than increasing DA synthesis, the use of DA agonists to mimic DA bound at post-synaptic DA receptors has been accepted and shown to be the most effective for treatment during the early stages of Parkinson's disease.

> ➢ **Decrease DA metabolism**

Previously, I have discussed that the release of DA from the presynaptic terminals can be metabolized by certain enzymes, including MAO and COMT. With the decreased levels of DA in Parkinson's disease, the use of MAO inhibitor and COMT inhibitor could prevent further DA metabolism and increase DA availability.

12.2 Huntington's disease

Huntington's disease is an inherited disorder that causes motor impairment. The motor impairment is due to the initial loss of medium spiny neurons in the striatum. Movement disturbance in Huntington's disease can be characterized as a hyperkinetic phase, with chorea as the prominent symptom. This symptom can be found in the early stages. This is followed by a hypokinetic phase, characterized by bradykinesia, dystonia (uncontrolled muscle contractions) and balance

disturbances, which is found in the later stages. In addition to the motor impairments, patients suffering from Huntington's disease also experience a wide variety of neuropsychiatric symptoms including apathy, anxiety, depression, obsessive-compulsive behavior and psychosis. Huntington's disease is caused by a mutation in the gene for a protein called huntingtin. The defect causes the abnormal repetition of cytosine, adenine, and guanine triplet, which are the building blocks of deoxyribonucleic acid (McColgan & Tabrizi, 2018). This results in the production of a mutant Huntington protein with an abnormally long polyglutamine repeat.

At the moment, there is no curative treatment for Huntington's disease. Currently approved drugs include TBZ for the treatment of chorea associated with Huntington's disease (S. Frank, 2009; Huntington Study Group, 2006). Other than TBZ, deuterabenazine is also used for the treatment. Unlike TBZ, deuterabenazine contains deuterium atoms to prolong its half-life. Commonly reported side effects include drowsiness, hypertonia, muscle rigidity, depression, akathisia, and restlessness. Several antipsychotics have also been used for the treatment of chorea: these include SUL, olanzapine and risperidone (Coppen & Roos, 2017).

12.3 ADHD

ADHD is characterized by a series of symptoms, including inappropriate hyperactivity, inattention and impulsiveness (Thapar et al., 2012). These symptoms arise from a deficiency in cognitive functions, especially the executive system functions (e.g. attentional control, inhibitory control and working memory) (Brown, 2008), and are related to the dysfunction of the mesocortical pathway in the dopaminergic system. ADHD commonly causes developmental and learning problems, resulting in difficulties with speech and language, motor co-ordination and reading. Patients with

ADHD also suffer from a range of psychiatric disorders including anxiety, antisocial personality disorders and depression. Until now, similarly to other psychiatric disorders (e.g. schizophrenia), the exact cause that contributes to the development of the illness is still unknown, although it has been hypothesized that there is a complex association between genetic factors and environmental risks. Several studies have shown that ADHD is related to the impairment of DA, 5-HT and norepinephrine neurotransmission in the brain. This reduction could be due to a dysfunction of the transporter including DAT. This is supported by a DAT knockout mouse that exhibits symptoms of hyperactivity and deficits in inhibitory behavior (Gainetdinov, 2008). Also, among different DA receptor subtypes, it is suggested that the impairments reflect both the D_4 and D_5 receptor subtypes (Faraone et al., 2001; Gizer et al., 2009).

With ADHD association resulting in the impairment of DA, 5-HT and norepinephrine neurotransmission in the brain, the treatment with psychostimulants is suggested to possess treatment efficacy because of its ability to increase neurotransmitter levels and receptor stimulation. The two most commonly prescribed drugs for ADHD are AMPH and MPH. Patients with long-term treatment with AMPH present fewer abnormalities in functional brain networks and improve the brain's functions (Hart et al., 2013). However, chronic administration of these drugs leads to the up-regulation of DAT expressions, due to the differences in initial responsiveness, which may possibly change risk for subsequent substance abuse (Zahniser & Sorkin, 2004).

12.4 Reinforcement disorders

I have discussed the mechanisms of action of some psychostimulants (e.g. MPH) that block the action of DAT at the presynaptic terminals. This action is based on the synaptic properties on an action potential-dependent DA release. Other psychostimulants including AMPH induce DA release from presynaptic terminals, and this action is independent of the properties of the neurons under action potential modulation. It is also thought that AMPH-induced DA release through DAT-mediated reverse transport in addition to its interference with DA reuptake activity (Sulzer et al., 2005). However, both mechanisms also lead to an increase in DA availability. This is supported by studies that have found that psychostimulants increase extracellular DA concentrations in *in-vivo* (Carboni et al., 1989; Di Chiara & Imperato, 1988). As chronic administrations of AMPH leads to up-regulation of DAT expressions, at high dose AMPH will also increase various transcription factors expressions, i.e. ΔFosB and CREB, a protein that contributes to addictive behavior (Nestler, 2013) (fig. 1-27). This describes general mechanisms in which psychostimulants regulate reinforcement properties. In addition to its action on DAT, psychostimulants such as cocaine also regulate DA release via other neuromodulators, including inhibition of 5-HT transporter (Mateo et al., 2004). I will discuss this regulation later on in the section on cross-talk between DA and 5-HT.
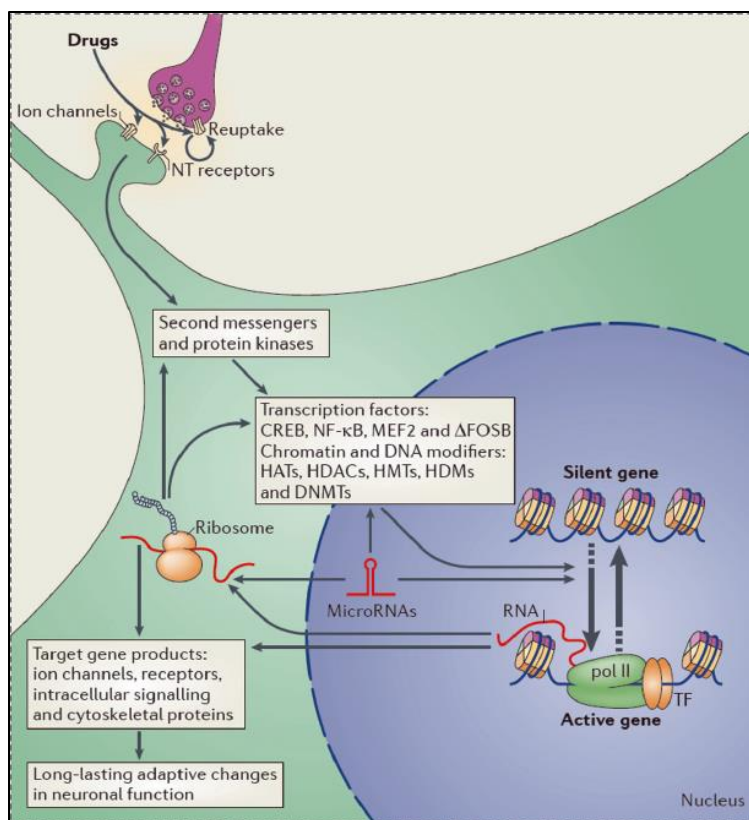
Fig. 1-27. Psychostimulants alter intracellular signaling cascade which leads to the activation or inhibition of various transcription factors (Nestler, 2013).

13.   *Pathophysiology of schizophrenia*

With the end of the basic discussions on dopaminergic and serotonergic systems in the brain and general knowledge of some of the DA-related neurological disorders, I would like to proceed with the second part of the introduction of this thesis. In this section, I would like to explore and understand the pathological aspects of schizophrenia, and later describe the action of approved antipsychotics and their mechanisms of action in the brain. Schizophrenia is characterized by relapse episodes of psychosis. Psychosis defines as an abnormal condition of the mind that results in the difficulties to determine between reality and hallucinations. Since no biological marker for schizophrenia has been found so far, the diagnosis of the illness is based on the assessment of the

symptoms, as was proposed by Eugene Bleuler; the assessment was printed in the first edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-I). In addition to the diagnosis, this assessment also makes suggestions for treatment, and has been continuously updated on the basis of new findings, with the latest edition, the fifth (DSM-V), published in 2013. With this criterion, the schizophrenia symptoms were first divided into two main categories: positive and negative symptoms. It is characterized by having two or more of these symptoms (Substance abuse and mental health service administration, 2016). The positive symptoms are episodic and associated with acute psychosis. These include hallucinations, delusions, disorganized speech and behavior. The negative symptoms generally represent a loss of function and include social withdrawal, slowness of thinking and movement, and lack of drive. The latest DSM-V excluded emotional expression for the assessment of the negative symptoms. In addition to the positive and negative symptoms, the third category, which includes the cognitive deficits, is also recognized among the symptoms. Cognitive deficits are the earliest and most constant symptoms found in schizophrenia. The cognitive deficits associated with schizophrenia include lack of abstraction, verbal memory, attention, working memory and executive functions. Other criteria including social dysfunction were also included in the assessment for schizophrenia symptoms.

13.1 Etiology of schizophrenia

The word "hyperdopaminergia" or hyperactivity of DA neurotransmission is synonymous with the development of psychosis. DA dysfunctions that contribute to the development of schizophrenia were traced in the 1950s with the discovery that chlorpromazine had antipsychotic properties, after a search for sedative drugs (Ban, 2007). In 1952, chlorpromazine was approved as an antipsychotic. In 1954, reserpine, which is isolated from Rauwolfia roots, was also used for the

treatment of "insanity" (psychosis). The use of reserpine, a TH inhibitor that leads to the inhibition of DA synthesis, showed benefits for treating psychosis (A Carlsson et al., 1972, 1973). Besides, psychostimulant drugs such as AMPH or MPH, which increase monoamine neurotransmitter levels, as discussed before, were found to induce psychotic symptoms or increase psychotic symptoms suffered by schizophrenics (J. A. Lieberman et al., 1987). The effect of AMPH-induced DA release in schizophrenics is generally twice that of the control subjects (Laruelle et al., 1999). Thus, over-stimulation of DA receptors has been accepted to contribute to the etiology of psychotic symptoms.

With the advancement of medical technology, more findings have been discovered to support this theory. Over-stimulation of DA receptors may come from the dysfunction of presynaptic (synthesis and release) or/ and postsynaptic (higher susceptibility or sensitivity of DA receptors) dopaminergic terminals (Seeman, 2013). Higher presynaptic DA synthesis, availability and release found in the striatum of schizophrenics confirm that the major abnormalities in DA transmissionism are from the presynaptic terminals (Howes et al., 2012) (fig. 1-28). Another study also supports this theory by reporting that a 14% increase in the DA synthesis capacity was found in the striatum of schizophrenics (Fusar-Poli & Meyer-Lindenberg, 2013). Although most of the abnormalities in schizophrenics are from the presynaptic terminals, a slight increase of $5.8 \pm 2.7\%$ (means $\pm$ S.E.) in DA receptors density was also found at the postsynaptic terminals, which contributes for the higher stimulations (Seeman, 2013).
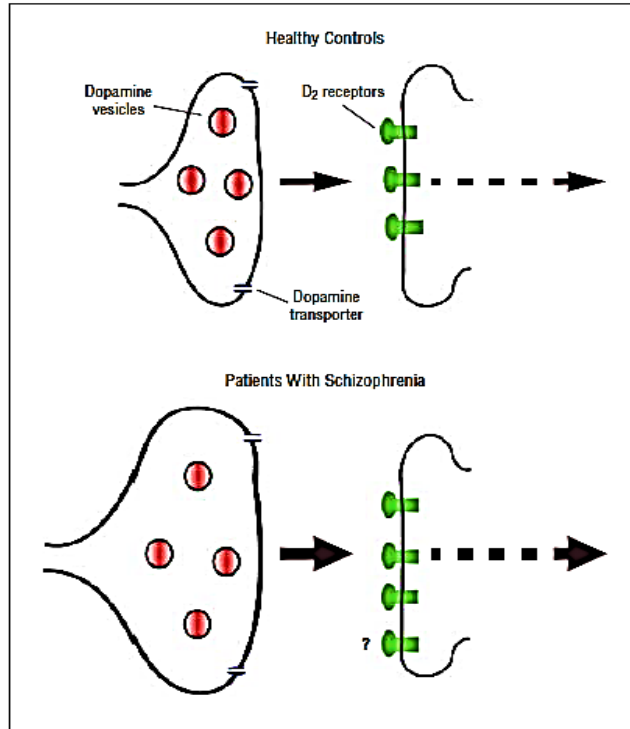
Fig. 1-28. Higher presynaptic DA synthesis, availability and release found in schizophrenics (Howes et al., 2012).

The GPCRs (in this case the D_2 receptor) can exist in high (D_2H)- or low (D_2L)- affinity states for DA; such conformation changes can rapidly convert into each other in a matter of seconds or less. D_2H has been hypothesized to be the functional state related to the action on DA (Seeman, 2013). In schizophrenics, there is an increase in the apparent D_2H state as compared with normal persons (fig. 1-29). However, at the D_2H state, the effect of DA on the receptor is not affected even with the presence of an antagonist (Boundy et al., 1995). This is interesting because both typical and typical antipsychotics are antagonists at the D_2 receptor and may be affecting the therapeutic efficacy of antipsychotics.
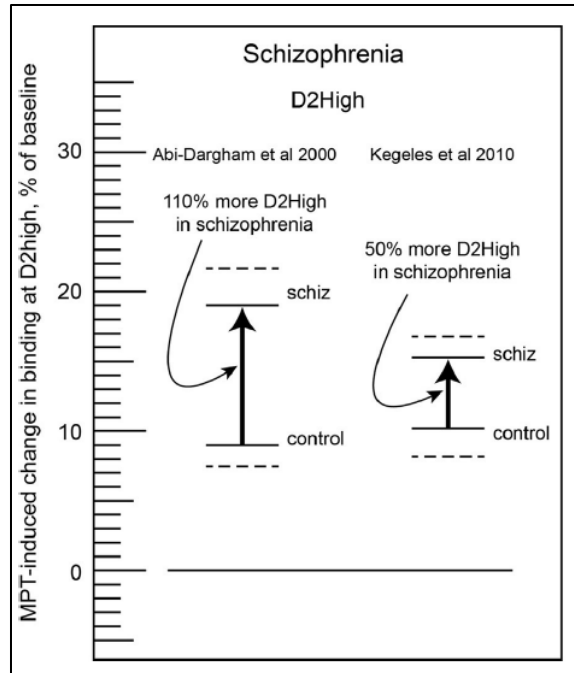
Fig. 1-29. Elevation of apparent D_2H receptor states in schizophrenics (Seeman, 2013).

These high levels of DA in the striatum, however, do not emerge to the same levels as in other brain regions, as there is an inverse relationship of DA release between the striatum and the prefrontal cortex. Higher striatal DA release and deficit capacity of prefrontal cortex DA release have been found in schizophrenics (Slifstein et al., 2015). There is a direct regulation from the prefrontal cortex, as the lesion of DA neurons in the prefrontal cortex results in an increase of DA levels and its metabolite in the striatum (Pycock et al., 1980). These lead to the concept of schizophrenia, which is characterized by hyperdopaminergia in mesolimbic and hypodopaminergia in mesocortical pathways. Furthermore, it is also suggested that the schizophrenia positive symptoms arise as a result of striatal hyperdopaminergia, based on the finding that higher dopamine metabolite levels are related to greater positive symptoms and response to antipsychotic treatment. In turn, it has been hypothesized that both the negative symptoms and the cognitive deficits of schizophrenia are a result of frontal cortex

hypodopaminergia. This was based on the similarities between behavior exhibited by animals and humans with frontal lobe lesions and the development of the negative symptoms.

13.2 Cross-talk between DA and other neurotransmitters contribute to schizophrenia

The fact that over-stimulation of DA receptors aggravates the positive but not the negative symptoms of schizophrenia indicates that the DA receptors are not the only receptors accountable for schizophrenia. In this section, I would like to discuss the interaction between DA and other neurotransmitters which may contribute to the development of the negative symptoms and cognitive deficits of schizophrenia.

13.2.1 DA and glutamate

L-glutamate is an amino acid used for the synthesis of proteins. L-glutamate can also act as one of the excitatory neurotransmitters or as a precursor for the synthesis of the inhibitory neurotransmitter, GABA. L-glutamate is involved in cognitive functions such as learning and memory (McEntee & Crook, 1993). In the central nervous system, L-glutamate can bind and activate four different types of receptors; α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartic acid (NMDA), kainate and metabotropic glutamate receptors. The first three are categorized as the ionotropic receptors that upon activation allow the passage of Na^+, Ca^{2+} and K^+ through the cell membrane, while the metabotropic receptors are coupled to the G-proteins and activate intracellular second messenger systems. Among these four receptors, NMDA is hypothesized to be the receptor that contributes to the full range of psychosis effects, including

the negative and cognitive deficits in schizophrenia (Coyle et al., 2012). NMDA receptor is located throughout the brain, with the highest densities in the frontal cortex, hippocampus and nucleus accumbens (Monaghan & Cotman, 1985). The consumption of phencyclidine, with its primary pharmacological properties, as an antagonist to the NMDA receptor will produce hypoglutamatergic mechanisms (Allen & Young, 1978). This occurs through GABAergic inhibitory interneurons to inhibit glutamatergic projection neurons and could induce a psychotic state similar to schizophrenia (Homayoun & Moghaddam., 2007). These findings led to the use of NMDA receptor antagonists as a model for schizophrenia in *in-vivo* studies.

The interaction between dopaminergic and glutamatergic neurons has been discussed in the literature. Based on the hypodopaminergic prefrontal cortex and hyperdopaminergic striatum, it is predicted that there is a direct regulation from the prefrontal cortex and the striatum. It is hypothesized that this regulation may involve glutamatergic neurons. The prefrontal cortex regulates the activity of midbrain DA neurons via both activating and inhibitory pathways (A Carlsson et al., 1999). The activating pathway consists of a direct projection to midbrain dopaminergic neurons, while the inhibitory pathway involves GABAergic neurons. These dual regulations have been demonstrated in rodents, in which higher prefrontal cortex glutamatergic stimulation increases but lower stimulation decreases DA release in nucleus accumbens (Jackson et al., 2001). In addition, blockade of glutamatergic activity in the ventral tegmental area produces an increase of DA release in nucleus accumbens, but a decrease in the prefrontal cortex (Takahata & Moghaddam, 2000). This indicates that a loss of glutamatergic receptor functions would result in indirect regulations of DA activity in neurons projecting to the prefrontal cortex as well as to the mesolimbic pathway.

13.2.2 DA and 5-HT

In addition to the glutamatergic neurons, serotonergic systems have been shown to have an interaction with dopaminergic systems. Several studies have shown that 5-HT receptors may also regulate DA transmission in the brain. Generally, an excess of 5-HT may potentiate the effect of D_2 agonist on the inhibition mediated by DA (Brodie & Bunney, 1996). Another study on the blockade of 5-HT transporter increases extracellular DA concentration and as showed with the effects of cocaine (Mateo et al., 2004). Specific 5-HT receptor subtypes involved in this regulation have also been studied. This includes the 5-HT_2 receptor subtypes family, where the activation of the 5-HT_{2A} receptor increases DA release (Gobert & Millan, 1999), while the 5-HT_{2A} antagonist will attenuate DA release (Pehek et al., 2001). This is different from the 5-HT_{2C} receptor, where the agonist suppresses, but antagonists increase DA release (Porras et al., 2002). Other than the 5-HT_2 receptor subtypes family, the 5-HT_{1A} receptor has also been shown to regulate DA release. The 5-HT_{2A} antagonist alone appears to suppress DA release, but the combination with the 5-HT_{1A} receptor subtype agonist increases DA release (Ichikawa et al., 2001). Furthermore, the 5-HT_4 receptor subtype also appears to be involved in this regulation where its activation has been shown to increase DA firing activity (Bonhomme et al., 1995). These findings have shown that serotonergic systems may also modulate DA in the brain, but the exact mechanisms on how this regulation mediates DA neurotransmission are still unknown. However, given that the phencyclidine-model of schizophrenia in rats showed an increase of 5-HT and its metabolite 5-HIAA levels (Martin et al., 1998), it is assumed that this regulation may involve NMDA receptors.

In addition to the indirect regulation of 5-HT on DA, the dysfunction of the 5-HT systems also plays an important role in the pathophysiology of schizophrenia, and has been implicated with

the negative symptoms. Between different types of 5-HT receptor subtypes, it has been suggested that 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 are the strongest receptor candidates for producing impairments that contribute to the development of the negative symptoms of schizophrenia (Richtand et al., 2007; Schotte et al., 1996). Due to an important aspect of 5-HT receptors in the pathophysiology of schizophrenia, I will discuss in the next section how antipsychotics incorporate their affinity on 5-HT receptors other than acting on the D_2 receptor alone in parts of their properties.

13.3 Antipsychotics: their classification and mechanisms of action

There are a wide variety of antipsychotics that have been approved for the treatment of schizophrenia over the years, starting from chlorpromazine, which has been approved by U.S. F.D.A. in 1973, to the latest lumateperone (LUMA), approved in 2019. All currently approved antipsychotics target the D_2 receptor. They have been grouped according to their mechanisms of action (Mailman & Murthy, 2010). The original antipsychotic drugs, including chlorpromazine, fluphenazine, haloperidol, etc., have been called as typical or the first generation. The clinical effectiveness of these antipsychotics is highly correlated to their affinity for the D_2 receptor (Creese et al., 1976). Despite their effectiveness, treatment with this type of antipsychotics may cause many side effects, including sedation, autonomic and cardiovascular effect, weight gain, extrapyramidal side effects (parkinsonism, dystonia, akathisia), tardive dyskinesia and neuroendocrine effects (Mailman & Murthy, 2010).

Further development of antipsychotics has focused on improving treatment and on reducing side effects. These drugs should have at least equal antipsychotic efficacy as the typical antipsychotics, and without producing the extrapyramidal and neurological side effects or

sustaining prolactin elevation. In the 1970s, clozapine was recognized as showing superior effects than typical antipsychotics in the treatment of schizophrenia. Clozapine induces less extrapyramidal side effects and tardive dyskinesia, and causes far less of an increase in prolactin relative to typical antipsychotics (J. Lieberman et al., 1989). Clozapine possesses many characteristics that distinguish it from the typical antipsychotics. The drug has a modest affinity for not only the D_2 receptor, but also for the 5-HT and many other neurotransmitter receptors including histaminergic, muscarinic, and α-adrenergic receptors (Bymaster et al., 1996). Due to the proven benefits of clozapine, the mechanism of action of antipsychotics had incorporated the affinity on many other neurotransmitter receptors in their properties. Subsequently, all approved antipsychotics have shared common properties with high affinity to the 5-HT and other neurotransmitter receptors, rather than acting on the D_2 receptor alone, which differentiates them from typical antipsychotics. These include risperidone, quetiapine, olanzapine, ziprasidone, paliperidone, asenapine, iloperidone and lurasidone, which were grouped as atypical or second generation of antipsychotics. It was then hypothesized that the benefits of these atypical antipsychotics to treat the negative symptoms and cognitive deficits enhance with a higher affinity towards $5\text{-}HT_{2A}$ than D_2 receptors (Schotte et al., 1996). Such characteristics also share a greater ability to treat cognitive functions associated with lower incidence of producing extrapyramidal side effects as compared with typical antipsychotics (Meltzer & Mcgurk, 1999). The similarities between typical and atypical antipsychotics were their antagonism properties on the D_2 receptor. These antipsychotics were aimed to block the activation of the D_2 receptor, and primarily targeted the postsynaptic dopaminergic receptors.

The newest generation of antipsychotics has shifted its mechanisms of action, targeting the presynaptic dopaminergic terminals associated with the finding of the dysfunctional presynaptic

dopaminergic terminals that contribute to the development of the positive symptoms of schizophrenia (as discussed in the etiology of schizophrenia section). In 2012, ARI was approved as the first antipsychotic of this class which acts as a partial agonist on the D_2 receptor. Since then, the next approved D2R partial agonist antipsychotics were CARI and BREX, both of which were approved in 2015, and the latest LUMA, approved in 2019. The similarities and the differences between D2R partial agonist antipsychotics will be discussed in the next section. The overall list and classification of antipsychotics approved by the U.S. F.D.A. are shown in fig. 1-30.
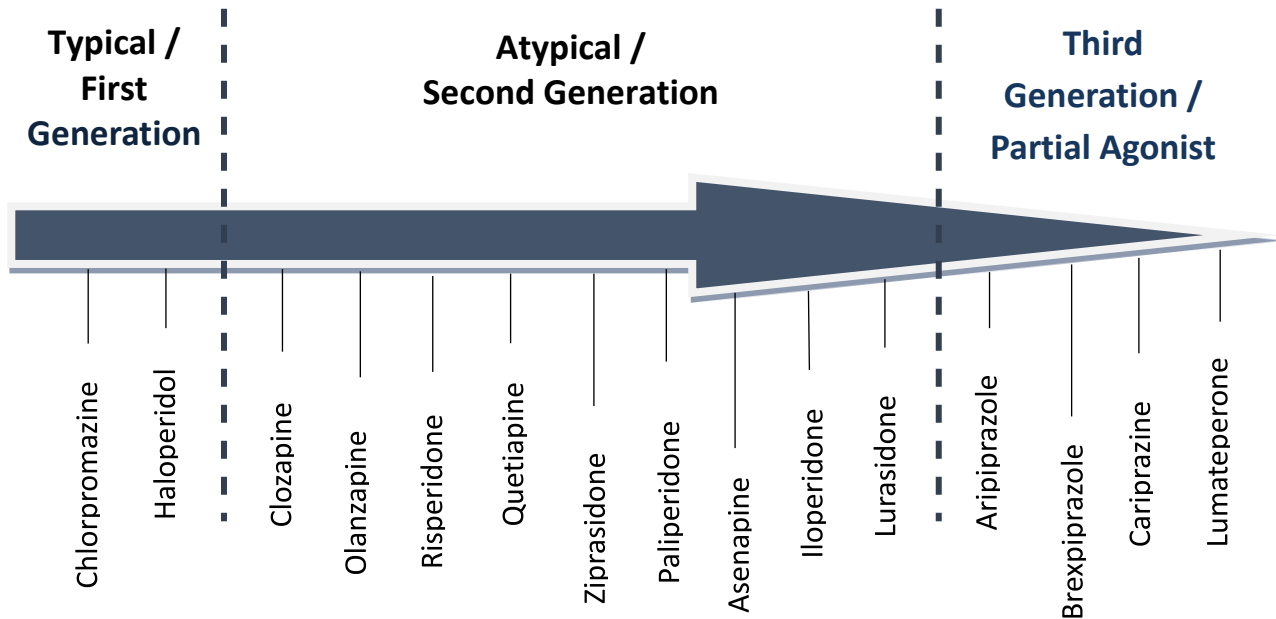
Fig. 1-30. Some of the list of oral antipsychotics approved by the U.S. F.D.A. for the treatment of schizophrenia. The mechanisms of action of antipsychotics have shifted from acting on D_2 antagonist alone (typical) to incorporating affinity on 5-HT receptors and other neurotransmitter receptors in addition to acting on D_2 antagonist (atypical). The latest development of antipsychotics showed a D_2 partial agonist property with a higher affinity on 5-HT receptors and other neurotransmitter receptors. The diagram is developed from Mailman & Murthy, 2010 and the U.S. F.D.A. website.

13.4 D₂R partial agonist antipsychotics

At the moment, there are four D_2R partial agonist antipsychotics. ARI was the first antipsychotic approved by the U.S. F.D.A., followed by BREX, CARI and the latest LUMA. The chemical structures for all four D_2R partial agonist antipsychotics are shown in fig. 1-31.
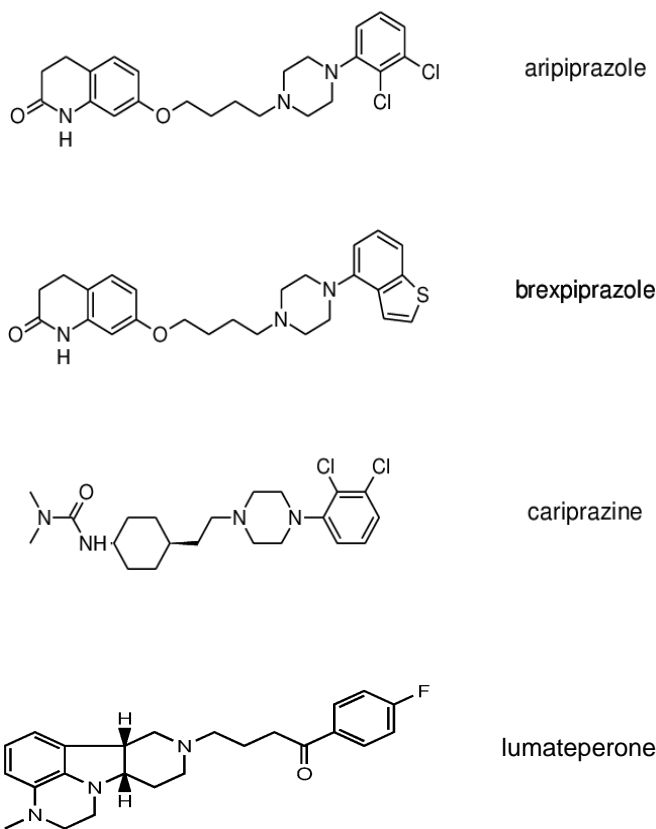
Fig. 1-31. Chemical structure of D_2R partial agonist antipsychotics.

To understand the effect and properties of D_2R partial agonist antipsychotics, most of the research published in the literature was done with ARI as a reference for comparison with other typical and atypical antipsychotics. This may be due to the fact that ARI was the only available candidate for this class for more than a decade. One of the unique properties of ARI in contrast to other typical

and atypical antipsychotics is its ability to stimulate or block presynaptic D_2R depending on endogenous DA levels (Ma et al., 2015). At high DA concentrations, the property of ARI changes from a partial agonist to an antagonist at D_2R. These properties are consistent with the lower intrinsic activity of ARI as a partial agonist as compared to DA at D_2R. This mechanism of action underlines the key role of its unique "DA stabilizer" property on dopaminergic neurotransmission. The goal of the intermediate level of D_2R stimulation is to remain beneath the threshold for the development of positive symptoms induced by excessive DA concentrations, but at the same time above the minimum levels to avoid causing adverse effects. ARI has also proved to have the capacity to display functional selectivity properties that may cause selective changes to different signaling pathways (Tuplin & Holahan, 2017). Therefore, with the activation of D_2R, ARI does not only activate the G-protein-dependent PKA signaling pathway, but can also simultaneously activate glycogen synthase kinase 3β-dependent signaling pathway (Pan et al., 2016).

Excluding LUMA, which has currently just received approval as an antipsychotic, our knowledge of the relationship between the other three D_2R partial agonist antipsychotics is still lacking. Are the properties which have been shown in ARI (describe above) can also be demonstrated on CARI and BREX are still unknown. To further differentiate between D_2R partial agonist antipsychotics, I would like to take this opportunity to highlight the similarities and differences in terms of their properties and efficacies between all D_2R partial agonist antipsychotics. The differences between D_2R partial agonist antipsychotics can be related to their binding affinities and efficacies towards D_2, D_3 and other receptors, including 5-HT, adrenergic, histamine, etc. as follows:

D₂-like receptors:

> **D₂S receptor:**

ARI, CARI, BREX and LUMA possess partial agonists activity at D_2R, with BREX having higher affinities for the receptor (0.30nM), followed by ARI (0.34nM) and CARI (0.69nM) and LUMA (32nM) at much less affinity (Kiss et al., 2010; Kumar & Kuhad, 2018; K. Maeda et al., 2014; Shapiro et al., 2003).

> **D₂L receptor:**

LUMA, on the other hand, is the only antipsychotic of this class reported to act differently on both presynaptic D_2R and postsynaptic D_2 receptors. While LUMA acts as a partial agonist at the presynaptic D_2R, it can also behave differently (as an antagonist) at the postsynaptic D_2 receptor (Kumar & Kuhad, 2018).

> **D₃ receptor:**

In addition to the D_2 receptor, ARI, CARI and BREX act as partial agonists at D_3 receptor where CARI (0.085nM) has 10-fold higher affinity than ARI (0.8nM) (Kiss et al., 2010; Shapiro et al., 2003), followed by BREX (1.1nM) at much less affinity (K. Maeda et al., 2014). CARI has also been shown to have rapid binding kinetics (Frank et al., 2018) towards the D_3 receptor. At low concentrations, CARI occupies most of the D_3 receptor in the striatum and cerebellum, as compared with ARI (Gyertyán et al., 2011). D_3 receptor has been hypothesized to be involved in social interactions and cognitive functions (Watson et al., 2012), and CARI appears to show greater improvement in negative symptoms compared with ARI (Earley et al., 2019), and risperidone (Németh et al., 2017).

5-HT receptors:

Due to the important role of 5-HT receptors in schizophrenia, as discussed in the section on cross-talk between DA and 5-HT, all D_2R partial agonist antipsychotics have a higher affinity to these receptors.

> **5-HT$_{1A}$ receptor:**

ARI, CARI and BREX possess a partial agonist activity at 5-HT$_{1A}$ receptor, with BREX having a higher affinity for this receptor (0.12nM) followed by ARI (1.7nM) and CARI (2.6nM) (Kiss et al., 2010; Kenji Maeda et al., 2014; Shapiro et al., 2003).

> **5-HT$_{2A}$ receptor:**

ARI, CARI, BREX and LUMA are also antagonists at the 5-HT$_{2A}$ receptor, with BREX having the highest affinity for this receptor (0.47nM) followed by LUMA (0.54nM), ARI (3.4nM) and CARI (18.8nM) (Kiss et al., 2010; Kumar & Kuhad, 2018; Kenji Maeda et al., 2014; Shapiro et al., 2003).

> **5-HT$_{2C}$ receptor:**

ARI, CARI and BREX are also antagonists at the 5-HT$_{2C}$ receptor, with BREX possessing the highest affinity for this receptor (12nM) followed with ARI (15nM) and CARI (134nM) (Kiss et al., 2010; Kenji Maeda et al., 2014; Shapiro et al., 2003).

> **5-HT$_7$ receptor:**

Other than 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2c}$, ARI, CARI and BREX are also antagonists at the 5-HT$_7$ receptor, with BREX having the highest affinity for this receptor (3.7nM) followed by ARI (9.6nM) and CARI (111nM) (Kiss et al., 2010; Kenji Maeda et al., 2014; Shapiro et al., 2003).

> **Histamine H$_1$ receptor:**

Other than all the receptors mention above, ARI, CARI and BREX also act as antagonists at the histamine H$_1$ receptor, with BREX having the highest affinity for this receptor (19nM) followed by ARI (27.9nM) and CARI (23.2nM) (Kiss et al., 2010; Kenji Maeda et al., 2014; Shapiro et al., 2003).

> **Adrenergic α$_{1A}$ receptor:**

Furthermore, ARI, CARI and BREX also act as antagonists at the α$_{1A}$-adrenergic receptor, with BREX having the highest affinity for this receptor (3.8nM) followed with ARI (25.9nM) and CARI (155nM) (Kiss et al., 2010; Kenji Maeda et al., 2014; Shapiro et al., 2003).

This kind of properties are probably important as it has been associated with low incidences of neurological side effects, weight gain and prolactin elevation in patients receiving treatment with D$_2$R partial agonist antipsychotics compared with other approved antipsychotics (Taylor et al., 2018). In addition, as noted, BREX has the highest affinity for all the 5-HT receptor subtypes mentioned above, together with the histamine H$_1$ and the α$_1$-adrenergic receptors, as compared with ARI and CARI. Also, BREX shows a modest reduction in impulsivity and significantly low incidence to develop akathisia compared with ARI (Citrome et al., 2016).

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II. AIM AND OBJECTIVES

Aim

This thesis aims to expand our knowledge on the homeostatic regulation of presynaptic terminals controlling DA synthesis and storage in the striatum. This regulation is of interest since impairments at the presynaptic terminals may have neurological consequences in diseases such as schizophrenia, Parkinson's, ADHD, etc. In addition, the definition of mechanisms of action of pharmacological treatments for these diseases are based on their synaptic actions. Thus, I have given special relevance to clinically useful treatments and their possible combinations in search for future treatments.

Objectives

The **first objective** of this thesis is to increase our understanding of the overall homeostatic mechanisms toward regulation of DA accumulation at the presynaptic terminals in the striatum. Synaptic mechanisms related to the DA synthesis, vesicular uptake, storage, release, reuptake, metabolism and feedback regulations by D_2R need to be studied to understand how several drugs such as QUIN, ARI, TBZ, OKA, PAOPA, AMPH and MPH act. Part of this data continues research done by a previous Ph.D. student (Marta González-Sepúlveda) for the manuscript in the Appendix. In addition, the combination of different clinically useful drugs (e.g. TBZ with ARI and PAOPA -experimental- with QUIN) is of relevance in search of a synergistic effect that could lead to better treatment approaches.

The **second objective** of this thesis is to examine the mechanism of actions of D_2-like partial agonist antipsychotics at presynaptic D_2R. We assumed all three D_2-like partial agonist antipsychotics should have similar effects but with different efficacies on brain presynaptic D_2R. Thus, these present experiments will:

- ➢ compare the similarities and differences in the functional properties and efficacy of D_2-like partial agonist antipsychotics in modulating DA feedback-regulations at presynaptic D_2R;
- ➢ assess if both agonist and antagonist properties under different dopaminergic tone previously documented with ARI (Ma et al., 2015), can also be observed with CARI and BREX at presynaptic D_2R; and
- ➢ contribute to understanding the discrepancies in their functional properties and efficacy between all three D_2-like partial agonist antipsychotics at presynaptic D_2R.

The **third objective** of this thesis is to explore the implication of non-D_2 receptor components in the properties of D_2-like partial agonist antipsychotics on DA dynamics. In my introduction, I have discussed that all three D_2-like partial agonist antipsychotics share common properties with distinct affinities at different receptors other than on the D_2 receptor. Thus, some of these properties are worth studying to assess if there is an interaction from the corresponding receptor of interest that may indirectly modulate DA accumulation and enhance the efficacy of D_2-like partial agonist antipsychotics at presynaptic D_2R. These include:

➢ the higher affinity of CARI than ARI and BREX on D_3 receptor; and

➢ the high affinity of D_2-like partial agonist antipsychotics towards certain 5-HT receptor subtypes. For this, the use of more specific agonists and antagonists of these receptor subtypes could provide an indicator of whether there is any effect that could be suggestive of a cross-talk between D_2 and selected 5-HT receptor subtypes.

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III. METHODOLOGY

1.   Materials

1.1   Chemicals

Optiphase 'Hisafe'-III liquid scintillation cocktail and [3,5-^{3}H]-l-tyrosine ([^{3}H]-Tyr), 40–60 Ci/mmol, were purchased from PerkinElmer, Boston, MA, USA. Natrium hydrogen carbonate, potassium dihydrogen phosphate and magnesium sulfate heptahydrate were obtained from Merck Biosciences, Darmstadt, Germany. Sodium disulphite and d-(+)-glucose anhydrous were purchased from Panreac Quimica S.A.U., Spain. Perchloric acid 70% and triethylamine were obtained from Fluka Biochemika, Switzerland. Sodium chloride and sodium hydroxide were purchased from Scharlau S.L., Spain. Octane-1-sulphonic acid sodium salt, HPLC grade was obtained from Romil Ltd, Cambridge, UK. Methanol and acetonitrile, both with ultra-gradient HPLC grade were from J.T. Baker, Netherlands. Other chemicals include l-ascorbic acid, calcium chloride dihydrate 99%, potassium chloride, sodium phosphate monobasic monohydrate 98%, ethylenediaminetetraacetic acid disodium salt dihydrate 99%, DA hydrochloride, dimethyl sulfoxide 99.5% (DMSO), citric acid and trichloroacetic acid 99% were purchased from Sigma-Aldrich, Steinheim, Germany.

1.2   Drugs

ARI was kindly provided by Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan. CARI hydrochloride and BREX were purchased from MedChem Express. (-)-quinpirole hydrochloride (QUIN), (s)-(-)-sulpiride (SUL), SB 277011-A dihydrochloride (SB 277011-A), okadaic acid (OKA), PAOPA, MDL 100907, 8-OH DPAT and SB 258719 were obtained from Tocris Bioscience, UK. D-amphetamine sulfate (AMPH), methylphenidate hydrochloride (MPH) and 3-hydroxybenzylhydrazine dihydrochloride (NSD-1015) were from Sigma-Aldrich, Steinheim, Germany. ARI, CARI, BREX and SUL were dissolved in DMSO, while QUIN, NSD-1015, AMPH, MPH, PAOPA, SB 277011-A, MDL 100907, 8-OH DPAT and SB 258719 were directly dissolved in milli-q water before all were further diluted with modified Krebs-Ringer-bicarbonate medium (Krebs-Ringer buffer) as described below:

1.3   Preparation of Krebs-Ringer buffer

Krebs-Ringer buffer was prepared with the following composition: 120mM sodium chloride, 0.8mM potassium chloride, 2.6mM calcium chloride dihydrate, 0.67mM magnesium sulfate heptahydrate, 1.2mM potassium dihydrogen phosphate, 27.5mM sodium hydrogen carbonate, and 10mM d-(+)-glucose anhydrous dissolved in milli-q water. Half of the chemicals (sodium chloride, potassium chloride, potassium dihydrogen phosphate and magnesium sulfate heptahydrate) were dissolved in milli-q water and prepared earlier, and the rest of the chemicals, consisting of calcium chloride dihydrate, sodium hydrogen carbonate and d-(+)-glucose anhydrous, were added into the

solution right before the experiments were done. The buffer was saturated with 95% O_2/5% CO_2 (carbogen) and the pH was adjusted into pH 7.4 with diluted NaOH solution.

1.4 Mobile Phase used in HPLC system

1.4.1 Preparation the mobile phase for [^{3}H]-Tyr purifications

The mobile phase used for the purification of [^{3}H}-Tyr with HPLC-UV is an ion-pair mobile phase that consists of the following compositions: 100mM sodium phosphate monobasic monohydrate and 0.19mM octane-1-sulphonic acid sodium salt dissolved in milli-q water. The pH was adjusted into pH 3.4 with a diluted phosphoric acid solution. The mobile phase solution was then filtered and degassed before 1% (vol/vol) methanol was added into the solution prior to the analysis.

1.4.2 Preparation the mobile phase for quantification of [^{3}H]-DA synthesis, [^{3}H]-DA release and [^{3}H}-DA storage

The mobile phase used for the determination of [^{3}H]-DA synthesis with *HPLC-UV* is an ion-pair mobile phase that consists of the following compositions: 100mM sodium phosphate monobasic monohydrate and 0.75mM octane-1-sulphonic acid sodium salt dissolved in milli-q water. The pH was adjusted into pH 4.5 with a diluted hydrochloric acid solution. The mobile phase solution was then filtered and degassed before 15% (vol/vol) methanol was added into the solution prior to the analysis.

1.4.3 *Preparation the mobile phase for quantification of endogenous DA, DOPAC, 5-HT and 5-HIAA concentrations*

The mobile phase used for the determination of endogenous DA, DOPAC, 5-HT and 5-HIAA with HPLC-EC is an ion-pair mobile phase that consists of the following compositions: 0.055M citric acid, 0.063mM ethylenediaminetetraacetic acid disodium salt dihydrate and 1.217mM octane-1-sulphonic acid sodium salt dissolved in milli-q water. The pH was adjusted into pH 7.2 with a diluted triethylamine solution. The mobile phase solution was then filtered and degassed before 0.5% (vol/vol) acetonitrile was added into the solution prior to the analysis.

2. Methods

Animals were handled in accordance with the Ethics Committee for Human and Animal Research (Universitat Autònoma de Barcelona), compliance with the guidelines established by the Ethical Committee for the use of Laboratory Animals in Spain (53/2013) and European Community's Council Directive 1986(86/609/EEC). The experimental protocols were performed as previously described (González-Sepúlveda et al., 2013; Ma et al., 2015) as below:

2.1 Striatal tissue preparations

A total of 69 male (aged (week) 8.5 ± 0.9 (mean $\pm$ SD), with approximate weight (g) 329.2 ± 54.1, (mean $\pm$ SD)) and 9 female (aged (week) 9.1 ± 1.8 (mean $\pm$ SD), with approximate weight (g) 231.6 ± 29.7, (mean $\pm$ SD)) of Sprague-Dawley rats (Charles River laboratories, France) were used in this study. The rats were euthanized via carbon dioxide asphyxiation in the animal service, Universitat Autònoma de Barcelona, between 0800-1000hrs. The brain was immediately removed and chilled in ice-cold Krebs-Ringer buffer. Striatal tissue (from both hemispheres) was dissected and minced using McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Surrey, UK), with an approximate cube shape of 0.3x0.3mm/side. This procedure was done in a 4^oC room to facilitate cooling of the tissue. Tissue minces were then suspended in ice-cold Krebs-Ringer buffer followed with centrifugation (1000rpm, 1-min, 3x) to remove the cell debris and molecules released during tissue processing, which includes intracellular proteases and DA (Anna Galán, unpublished observation). The supernatant was discarded for each centrifugation and the final volume of Krebs-Ringer buffer added was according to the total number of samples for each

experiment. Striatal tissue from a single rat yielded up to 28 aliquots of 25µl (0.3-0.7mg protein each) corresponding to 24 incubation samples and 4 blank samples inside a 2ml polypropylene tube containing 225µl of Krebs-Ringer buffer. Overall steps for striatal tissue preparations are illustrated in fig. 3-1.

Sprague-Dawley rats were euthanized with CO_2 asphyxiation.

Brain was immediately extracted and suspended in ice-cold Krebs-Ringer buffer.

Striatal tissue as shown with an arrow (from both brain hemisphere) was dissected.

Striatal tissue was then minced using McIlwain tissue chopper, with an approximate cube shape of 0.3x0.3mm/side.

Tissue minces were then suspended in ice-cold Krebs-Ringer buffer followed with a centrifugation (1000rpm, 1-min, 3x) to remove the cell debris.

Fig. 3-1. Overall steps for striatal tissue preparations.

2.2 Determination of [^{3}H]-DA synthesis, [^{3}H]-DA release and [^{3}H]-DA storage

2.2.1 [^{3}H}-Tyr purification using high-performance liquid chromatography with ultraviolet detector (HPLC-UV)

The radio-labeled [^{3}H]-Tyr precursor, 40–60 Ci/mmol, was purified before use to maintain a high degree of purity after storage due to its degradation (rate of 1–3 % per month). The HPLC system used for this purification consisted of a reverse-phase C18 column (Tracer Extrasil ODS2, 5µm particle size, 25x0.46cm; Teknokroma, Spain) and equipped with a UV detector set at 285nm. Ion-pair mobile phase was used as described above and the flow rate was set at 1.0 ml/min. Under these conditions, tyrosine was eluted between 9–10 min. For each purification, 0.4mCi of [^{3}H]-Tyr was injected into the HPLC, and the whole [^{3}H]-Tyr fraction (1.5–1.8ml) was collected. An aliquot from the [^{3}H]-Tyr fraction was mixed with optiphase 'Hisafe'-III liquid scintillation cocktail in a scintillation vial for quantification using a liquid scintillation counter (Perkin Elmer Tri-Carb 2810TR, USA). Results were recorded as disintegration per min (DPM) counts. The concentration of [^{3}H]-Tyr was determined based on the DPM counts and area under the curve against an external standard calibration curve of non-radiolabeled tyrosine injected with the final concentration of 2.5-20nmol/µl prior to the analysis. This concentration was used to determine the amount of [^{3}H]-Tyr to be added into each sample for the synthesis of [^{3}H]-DA.

2.2.2 Drug(s) treatment and samples incubation for the determination of [^{3}H]-DA synthesis

Striatal tissue samples which had been prepared were incubated for 120-min (incubation-time-dependent effects in our experimental conditions: González-Sepúlveda et al., submitted) at 37°C, 400 rpm with discontinuous 15s/10s of agitation/interval in an Eppendorf Thermomixer Comfort (5 Prime, Inc., Boulder, CO) under carbogen atmosphere. SUL, if used, was added into the samples at the latest 20-min prior to the treatment with the drug (QUIN, ARI, CARI, BREX, or DMSO), which was added at 10-min before the end of incubation time. Depolarizing condition was obtained by increasing potassium (K$^+$) concentrations in the Krebs-Ringer buffer with concentrated potassium chloride, added 10-min before drug treatments. The radio-labeled [^{3}H]-Tyr was added with a final concentration of 0.10μM to all the samples, and the incubation was continued for another 10-min to synthesize [^{3}H]-DA. The experiment ended with the addition of a deproteinizing solution that contains the following compositions: 7% w/v trichloroacetic acid, 32.2nMol l-ascorbic acid and 25nMol DA hydrochloride as an internal standard. The internal standard was used to quantify the recovery efficiency during sample incubations as well as to facilitate the collection of [^{3}H]-DA by providing the peak signal for DA to be detected via HPLC-UV. For the blank samples (non-incubated), the deproteinizing solution was added before [^{3}H]-Tyr; the samples were kept on ice throughout the experiments. The acidic condition of deproteinizing solutions will reduce pH and stop the reactions by denaturing enzymes in the tissue samples. Overall experimental design of drug incubation time for the determination of [^{3}H]-DA synthesis is indicated as a timeline in fig. 3-2. At the end of the incubations, all samples were kept on ice before being further homogenized using Dynatech/Sonic Dismembrator (Dynatech Labs, Chantilly, VA) for approximately 5s. This sonication will allow the liberation of intracellular [^{3}H]-

DA for quantifications. An aliquot (10µl) was taken for protein quantifications using Pierce BCA protein assay (Thermo Scientific, IL, USA), to consider the variability of tissue contents in each sample tube. Samples were then centrifuged (12,000rpm, 10-min, 4°C), and supernatants were recovered. [^{3}H]-DA was analyzed with HPLC-UV.

(a)

Time (min) t (0)   t (100)   t (110)   t (120)   t (130)

| Incubation start | SUL | QUIN/ ARI/ CARI/ BREX/ DMSO | [^{3}H]-Tyr | Incubation end |

Deproteinizing solution

(b)

Time (min) t (0)   t (100)   t (110)   t (120)   t (130)

| Incubation start | K^+ | QUIN/ ARI/ CARI/ BREX/ DMSO | [^{3}H]-Tyr | Incubation end |

Deproteinizing solution
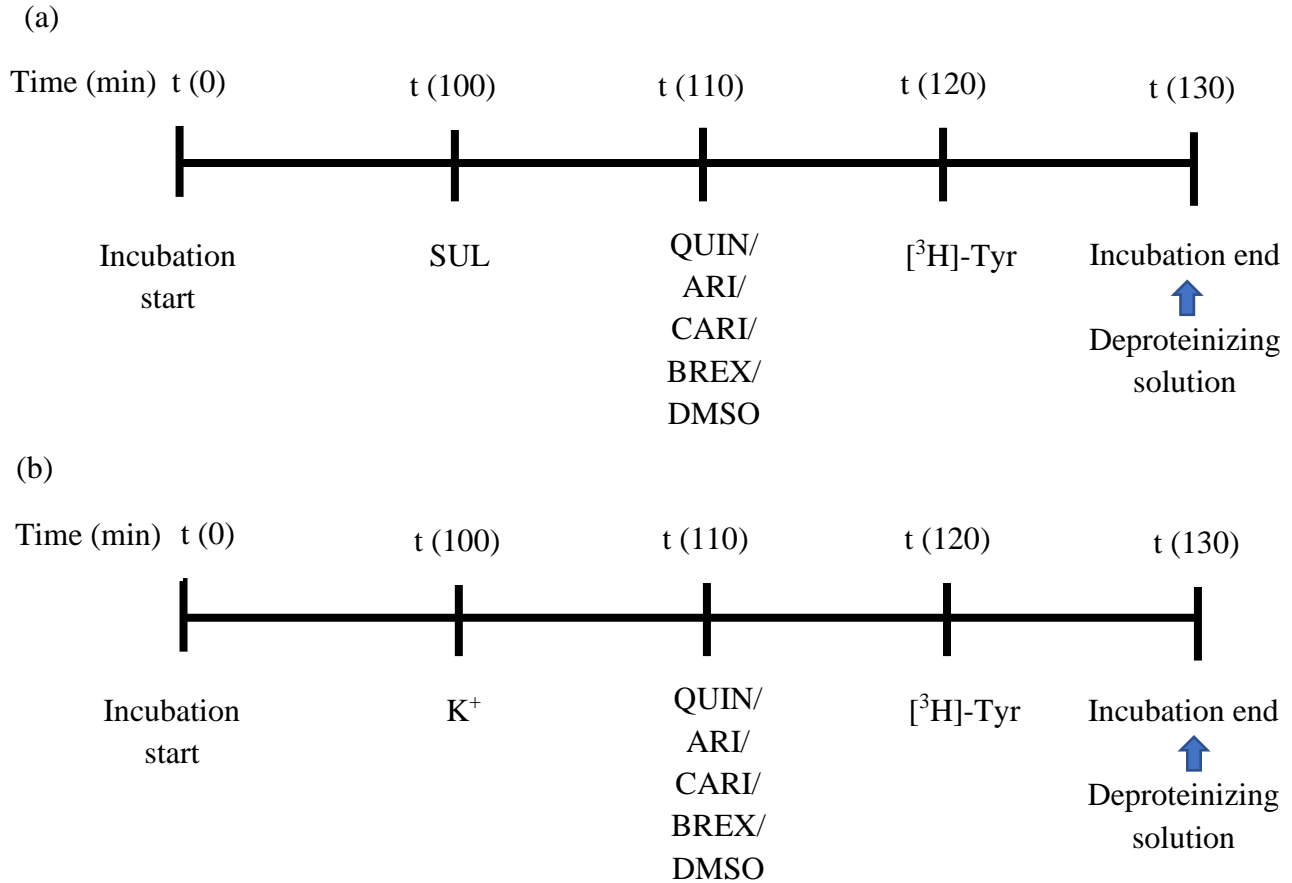
Fig. 3-2. Experimental design of drug incubation time for the determination of [^{3}H]-DA synthesis is illustrated in a timeline under (a) non-depolarized condition of 2mM K^+ and (b) depolarizing condition with concentrated K^+.

2.2.3 Drug(s) treatment and samples incubation for the determination of [^{3}H]-DA release and [^{3}H]-DA storage

For the determination effect of the drugs on [^{3}H]-DA release and [^{3}H]-DA storage, the same striatal tissue samples preparation was used. Similar pre-incubation time and conditions were applied. However, the radio-labeled [^{3}H]-Tyr with a final concentration of 0.10μM was added to all the samples at the beginning of samples incubation to newly synthesize [^{3}H]-DA. Later, NSD-1015 with a final concentration of 10μM was added into all the samples at the latest 40-min prior to the treatment with the drug (AMPH, ARI, CARI or BREX), which was added at 30-min before the end of pre-incubation time. NSD-1015 may block the activity of aromatic amino acid decarboxylase, and prevent new [^{3}H]-DA synthesis production or any effect towards increasing or decreasing [^{3}H]-DA synthesis from the drugs treatment. Thus, any changes in [^{3}H]-DA concentrations in the tissue may indicate the effect of [^{3}H]-DA depletion from storage. The supernatant was then carefully isolated (reflecting the amount of [^{3}H]-DA being released), and a new Krebs-Ringer buffer was added into the tissue samples with the same final volume. The experiment ended with the addition of the same deproteinizing solution as above into both the supernatant and the tissue samples. For the blank samples (non-incubated), the deproteinizing solution was added before the addition of [^{3}H]-Tyr, which took place prior to the separation between the supernatant and the tissue samples. Both the supernatant and the tissue from the blank samples were kept on ice throughout the experiments. Overall experimental design of drug incubation time for determination of [^{3}H]-DA release and [^{3}H]-DA storage is shown in a timeline in fig. 3-3. At the end of the incubation, all samples were kept on ice before further homogenized using Dynatech/Sonic Dismembrator (Dynatech Labs, Chantilly, VA) for approximately 5s. This

sonication will allow the liberation of intracellular [^{3}H]-DA for quantifications. An aliquot (10µL) was also taken for protein quantifications using Pierce BCA protein assay (Thermo Scientific, IL, USA), to consider the variability of tissue contents in each sample. All samples were then centrifuged (12,000rpm, 10-min, 4°C), and supernatants were recovered. [^{3}H]-DA in both supernatant and tissue samples were analyzed with HPLC-UV.

Time (min) t (0)   t (80)   t (90)   t (120)

Incubation start   NSD-1015   AMPH/   Incubation end
                                ARI/
[^{3}H]-Tyr   CARI/   Separate supernatant with the
                                BREX/   tissue samples
                                DMSO
                                             Deproteinizing solution

Fig. 3-3. Experimental design of drug incubation time for the determination of [^{3}H]-DA release and [^{3}H]-DA storage is illustrated in a timeline.

2.2.4 Introduction of the HPLC system

The HPLC system is one of the techniques used for purification and to separate each component in mixtures. The HPLC is mainly constituted of an injector, a pump, a column (stationary phase) and a liquid form of a mobile phase (solvent), as illustrated in fig. 3-4. The HPLC is usually connected with a detector to visualize, identify and quantify each component in the compound. Each component is visualized in a peak with its concentration quantified based on the area under the curve. The HPLC system relies on the pump to control and pressurize the liquid form of the mobile phase, which carries the injected sample through a column. This column consists of an

adsorbent (e.g. silica in a reverse-phase column) to separate each component from the mixtures depending on the degree of interaction with the adsorbent particles. The composition and the pH of the mobile phase together with the flow rate, which can be controlled via the pump, play an important role to regulate the retention time for an analyte.
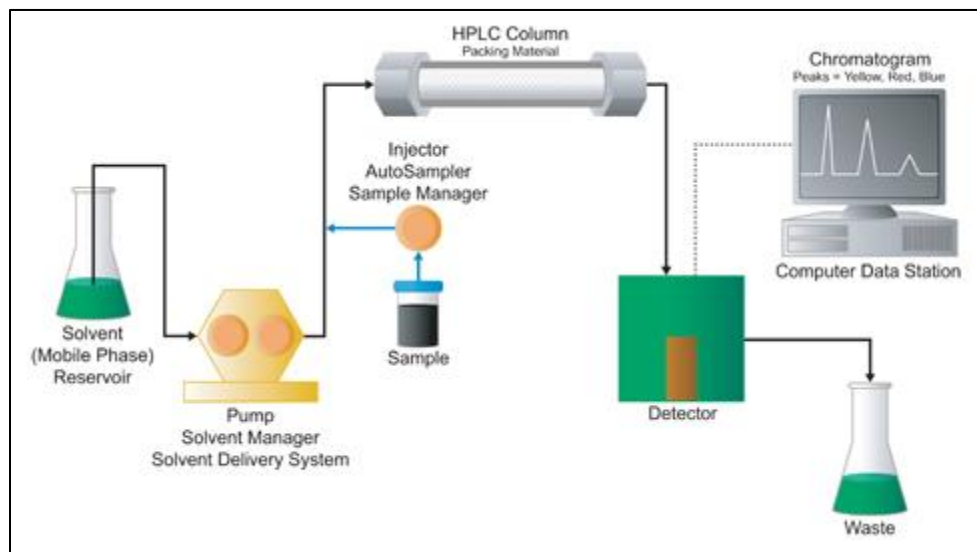
Fig. 3-4. The HPLC system. The image is extracted from https://www.waters.com/waters/en_US/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=en_US

2.2.5 [^{3}H]-DA analysis with HPLC-UV

The chromatographic system used for [^{3}H]-DA analysis consists of a reverse-phase C18 column (Tracer Extrasil ODS2, 5μm particle size, 25x0.46cm; Teknokroma, Spain). The C18 column contains a hydrophobic string with 18 carbons. The longer carbon strings enhance the interaction area, thus increasing the retention, to isolate and detect DA in the tissue samples. An ion-pair mobile phase was used as described above, and the flow rate was set at 1.0 ml/min. The hydrophobic moiety of octane-1-sulphonic acid sodium salt used in the mobile phase interacts with

hydrophobic strings in C18 column and together with the negative charge from octane-1-sulphonic acid sodium salt enhances the retention time of positively charged amines, such as DA. The HPLC system is connected with a UV detector, set at 285nm to detect DA used as an internal standard. This is due to the low sensitivity of the UV detector, which is not able to detect endogenous DA in the tissue samples (fig. 3-5). Thus, the DA fraction detected by the UV detector was a mixture of three sources of DA, including the endogenous DA, the [3H]-DA which was synthesized from the [3H]-Tyr, and DA which was used as the internal standard. Since both DA from endogenous and internal standards were not radioactively labeled, their presence was not quantitatively measured by liquid scintillation and was not affect the calculation of [3H]-DA concentration.
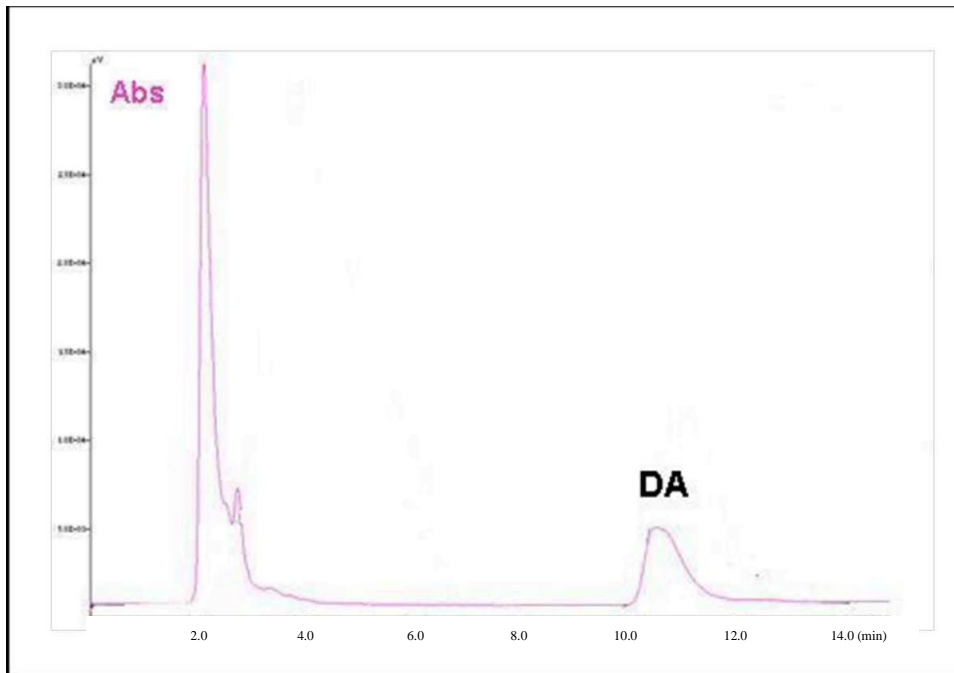
Fig. 3-5. Typical peaks of a UV chromatogram obtained from the tissue samples. The first peak is ascorbic acid while the second peak is the DA fraction (Ma G.F., Ph.D. thesis 2014).

The same volume of 50μL for each sample and the internal standard were injected into the HPLC-UV and the recovery of the internal standard was quantified via internal/external standard peak area. Besides, since the [3H]-Tyr was used as a precursor for the synthesis of new [3H]-DA, there was an excess of [3H]-Tyr in each sample (in order to ensure enough [3H]-Tyr available for the synthesis of [3H]-DA). In our protocols, we did not use tyrosine as an internal standard and this [3H]-Tyr peak could not be detected by the UV detector and did not appear in the chromatogram. Therefore, this [3H]-Tyr can be excluded in the measurements by ensuring there is enough separation in the retention time between the [3H]-Tyr and [3H]-DA fractions as shown in fig. 3-6; this was done at the beginning of the study.
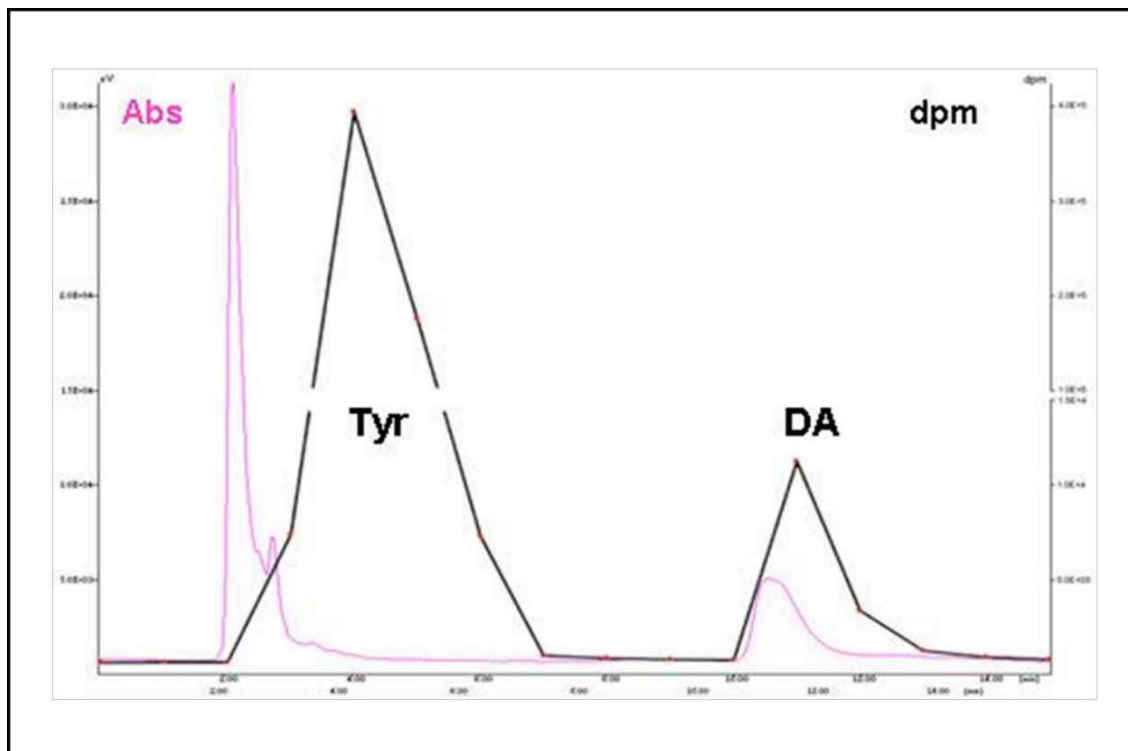
Fig. 3-6. Temporal outline of radioactivity present in the eluates in comparison with DA chromatogram. The black peaks represent the DPM counts while the pink peaks indicate the absorbance value obtained from the UV detector (Ma G.F., Ph.D. thesis 2014).

78

The retention time of DA, based on the conditions used in our HPLC system, was around 10-min. The fractions of DA were collected in scintillation vials; this sampling took approximately 2.70-min. This eluent, which consists of DA fractions, was later mixed with an optiphase 'Hisafe'-III liquid scintillation cocktail (to convert the kinetic energy of nuclear emissions and provide better counting and detection efficiency) to a final volume of 8ml. [^{3}H]-DA concentration was then quantified using a liquid scintillation counter (Perkin Elmer Tri-Carb 2810TR, USA).

2.2.6 Results

Results were recorded as DPM counts and were corrected with the following variables: (1) DA internal standard recovery, (2) DPM counts in blank samples, and (3) amount of protein in each incubated sample tubes. Results were presented in the form of concentration-response curves or histogram after normalization of the data to the percent of control in the same brain incubations and pooling of data from more than one brain with the number of samples (n) indicated in the graph/ bar/ figure legends.

2.3   Determination of endogenous DA, DOPAC, 5-HT and 5-HIAA concentrations

2.3.1 Drug treatments and sample incubations

For the determination of endogenous DA and DOPAC concentrations, the preparation of striatal tissue minces was similar to above. However, the drug(s) (QUIN, ARI, CARI, BREX, SUL, SB 277011-A, etc.) treatment was added at the beginning of the sample incubations. Similar pre-incubation time and conditions were applied. The incubation ended with the addition of 10

volumes (w/v) deproteinizing solution of 0.25M perchloric acid containing 0.25mM ethylenediaminetetraacetic acid disodium salt dihydrate and 0.1mM sodium disulphite. For showing basal values (non-incubated) in the samples, the deproteinizing solution was added at the beginning of sample incubations and they were kept on ice throughout the experiments. The experimental design of drug incubation time for the determination of endogenous DA and DOPAC accumulation is described in a timeline in fig. 3-7. During the first 120-min of sample incubations, a typical 4-fold increase of endogenous DA occurs, thought to reflect new DA formation and storage without release in non-depolarizing conditions (González-Sepúlveda et al., submitted). Thus, in this thesis I have called this increase "spontaneous DA accumulation". At the end of the incubations, all samples were kept on ice before being further homogenized using Dynatech/Sonic Dismembrator (Dynatech Labs, Chantilly, VA) for approximately 5s. An aliquot (10μl) was taken for protein quantifications using Pierce BCA protein assay (Thermo Scientific, IL, USA), to quantify tissue in each sample tubes. Samples were then centrifuged (13,000rpm, 5-min, 4°C), and the supernatants were recovered. The concentrations of DA, DOPAC, 5-HT and 5-HIAA were quantified using high-performance liquid chromatography with electrochemical detector (HPLC-EC).

By using the above sample preparations, neither the endogenous 5-HT nor the 5-HIAA concentrations were able to be detected and quantified by our HPCL-EC. For the determination of endogenous 5-HT and 5-HIAA concentrations in each sample, the preparation of striatal tissue minces was slightly modified to increase the tissue content in each sample tube. Therefore, the number of samples prepared from a single rat brain was reduced to a total of 24 samples. Furthermore, to concentrate the amount of endogenous 5-HT and 5-HIAA concentrations to be detected, the total volume for each sample was also reduced. For this experiment, each sample

consisted of 25µl of striatal tissue samples in 100µl of Krebs-Ringer buffer inside a 2ml polypropylene tube containing 100µl of Krebs-Ringer buffer. The same experimental design as for the determination of endogenous DA and DOPAC concentrations was followed, with the drug added at the beginning of the sample incubations. At the end of the incubations, similar protocols including homogenization, protein determination and centrifugations were followed as above.

Time (min) t (0)   t (120)

Incubation start   Incubation end

Drug(s)   Deproteinizing
                                                                        solution
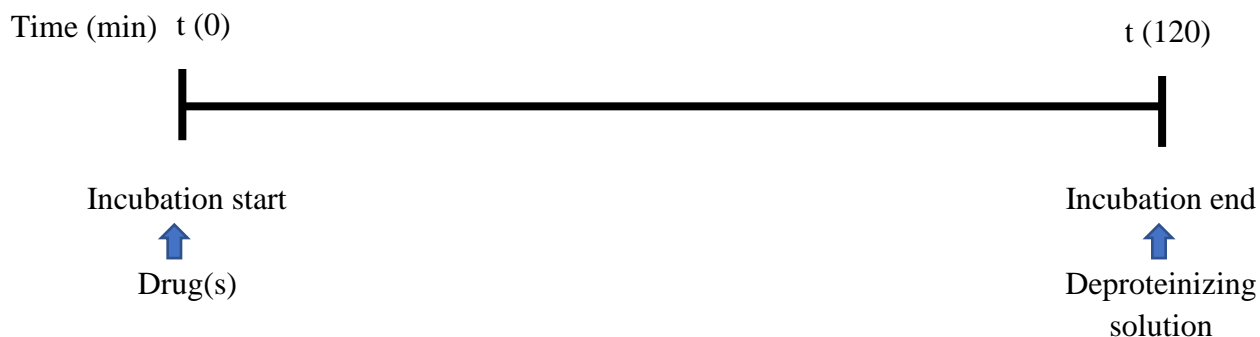
Fig. 3-7. Experimental design of drug incubation time for determination of endogenous DA, DOPAC, 5-HT and 5-HIAA accumulation is illustrated in a timeline.

2.3.2 Analysis of DA, 5-HT and its metabolites by HPLC-EC

For the determination of DA, DOPAC, 5-HT and 5-HIAA concentrations, the HPLC-EC was used for higher sensitivity. The chromatographic system used consists of a reverse-phase C18 column (2.5µm particle Fortis C18, 10 x 0.46 cm, Sugelabor, Spain). The flow rate was set at 1.5ml/min. The HPLC system was equipped with Coulochem II (ESA) detector (model 5011 dual-electrode analytical cell with porous graphite electrodes) to detected, separated and quantified DA, DOPAC, 5-HT and 5-HIAA concentrations in the samples (fig. 3-8). The potential of electrodes 1 and 2 was set at −0.05V and +0.4V respectively. The concentration of endogenous DA, DOPAC, 5-HT and 5-HIAA in the tissue samples was determined on the basis of the area under the curve against an

external standard of the calibration curve (final concentrations of 10-200nmol/µl obtained from 20 µl of injections) which was done prior to the analysis. The same volume for the samples (20µl) was injected into the HPLC-EC for the analysis of endogenous DA and DOPAC, while the amount of aliquot injected for the analysis of endogenous 5-HT and 5-HIAA was doubled.
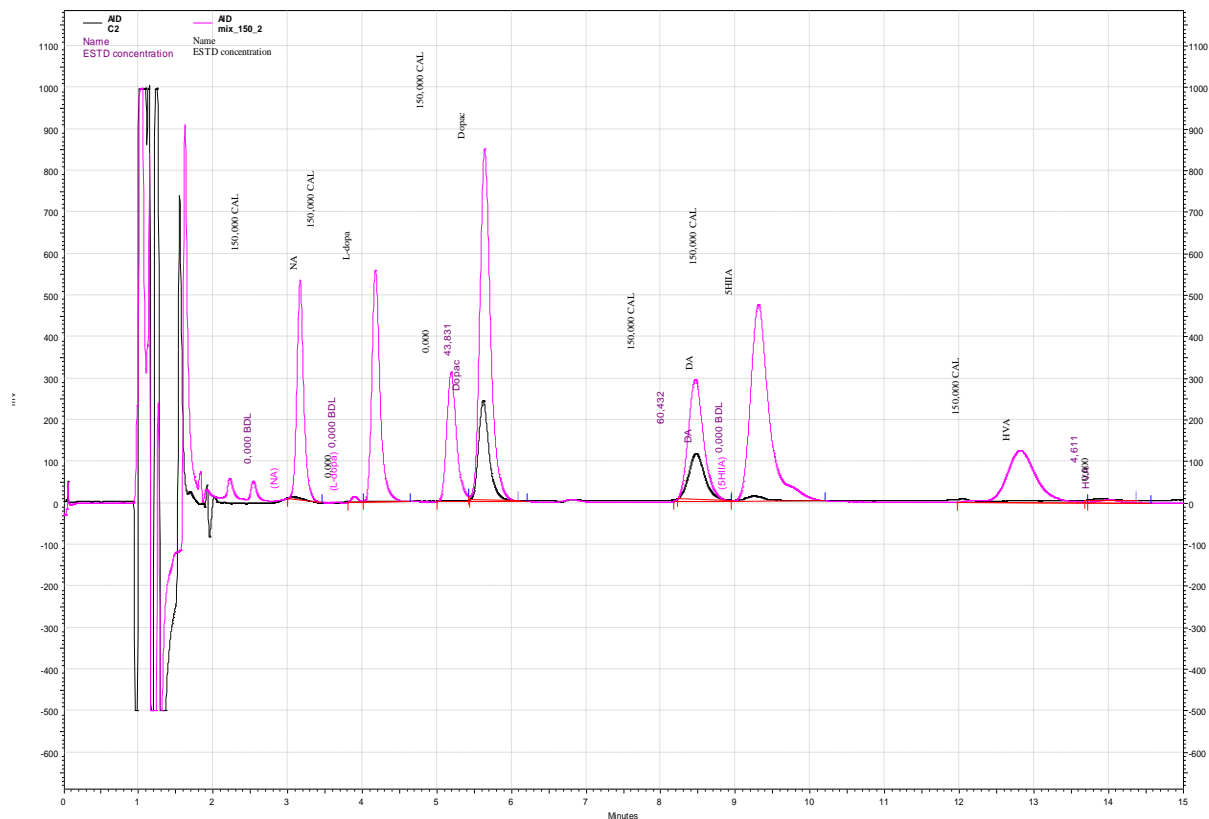
Fig. 3-8. Typical peaks of a chromatogram obtained from the tissue samples using HPLC-EC. The black peaks represent both DA and DOPAC fractions in the tissue samples while the pink peaks indicate the external standard used for quantifications.

2.3.3 *Results*

Results were expressed via the concentration of DA, DOPAC, 5-HT and 5-HIAA versus mg protein in each sample. While the concentration-response curves obtained from more than one brain incubations were normalized to percent of control in the same brain incubation and pooling of data from more than one brain with the number of samples (n) indicated in the graph/ bar/ figure legends. The relationship between the DOPAC/ DA and the 5-HIAA/ 5-HT concentration ratios was presented as a release or metabolism index.

2.4   Statistical analysis

Statistical analysis was carried out with GraphPad Prism software (version 4) (GraphPad Software Inc, USA). Data were presented as mean ± standard error of the mean (SEM). Interaction between 2 factors was assessed by two-way analysis of variance (ANOVA) followed by Bonferroni test for post-hoc comparison. One-way ANOVA was applied with Dunnett´s post-hoc test for comparison against control or Bonferroni post-hoc test for direct comparison between tested groups. Statistical analysis of treated drug and the respective control was performed by the unpaired Student´s t-test. A difference was considered to be statistically significant at $*p<0.05$, $**p<0.01$ and $***p<0.001$.

(Blank)

IV. RESULTS

Results

1. First objective: Increase our understanding of the overall homeostatic mechanisms toward regulation of DA accumulation at presynaptic neurons in the striatum.

In our *ex-vivo* experimental method, the mincing of brain striata on ice during tissue preparations releases DA from synaptic vesicles, which leads to a decrease of DA concentration and increase its metabolism. This was discovered in our group in 2020 by Anna Galán, who found a decrease of DA levels but an increase in the DOPAC and the DOPAC/ DA concentration ratio in minced vs. unminced striatal tissue, thus indicating transiently higher cytosolic DA levels in striatal tissues which undergo such processing, despite the low temperature (0-4ºC) maintained. However, during tissue sample incubations at 37ºC, there is a 3-4-fold spontaneous increase in endogenous DA levels in the first 120-min of samples incubation, reaching higher DA levels than previously to mincing. During this process, endogenous DA can actively be synthesized, reaccumulated and refilled inside the synaptic vesicles. The increase of DA synthesis was highly dependent on a negative-feedback mechanism on TH primarily via cytosolic DA, until it reached a plateau state where the equilibrium between DA storage and leak from vesicles was achieved, probably due to the maximal storage capacity (González-Sepúlveda et al., submitted). Such an effect also occurs without any external stimulation at low K^+ concentration (2mM K^+), which prevents DA release. This observation opens a new framework of research towards understanding the homeostatic mechanisms on the regulation of DA storage dynamics at presynaptic terminals and its pharmacological utility as a test of the synaptic effects of dopaminergic drugs.

1.1 *Spontaneous increase of endogenous DA accumulation over incubation time*

In order to understand the overall homeostatic mechanisms toward regulation of DA accumulation at presynaptic neurons in the striatum, a similar experiment with the same experimental conditions than in González-Sepúlveda et al. was done to prove that such an increase of spontaneous DA can be replicated. Similarly, an increase of spontaneous endogenous DA accumulation was shown in 120-min sample incubations (fig. 4-1). In line with the increase of DA accumulation, an increase of its metabolite, DOPAC levels were observed. DA is likely transformed into DOPAC when storage in vesicles approaches saturation. With a similar rate of increase between DA and DOPAC levels, I observed an apparent constant in the DOPAC/ DA concentration ratio in the first 120-min incubations. I conclude that our *ex-vivo* tissue incubations may provide an ideal environment to study the homeostatic mechanisms which accumulated neurotransmitter DA at the presynaptic terminals. This observation was extended to 5-HT for this thesis and it will be illustrated in the results of my third objective.
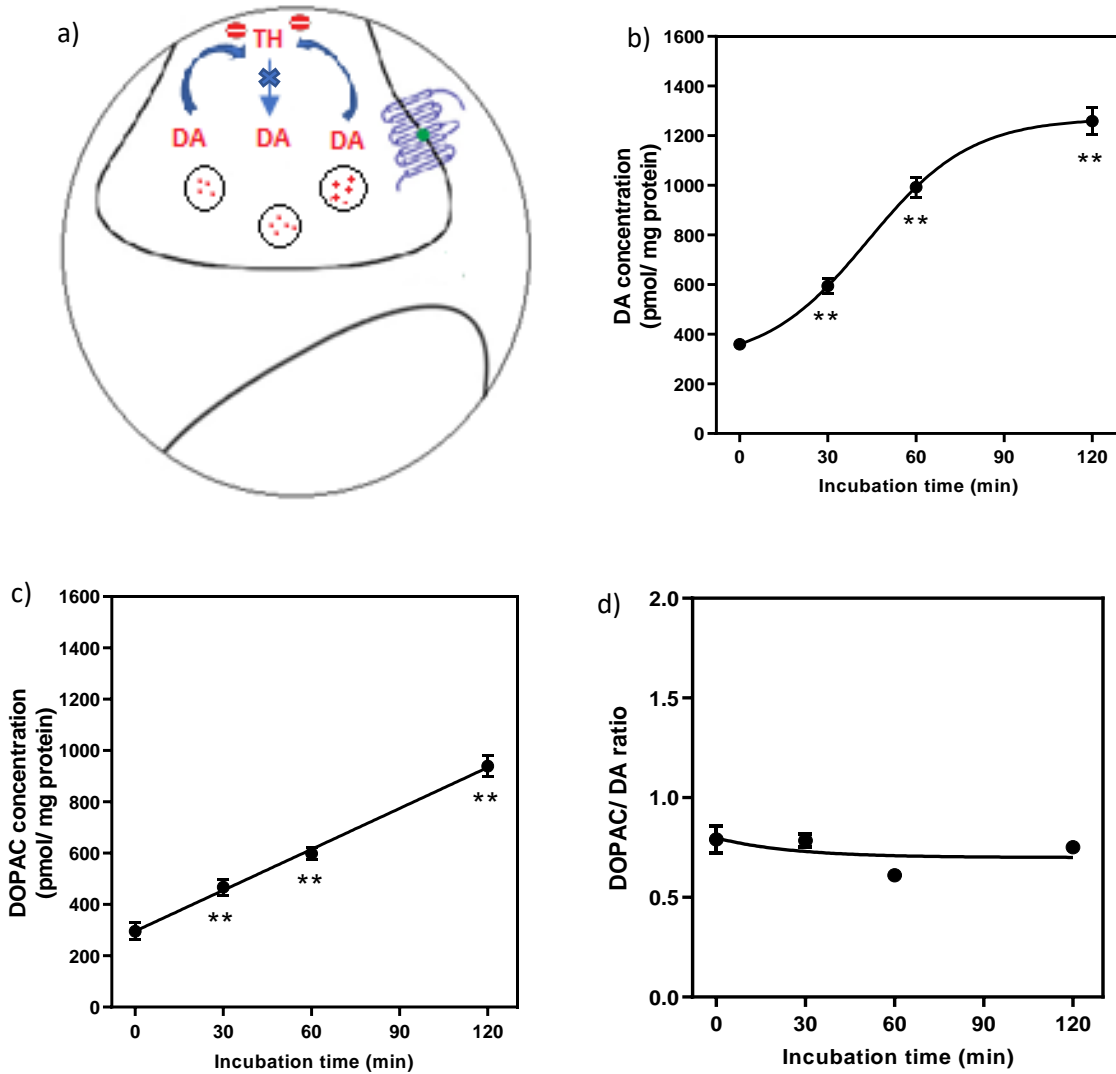
Fig. 4-1. (a) Schematic illustration of the increase of DA synthesis during tissue samples incubation, which was highly dependent on a negative-feedback mechanism on TH, primarily via cytosolic DA leak from synaptic vesicles. Such increases were translated in view of the effect of different tissue samples' incubation times on endogenous (b) DA accumulation, (c) DOPAC levels, and (d) the DOPAC/ DA concentration ratio at 2mM K^+. The data are expressed as mean $\pm$ SEM (n=16 incubations per data point) from the control group of four brains accumulated from four different experiments with D_2R agonist and partial agonist antipsychotics in Results Chapter 2. **$p<0.01$ vs basal of non-incubated samples which correspond to the 0-min value, one-way ANOVA followed with Dunnett's post-hoc test. The data were adjusted into the sigmoidal dose-response curve for both DA accumulation and DOPAC levels, one-phase exponential decay for the DOPAC/ DA concentration ratio.

1.2 D₂R agonist and partial agonist decrease endogenous DA and DOPAC accumulations

With the aim to understand the homeostatic mechanisms and regulations at presynaptic D_2, I first evaluated the effect of QUIN, a full D_2R agonist, and ARI, a D_2R partial agonist on DA accumulation. This effect was studied under non-depolarized conditions (2mM K^+), which prevent DA release and thus D_2 receptor activation by endogenous DA. Under this condition, 1µM QUIN significantly blocked endogenous DA accumulation as early as 30-min and the blockade was maintained for 120-min incubations, shown by a statistically significant ANOVA interaction with control data under incubation-time-dependent effects, $p<0.001$ (fig. 4-2b). The same effect was also observed with 1µM ARI but with less efficacy, since the significant decrease in DA accumulation can only be seen after 60-min incubations and remains lower than that of QUIN for 120-min incubations, $p<0.01$ (fig. 4-2e). As this experiment was done under non-depolarizing conditions, the effect for both the D_2 agonist and the partial agonist is thought to modulate a negative-feedback inhibition on TH by stimulating D_2R, probably void of extracellular DA as 2mM K^+ prevents DA release. This condition may also affect DOPAC levels as a TH inhibition would also prevent new DOPAC formation. As expected, a decrease in DOPAC concentration by 1µM QUIN was observed with a statistically significant interaction by 1µM QUIN as early as 30-min and remains the same for 120-min incubations, $p<0.001$ (fig. 4-2c). A decreased in DOPAC concentration can also be observed with 1µM ARI but with less efficacy, as the significant decrease can only be seen at 120-min incubations, $p<0.05$ (fig. 2f). Differently to QUIN, ARI showed a lower decrease of DOPAC levels, which led to a non-statistically significant increase in the DOPAC/ DA concentration ratio, $p<0.05$ (QUIN) and $p>0.05$ (ARI) (fig. 2d and 2g). These results show that D_2R agonist and partial agonist at the presynaptic terminals lead to a decrease of

DA synthesis, accumulation and metabolism. Thus, a decrease of DOPAC levels follows the decrease of DA concentration elicited by QUIN and ARI.
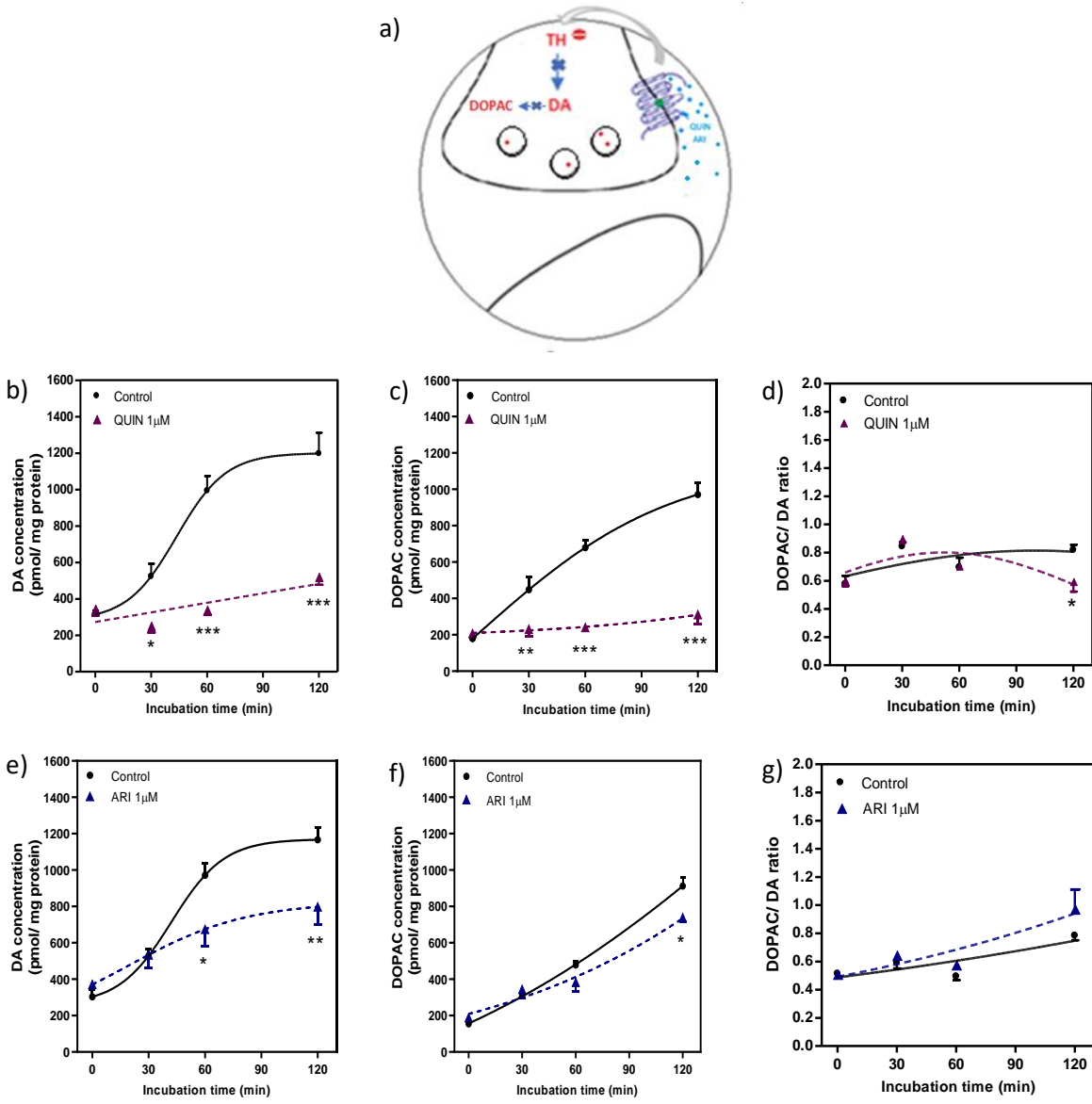
Fig. 4-2. (a) Schematic illustration of the mechanism of QUIN and ARI at 2mM K^+, which is thought to modulate a negative-feedback inhibition on TH via stimulating D_2R. Such an activation leads to a decrease in DA accumulation (b and e) followed by DOPAC levels (c and f). The DOPAC/ DA concentration ratio was plotted for both QUIN and ARI (d and g) as a metabolism index. The data are expressed as mean ± SEM (n=4 incubations per data point). All incubations from one graph were obtained from a single brain. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs control data under incubation-time-dependent effects, two-way ANOVA followed with Bonferroni's post-hoc test. The data were adjusted into the following analysis parameters: panel (b) sigmoidal dose-response curve for control and one-phase exponential decay for QUIN; panel (c) sigmoidal dose-response for both control and QUIN curve; panel (d) gaussian for both control curve and QUIN curve; panel (e) sigmoidal dose-response for both control and ARI curve; panel (f) sigmoidal dose-response for both control and ARI curve; and panel (g) gaussian for both control curve and ARI curve.

91

1.3 Independent effects of D_2R stimulation and DA inhibition on TH to inhibit $[^3H]$-DA synthesis

Previously, I showed that both QUIN and ARI significantly decrease DA accumulation; this effect was studied under non-depolarized conditions that prevent DA release (fig. 4-2). Both QUIN and ARI actions were assumed via the activation of presynaptic D_2R, which can modulate a negative-feedback mechanism to decrease TH phosphorylation. To assess whether exogenous DA application to slice incubations inhibited TH through D_2R, as was thought, by D_2 agonists or via direct DA inhibition of the enzyme, I studied the effect of the addition of $1\mu M$ DA into the samples on $[^3H]$-DA synthesis in comparison with the full D_2 agonist, QUIN in the presence or absence of the D_2 antagonist, SUL (fig. 4-3). The technique of $[^3H]$-DA synthesis from $[^3H]$-Tyr was used in order to be able to differentiate between exogenously applied DA and endogenously synthesized DA. As reported in González-Sepúlveda et al., submitted, I demonstrated that the addition of additional extracellular DA significantly decreases $[^3H]$-DA synthesis. To know if this action was due to the activation of presynaptic D_2R, I further combined with the D_2 antagonist, SUL. The presence of SUL was shown to block the effect of QUIN, but not DA, suggesting a prominent effect of DA by other mechanisms, probably involving DAT, which transports extracellular DA into the presynaptic terminals. Such a mechanism increases cytosolic DA levels which compete with the BH_4 cofactor to limit TH enzymatic activity. Cytosolic DA could regulate its own inhibition on $[^3H]$-DA synthesis (as was proven by González-Sepúlveda et al.,-submitted), as compared with the effect of QUIN via the activation of D_2R alone.
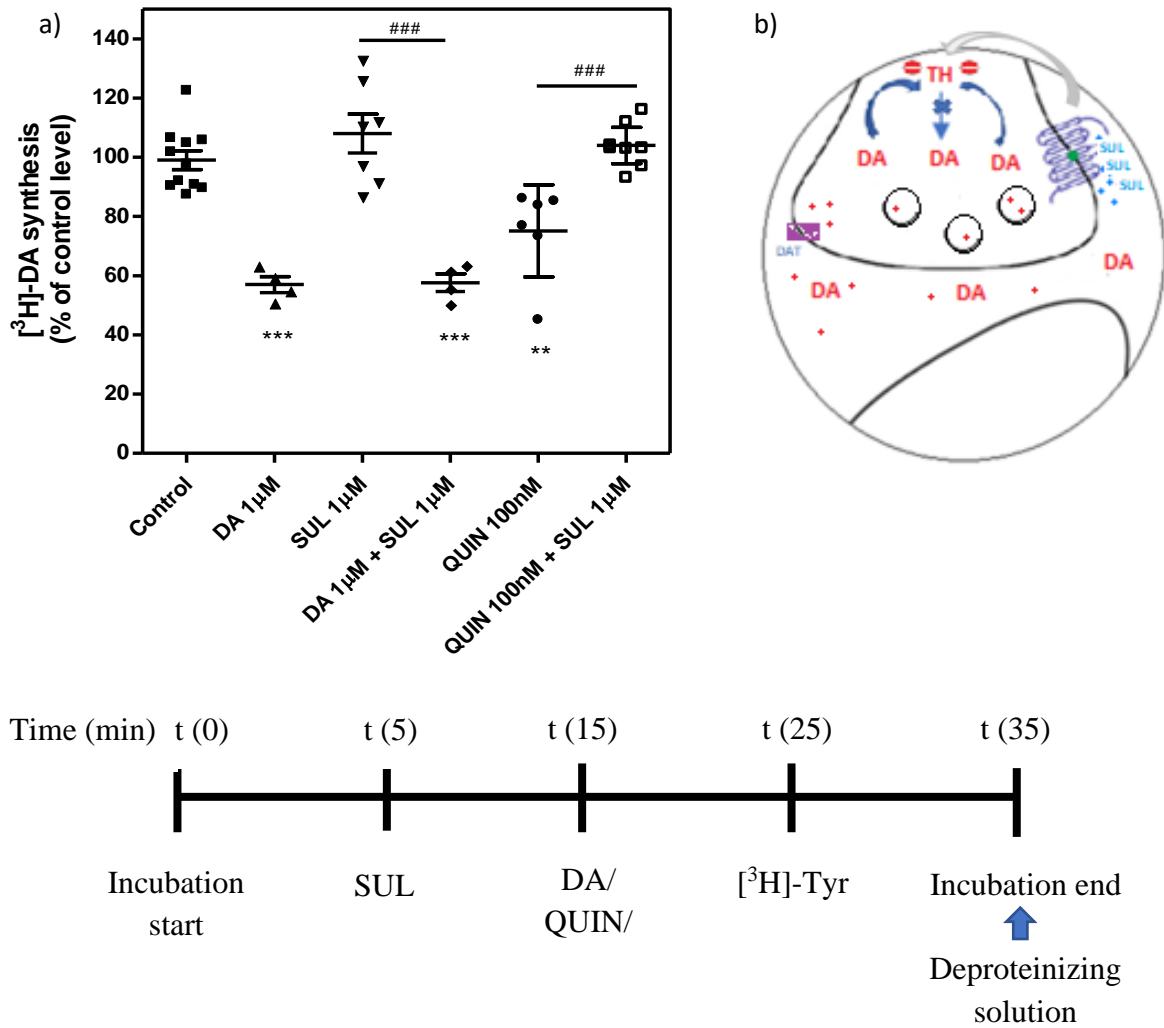
Fig. 4-3. (a) D_2 antagonism blocks the effect of QUIN but not DA on the inhibition of $[^3H]$-DA synthesis under 2mM K^+, as signified in the schematic diagram illustrating that DA can directly inhibit TH (b), and unlike QUIN this effect is not mediated by D_2R. Contrarily to other experiments, this study was done in 25-min tissue incubations, as shown in the timeline above, to match the experimental design in González-Sepúlveda et al., submitted. The data are expressed as mean ± SEM (n=4-6 incubations per data point) from two brains. **p<0.01 and ***p<0.001 vs control, one-way ANOVA followed with Dunnett's post-hoc test; and ###p<0.001, one-way ANOVA followed with Bonferroni's post-hoc test for comparison between DA and QUIN effects with and without pretreatment with SUL.

1.4 OKA increases endogenous DA accumulation

In the introduction I have mentioned that short-term TH activity can be modulated with the phosphorylation. The phosphatase inhibitor OKA was shown to increase the phosphorylation of TH at Ser^{19}, Ser^{31} and Ser^{40} (Haycock, 1990). With the increase of TH phosphorylation, I hypothesized OKA would increase DA accumulation. As predicted, OKA significantly increased endogenous DA accumulation; this could only be observed at the higher concentrations used in this study, with the EC_{50} of 2.3μM, $p<0.01$ (fig. 4-4). My results are in agreement with Ma G.F., Ph.D. Thesis 2014, that OKA significantly enhanced $[^{3}H]$-DA synthesis. I believe this effect may be due to the phosphorylation of Ser^{31} and Ser^{40}, since phosphorylation of Ser^{19} has been shown to have no direct effect on TH activity (Dunkley et al., 2004). This is supported by González-Sepúlveda et al., submitted, where the authors found that OKA increased Ser^{31} and Ser^{40} TH phosphorylation. In line with the increase of DA accumulation, an increase of DOPAC levels was also observed at the same concentration, with the EC_{50} of 3.3μM, $p<0.01$. Interestingly, the levels of DOPAC were tremendously increased at a much higher magnitude than DA accumulation, leading to a significant increase in the DOPAC/ DA concentration ratio, $p<0.01$. This result suggests that OKA did not only increase DA accumulation but simultaneously increased DA metabolism more than it accumulated.
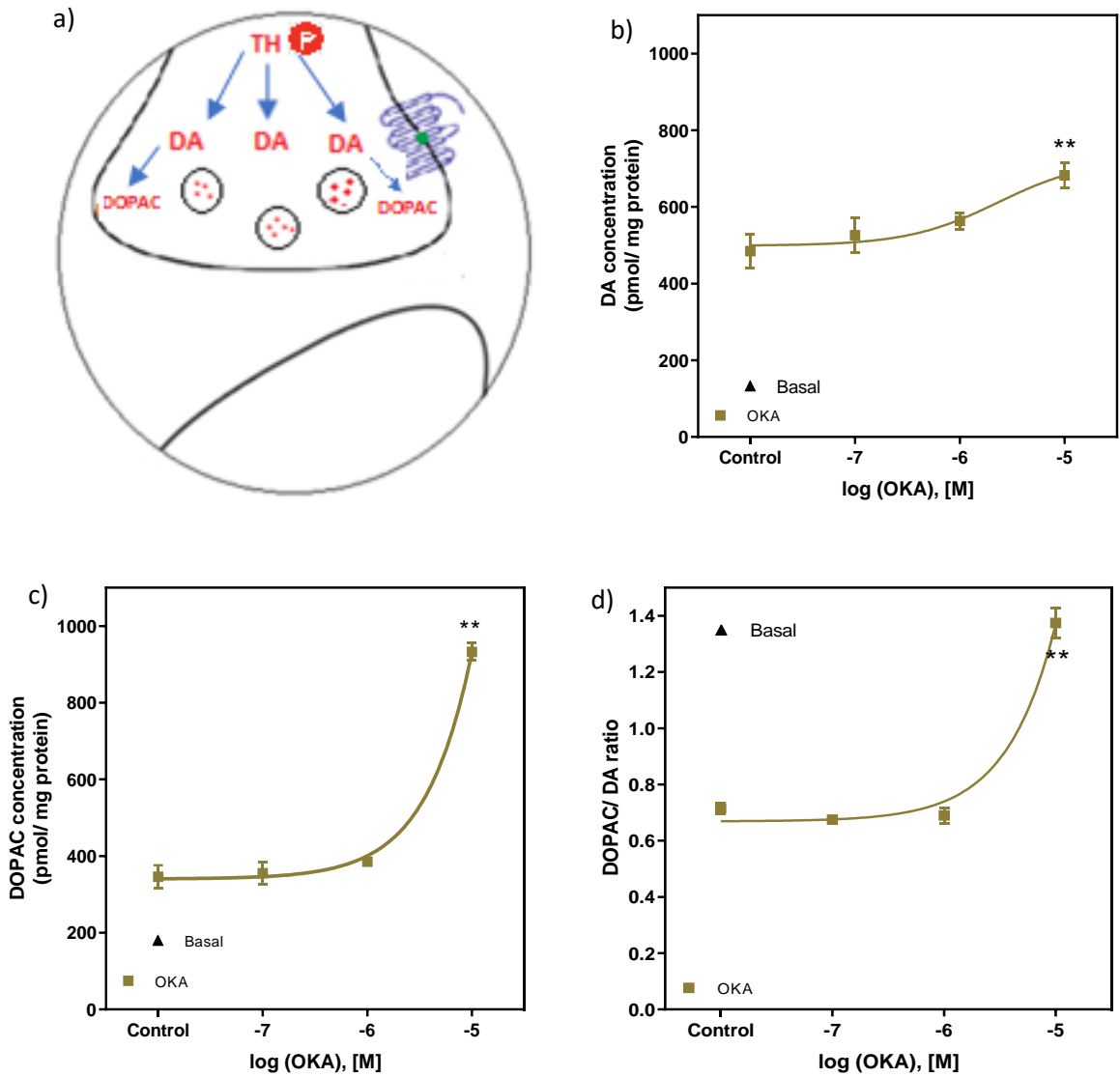
Fig. 4-4. (a) Schematic illustration of the effect of OKA to increase DA accumulation. but simultaneously enhance its metabolite levels, indicating higher DA metabolism. This is manifested by the concentration-response curves on the effects of OKA on endogenous (b) DA accumulation, (c) DOPAC levels, and (d) the DOPAC/ DA concentration ratio under 2mM K^+ in 120-min incubations. The data are expressed as mean $\pm$ SEM (n=6 incubations per data point) from one brain. Basal levels in non-incubated samples are shown by ▲. **$p<0.01$ vs control, one-way ANOVA followed with Dunnett's post-hoc test. The data were adjusted into a sigmoidal dose-response curve for DA accumulation, DOPAC levels, and the DOPAC/ DA concentration ratio.

1.5 Lack of effects by **PAOPA** *on endogenous DA accumulation*

As I discussed in the introduction, the orthosteric site at GPCRs is the primary and active binding site for the ligand at the receptor including D_2R. This includes QUIN, a D_2 agonist. Other than the orthosteric site, the allosteric binding site provides an alternative for ligand (known as allosteric modulators) to bind at GPCRs. PAOPA is the positive allosteric modulator at the D_2 receptor (Basu et al., 2013). Under non-depolarized conditions (2mM K^+) and without any orthosteric stimulation, PAOPA did not have any effects on endogenous DA accumulation, DOPAC levels and the DOPAC/ DA concentration ratio (fig. 4-5). This is consistent with the properties of an allosteric modulator, which does not activate the D_2 receptor and has no capacity to facilitate signal transductions on its own.
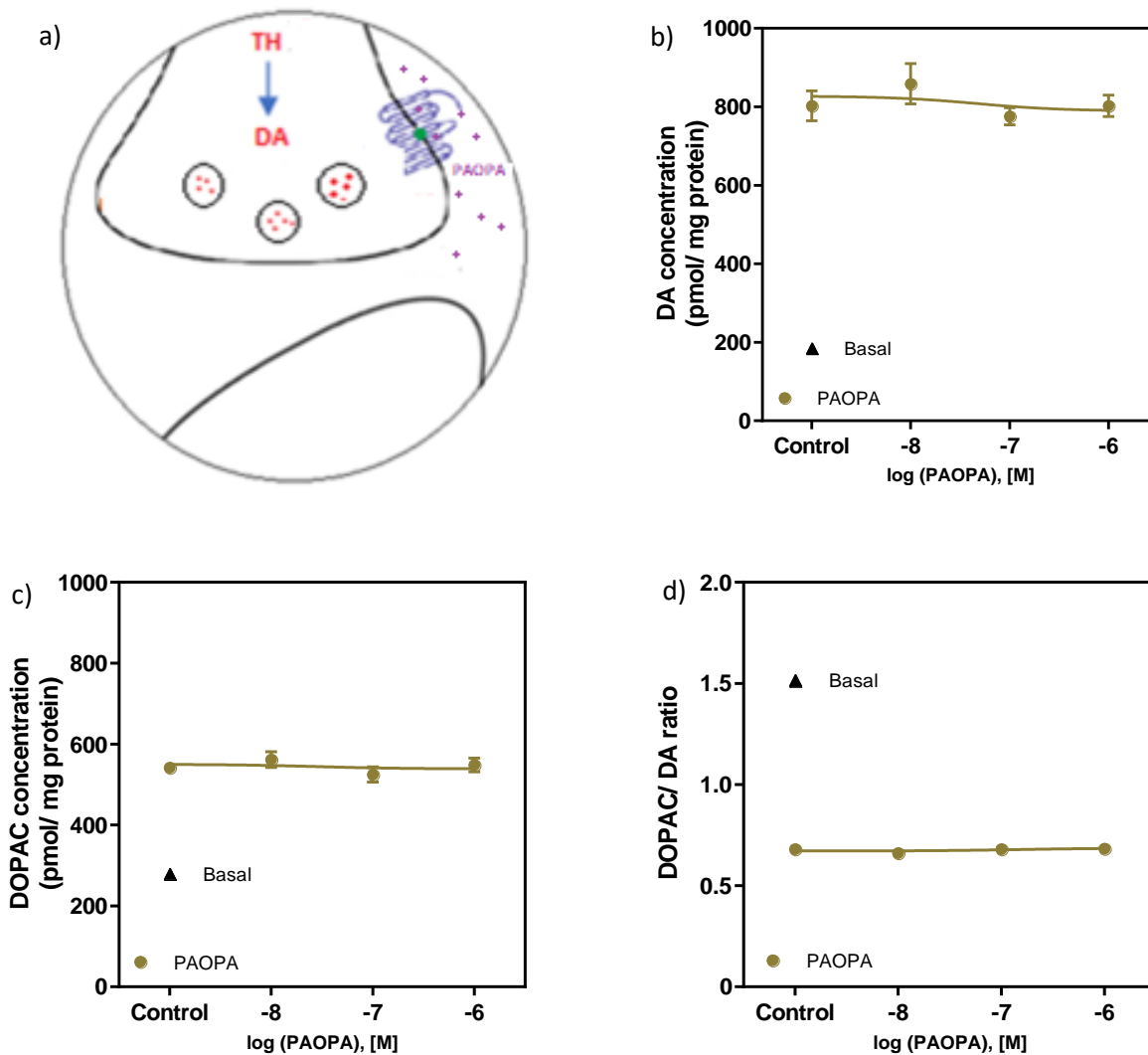
Fig. 4-5. (a) The presence of PAOPA did not regulate presynaptic DA accumulation, as shown by lack of effects in the concentration-response curves of PAOPA on endogenous (b) DA accumulation, (c) DOPAC concentration, and (d) the DOPAC/ DA concentration ratio under 2mM K^+. The data are expressed as mean ± SEM (n=6 incubations per data point) from one brain. Basal levels in non-incubated samples are shown by ▲. The data was adjusted into the sigmoidal dose-response curve for DA accumulation, DOPAC levels and the DOPAC/ DA concentration ratio.

1.6 PAOPA potentiates QUIN effect on endogenous DA accumulation

I have previously shown that PAOPA alone did not activate the D_2 receptor and had no action to facilitate signal transductions to decrease DA accumulation on its own (fig. 4-5). As a positive allosteric modulator, a few studies have shown that PAOPA can enhance the affinity of an agonist and increase its binding to D_2 receptor (Mishra et al., 1999; Verma et al., 2005). This was achieved by maintaining the D_2 receptor in high-affinity states. Thus, I hypothesized that the combination of QUIN, a D_2 agonist with PAOPA will increase its efficacy to inhibit DA accumulation. To do this, I used a low concentration of QUIN (10nM) and 10nM of PAOPA (a concentration that showed a significant increase of QUIN binding on the D_2 receptor by Verma et al., 2005). Similarly to before (fig. 4-5), PAOPA alone did not have any effect on DA accumulation. However, the presence of PAOPA significantly enhanced the QUIN effect to decrease DA accumulation, $p<0.05$ (fig. 4-6b). The same effect was observed on DOPAC levels (fig. 4-6c) but it did not reach statistical significance and led to no significant differences in the DOPAC/ DA concentration ratio (fig. 4-6d). This study indicates that the efficacy of D_2 agonists on the inhibition of DA synthesis via stimulating D_2R to mediate a feedback-inhibition on TH can be enhanced with the combination of a positive allosteric modulator. Given the low number of incubations and single PAOPA concentration used, this result could be considered preliminary, but potentially promising for further study.
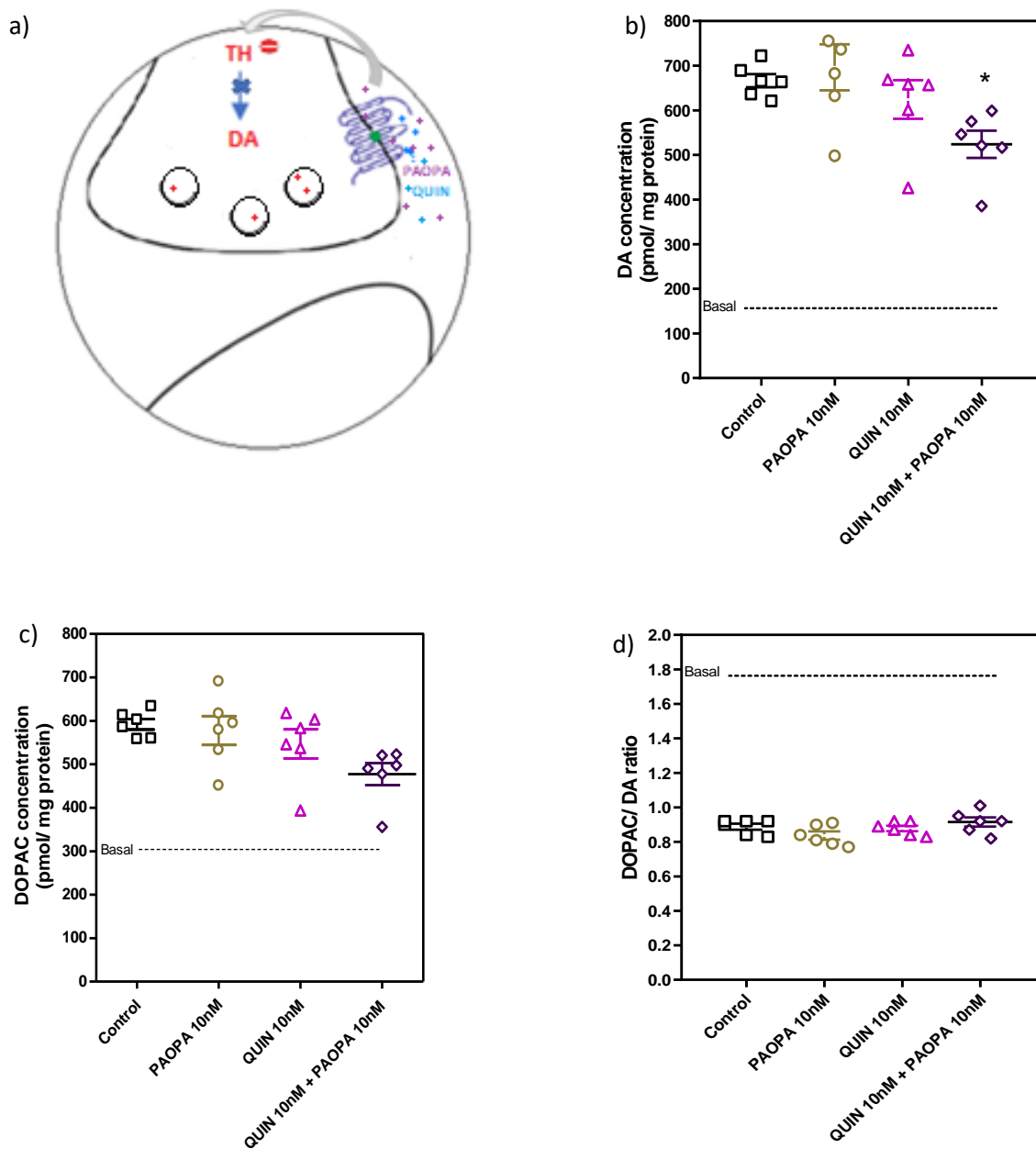
Fig. 4-6. (a) A schematic diagram showing that PAOPA increases D_2 agonist efficacy at presynaptic terminals. This is revealed by the combination of PAOPA and QUIN to decrease endogenous (b) DA accumulation. The same effect was observed on DOPAC levels (c) but it did not reach statistical significance, leading to no significant differences in the DOPAC/ DA concentration ratio (d). The basal levels in non-incubated samples are shown by the dotted line. The data are expressed as mean ± SEM (n=6 incubations per data point) from one brain. *p<0.05, one-way ANOVA followed with Bonferroni's post-hoc test for comparison between "PAOPA" and "PAOPA + QUIN" effects.

1.7 VMAT-2 inhibition decreases DA accumulation inside synaptic vesicles

I have shown the increase of DA accumulation in presynaptic neurons, which was due to the continuity of DA synthesis during tissue samples incubations, as documented by González-Sepúlveda et al. (fig. 4-1). The DA synthesized is then uptaken, encapsulated and stored inside the synaptic vesicles by an integral membrane protein VMAT-2 (German et al., 2015). Here, I would like to analyze quantitatively the role of VMAT-2 controlling this activity on DA accumulation with TBZ, a VMAT-2 inhibitor. In agreement with previous results by González-Sepúlveda et al. using [^{3}H]-Tyr to [^{3}H]-DA synthesis, my quantitative analysis shows that TBZ significantly decreases uptake of endogenous DA into synaptic vesicles, resulting in a decrease of DA levels with the maximal effects (E_{max}) of 87.0% and EC_{50} at 41.3nM, $p<0.01$ (fig. 4-7b). The significant effect of TBZ to deplete DA storage can be seen with a decrease of DA concentration beyond the basal level, which is the concentration of DA detected in the minced tissue before being accumulated during sample incubations. Such conditions lead to an increase of cytosolic DA that will: (1) modulate a negative-feedback on TH (as was proven by González-Sepúlveda et al., submitted); and (2) be metabolized by MAO and aldehyde dehydrogenase, as shown, with a significant increase of DOPAC levels, EC_{50} at 106.0nM, $p<0.01$ (fig. 4-7c). This effect leads to an increase in the DOPAC/ DA concentration ratio (fig. 4-7d). By comparing both the EC_{50} to decrease of DA accumulation and to increase DOPAC levels, it is concluded that TBZ action on VMAT-2 happens at slightly lower concentrations than its effect to increase DOPAC levels. This suggests a primary action of DA to exert a negative-feedback inhibition on TH than a secondary effect to be metabolized into DOPAC, the DA that cannot be stored.
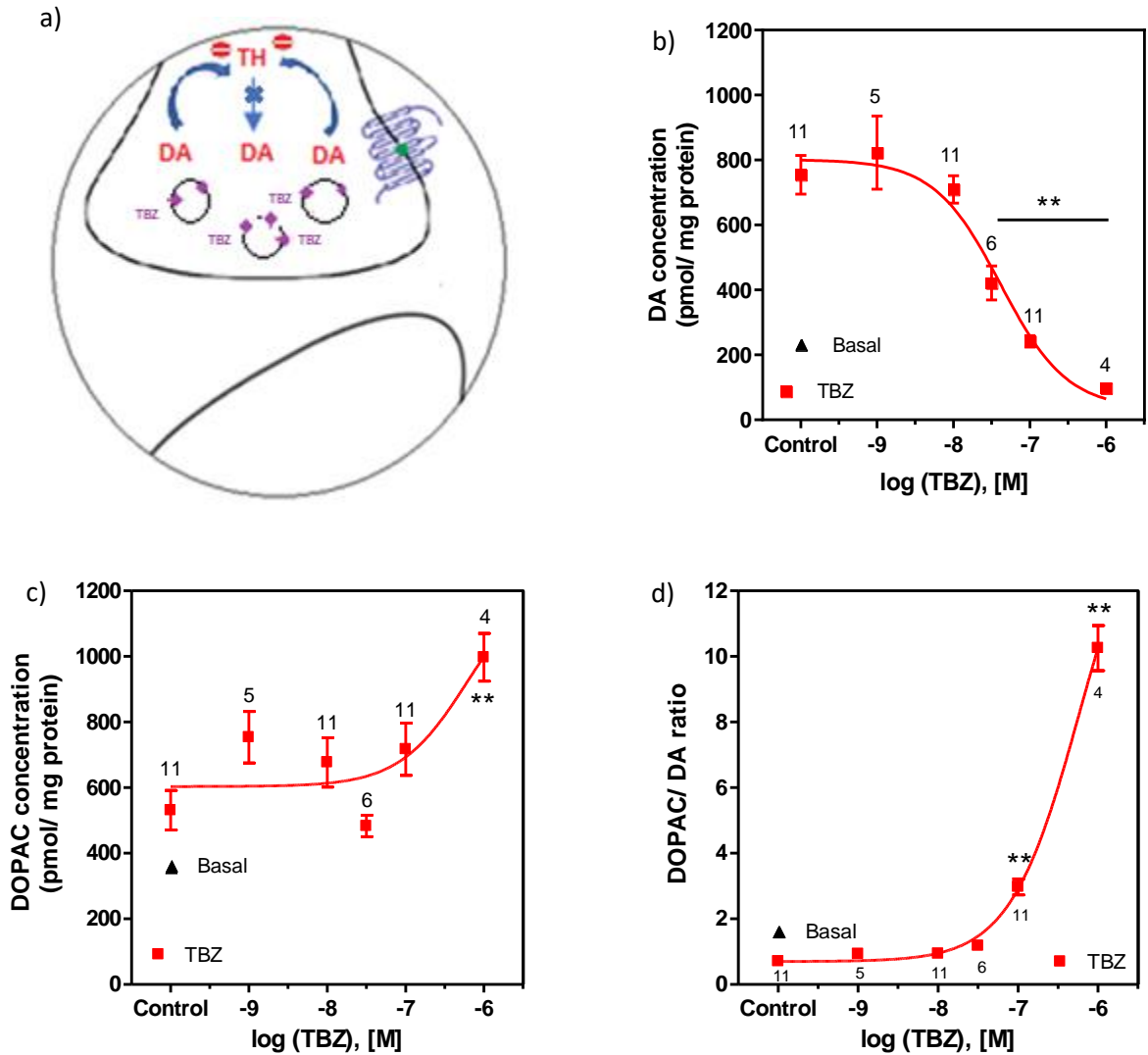
Fig. 4-7. (a) Schematic illustration on the effect of TBZ to deplete DA storage via VMAT-2 inhibition, thus increasing cytosolic DA which can modulate a negative-feedback inhibition on TH. This mechanism is shown by the concentration-response curves of TBZ on endogenous (b) DA accumulation, (c) DOPAC levels and (d) DOPAC/ DA concentration ratio. The data are expressed as mean ± SEM from two brain incubations with n shown in the graph. Basal levels in non-incubated samples are shown by ▲. **$p<0.01$ vs control, one-way ANOVA followed with Dunnett's post-hoc test. The data were adjusted into the sigmoidal dose-response curve for DA accumulation, DOPAC levels and the DOPAC/ DA concentration ratio.

1.8 VMAT-2 inhibition by TBZ at different incubation times

As mentioned in the methodology, the effect of DA accumulation was studied with the addition of TBZ at the beginning of the 2h sample incubations. To know the minimum time required for TBZ to block VMAT-2 action, different incubation times were used. For this, 30nM concentration was chosen for TBZ, because this concentration showed an intermediate and significant decrease in endogenous DA accumulation (fig. 4-7). I found that TBZ significantly inhibited VMAT-2 action, caused a decrease in endogenous DA accumulation in as short as 10-min drug incubation time, and this effect was increased with longer drug incubations (fig. 4-8a). Similarly to the previous result (fig. 4-7), higher DOPAC concentration was observed from DA metabolism (fig. 4-8b). The DOPAC levels were found to be significantly increased at 30-min drug incubation time. This result also indicates that the prominent action of cytosolic DA in modulating negative-feedback inhibition on TH to decrease DA accumulation was faster than the time required for DA to be metabolized. Overall, from both data on the effect of DA and DOPAC concentrations, I estimate a significantly delayed increase in the DOPAC/ DA concentration ratio, indicating higher efficacy of TBZ on the inhibition of DA accumulation, with longer time incubations and a delayed DA metabolism as a consequence of the previous effect (fig. 4-8c)
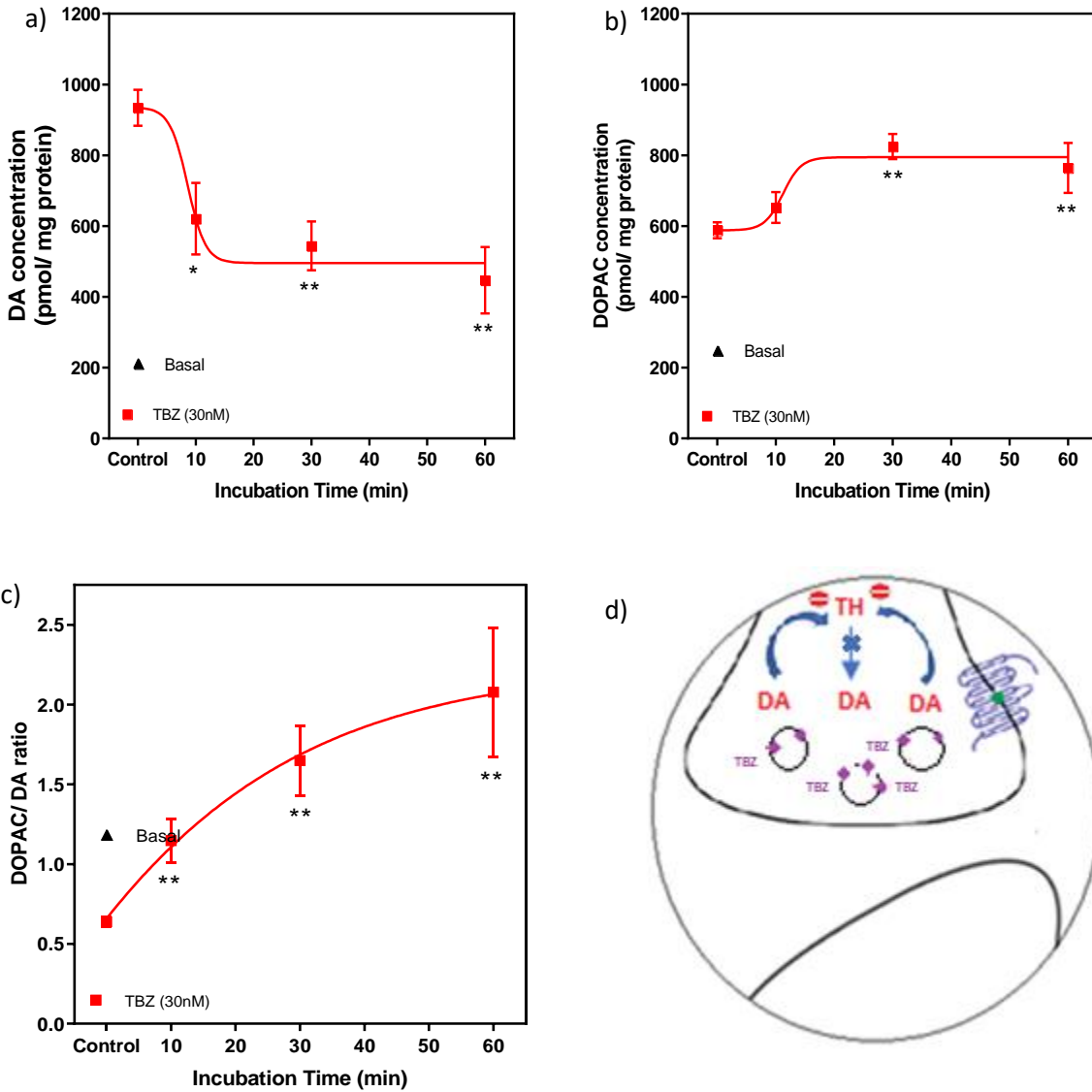
Fig. 4-8. Effects of different TBZ incubation times on endogenous (a) DA concentration, (b) DOPAC levels, and (c) the DOPAC/ DA concentration ratio at 2mM K^+. (d) A schematic diagram describing the effect of TBZ to deplete DA storage via VMAT-2 inhibition, thus increasing cytosolic DA, which can exert a negative-feedback inhibition on TH. The data are expressed as mean $\pm$ SEM (n=6 incubations per data point) from one brain. Basal levels in non-incubated samples are shown by ▲. *p<0.05 and **p<0.01 vs control, one-way ANOVA followed with Dunnett's post-hoc test. The data were plotted into the sigmoidal dose-response curve for both DA accumulation and the DOPAC/ DA concentration ratio, sigmoidal dose-response with variable slope curve for DOPAC levels.

1.9 Cytosolic DA negative-feedback overcomes D₂R activation effects on DA accumulation

I have previously shown that ARI significantly decreased endogenous DA accumulation via the activation of D_2R (fig. 4-2), but TBZ differently decreased DA accumulation via the inhibition of VMAT-2 to increase cytosolic DA, which can modulate a negative-feedback inhibition on TH (fig. 4-7). While the relationship between the effect of decreasing DA accumulation was dependent on the concentration of ARI (as I will show in fig. 4-18), the dose-response effect of TBZ was not obvious. At low concentrations (≤10nM), TBZ did not mediate the decreasing effects, suggesting a narrow window separating clinical benefits from excessive unwanted side effects (fig. 4-7a, and similarly to previous results by González-Sepúlveda et al. using [^{3}H]-Tyr to [^{3}H]-DA synthesis). Therefore, it may be interesting to study the effects of the combination of ARI and TBZ on DA accumulation. The hypothesis behind this combination may be clinically relevant, due to the TBZ treatment of hyperkinetic disorders alone giving rise to a certain side effect. In clinical patients, TBZ is titrated slowly to bypass the threshold levels and find the most effective dose for each patient, and at the same time to avoid the side effects. In theory, such co-treatment with ARI may give the advantage of reducing TBZ doses if the combination will enhance its efficacy to decrease DA accumulation. Unfortunately, I observed no synergistic effect and interactions between both the combination of a subthreshold (1nM) and a standard (100nM) concentration, of ARI with TBZ on decreasing DA accumulation as no obvious displacements of TBZ concentration-response curve to the left was observed (fig. 4-9). This suggests VMAT-2 inhibition exerts a powerful inhibition of DA synthesis through cytosolic DA negative-feedback on TH, which overcomes the D_2R activation mediated effects. Although ARI has not been approved for the treatment of chorea, one study has shown that a high dose of ARI improves some of the symptoms of Huntington's

disease patients (Ciammola et al., 2009). I noticed the limitation of this study with regard to ARI dosage, where it was prescribed at a much higher dose in clinical patients as compared with other partial agonist antipsychotics. The fact that there is no synergism on DA accumulation does not refute the combination. Therefore, this experiment deserves to be studied further using higher concentrations of ARI, or with other partial agonist antipsychotics.
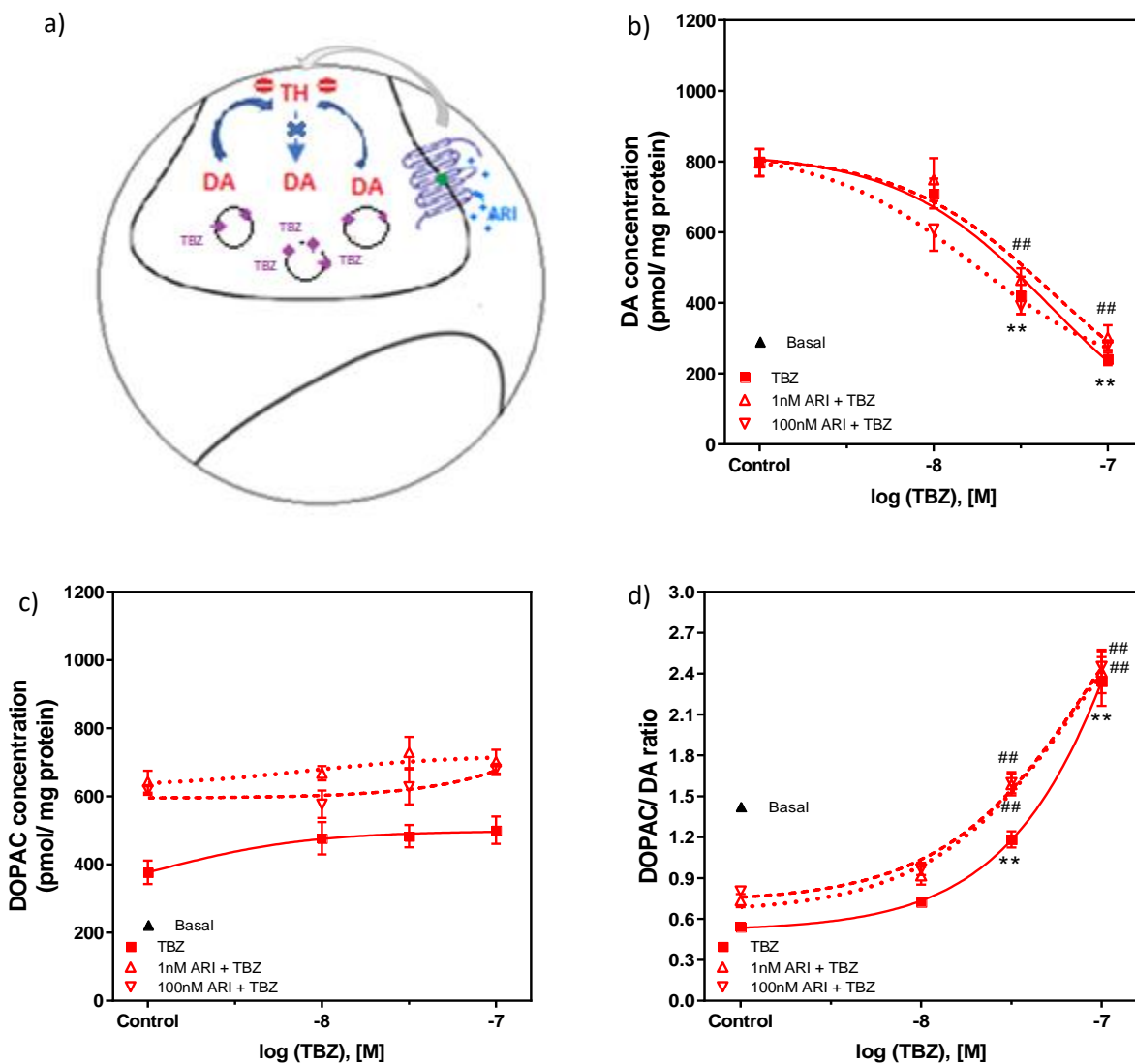
Fig. 4-9. (a) Schematic diagram illustrating the prominent effect of cytosolic DA to inhibit TH, which overcomes the simultaneous D_2R activation by ARI, as shown by the lack of synergistic effects between concentration-response curves of TBZ with and without the presence of 1nM or 100nM ARI on (b) DA accumulation, (c) DOPAC levels, and (d) the DOPAC/ DA concentration ratio. The data are expressed as mean ± SEM (n=6 incubations per data point). All incubations for each concentration-response curve were obtained from a single brain. Basal levels in non-incubated samples are shown by ▲. **p<0.01, one-way ANOVA followed with Dunnett's post-hoc test for TBZ alone vs control; ##p<0.01, one-way ANOVA followed with Dunnett's post-hoc test for comparison on the addition of 1nM or 100nM ARI and TBZ on decreasing DA accumulation vs control in the same brain incubation. The data were adjusted into the same sigmoidal dose-response curve for all analysis parameters of DA accumulation, DOPAC levels and the DOPAC/ DA concentration ratio.

1.10 AMPH decreases endogenous DA accumulation

Other than TBZ, another drug that indirectly interrupts DA storage is AMPH (Floor & Meng, 1996). As compared with TBZ, which may increase cytosolic DA levels, AMPH will induce DA release from presynaptic terminals and increase extracellular DA (Carboni et al., 1989). With the interruption of DA storage and increased DA release from dopaminergic terminals, I hypothesized that AMPH would reduce DA accumulation. This is putatively shown by the action of cytosolic DA in addition to the D_2R activation from exogenous DA to modulate a negative-feedback inhibition on TH, although a direct blocking of TH activity by AMPH by raising cytosolic DA was not documented. As expected, AMPH significantly decreased endogenous DA accumulation under non-depolarized conditions (2mM K^+) with the maximal effects (E_{max}) of 72.0% and EC_{50} at 147nM, $p<0.01$ (fig. 4-11b). In contrast, AMPH did not increase but slightly decreased DOPAC levels, $p<0.01$ (fig. 4-11c) indicating that less cytosolic DA was formed as compared with TBZ (fig. 4-7 and 4-8). This may be due to the combined effects of AMPH on the impairment of DA storage together with its action to block the reuptake activity of DAT and released DA stimulating D_2R (Sulzer et al., 2005). Overall, the well-known releasing action of AMPH was evidenced by an increase in the DOPAC/ DA concentration ratio, $p<0.01$ (fig 4-11d), but the small extent of this increase suggests D_2R stimulation by released DA, limiting both DA and DOPAC formation.
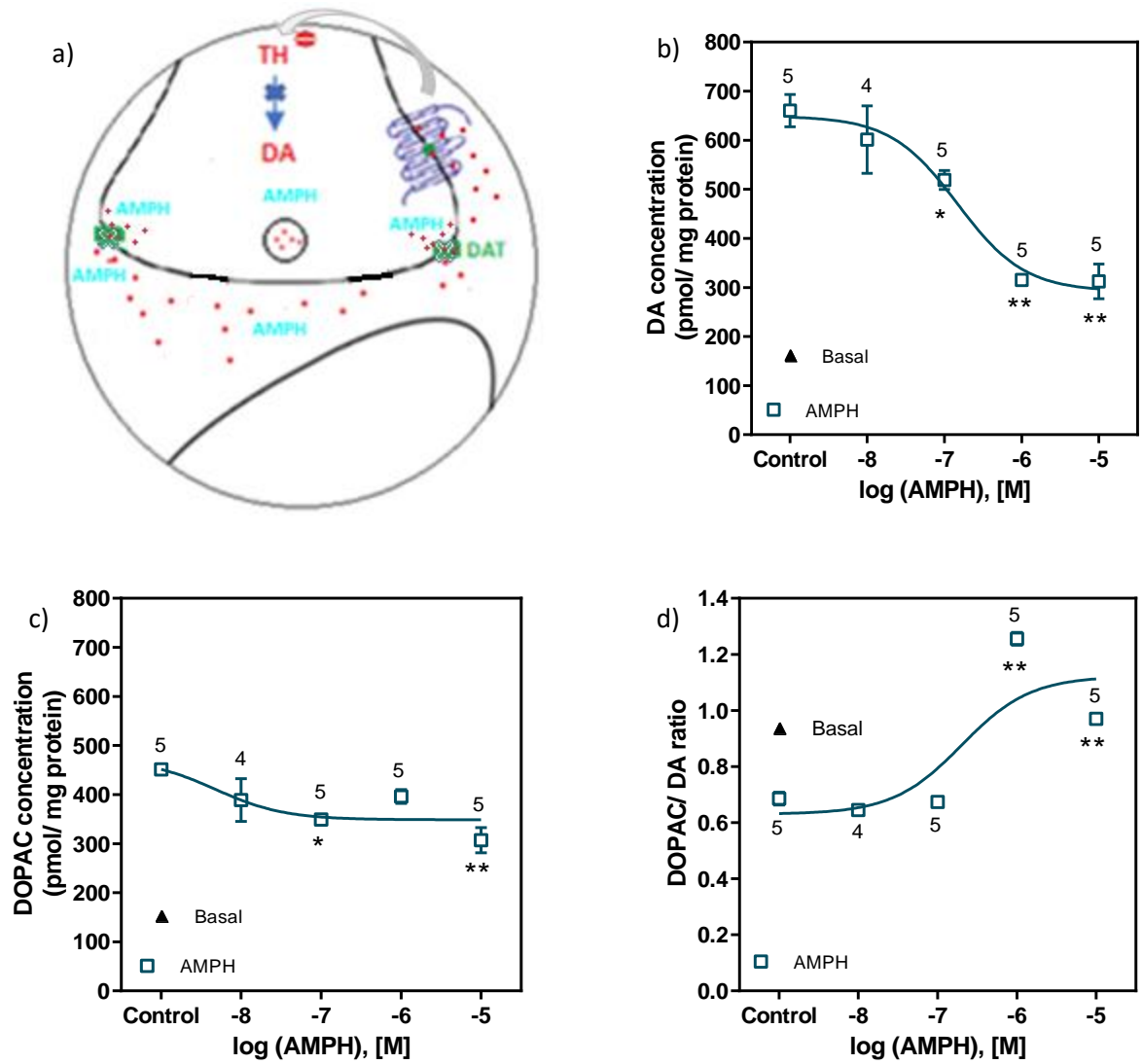
Fig. 4-10. (a) Schematic illustration of the effects of AMPH to decrease DA accumulation primarily via released DA stimulating D_2R, as shown by the concentration-response curves of AMPH during 120-min incubations under 2mM K^+ on endogenous (b) DA accumulation, (c) DOPAC concentration, (d) the DOPAC/ DA concentration ratio. The data are expressed as mean $\pm$ SEM from one brain incubation with n shown in the graph. Basal levels in non-incubated samples are shown by $\blacktriangle$. Asterisks indicate **$p<0.01$ vs control, one-way ANOVA followed with Dunnett's post-hoc test. The data were adjusted into the sigmoidal dose-response curve for DA accumulation, DOPAC levels and the DOPAC/ DA concentration ratio.

1.11 Effect of depolarization-induced by higher K$^+$ concentrations on DA accumulation

Neuronal depolarization via elevated K$^+$ concentrations is regularly used in *in-vitro* studies. In our *ex-vivo* studies, under basal conditions (2mM K$^+$) will produce what we call "a low dopaminergic tone". Increasing K$^+$ concentration to 15mM will induce depolarization and mimic a high dopaminergic tone, with approximately 6.6-fold DA release into the medium (Ma et al., 2015). Previously, I have shown that DA application into slice incubations inhibited TH, leading to a decrease in [^{3}H]-DA synthesis (fig. 4-3). Similarly, depolarization induced by higher K$^+$ concentrations significantly decreased DA accumulation, as shown by a significant interaction between incubation-time-dependent effects at both 2mM K$^+$ and 15mM K$^+$, p<0.001 (fig. 4-11b). Conversely, the levels of DOPAC did not decrease as depolarization increased DA release from dopaminergic terminals and increased its availability to be metabolized (fig. 4-11c). With high levels of DOPAC accumulated throughout the incubation in contrast with a decrease of DA accumulation, leading to an increase in the DOPAC/ DA concentration ratio under K$^+$-evoked depolarization (display by a significant interaction between incubation-time-dependent effects at both 2mM K$^+$ and 15mM K$^+$, p<0.001) (fig. 4-11d). These results are consistent with DA inhibition of its own synthesis and potentiation of metabolism after being released.
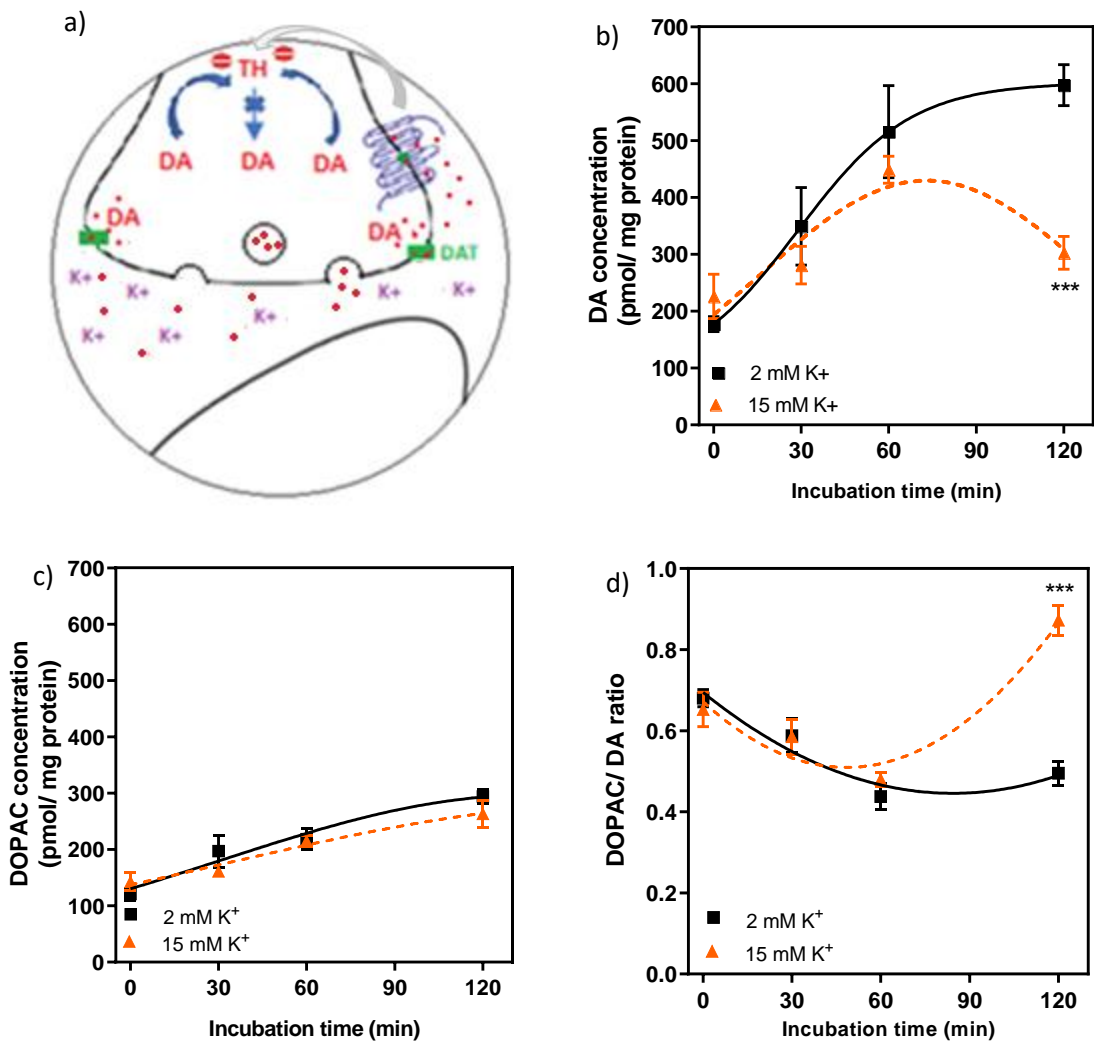
Fig. 4-11. (a) Schematic diagram displaying the effects of depolarization induced by increasing K+ concentrations, thus producing a high dopaminergic tone with DA mediated inhibition mechanisms of its own synthesis. This illustration describes these effects on endogenous (b) DA accumulation, (c) DOPAC levels, and (d) the DOPAC/ DA concentration ratio. The data are expressed as mean ± SEM (n=6 incubations per data point) from one brain for each group (2mM K+ or 15mM K+ group). ***p<0.001 vs 2mM K+ curve under incubation-time-dependent effects, two-way ANOVA followed with Bonferroni's post-hoc test. The data were adjusted into the following analysis parameters: panel (b) sigmoidal dose-response curve for both 2mM K+ and 15mM K+; panel (c) gaussian curve for 2mM K+ and 15mM K+; and panel (d) polynomial second-order curve for 2mM K+ and 15mM K+. The experiment was done in part of the work carried out by Nayadoleni Nieves Rivera for her Master's degree in Neurosciences in 2019, under the supervision of Dr. Jordi Ortiz and myself.

1.12 Effects of MPH under different experimental conditions induced by K^+ concentrations

Both AMPH and MPH block the reuptake of catecholamine neurotransmitters, including DA, by DAT. Previously, I have shown the effect of AMPH at presynaptic terminals in regulating DA accumulation (fig. 4-10). While the effects of AMPH at presynaptic terminals were independent of the properties of neurons under an action potential, other psychostimulants, including MPH, are known to act differently. The action of MPH, for instance, can be demonstrated subjected to the synaptic properties of membrane potential-dependent DA release, as shown in fig. 4-12. Under non-depolarized conditions (2mM K^+), MPH did not have any significant effect on presynaptic terminals based on its effect on DA accumulation, DOPAC and the DOPAC/ DA concentration ratio. As I induce depolarization with higher K^+ concentrations (based on the effect in fig. 4-11 and Ma et al., 2015), there is a significant decrease of DA accumulation (the same finding, as shown in fig. 4-11), thought by the action of cytosolic DA and D_2R stimulation by released DA, both mediate a negative-feedback inhibition on TH. Such depolarization also shown to increase DOPAC levels, indicating higher DA metabolism leading to an increase in the DOPAC/DA concentration ratio. In the presence of MPH, however, the highest MPH concentrations used clearly reverse the effects shown on DA, DOPAC and the DOPAC/ DA concentration ratio, thus indicating a prominent action by DAT to inhibit TH through cytosolic DA as compared with stimulating D_2R by DA-released mechanisms. Overall, the effects of MPH at presynaptic terminals in regulating DA accumulation are explicitly dependent on membrane potential induced by depolarization at higher K^+.

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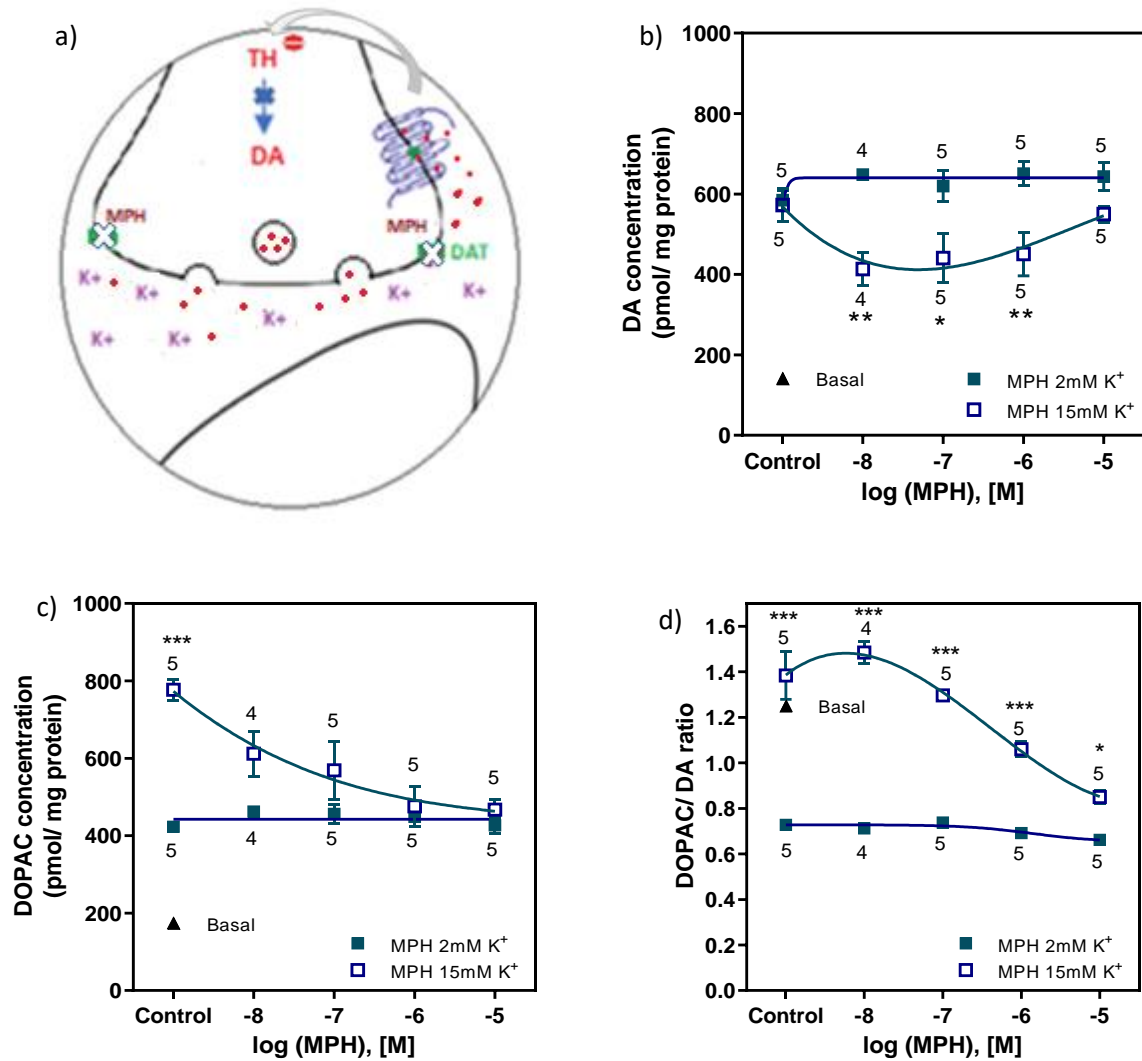
Fig. 4-12. (a) Schematic diagram displaying the action of MPH to block the reuptake of DA from extracellular milieu under depolarization induced by 15 mM K$^+$. This mechanism shows that MPH reverses the effects of depolarization induced by 15mM K$^+$ on (b) DA accumulation, (c) DOPAC levels, and (d) the DOPAC/ DA concentration ratio. The data are expressed as mean ± SEM from one brain incubation for each group (2mM K$^+$ or 15mM K$^+$ group) with n shown in the graph. Basal levels in non-incubated samples are shown by ▲. Asterisks indicate *p<0.05 and **p<0.01, two-way ANOVA followed with Bonferroni's post-hoc test between the two groups. The data were adjusted into the sigmoidal dose-response curve for 2mM K$^+$ curves and polynomial third order for 15mM K$^+$ curves.

Results

2. **Second objective: Examine and compare the mechanism of actions of D_2-like partial agonist antipsychotics at presynaptic D_2R.**

The properties of QUIN and ARI at presynaptic D_2R have been previously documented using the same $[^3H]$-DA synthesis method in rat striatal brain samples incubated *ex-vivo* (Ma et al., 2015). For this reason, these compounds were used as references in this study, where ARI, CARI and BREX, D_2R partial agonist antipsychotics have been compared. LUMA, however, was not included because it was only recently approved by the U.S. F.D.A. in December 2019, after the planning and start of this Ph.D. thesis work. In addition, I have taken advantage of the spontaneous accumulation of endogenous DA and DOPAC in the samples when they are incubated under non-depolarizing conditions, as shown in the section above. Obtaining more information about the effects of all three D_2-like partial agonist antipsychotics will increase our understanding of the mechanisms contributing to the discrepancies in their functional properties and efficacy at presynaptic D_2R.

2.1 D₂R agonist and partial agonist antipsychotics decrease [³H]-DA synthesis

The similarities and the differences in the functional properties and efficacy of D_2R partial agonist antipsychotics were examined by monitoring the changes in [³H]-DA synthesis in rat brain striatum incubated *ex-vivo*. At non-depolarizing conditions (2mM K^+), QUIN activated D_2R and significantly inhibited [³H]-DA synthesis with the E_{max} of 51%; $p<0.001$. Under the same experimental condition, the activation of D_2R by partial agonist antipsychotics inhibited [³H]-DA synthesis dose-dependently, as shown in fig. 4-13. ARI significantly inhibited [³H]-DA synthesis at much lower concentration (10nM) with the E_{max} of 51%; $p<0.01$. However, CARI and BREX showed this effect at much higher concentrations (100nM for CARI and 1μm for BREX) with the E_{max} of 52%; $p<0.01$; (CARI), and 75%; $p<0.01$ (BREX). I concluded that BREX shows higher efficacy on the inhibition of [³H]-DA synthesis based on the E_{max} at the maximum concentrations used in this experiment, while ARI and CARI have almost equal efficacy with an intermediate efficiency in inhibiting [³H]-DA synthesis. In this method, I also noted that QUIN, which is a full D_2R agonist, was not able to completely inhibit [³H]-DA synthesis (similarly to the report by Ma et al., 2015); this may be due to the limitation of the higher QUIN concentrations used, which did not give higher effects or the incubation time (10-min) for the drug in this experiment.
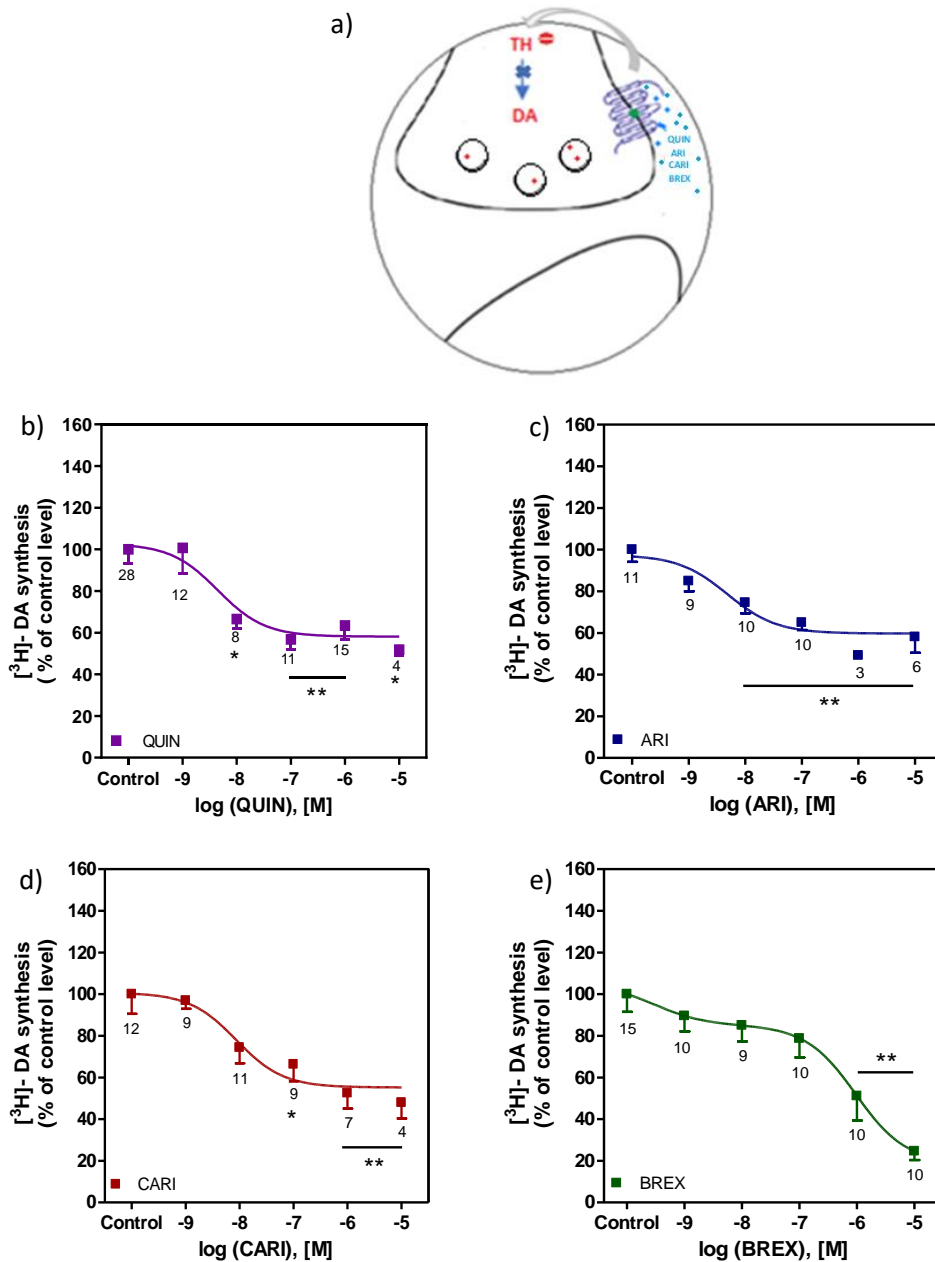
Fig. 4-13. (a) Schematic illustration of the mechanism of D_2 agonist and partial agonist antipsychotics at 2mM K^+ is thought to modulate a negative-feedback inhibition on TH via stimulating D_2R. Results represent the concentration-response curves of D_2R agonist and partial agonist antipsychotics on striatal $[^3H]$-DA synthesis *ex-vivo* for (b) QUIN, (c) ARI, (d) CARI, and (e) BREX. The data are expressed as mean ± SEM for each brain incubation normalized to the control values with n shown in the graph. Asterisks indicate *p<0.05 and **p<0.01 vs control, one-way ANOVA followed with Dunnett's post-hoc test. The data were adjusted into a sigmoidal dose-response curve for QUIN, ARI and CARI; two-site competition for BREX. The effect of QUIN on $[^3H]$-DA synthesis was in part of the work carried out by Anna Martínez Rivas for her Master's degree in Biochemistry, Molecular Biology and Biomedicine in 2018 under the supervision of Dr. Jordi Ortiz and myself.

115

2.2   *D_2R selectivity for agonist and partial agonist antipsychotics on [^3H]-DA synthesis*

I have discussed D_2R partial agonist antipsychotics' property to bind with modest affinities to different receptors (Kiss et al., 2010; K. Maeda et al., 2014; Shapiro et al., 2003), which may indirectly regulate DA synthesis. To determine D_2R specificity for D_2R agonist and partial agonist antipsychotics on the inhibition of [^3H]-DA synthesis, we further combined the D_2R agonist and partial agonist antipsychotics with 1μM SUL, a selected D_2R antagonist (fig. 4.14). We found that under the same conditions, the presence of 1μM SUL could significantly block QUIN, ARI and CARI effects on the inhibition of [^3H]-DA synthesis at the concentrations up to 1μM, as shown by a significant interaction between SUL and non-SUL groups, $p<0.05$ (QUIN vs QUIN+SUL), $p<0.001$ (ARI vs ARI+SUL) and $p<0.05$ (CARI vs CARI+SUL). Interestingly, 1μM SUL fully blocked the effects of BREX on the inhibition of [^3H]-DA synthesis at much lower concentrations (up to 10nM only), but with no significant interaction between the two groups (BREX vs BREX+SUL), $p>0.05$. 1μM SUL could not eliminate completely the effects of either QUIN, ARI or CARI at 10μM, probably due to competition against a molecule with a higher affinity for D_2R; however, their concentration-response curves can be seen as a displacement to the right. The effects of BREX at the concentration of 100nM and above were not blocked by 1μM SUL. Therefore, the concentration-response curve of BREX best fitted with a two-site competition model, considering the higher efficacy at the highest concentrations. This model described the specificity of BREX, involving more than one mechanism or receptor. However, for ARI, CARI and QUIN this effect was found at higher concentrations and fitted well in the sigmoidal dose-response curve. To conclude, the comparison between two concentration-response curves of D_2R agonist and partial agonist antipsychotics with and without prior treatment with 1μM SUL

demonstrates the concentrations related to D_2R specificity, mediated by the activation of D_2R mechanisms (D_2R-dependent)
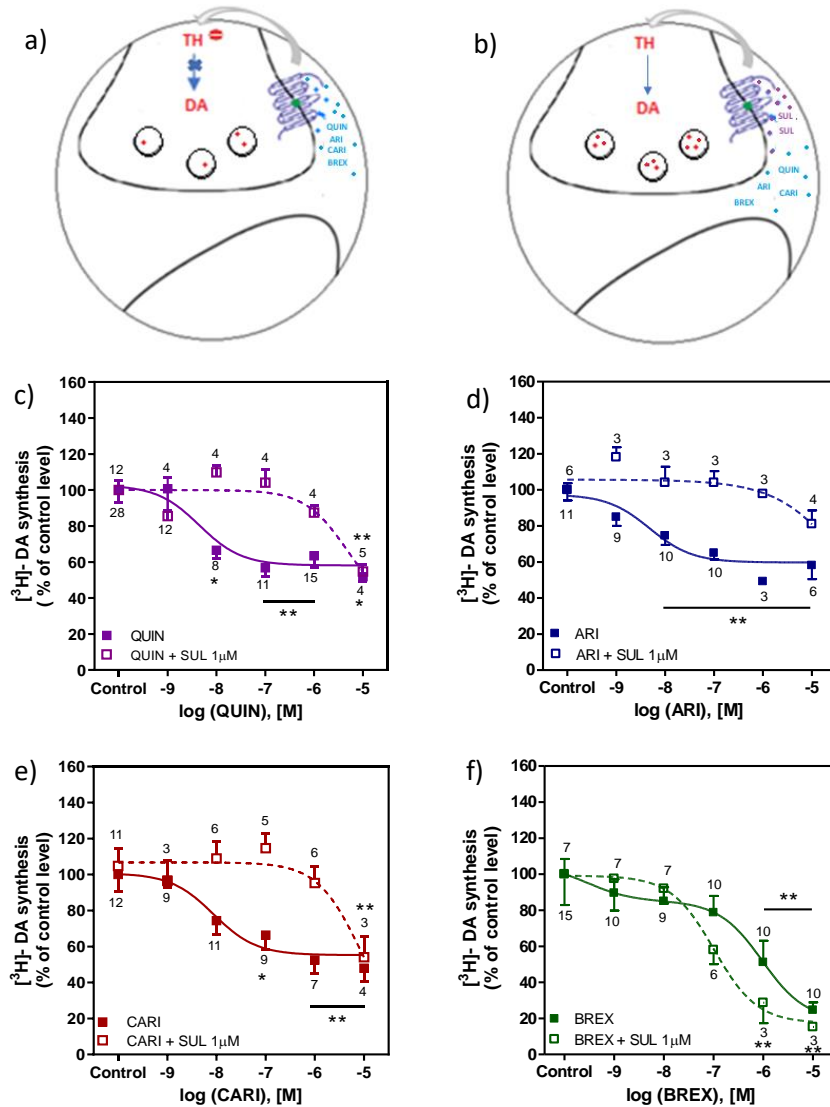
Fig. 4.14. Schematic diagrams illustrating the specificity of the effect of D_2 agonist and partial agonist antipsychotics to decrease [^{3}H]-DA synthesis. This was tested with (a) and without (b) in the presence of 1μM SUL to block D_2R stimulations. Results are represented as a comparison between concentration-response curves on the effects of D_2R agonist and partial agonist antipsychotics, with and without pretreatment with 1μM SUL on striatal [^{3}H]-DA synthesis *ex-vivo* for (c) QUIN, (d) ARI, (e) CARI, and (f) BREX. The data are expressed as mean ± SEM for each brain incubation, normalized to the respective control values with n shown in the graph. Asterisks indicate *p<0.05 and **p<0.01 of QUIN/ARI/CARI/BREX vs control (DRUG or DRUG+SUL group), one-way ANOVA followed with Dunnett's post-hoc test from the same group. The data were adjusted into the sigmoidal dose-response curve for both QUIN and QUIN+SUL in panel (c), ARI and ARI+SUL in panel (d), and CARI and CARI+SUL in panel (e). The data were adjusted into two-site competition for BREX and sigmoidal dose-response curve for BREX+SUL in panel (f). The effect of QUIN with and without pretreatment with 1μM SUL on [^{3}H]-DA synthesis was in part of the work carried out by Anna Martínez Rivas for her Master's degree in Biochemistry, Molecular Biology and Biomedicine in 2018 under the supervision of Dr. Jordi Ortiz and myself.

2.3 *Sustained effects of D₂R agonist and partial agonist antipsychotics on endogenous DA accumulation*

To gain further insight into the inhibition of [^{3}H]-DA synthesis by all D₂R agonist and partial agonist antipsychotics, I quantified endogenous DA levels, under the hypothesis that a decrease of DA synthesis will affect storage. To study the effect of the drug on DA accumulation, the samples were treated at the beginning of the tissue samples' 120-min incubation. Thus, to compare the efficacy and the properties of D₂R agonist and partial agonist antipsychotics on endogenous DA accumulation, it was necessary to see if the effect of the drugs was sustained over the end period of time incubations. 1µM concentration was chosen for all the drugs related to the concentration which showed significant inhibition of [^{3}H]-DA synthesis (fig. 4-13). As shown in fig. 4.2, 1µM QUIN and 1µM ARI were shown to significantly block endogenous DA accumulation with different efficacy and remain blocked for 120-min incubations. Under the same experimental condition, a similar effect was also observed with 1µM BREX and 1µM CARI. BREX showed a significant decrease in DA accumulation after 60-min incubations that remained for 120-min incubations, $p<0.01$ and $p<0.001$ respectively, while 1µM CARI only showed this effect at 120-min incubations, $p<0.001$ (fig. 4-15). Provided that this experiment was done under non-depolarizing conditions, the effect for both D₂ agonist and partial agonist antipsychotics is thought to modulate a negative-feedback inhibition on TH by stimulating D₂R, probably void of extracellular DA, as 2mM K$^+$ prevent DA release. The same effect was also observed on DOPAC concentration, as shown earlier for both 1µM QUIN and 1µM ARI (fig. 4-2). Similar to 1µM ARI, a decreased DOPAC concentration can also be observed with 1µM CARI, $p<0.01$ at 120-min incubations (fig. 4-16). Contrary to 1µM QUIN, 1µM ARI and 1µM CARI, 1µM BREX increased

the DOPAC concentration, as shown by a statistically significant interaction with control curves at 120-min incubations, p<0.001. In order to show the relation of the ligands' activity towards both decreasing DA synthesis and its metabolisms at the same time, I plotted the DOPAC/ DA concentration ratio. Under this correlation, I found that only QUIN significantly decreased the DOPAC/ DA concentration ratio, while D_2R partial agonist antipsychotics increased it, with CARI and BREX showing a significant effect, p<0.001 (fig. 4-17). These results showed the sustained effect of D_2R agonist and partial agonist antipsychotics at presynaptic terminals in 120-min incubations to decrease DA synthesis, which will lead to a decrease in DA accumulation. Thus, a decrease or increase of DOPAC levels in relation to a decrease of DA accumulation elicited by D_2R agonist and partial agonist antipsychotics respectively indicates a different mechanism involved.

Fig. 4-15. (a) Schematic illustration of the mechanism of D_2 agonist and partial agonist antipsychotics to modulate a negative-feedback inhibition on TH via stimulating D_2R. Such activation leads to a decrease in DA accumulation, as indicated by (b) QUIN, (c) ARI, (d) CARI, and (e) BREX under different incubation times. The data are expressed as mean $\pm$ SEM (n=4 incubations per data point). All incubations from one graph were obtained from a single brain. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs control curve under incubation-time-dependent effects, two-way ANOVA followed with Bonferroni's post-hoc test. The data were adjusted into the following analysis parameters: panel (b) sigmoidal dose-response for control curve and one-phase exponential decay for QUIN curve; panel (c) sigmoidal dose-response for both control and ARI curve; panel (d) sigmoidal dose-response for control curve and gaussian for CARI curve; and panel (e) sigmoidal dose-response for control curve and gaussian for BREX curve.

121

Fig. 4-16. (a) Schematic illustration of the mechanism of D_2 agonist and partial agonist antipsychotics to modulate a negative-feedback inhibition on TH via stimulating D_2R. This activation also leads to a decrease in DOPAC levels, as indicated by (b) QUIN, (c) ARI, and (d) CARI, but not (e) BREX under different incubation times. The data are expressed as mean ± SEM (n=4 incubations per data point). All incubations from one graph were obtained from a single brain. *p<0.05, **p<0.01 and ***p<0.001 vs control curve under incubation-time-dependent effects, two-way ANOVA followed with Bonferroni's post-hoc test. The data were plotted into sigmoidal dose-response in all control and ligands' curves.
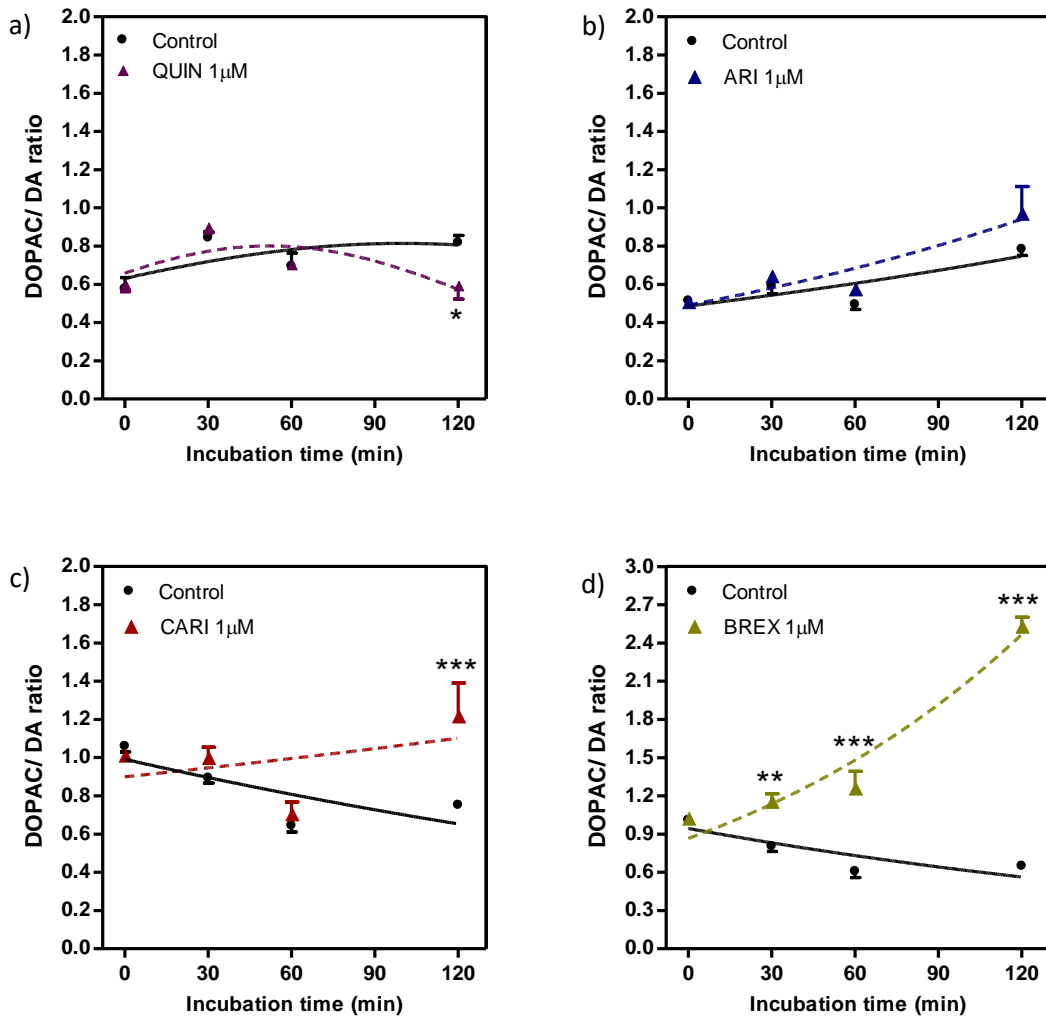
Fig. 4-17. The DOPAC/ DA concentration ratio over different incubation times with the effect of (a) QUIN, (b) ARI, (c) CARI, and (d) BREX. The data are expressed as mean ± SEM (n=4 incubations per data point). All incubations from one graph were obtained from a single brain. *p<0.05, **p<0.01 and ***p<0.001 vs control curve under incubation-time-dependent effects, two-way ANOVA followed with Bonferroni's post-hoc test. The data were plotted into the following analysis parameters: panel a) gaussian for both control and QUIN curve; panel b) gaussian for both control and ARI curve; panel c) exponential growth for both control and CARI curve; and panel d) exponential growth for both control and BREX curve.

2.4 D₂R-dependent effects of agonist and partial agonist antipsychotics decrease endogenous DA accumulation

With a sustained effect of the drugs on DA accumulation and DOPAC levels in 120-min incubation, as shown in fig. 4-15, 4-16 and 4-17, the effect of D_2R agonist and partial agonist antipsychotics on the spontaneous DA accumulation were examined by monitoring the changes in the concentration of endogenous DA and DOPAC with and without the combination of 1μM SUL at 2mM K^+ to match the experiments on $[^3H]$-DA synthesis. In line with a decrease in $[^3H]$-DA synthesis, as shown in fig. 4-13, D_2R agonist and partial agonist antipsychotics significantly decreased DA accumulation dose-dependently, $p<0.01$ (fig. 4-18). I also found that under the same conditions the presence of 1μM SUL could significantly block QUIN, ARI and CARI effects on DA accumulation, as shown by a significant interaction between SUL and non-SUL groups, $p<0.01$ (QUIN vs QUIN+SUL), $p<0.001$ (ARI vs ARI+SUL) and $p<0.05$ (CARI vs CARI+SUL), up to 1μM concentration. Similarly to the effect on $[^3H]$-DA synthesis, 1μM SUL can also block the effect of BREX on DA accumulation at much lower concentrations (up to 100nM only), but no significant interactions between BREX vs BREX+SUL groups were found, $p>0.05$. I conclude that D_2R agonist and partial agonist antipsychotics had a similar effect of inhibiting $[^3H]$-DA synthesis and decreasing endogenous DA accumulation, and that the presence of 1μM SUL did not block the effect of QUIN, ARI and CARI at 10μM, with BREX at much lower concentrations (>100nM).
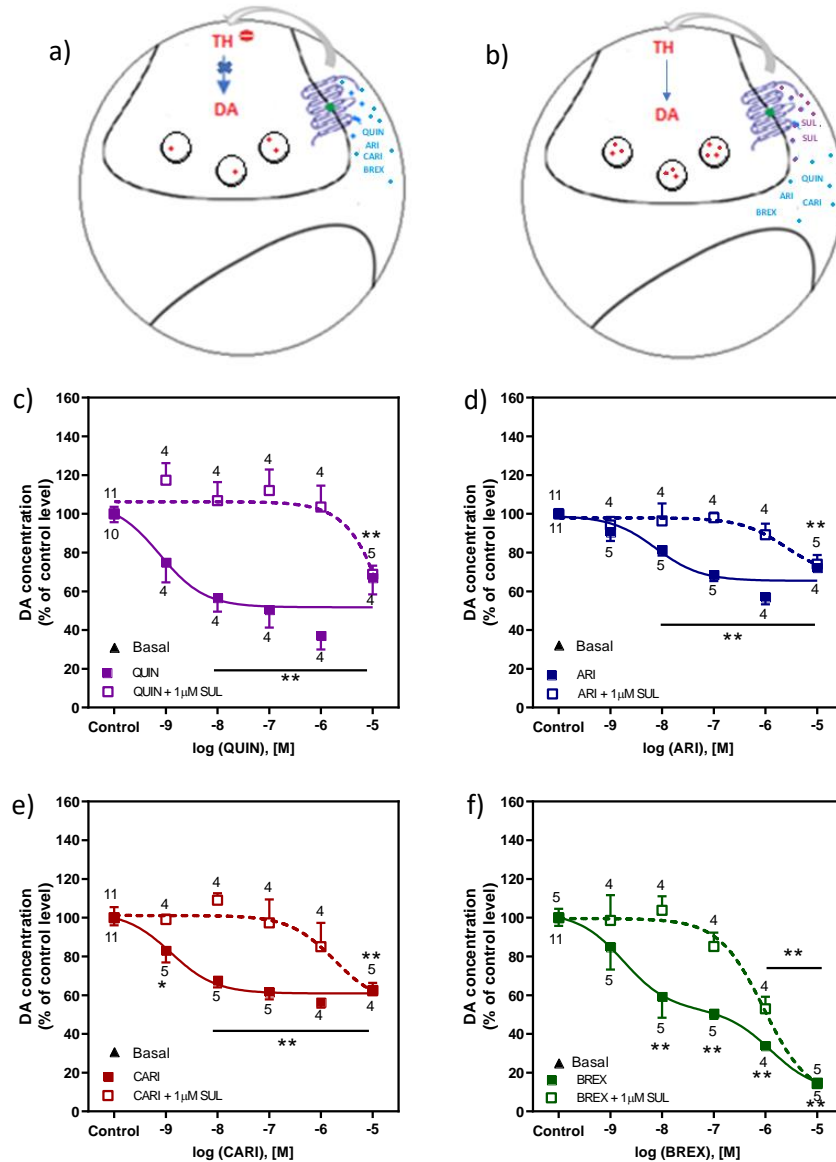
Fig. 4-18. Schematic diagrams illustrating the specificity of the effect of D_2 agonist and partial agonist antipsychotics to decrease DA accumulation, which was tested with (a) and without (b) in the presence of 1μM SUL to block D_2R stimulations. The results are represented as a comparison between concentration-response curves on the effects of D_2R agonist and partial agonist antipsychotics with and without pretreatment with 1μM SUL on the spontaneous accumulation of endogenous DA during 120-min incubations with (c) QUIN, (d) ARI, (e) CARI, and (f) BREX. The data are expressed as mean ± SEM from two brain incubations for each group (DRUG or DRUG+SUL group), normalized to the control values, with n shown in the graph. Basal DA levels in non-incubated samples are shown by ▲. Asterisks indicate *p<0.05 and **p<0.01 vs control (DRUG or DRUG+SUL group), one-way ANOVA followed with Dunnett's post-hoc test from the same group. The data were adjusted into the sigmoidal dose-response curve for both QUIN and QUIN+SUL in panel (c), ARI and ARI+SUL in panel (d), and CARI and CARI+SUL in panel (e). The data were adjusted into two-site competition for BREX and sigmoidal dose-response curve for BREX+SUL in panel (f).

2.5 Similar D$_2$R antagonist property of CARI and ARI which depends on dopaminergic tone differently to BREX

I have shown that under basal conditions of 2mM K$^+$, D$_2$R partial agonist antipsychotics significantly inhibit [^{3}H]-DA synthesis (fig. 4-13), but ARI loses this property as the K$^+$ concentration increases, becoming an antagonist due to its lower efficacy as agonist than QUIN or DA (Ma et al., 2015). In order to assess if the antagonist properties of ARI at high dopaminergic tone were unique to this compound or can also be observed for both CARI and BREX, 1µM concentration was chosen for CARI and BREX, while 100nM was chosen for ARI and QUIN, as these concentrations significantly inhibit [^{3}H]-DA synthesis under 2mM K$^+$ (fig. 4-13). Interestingly, I observed that the properties of 1µM CARI as an agonist disappeared as I gradually increased K$^+$ concentrations (2mM K$^+$, $p<0.01$, 4mM K$^+$, $p<0.05$, 6mM K$^+$, $p>0.05$, 10mM K$^+$, $p>0.05$, and 15mM K$^+$, $p>0.05$ (fig. 4-19c)), thus showing similar properties as 100nM ARI (Ma et al., 2015). Such conditions suggest that – similarly to ARI (Ma et al., 2015) – CARI acts as an antagonist due to its property of having lesser agonist efficacy than extracellular DA being released at this condition. Further combination with the full agonist QUIN proved that CARI behaves as an antagonist and fully blocks QUIN effect under 15mM K$^+$ dopaminergic tone, $p<.001$ (fig. 4-19d).

Under the same experimental conditions, however, 1µM BREX significantly decreased [^{3}H]-DA synthesis irrespective of K$^+$ concentrations: 2mM K$^+$, $p<0.05$, 4mM K$^+$, $p<0.01$, 6mM K$^+$, $p<0.001$, 10mM K$^+$, $p<0.001$, and 15mM K$^+$, $p<0.001$ (fig. 4-19e). This result suggests that the [^{3}H]-DA synthesis inhibition mediated by D$_2$R-independent effects of BREX enhances its apparent intrinsic activity on DA synthesis, producing a tonic effect under 15mM K$^+$. 100nM QUIN was also studied for comparison. Consistently with the typical D$_2$R agonist effects observed

on the inhibition of [^{3}H]-DA synthesis under 2mM K$^+$ (fig. 4-13), 100nM QUIN also acted as a stable agonist under 15mM K$^+$, $p>0.05$ (fig. 4-19f). I conclude that a distinctive pharmacodynamic property of 1μM BREX from other D$_2$R partial agonist antipsychotics on DA synthesis is that BREX effects are not dependent on D$_2$R and depolarization-induced DA release.
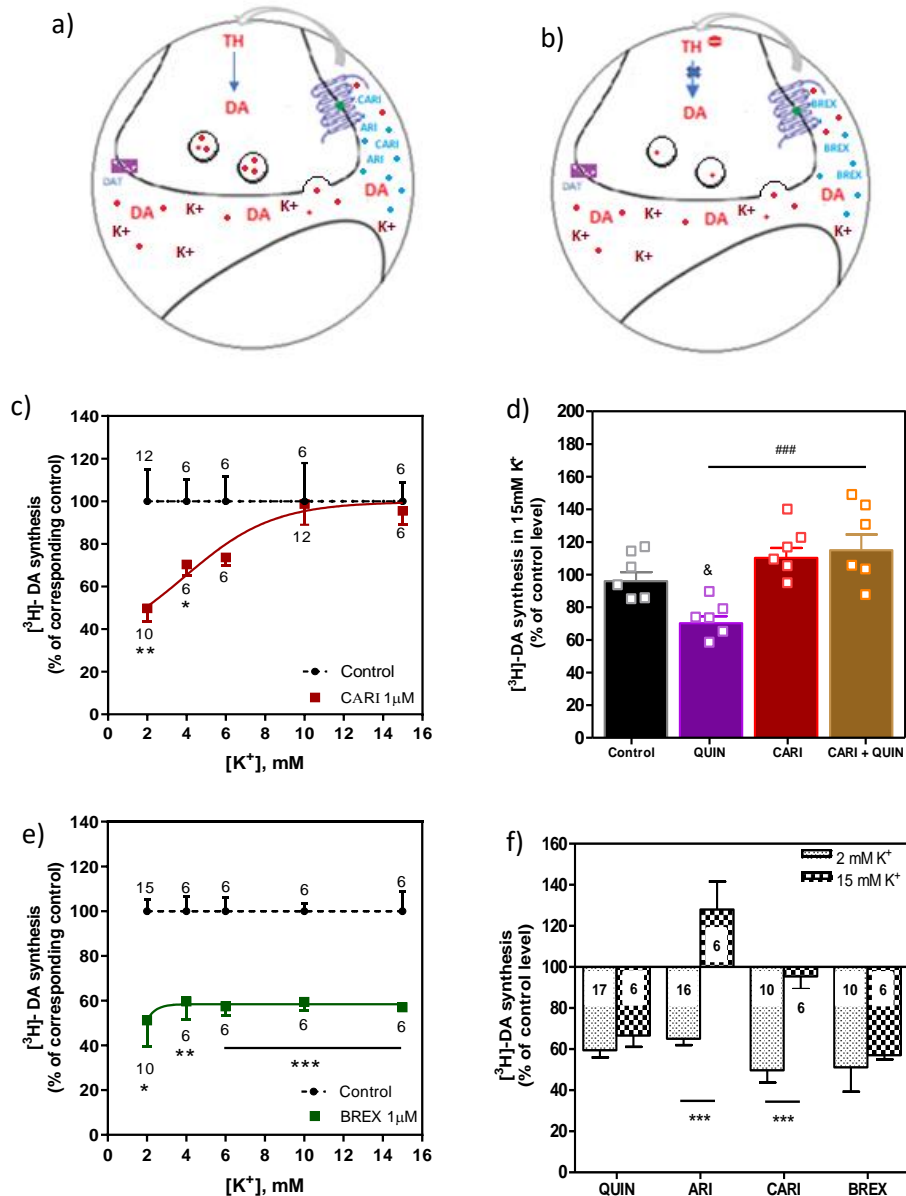
Fig. 4-19. Schematic illustration summarize that K^+ depolarization impairs CARI- and ARI (a)- but not BREX (b)-inhibition of $[^3H]$-DA synthesis. (c) K^+ concentration-response curves of 1μM CARI, (d) antagonist properties of 100nM CARI significantly block 100nM QUIN inhibition on DA synthesis at 15mM K^+, (e) K^+ concentration-response curves of 1μM BREX, and (f) comparison of 100nM QUIN, 100nM ARI, 1μM CARI and 1μM BREX between 2mM K^+ and 15mM K^+ conditions on the inhibition of DA synthesis. The data are expressed as mean ± SEM for each brain incubation normalized to the respective control group under the same K^+ concentrations with n shown in the graph/bar. **$p<0.01$ and ***$p<0.001$, unpaired Student's t-test for comparison between drug and respective control under the same K^+ concentrations; &$p<0.05$ vs control in the same brain incubations, one-way ANOVA followed with Dunnett's post-hoc test; and ###$p<0.001$, one-way ANOVA followed with Bonferroni's post-hoc test for comparison between "QUIN" and "QUIN+CARI" effects at 15mM K^+. The data were adjusted into the sigmoidal dose-response curve for both CARI and BREX in panel (c) and (e).

128

2.6 Biphasic effect of D_2R partial agonist antipsychotics on endogenous DOPAC concentration

I have shown that under non-depolarized conditions of 2mM K^+, D_2R agonist and partial agonist antipsychotics significantly decreased endogenous DA accumulation (fig. 4-18). I hypothesized the same effects could be observed with DOPAC under these conditions, as decreasing the level of DA being synthesized at presynaptic terminals will contribute to a decrease in the amount of cytosolic DA, which can be catabolized. Reinforcing previous data, this effect was observed only with QUIN, ARI and CARI, $p<0.01$, up to 1μM concentrations (fig 4-20). The biphasic effect of the DOPAC levels was observed with QUIN, ARI and CARI at the concentration of 10μM. This biphasic effect, however, can be seen on BREX at much lower concentrations (>100nM). Similar to the effect on DA accumulation, the presence of 1μM SUL could significantly block QUIN, ARI and CARI effects on DOPAC level up to 1μM concentrations, as shown by a significant interaction between SUL and non-SUL groups, $p<0.001$ (QUIN vs QUIN+SUL), $p<0.01$ (ARI vs ARI+SUL) and $p<0.001$ (CARI vs CARI+SUL). Interestingly, however, 1μM SUL could only block BREX decreasing effect on DOPAC concentration at lower concentrations (up to 10nM), since an increase of DOPAC level was observed at the concentrations not associated with D_2R specificity (no significant interaction between both BREX and BREX+SUL groups, $p>0.05$). These results indicate a D_2R-independent effect of partial agonist antipsychotics which leads to an increase in DOPAC concentration.

Fig. 4-20. Schematic diagrams illustrating the specificity of the effect of D_2 agonist and partial agonist antipsychotics at presynaptic D_2R was tested with (a) and without (b) in the presence of 1μM SUL to block D_2R stimulations. Results were represented as a comparison between concentration-response curves on the effects of D_2R agonist and partial agonist antipsychotics with and without pretreatment with 1μM SUL on endogenous DOPAC concentration for (a) QUIN, (b) ARI, (c) CARI, and (d) BREX. The data are expressed as mean ± SEM from two brain incubations for each group (DRUG or DRUG+SUL group) normalized to the control values with n shown in the graph. Basal DOPAC levels in non-incubated samples are shown by ▲. Asterisks indicate *p<0.05 and **p<0.01 vs control (DRUG or DRUG+SUL group), one-way ANOVA followed with Dunnett's post-hoc test from the same group. The data were adjusted into the following analysis parameters: panel (c) sigmoidal dose-response curve for both QUIN and QUIN+SUL; panel (d) polynomial third order for ARI and sigmoidal dose-response curve for ARI+SUL; panel (e) polynomial third order for CARI and sigmoidal dose-response curve for CARI+SUL; and panel (f) polynomial third order for BREX and sigmoidal dose-response curve for BREX+SUL.

2.7 Lack of D_2R antagonist effect on [^{3}H]-DA synthesis and accumulation

To study the specificity of D_2R agonist and partial agonist antipsychotics on the inhibition of [^{3}H]-DA synthesis and endogenous DA accumulation, the tissue samples were pre-treated with 1μM SUL to block the activation of D_2R. Therefore, the effect of 1μM SUL alone was done in parallel with the rest of the drug combinations to shows the effect of SUL alone. I found that 1μM SUL as a D_2R antagonist had no significant effect on [^{3}H]-DA synthesis, $p>0.05$, endogenous DA accumulation, $p>0.05$, DOPAC concentration, $p>0.05$, and the DOPAC/DA concentration ratio, $p>0.05$ (fig. 4-21), consistently with the absence of DA release under non-depolarizing conditions (2mM K^+).
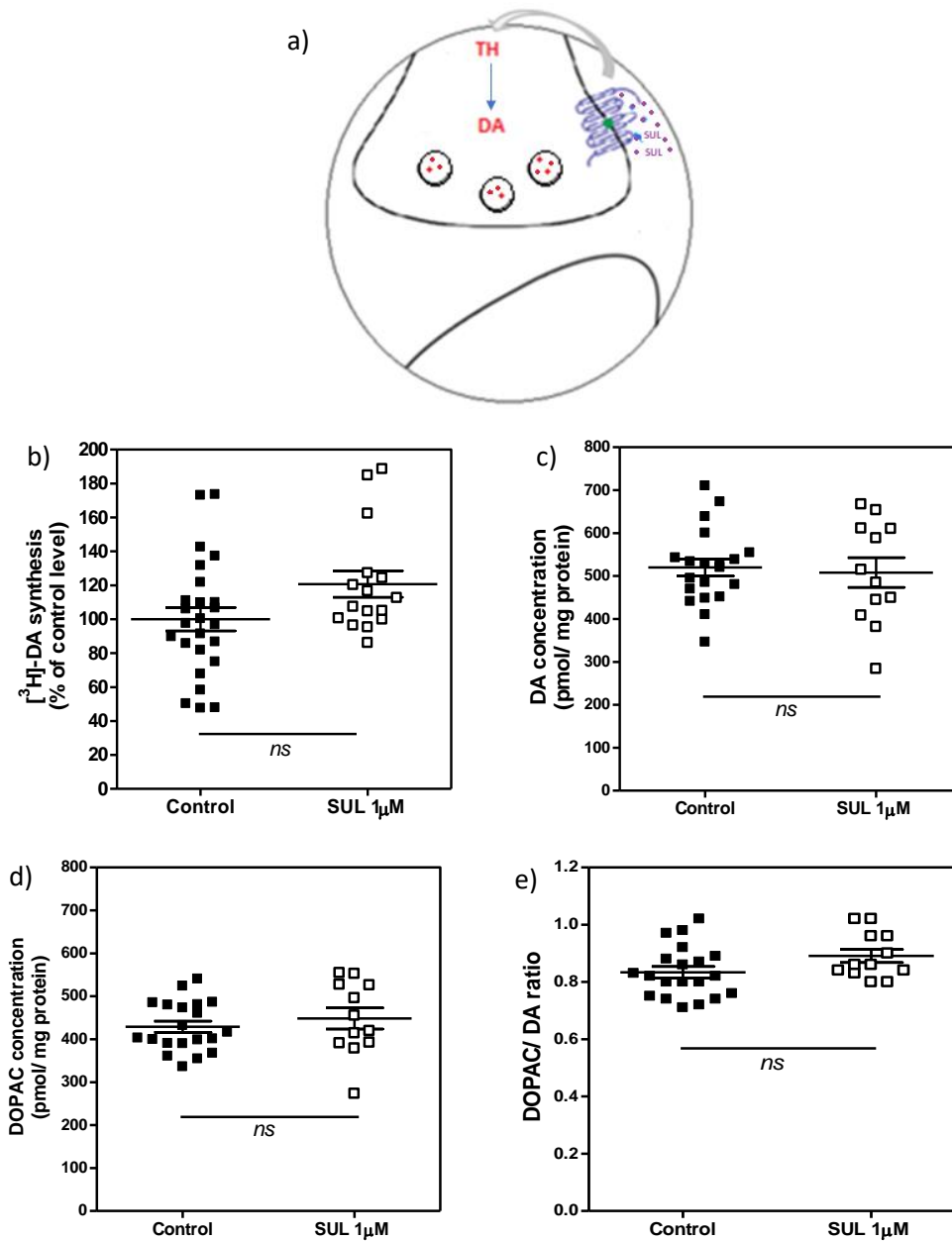
Fig. 4-21. (a) Schematic illustration of the lack of effect by D_2 antagonist at presynaptic D_2R, as shown by 1μM SUL on (b) [^{3}H]-DA synthesis, (c) DA accumulation, (d) DOPAC levels, and (e) the DOPAC/ DA concentration ratio at 2mM K^+. The data were obtained from four brain incubations (n=3-4 incubations each) which run in parallel with the combination with D_2R agonist and partial agonists antipsychotics. The data are expressed as mean ± SEM, unpaired Student´s t-test.

2.8 Increase in the DOPAC/ DA concentration ratio mediated by D_2R-independent activation mechanisms of partial agonist antipsychotics

I have previously shown that all D_2R agonist and partial agonist antipsychotics decreased DA accumulation under 2mM K^+ of non-depolarized conditions (fig. 4-18). However, the decrease of DOPAC levels was not in line with DA as the biphasic effect was observed (fig. 4-20). For further understanding of this effect, I plotted the relationship between the DOPAC/ DA concentration ratio as a metabolism or release index. As revealed by this ratio, D_2R partial agonist antipsychotics probably promote DA release or metabolism from the synaptic vesicles and increased cytosolic DA and its metabolism at high concentrations, as shown by a significant increase in the DOPAC/ DA concentration ratio as compared with D_2R agonist (fig. 4-22). The effect of BREX on the increase of the DOPAC/ DA concentration ratio could be seen at much lower concentrations (from 100nM) and was of much higher magnitude at the highest concentration used (10μM). As mentioned earlier, this elevation was not blocked in the presence of 1μM SUL, suggesting that an increase of DA release or metabolism induced by D_2R partial agonist antipsychotics can be interpreted as an indicator of D_2R-independent activation mechanisms.
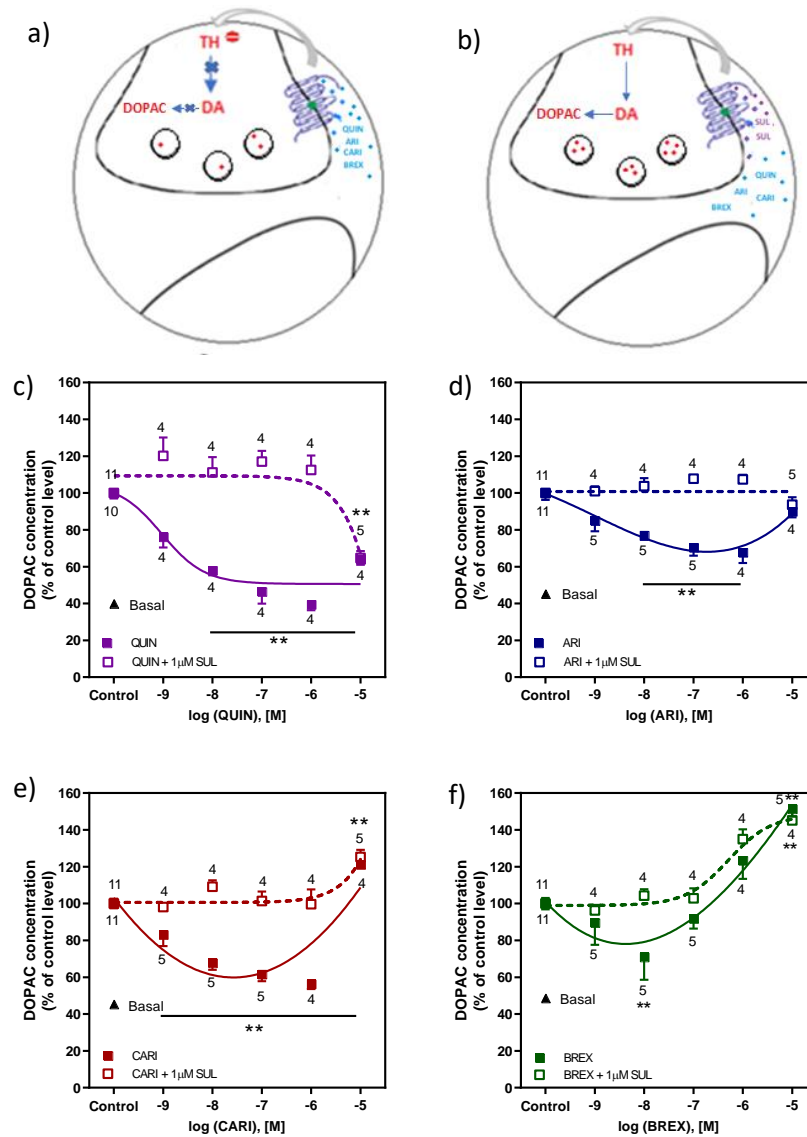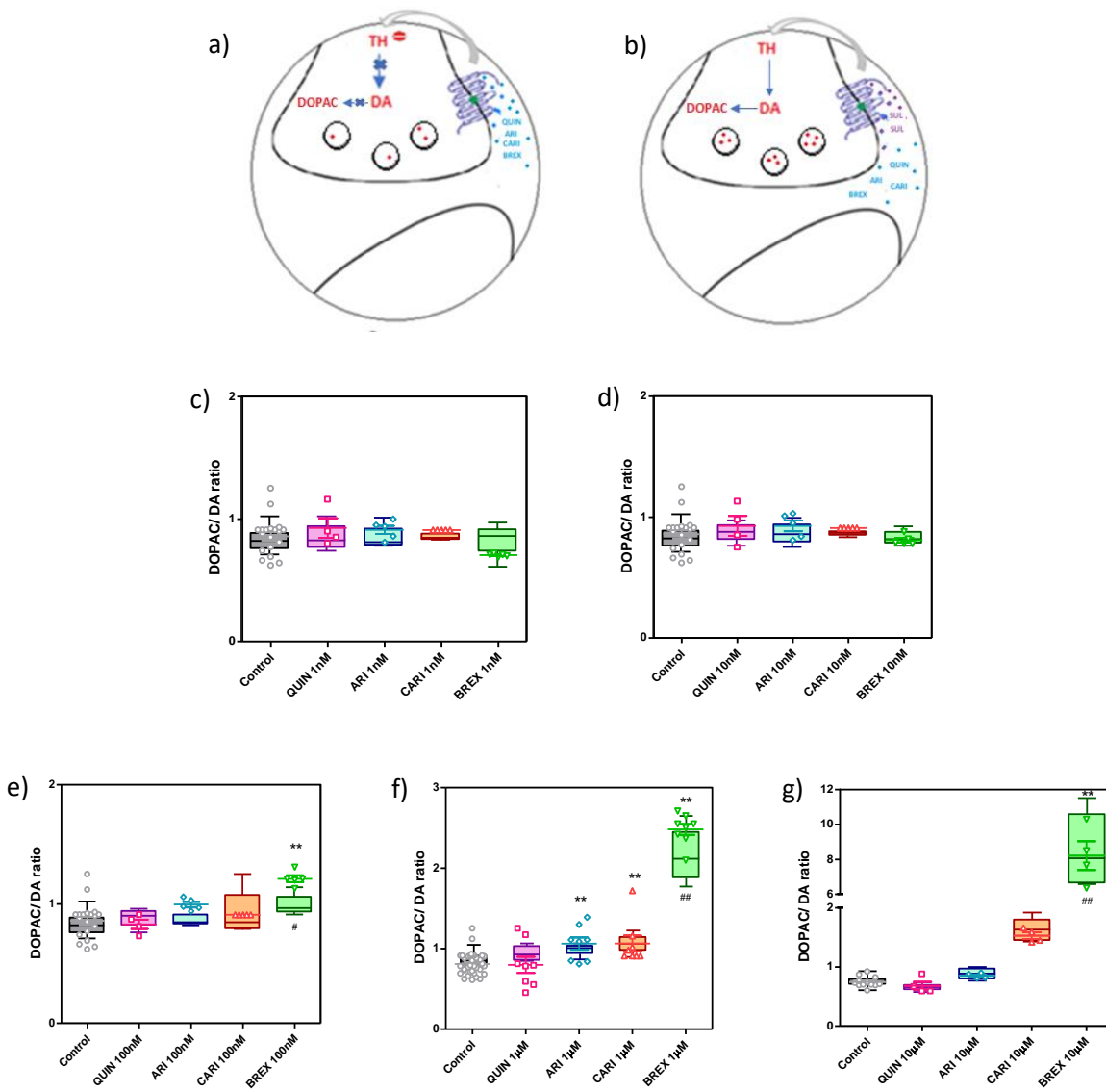
Fig. 4-22. Schematic diagrams illustrate the specificity of the effect of D_2 agonist and partial agonist antipsychotics at presynaptic D_2R, which was tested with (a) and without (b) in the presence of 1μM SUL to block D_2R stimulations. Results were represented as a comparison between D_2R agonist and partial agonist antipsychotics on the DOPAC/ DA concentration ratio at different concentrations after 120-min incubations under 2mM K^+. Under the absence (scatter dot plot) and the presence (box plot and whiskers) of 1μM SUL, the DOPAC/ DA concentration ratio can be interpreted as an indicator of D_2R-independent activation as its elevation was not blocked by SUL. Differently to the D_2R full agonist QUIN, all three D_2R partial agonist antipsychotics increased the DOPAC/ DA concentration ratio, however, the effect of BREX was observed at much lower concentrations (100nM) and was of much higher magnitude at the highest concentration used (10μM). The data are expressed as mean ± SEM from two brains with n=3-6 incubations for each group (DRUG or DRUG+SUL group). **$p<0.01$ vs control (non-SUL group); #$p<0.05$ and ##$p<0.01$ vs control (DRUG+SUL group), one-way ANOVA followed with Dunnett´s post-hoc test from the same group.

134

2.9 D2R-independent activation mechanisms of partial agonist antipsychotics could be mediated by [3H]-DA release

The mechanism by which D_2R-independent inhibition of [3H]-DA synthesis occurs also leads to an increase in the DOPAC/ DA concentration ratio (fig. 4-22). Thus, to know if this mechanism was due to the effect of disrupting vesicular storage, leading to DA leakage either into the cytosol or directly facilitating DA release from dopaminergic terminals, I quantitatively measured [3H]-DA concentration in the supernatant and the tissue samples simultaneously. I found that, at the concentration mediated by D_2R-independent activation mechanisms (10µM), all D_2R partial agonist antipsychotics significantly elevated [3H]-DA efflux from dopaminergic terminals to a lesser extent than AMPH used as a reference compound, $p<0.001$ (fig. 4-23). [3H]-DA released was significantly increased by ARI, CARI and especially BREX, and conversely [3H]-DA storage was found to have decreased, $p<0.001$. This effect could contribute to the mechanism of D_2R-independent inhibition of DA synthesis by D_2R partial agonist antipsychotics, increasing DA feedback inhibition on TH, and may further enhance its efficacy on the inhibition of [3H]-DA synthesis.
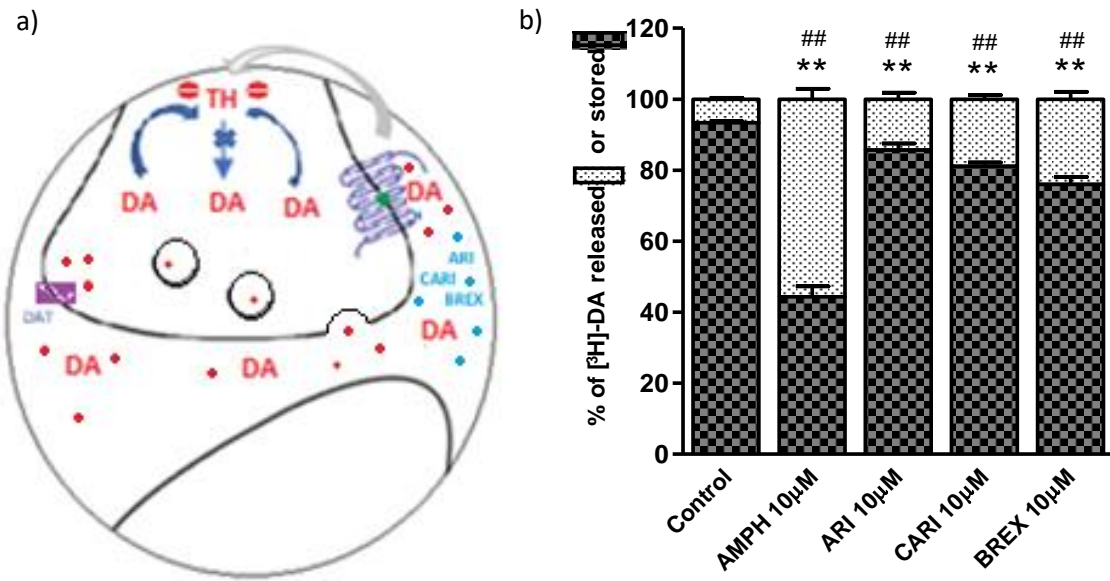
Fig. 4-23. (a) Schematic diagram illustrating that the D_2R-independent inhibition on DA synthesis by partial agonist antipsychotics could be mediated by feedback inhibition of TH due to impaired vesicular storage. (b) This effect is represented as the percentage of $[^3H]$-DA synthesis being released or stored with AMPH used as a positive control. The data are expressed as mean $\pm$ SEM (n=16 incubations for control and n=8 incubations for each drug tested) from two brains. **$p<0.01$ vs $[^3H]$-DA released in control group; ##$p<0.01$ vs stored $[^3H]$-DA in control group, one-way ANOVA followed with Dunnett's post-hoc test.

2.10 OKA-induced phosphorylation modifies DA dynamics elicited by QUIN and ARI – but not CARI and BREX – at high concentrations

With an increase of DA accumulation mediated by the phosphorylation of TH, probably on Ser^{31} and Ser^{40} via OKA phosphatase inhibition, as shown in fig. 4-4, I wanted to explore the phosphorylation-related mechanisms of D_2 agonist and partial agonist antipsychotics on TH that lead to the inhibition of DA accumulation. I found that, similarly to before (fig. 4-4), a significant increase of DA accumulation was observed with OKA alone. When combining D_2R agonist and partial agonist antipsychotics with OKA, only CARI and BREX showed a significant decrease of DA accumulation, $p<0.01$ and $p<0.001$ respectively (fig. 4-24a), but not QUIN and ARI, suggesting that the latter compounds lost their efficacy for the inhibition of DA synthesis, e.g. to decrease TH phosphorylation. My results are in agreement with the previous study done by Ma G.F., Ph.D. thesis 2014, on [^{3}H]-DA synthesis, where the combination of OKA and QUIN moved the QUIN-inhibition curve of [^{3}H]-DA synthesis to the right, increasing QUIN IC_{50}. However, the effects on DOPAC concentration and the DOPAC/ DA concentration ratio cannot be explained in the same way. An opposite effect was observed on DOPAC concentration, which led to the increase of the DOPAC/ DA concentration ratio by OKA alone. QUIN, however, significantly decreased, $p<0.01$, while CARI and BREX increased both DOPAC and the DOPAC/ DA concentration ratio, $p<0.05$ and $p<0.001$ respectively (fig. 4-24b and 4-24c). This is consistent with the effect of D_2R-independent mechanisms by CARI and BREX to increased DOPAC levels, as shown in fig. 4-22. Since both QUIN and ARI did not significantly decrease DA accumulation in the presence of OKA-mediated TH phosphorylation, a full concentration-response curve may

be needed to determine the mechanism of D_2 agonist and partial agonist antipsychotics in regulating TH phosphorylation.
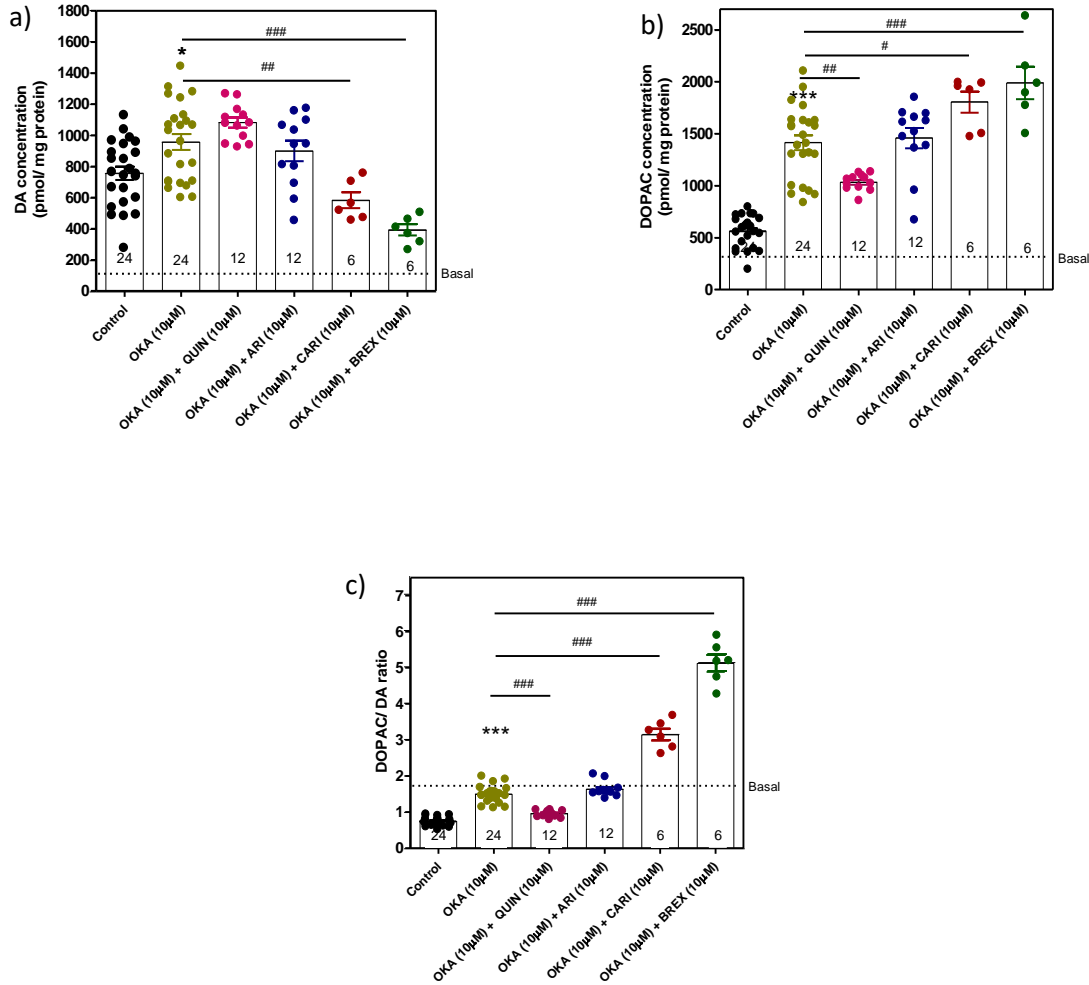
Fig. 4-24. Combination of OKA with QUIN, ARI, CARI and BREX effects on endogenous (a) DA accumulation, (b) DOPAC levels, and (c) the DOPAC/ DA concentration ratio. The basal levels in non-incubated samples are shown by the dotted line. Bars representing the mean ± SEM of individual points shown, obtained from three brain incubations with n shown in the bar. *p<0.05 or ***p<0.001 vs control, one-way ANOVA followed with Dunnett's post-hoc test; and ##p<0.01 or ##p<0.001, one-way ANOVA followed with Bonferroni's post-hoc test for comparison between "OKA" and "OKA + treated drug" effects.

Results

3. **Third objective: Explore the implication of non-D$_2$ receptor components in the properties of D$_2$-like partial agonist antipsychotics on DA dynamics.**

3.1 Lack of D$_3$ receptor involvements in CARI effects on endogenous DA

I have discussed earlier the CARI's property of higher affinity on the D$_3$ receptor as compared with ARI and BREX. To see if the higher affinity of CARI on the D$_3$ receptor may affect its efficacy in regulating DA synthesis, 100nM SB 277011-A was used to block the D$_3$ receptor (Reavill et al., 2000). We found that the D$_3$ receptor blockade by 100nM SB 277011-A alone did not have a significant effect on DA accumulation. With the combination of 100nM CARI (concentration related to D$_2$R-dependent activation mechanisms, which hypothetically may include D$_3$ receptor as D$_2$ and D$_3$ receptors are both blocked by 1µM SUL), the D$_3$ receptor blockade did not change 100nM CARI decrease of endogenous DA accumulation, $p<0.01$ (fig. 4-25b) and DOPAC levels, $p<0.05$ (fig. 4-25c). The limitation of this study is that there was no positive control that showed SB 277011-A truly blocked the D$_3$ receptor. Besides, no test has been done with a pure D$_3$ receptor agonist to assess the effect of the D$_3$ receptor in regulating DA accumulation. D$_3$ receptor was highly expressed in the substantia nigra and ventral tegmental area, where cell bodies are present, as mentioned in the introduction; thus, it could be doubted whether it would have effects at nerve endings. Therefore, on the basis of this study alone, it cannot be concluded that the D$_3$ receptor may not have any effects in regulating DA synthesis and

endogenous DA level in rat brain striatum. However, it does indicate that a higher affinity of CARI towards D_3 than D_2 receptors does not affect its efficacy at striatal presynaptic D_2R.
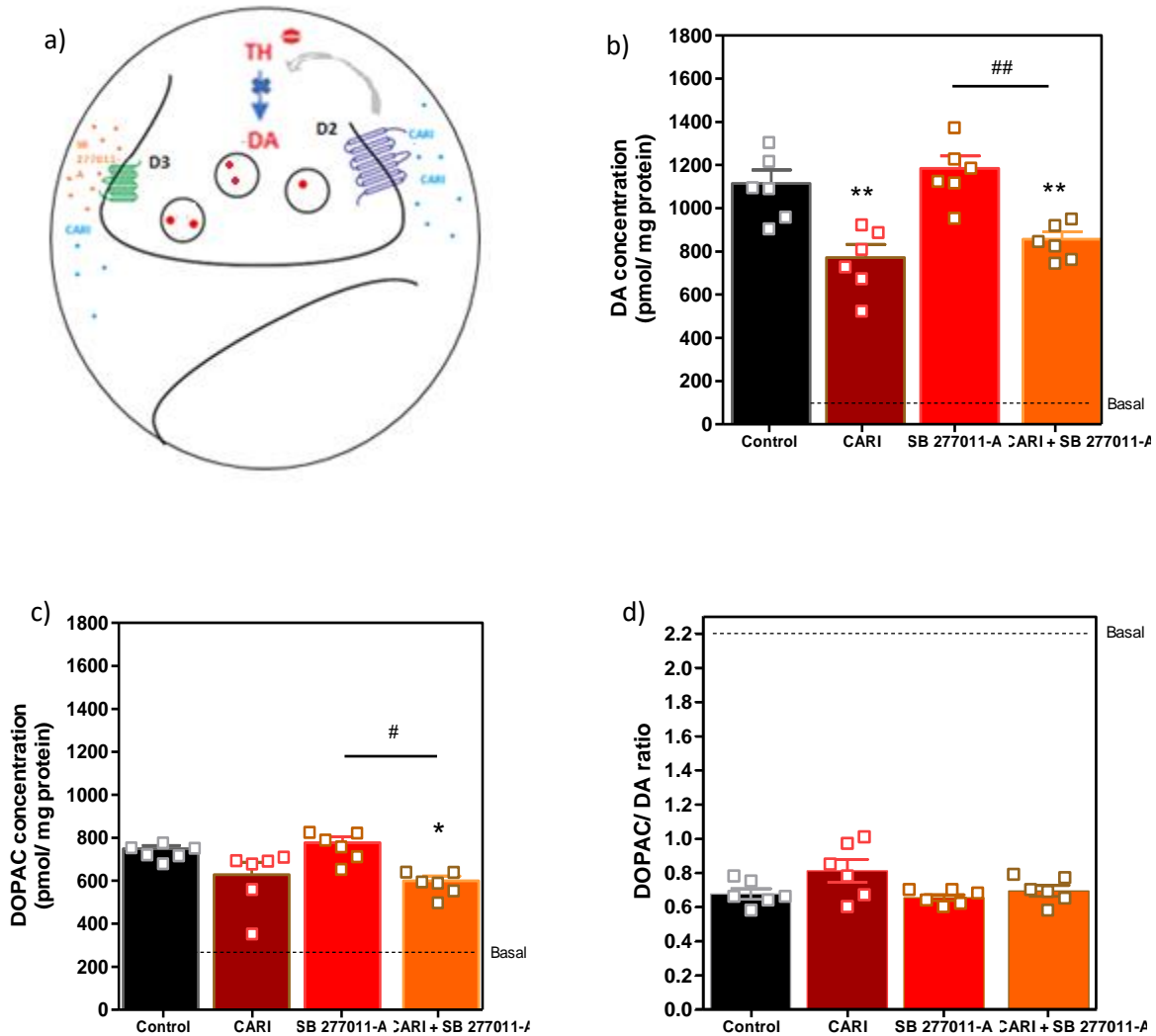
Fig. 4-25. (a) Schematic illustration of the lack of effect by a D_3 antagonist (SB 277011-A) at presynaptic D_2R, which did not affect CARI efficacy to decrease DA accumulation, as shown by the combination of (100nM SB 277011-A) with CARI (100nM) on endogenous (b) DA accumulation, (c) DOPAC levels, and (d) the DOPAC/ DA concentration ratio. The basal levels in non-incubated samples are shown by the dotted line. The data are expressed as mean ± SEM (n=6 incubations per data point) from one brain. *p<0.05 or **p<0.01 vs control, one-way ANOVA followed with Dunnett's post-hoc test; and #p<0.05 or ##p<0.01, one-way ANOVA followed with Bonferroni's post-hoc test for comparison between "SB 277011-A" and "SB 277011-A+CARI" effects.

3.2 Spontaneous increase of endogenous 5-HT accumulation over incubation time

In order to assess whether the spontaneous accumulation of DA in our *ex-vivo* studies (first documented by González-Sepúlveda et al.) was unique to this neurotransmitter or a trait of other neurotransmitters, I studied 5-HT levels over different incubation times. Similar to the incubation-time-dependent effects on DA accumulation, as shown in fig. 4-1, a spontaneous increase of endogenous 5-HT in the striatum was also observed in our *ex-vivo* study. I found a significant increase of 5-HT concentration from non-incubated samples which corresponds to 0-min sample incubations, $p<0.01$ (fig. 4-26b). In line with the increase of 5-HT accumulation, an increase of 5-HIAA levels was also observed, $p<0.01$ (fig. 4-26c). The levels of 5-HIAA were tremendously increased after 120-min incubations, indicating that the ability of 5-HT to be stored inside the synaptic vesicles may reach the maximum capacity leading to higher cytosolic 5-HT levels. Overall, an increase of the 5-HIAA/ 5-HT concentration ratio was observed, $p<0.01$ (fig. 4-26d) over incubation's time, due to the limitation of 5-HT capacity to be stored inside synaptic vesicles together with the continuity of 5-HT to be actively synthesized at the same time under non-depolarized conditions, which prevent 5-HT release. Such results can also be observed on 5-HT accumulation, $p<0.01$ (fig. 4-26e), 5-HIAA levels, $p<0.01$ (fig. 4-26f), and the 5-HIAA/ 5-HT concentration ratio, $p<0.01$ (fig. 4-26g) using rat brain hippocampus incubated *ex-vivo*.
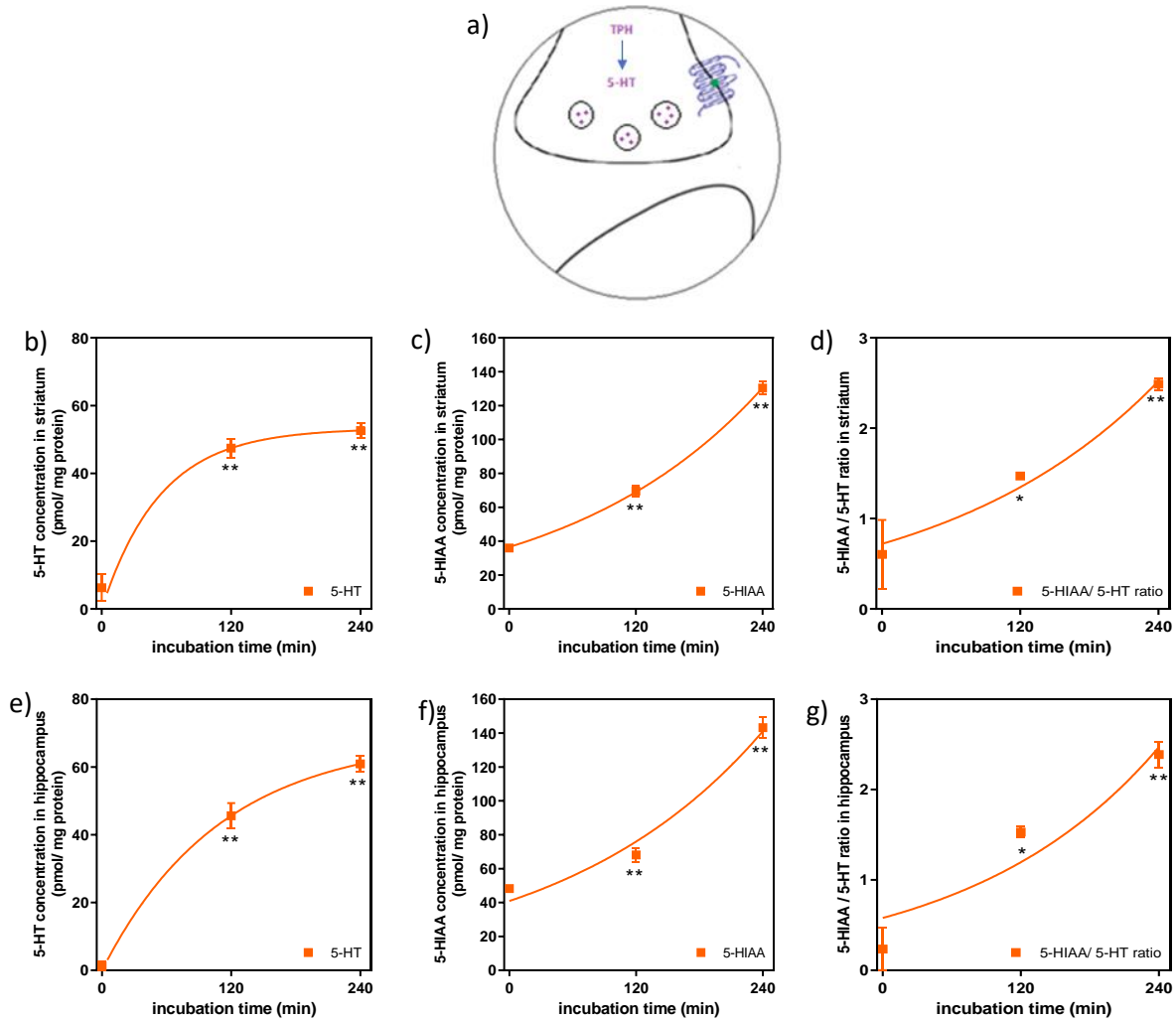
Fig. 4-26. (a) Schematic illustration of a similar phenomenon as was observed on DA accumulation on an increase of 5-HT synthesis, accumulation and metabolism during tissue samples incubation. This increase was translated in view of the effect of different tissue samples' incubation time on endogenous (b and e) 5-HT accumulation, (c and f) 5-HIAA levels, and (d and g) the 5-HIAA/ 5-HT concentration ratio at 2mM K^+ over 240-min incubation's time in the striatum and hippocampus incubated *ex-vivo*. (b and e) Endogenous 5-HT was increased spontaneously during the first 120-min under 2mM K^+ tending to saturation, which indicates that it cannot be fully stored. (c and f) A significant amount of 5-HIAA was found in incubated samples, which showed the existence of cytosolic 5-HT. The levels of 5-HIAA were tremendously increased after 120-min incubations, indicating higher cytosolic 5-HT levels being metabolized, which leads to an overall increase in the 5-HIAA/ 5-HT concentration ratio (d and g) over incubation times. The data are expressed as mean ± SEM (n=6 incubations per data point for the striatum from one brain) and (n=12 incubations per data point for the hippocampus from two brains). *$p<0.05$ and **$p<0.01$ vs level of 5-HT, 5-HIAA and the 5-HIAA/ 5-HT concentration ratio in non-incubated samples corresponding to the 0-min value, one-way ANOVA followed with Dunnett's post-hoc test. The data were adjusted into the following analysis parameters: panel (b) one-phase exponential association; panel (c) exponential growth; panel (d) exponential growth; panel (e) one-phase exponential association; panel (f) exponential growth; and panel (g) exponential growth.

142

3.3 Similar effects of D2R partial agonist antipsychotics on 5-HT and DA accumulations

Higher 5-HT receptors' selectivity is one of the properties of D_2R partial agonist antipsychotics. All D_2R partial agonist antipsychotics are partial agonists at 5-HT_{1A} and antagonists for both 5-HT_{2A} and 5-HT_7 receptor subtypes, with BREX having the highest affinity compared with ARI and CARI. To see if these characteristics may also affect the level of 5-HT, the effect of D_2R partial agonist antipsychotics on 5-HT and 5-HIAA levels was observed in comparison with the concentrations of DA and DOPAC in rat brain striatum. Antipsychotic concentrations that trigger D_2R-independent activation mechanisms were chosen for this study. I found a similar effect of partial agonist antipsychotics on 5-HT accumulation, but to a lesser extent on DA (fig. 4-27). All D_2R partial agonist antipsychotics showed the same trend to decrease endogenous 5-HT accumulation, but only BREX displayed a statistically significant effect, $p<0.01$. However, the decrease of 5-HT concentration did not run parallel to that of its metabolite, 5-HIAA levels. An increase of 5-HIAA concentration was observed with BREX, which displayed a statistically significant effect, $p<0.01$. Such elevation on 5-HIAA as compared with 5-HT concentration leads to an increase in the 5-HIAA/ 5-HT concentration ratio, with both CARI and BREX showing a statistically significant effect, $p<0.005$ (CARI) and $p<0.001$ (BREX). This suggests that the D_2R-independent activation mechanisms by partial agonist antipsychotics shared a similar mechanism to interfere with 5-HT storage and increased 5-HT release or metabolism, but to a lower degree than the action on DA in the striatum. This was indicated with similar effects observed for both 5-HT and DA accumulation (fig. 4-27c and 4-27f), 5-HIAA and DOPAC levels (fig. 4-27d and 4-27g), and between the 5-HIAA/ 5-HT and the DOPAC/ DA concentration ratio (fig. 4-27e and 4-27h).
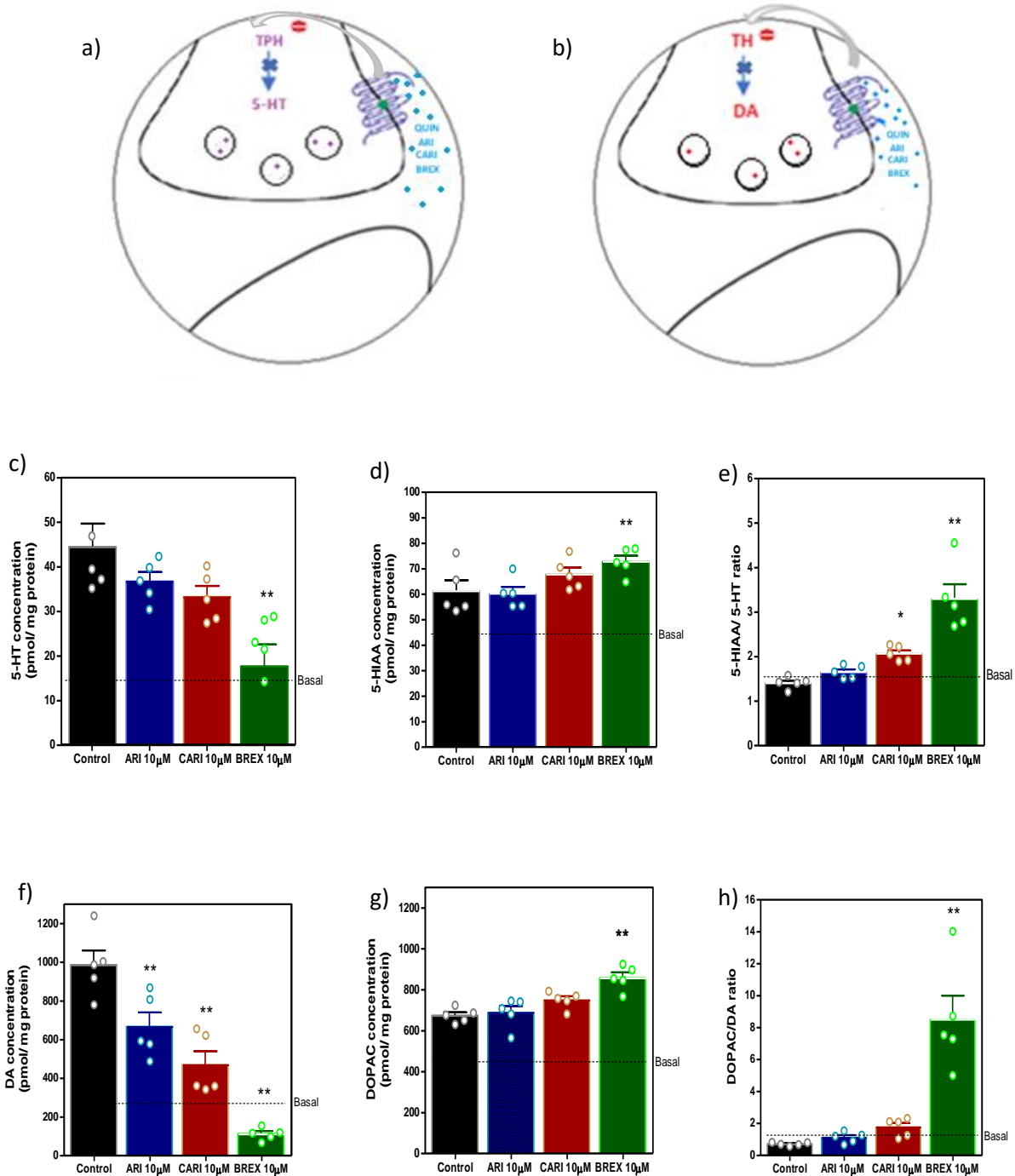
Fig. 4-27. Schematic illustration of similar effects mediated by D_2R-independent activation mechanisms of partial agonist antipsychotics on (a) 5-HT and (b) DA dynamics, but affecting 5-HT to a lesser extent than DA accumulation. This is revealed by the comparison of the effects of D_2R partial agonist antipsychotics between (c) 5-HT and (f) DA accumulation, (d) 5-HIAA and (g) DOPAC levels, and the (e) 5-HIAA/ 5-HT and (h) DOPAC/ DA concentration ratio from the same brain at 2mM K^+. The data are expressed as mean ± SEM (n=5 incubations per data point) from one brain. **p<0.01 vs control, one-way ANOVA followed with Dunnett's post-hoc test.

3.4 Lack of indirect regulation by the 5-HT$_{2A}$ receptor on the inhibition of DA accumulation

I have shown that the D$_2$R-independent activation mechanisms by partial agonist antipsychotics induce 5-HT release and/or metabolism as shown by an increase in the 5-HIAA/ 5-HT concentration ratio (fig. 4-27). Thus, I hypothesized that incorporating higher affinity towards certain 5-HT receptors in their properties might indirectly regulate the inhibition of DA synthesis, by affecting 5-HT release. This is supported by the literature, which shows that 5-HT can potentiate the effect of D$_2$ agonist on the inhibition mediated by DA (Brodie & Bunney, 1996).

5-HT$_{2A}$ receptor subtype plays an important role in antipsychotics action. It has been suggested that a potent blockade of 5-HT$_{2A}$ receptor subtype may contribute to its antipsychotic properties, since it was found in clozapine and has become the basis of pharmacological profile to the development of atypical antipsychotics (Rasmussen & Aghajanian, 1988). Antipsychotics such as risperidone, which also acts as an antagonist at 5-HT$_{2A}$ receptor subtype other than D$_2$, have been shown to increase extracellular 5-HT levels (Hertel et al., 1997). D$_2$R partial agonist antipsychotics also have a similar property on the 5-HT$_{2A}$ receptor subtype. To study the effect of 5-HT$_{2A}$ receptor subtype antagonism on endogenous DA accumulation, MDL 100907, a ligand with selective antagonism of 5-HT$_{2A}$ receptor subtype was used. Several studies have shown that blockade of 5-HT$_{2A}$ receptor subtype appears to increase DA release in the prefrontal cortex (Schmidt & Fadayel, 1995) and potentiate haloperidol-induce DA release (Bonaccorso et al., 2002). However, in my *ex-vivo* study using rat brain striatum, MDL 100907 did not have any effect on endogenous DA accumulation, DOPAC concentration, and the DOPAC/ DA concentration ratio (fig. 4-28) under 2mM K$^+$. These results suggested that higher selectivity and affinity of D$_2$R

partial agonist antipsychotics on 5-HT$_{2A}$ receptor subtype did not contribute to its D$_2$R-independent effects previously described. Thus, I excluded the possibility of an indirect regulation by 5-HT$_{2A}$ which leads to a decrease in DA synthesis. In addition, since there was no effect of the blockade of 5-HT$_{2A}$ on DA accumulation, the effect on 5-HT levels was not quantified for comparison.

Fig. 4-28. (a) Schematic illustrating the lack of effects of the 5-HT$_{2A}$ antagonism on the D$_2$ receptor, as shown by the concentration-response curves of MDL 100907 on endogenous (b) DA accumulation, (c) DOPAC concentration, and (d) the DOPAC/ DA concentration ratio under 2mM K$^+$. The data are expressed as mean ± SEM (n=6 incubations per data point) from one brain. Basal levels in non-incubated samples are shown by ▲. The data were adjusted into the sigmoidal dose-response curve for DA accumulation, DOPAC levels, and the DOPAC/ DA concentration ratio.

3.5 Lack of indirect regulation by the 5-HT$_{1A}$ receptor on the inhibition of DA accumulation

As discussed in the introduction, 5-HT$_{1A}$ receptor can be located at both pre- and post- synaptical terminals. Similar to the D$_2$R, this receptor may regulate 5-HT inhibition. However, the 5-HT$_{1A}$ presynaptic autoreceptor is located on the cell bodies; thus, it could be doubted whether it would affect DA accumulation determination at nerve endings. Given that D$_2$R partial agonist antipsychotics acted as partial agonists at the 5-HT$_{1A}$ receptor subtype, 8-OH DPAT, a ligand with an agonist property on 5-HT$_{1A}$ receptor subtype, was examined on endogenous DA accumulation. 8-OH DPAT did not only activate the 5-HT$_{1A}$ but also the 5-HT$_7$ receptors (Hedlund et al., 2004).

I found that under 2mM K$^+$, 8-OH DPAT did not have any effect on endogenous DA accumulation, DOPAC concentration, and the DOPAC/ DA concentration ratio (fig. 4-29). These results indicate that higher selectivity and affinity of D$_2$R partial agonist antipsychotics on the 5-HT$_{1A}$ receptor subtype do not contribute to its D$_2$R-independent effects on DA accumulation. Thus, I excluded the possibility that an indirect regulation by the activation of both 5-HT$_{1A}$ and 5-HT$_7$ leads to a decrease in DA synthesis. Similar to the previous experiment, since there were no effects of the activation of both 5-HT$_{1A}$ and 5-HT$_7$ receptors on DA accumulation, which is my interest, the effect on 5-HT levels was not quantified for comparison.
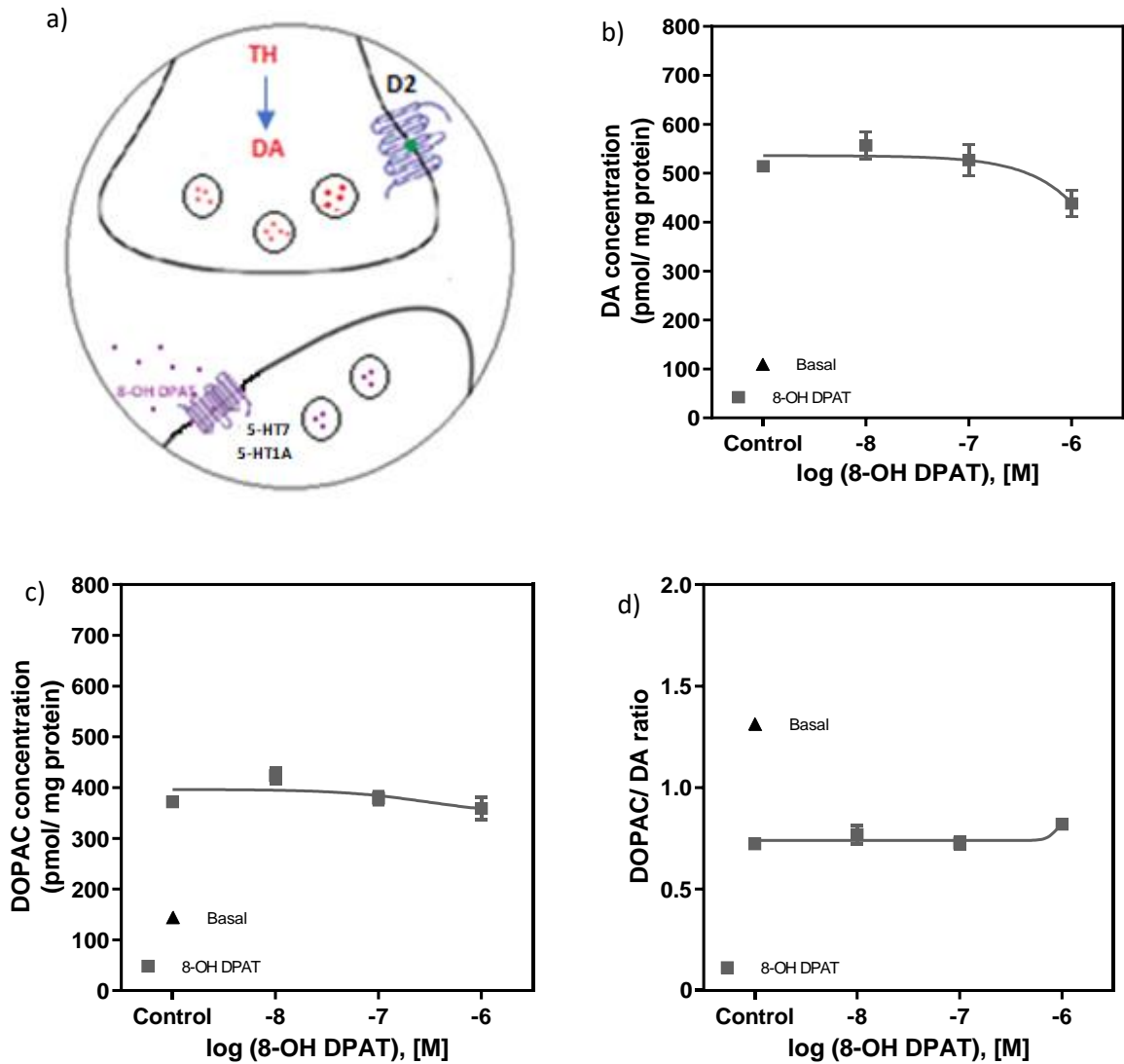
Fig. 4-29. (a) Schematic diagram illustrating the lack of effects of the 5-HT$_{1A}$ and 5-HT$_7$ agonism on the D$_2$ receptor, as shown by the concentration-response curves of 8-OH DPAT on endogenous (b) DA accumulation, (c) DOPAC concentration, and (d) the DOPAC/ DA concentration ratio under 2mM K$^+$. The data are expressed as mean ± SEM (n=6 incubations per data point) from one brain. Basal levels in non-incubated samples are shown by ▲. The data were adjusted into the sigmoidal dose-response curve for DA accumulation, DOPAC levels and the DOPAC/ DA concentration ratio.

3.6 Lack of indirect regulation by the 5-HT$_7$ receptor on the inhibition of DA accumulation

Pharmacological blockade of the 5-HT$_7$ receptor subtype has been implied to have therapeutic applications in schizophrenia. Several animal studies have shown antipsychotics-like benefits by blockade of 5-HT$_7$ receptor subtype with a decrease in AMPH-induced prepulse inhibition (Galici et al., 2008) and attenuated PCP-induced deficits in reversal learning (McLean et al., 2009). Given that D$_2$R partial agonist antipsychotics also act as antagonists at 5-HT$_7$ receptor subtype, I also hypothesized that this property may indirectly affect endogenous DA accumulation. Therefore, SB 258719, a ligand with an antagonism property on selective 5-HT$_7$ receptor subtype was used in this study. In my *ex-vivo* study using rat brain striatum, SB 258719 did not have any effect on endogenous DA accumulation, DOPAC concentration, and the DOPAC/ DA concentration ratio (fig. 4-30) under 2mM K$^+$. These results also suggest that higher selectivity and affinity of D$_2$R partial agonist antipsychotics on the 5-HT$_7$ receptor subtype did not contribute to its D$_2$R-independent activation mechanisms. Thus, I excluded the possibility that indirect regulation by the activation (as showed with 8-OH-DPAT in fig 4-29) or blockade of the 5-HT$_7$ receptor subtype leads to a decrease in DA synthesis. With no effects of 5-HT$_7$ receptor on DA accumulation, which is my interest, the effect on 5-HT levels was not quantified for comparison.
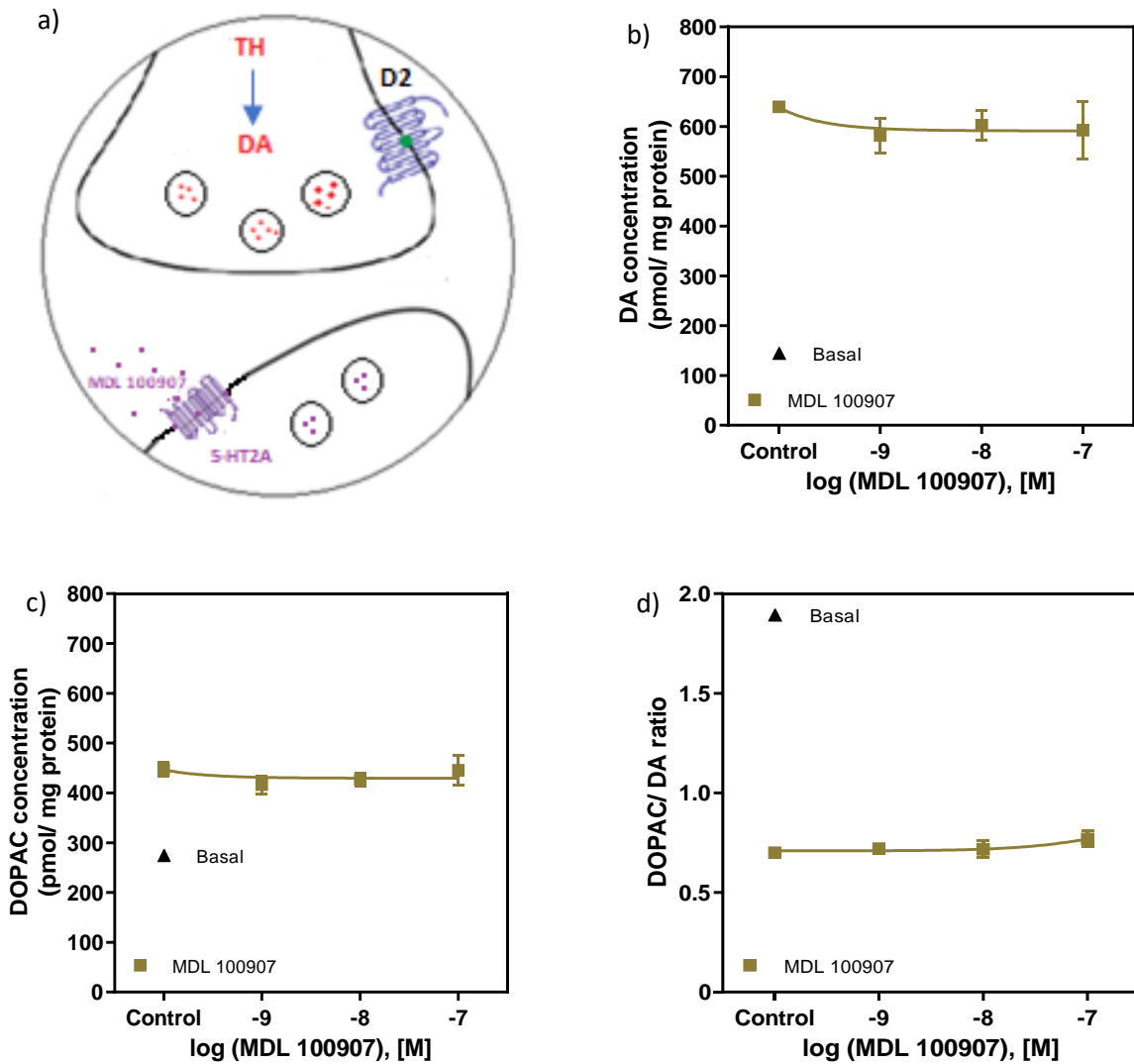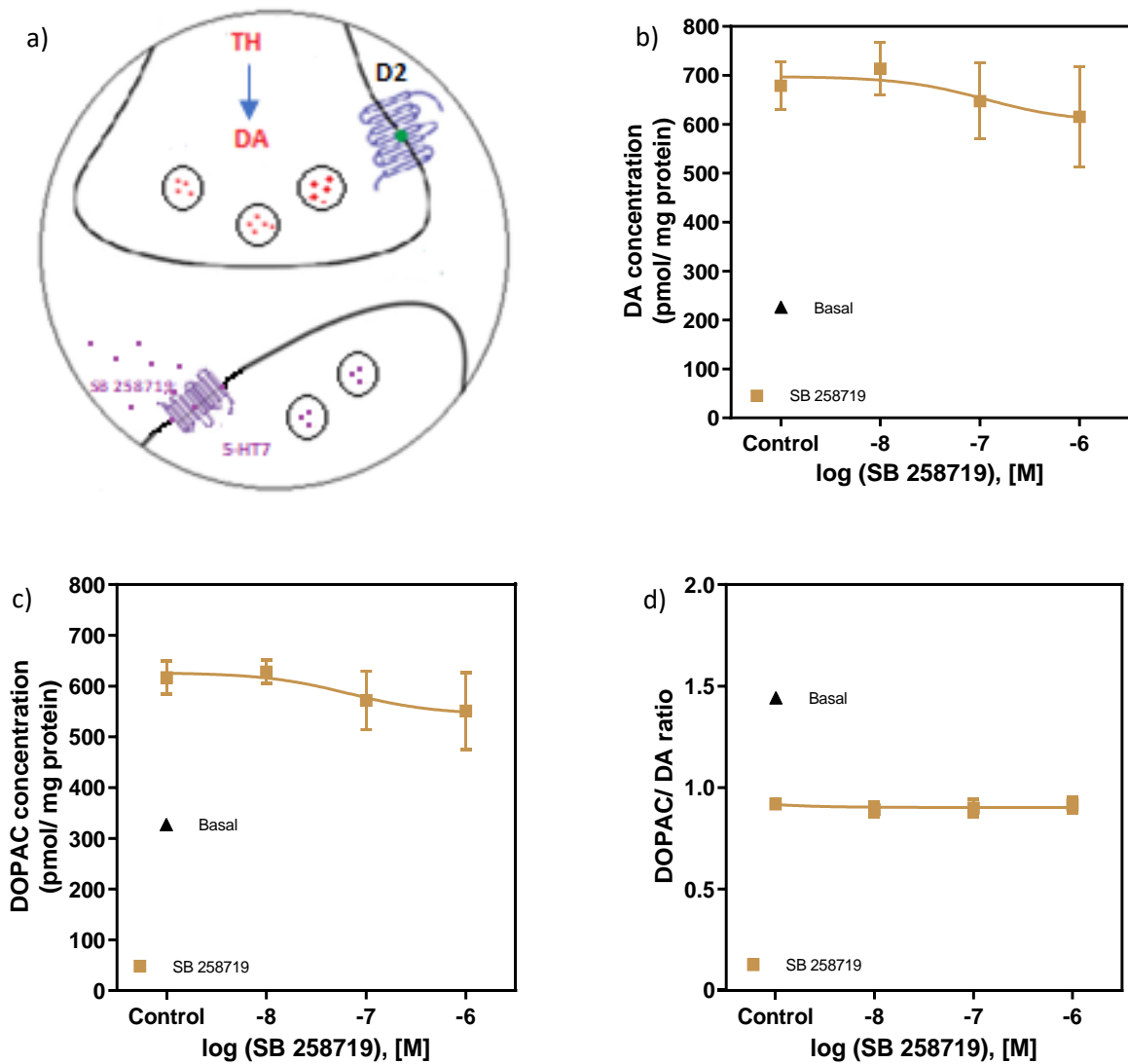
Fig. 4-30. (a) Schematic illustration of the lack of effects of 5-HT$_7$ antagonism on D$_2$ receptor, as shown by the concentration-response curves of SB 258719 on endogenous (b) DA accumulation, (c) DOPAC concentration, and (d) the DOPAC/ DA concentration ratio under 2mM K$^+$. Data are expressed as mean $\pm$ SEM (n=6 incubations per data point) from one brain. Basal levels in non-incubated samples are shown by $\blacktriangle$. Data was adjusted into the sigmoidal dose-response curve for DA accumulation, DOPAC levels and the DOPAC/ DA concentration ratio.

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V. DISCUSSIONS

Discussions

1.   **First objective: Increase our understanding of the overall homeostatic mechanisms toward regulation of DA accumulation at presynaptic neurons in the striatum.**

This thesis is focused on how drug treatments regulate DA synthesis, storage and metabolism. Towards this aim, I have used a similar methodology to González-Sepúlveda et al., with a clear focus on DA accumulation *ex-vivo*, a previously unreported phenomenon. The strength of this method is its simplicity and utility to describe various local synaptic mechanisms such as negative-feedback inhibition on TH that will affect DA synthesis and storage dynamics during tissue samples incubation; its limitation, the use of fresh brain partly altered by slicing long-range circuitry and some artificial neurotransmitter release. Dopaminergic neurotransmission at presynaptic neurons consists of synthesis, vesicular uptake and storage, release, reuptake from extracellular milieu, metabolism and feedback regulations. Presynaptic D_2R regulate homeostasis through the activation of signal transduction that inhibits phosphorylation changes needed to activate TH activity (Ford, 2014). My results show that both D_2 agonist, QUIN and partial agonist, ARI decrease DA accumulation (fig. 4-2). QUIN and ARI both stabilize the active conformational states of the receptor. Such action inhibits the formation of the second messenger cAMP, leading to the lack of activation of DA synthesis via regulating TH phosphorylation activity. The partial agonists, on the other hand, have less efficacy to stabilize the active conformational state of the receptor as compared with a full agonist. The intrinsic activity of partial agonists depends on the receptor reserve in tissue samples (Meller et al., 1987). Consequently, partial agonists such as ARI may need to occupy more receptors to reach a similar efficacy to QUIN. Therefore, even though

ARI has a higher affinity for the D_2 receptor than QUIN (0.34nM vs 4.8nM), it does not reflect its efficacy on DA, as we demonstrated that ARI has less efficacy than QUIN on DA accumulation (E_{max} 43% (ARI) vs 63% (QUIN) in table 2). The efficacy of D_2 agonists such as QUIN on DA accumulation can be increased in the presence of positive allosteric modulators (fig. 4-6b).

In contrast, binding of D_2R with a D_2 antagonist, SUL, does not change the conformational state, which could be mostly inactive in experiments performed in 2mM K^+ (1-2% signal ratio of phosphorylated/unphosphorylated Ser^{40} on TH, as reported by González-Sepúlveda et al.), but it will block other agonists from binding to the receptor. The effect of DA, however, differs from D_2 agonist in that it does not only modulate DA feedback-inhibition through D_2R, but it can also directly inhibit TH activity by competing with BH_4 for binding of the ferric iron at TH catalytic site (Dunkley et al., 2004). Provided that DA can regulate a negative-feedback inhibition on TH via various mechanisms, the effect of DA in regulating DA synthesis was not blocked in the presence of SUL, as is the case with QUIN (fig. 4-3). The overall effect of agonists' and antagonists' effects on DA accumulation is illustrated in fig 5-1. Understanding the effect of an agonist, partial agonist and antagonist on the receptor is important, because various pharmaceutical drugs have been based on these sometimes subtle differences.
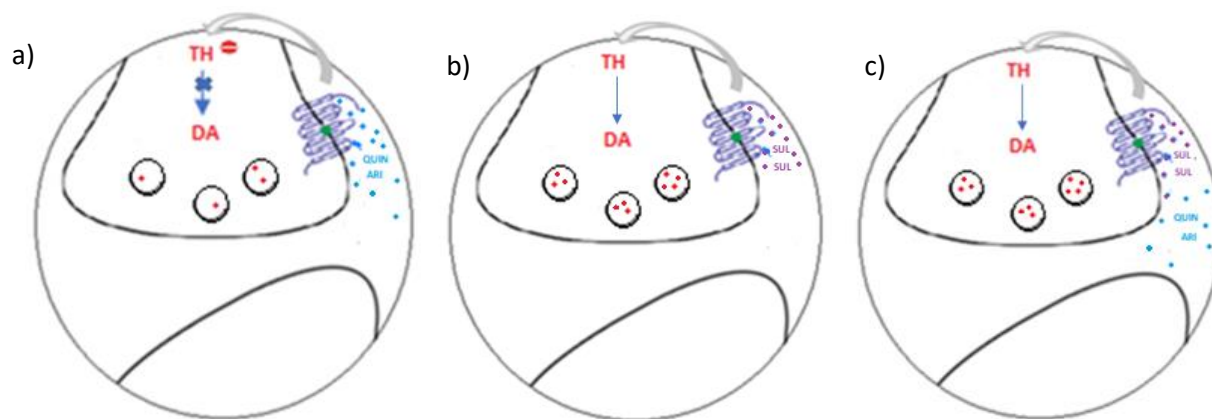
Fig. 5-1. . Illustration of the effects of DA accumulation by QUIN, ARI and SUL. The images represent that: (a) QUIN and ARI decrease DA accumulation, (b) SUL alone did not have any effect on DA accumulation, and (c) the presence of SUL blocks QUIN's and ARI's effect of decreasing DA accumulation.

I have discussed the effect of DA, which can directly inhibit TH activity. As found and discussed in detail by González-Sepúlveda et al., submitted, I have also shown that direct DA inhibition of TH decreases the capacity of DA storage at presynaptic neurons. Increases in cytosolic DA availability can negatively feedback on TH, showing an incubation-time-dependent effect (fig. 4-1 and González-Sepúlveda et al., submitted). The continuation of DA being synthesized during samples' incubation and transport by VMAT-2 inside synaptic vesicles leads to an increase of DA accumulation. VMAT-2 inhibition by TBZ significantly blocks its activity to transport monoamine neurotransmitters including DA into synaptic vesicles, thus decreasing DA accumulation (fig. 4-7). I have reported a significant depletion of DA levels with the E_{max} 87.0%. My result is compatible with another finding, which also found that TBZ depletes striatal DA content by approx. 90% (Reches et al., 1983). My finding is almost the same as that reported by González-Sepúlveda et al. on [^{3}H]-DA synthesis (EC_{50} of 61.0nM), suggesting that the effect of TBZ on DA accumulation is dependent on the amount of DA being synthesized, both being regulated by cytosolic DA on TH.

In addition to exerting a negative-feedback inhibition on TH, higher cytosolic DA will also lead to its metabolism with increased DOPAC levels. Similar to the increase of DOPAC concentration reported by Reches et al., 1983, I have also found that TBZ significantly increases DOPAC levels. This effect was observed at much higher concentrations (EC_{50} of 106.0nM) with almost 2-fold increase, reaching 10-fold if we consider the DOPAC/ DA concentration ratio. This result indicates the prominent action of cytosolic DA in modulating a negative-feedback inhibition on TH at lower cytosolic DA levels than needed to being metabolized, thus indirectly indicating higher DA affinity towards TH than MAO. This is supported by another experiment where I found that TBZ significantly inhibited VMAT-2 action, causing a decrease in endogenous DA accumulation in as short as a 10-min drug incubation time, while a significantly higher DOPAC concentration was observed at 30-min drug incubation time (fig. 4-8). These results were also consistent with the data used by a mathematical model where the reaction velocity of cytosolic DA on TH was higher than its metabolism by MAO (Best et al., 2009). Although the basal cytosolic DA levels have been reported to be normally quite low (Best et al., 2009), I demonstrated a prominent effect, that is, it can produce a significant inhibition of DA synthesis/accumulation. Interestingly, this powerful modulation by cytosolic DA on TH overcomes the addition of D_2R activation effects mediated by ARI (fig. 4-9).
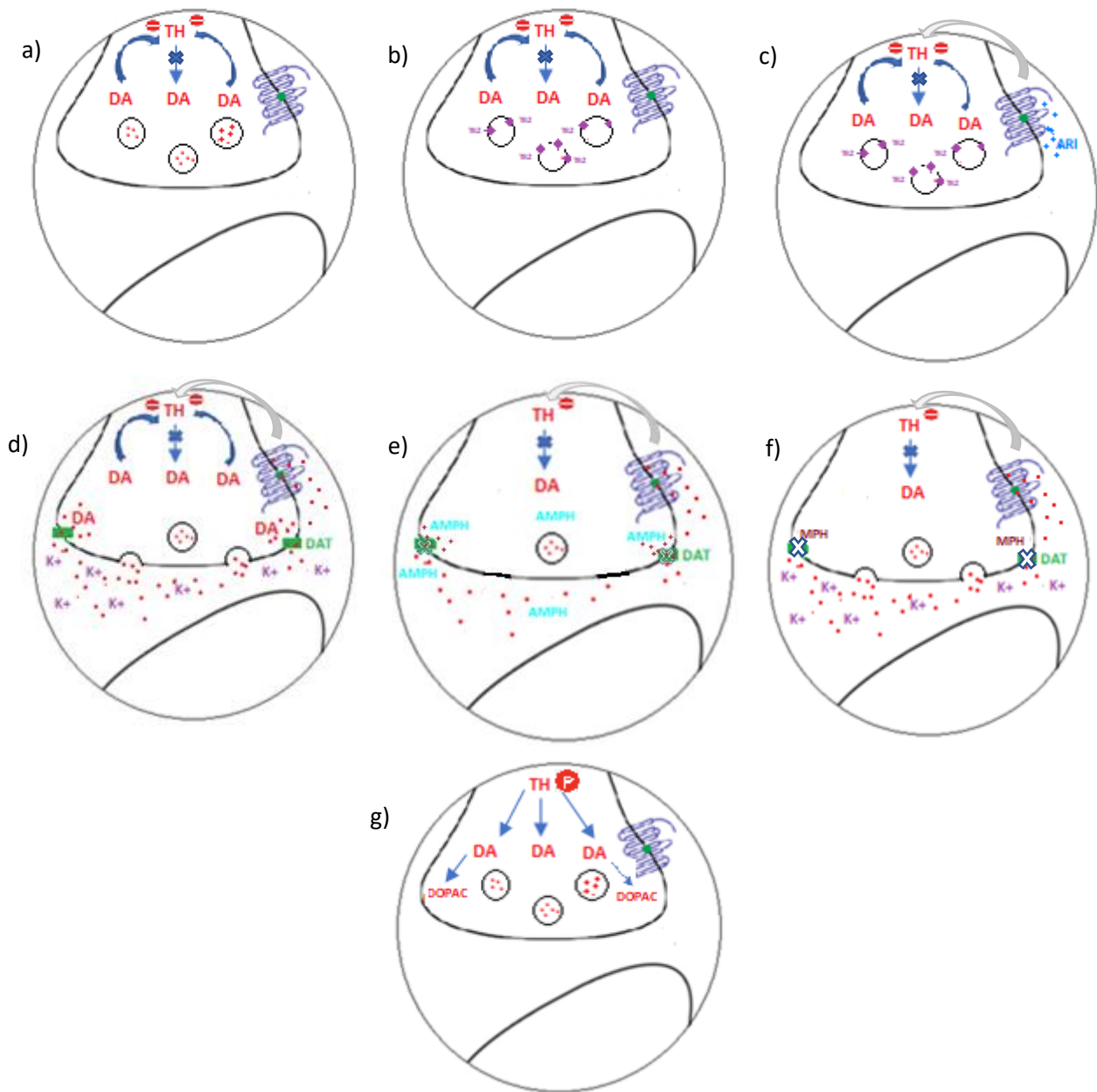
Fig. 5-2. Different experimental manipulations to demonstrate various homeostatic regulations at presynaptic terminals in modulating negative-feedback inhibition on TH by DA. a) Due to the limitation of DA storage capacity, excess of cytosolic DA can modulate a negative-feedback inhibition on TH under time-incubation effect; b) Presence of TBZ block the uptake of DA into synaptic vesicles, increasing cytosolic DA, which can regulate a negative-feedback inhibition on TH; c) A prominent effect of cytosolic DA on a negative-feedback inhibition on TH overcomes the effect of D_2R activation mediated by ARI; d) A high dopaminergic tone induced by high K^+ concentrations increases extracellular DA, which can exert a negative-feedback inhibition on TH both via D_2R and DA reuptake by DAT, contributing to the increase in cytosolic DA; e) Dual regulations of AMPH to induce DA release and block the reuptake of DA by DAT, thus increasing extracellular DA, which can modulate a negative-feedback inhibition on TH via D_2R; f) Presence of MPH presumably reduces cytosolic DA levels at high

158

dopaminergic tone, induced by higher K^+ concentrations, thus increasing extracellular DA. which can modulate a negative-feedback inhibition on TH via D_2R; and (g) Contrarily, excess of cytosolic DA by TH phosphorylation with OKA tends to increase its metabolism and difficulties to exert a negative-feedback inhibition on TH by both D_2R and direct DA inhibition of TH.

An increase of DA accumulation was observed with the effects on TH phosphorylation by the phosphatase inhibitor OKA (fig. 4-4). However, the presence of OKA at the same time increased DOPAC levels at a much higher magnitude, thus indicating an excess of cytosolic DA that overcomes its capacity to be stored. Differently from the effects of TBZ, where cytosolic DA can exert direct negative-feedback inhibition on TH to decrease DA accumulation, excess of cytosolic DA by OKA difficulted the feedback mechanisms. González-Sepúlveda et al. have reported that DA can directly inhibit TH irrespective of its phosphorylation status, thus indirectly suggesting that OKA may favor DA metabolism by MAO more than the phosphorylation relief of DA binding to the cofactor site, leading to inhibition on TH. Therefore, if two sites (low- or high-affinity)/conformation for DA inhibition of TH exist (as reported by González-Sepúlveda et al.), then we could assume that only one site depends on TH phosphorylation, increased by OKA which is the high-affinity site. In addition, OKA favors a phosphorylated state of TH, while D_2R activation favors a dephosphorylated state. Thus, in the presence of OKA, the ability to decrease DA synthesis by phosphorylation is compromised.

Higher dopaminergic tone was mimicked in our *ex-vivo* studies with different K^+ concentrations (Nayadoleni N.R, Master thesis 2019). As the extracellular K^+ concentrations increase, the chemical gradient between the intra and extra cellular become less steep, leading the voltage of the membrane to depolarize and to release DA. Such high levels of DA extracellular tone elicited by a K^+-induced depolarization exert a negative-feedback inhibition on TH. Since the sensitivity of this technique could not quantitatively measure the amount of DA being released, I

cannot know whether high K^+ concentration will produce extracellular DA levels approaching those of either tonic or phasic transmission (nM vs uM DA concentrations (Krenz et al., 2013)), or either low- or high- affinity state of the D_2R. However, these data indicate that: (1) the beneficial effect of D_2 agonist and partial agonist in modulating presynaptic D_2R could be clearly assessed at the low dopaminergic tone, and (2) the effect of changes in K^+ concentrations to 15mM will mimic a high dopaminergic tone, as mentioned by Ma et al., 2015, which may change the negative-feedback inhibition of TH. This is supported by another experiment, in which I found that AMPH significantly decreased DA accumulation, likely through D_2R stimulation by released DA (fig. 4-10). However, AMPH increased DA release and blocked the reuptake activity, both through DAT. This treatment must have decreased cytosolic DA levels as compared with TBZ, as shown by the lower DOPAC levels elicited by AMPH vs TBZ. A proof of the important regulation of DAT to increase the negative-feedback inhibition of TH by cytosolic DA under high dopaminergic tone is that the presence of MPH significantly reduced the effect of the DA tone (fig. 4-12). Such an effect indicates a less prominent regulation by D_2R than by cytosolic DA after reuptake via DAT in modulating a negative-feedback inhibition on TH at presynaptic terminals.

Discussions

2.   **Second objective: Examine and compare the mechanism of actions of D_2-like partial agonist antipsychotics at presynaptic D_2R.**

In this study, *ex-vivo* experiments were done with rat brain striatum to account for changes in presynaptic dopaminergic neurotransmission to antipsychotics drugs. I used two different methods to determine the amount of $[^3H]$-DA being synthesized by using $[^3H]$-Tyr as a precursor and quantifying endogenous DA or DOPAC levels. Most experiments were performed under non-depolarizing conditions that maintain D_2R free from released DA. Under these conditions, presynaptic D_2 receptor stimulation with D_2R partial agonist antipsychotics decreased DA synthesis efficiently, allowed less endogenous DA being stored and, in some cases, it also reduced the amount of DOPAC formed – with similar efficacy as the full D_2 agonist, QUIN –. Since the etiopathology that contributes to the development of schizophrenia symptoms appears to increase the DA synthesis capacity, its availability and release from presynaptic terminals in the striatum (Howes et al., 2012), it is tempting to speculate that the DA synthesis and storage in schizophrenics' brains could also be regulated similarly by treatment with D_2R partial agonist antipsychotics, thus contributing to their mechanisms of action during psychosis.

In these *ex-vivo* studies, I demonstrate the ability of D_2R partial agonist antipsychotics to inhibit $[^3H]$-DA synthesis and to decrease endogenous DA accumulation, while SUL did not produce any significant changes at presynaptic dopaminergic terminals under non-depolarizing conditions (fig. 4-21). This is consistent with the properties of SUL, which acted as a D_2 antagonist and was aimed to block D_2 receptors, the classic mechanisms of action of all typical and atypical

antipsychotics. It may be interesting to compare my results with the effect of antipsychotics on DA synthesis capacity in clinical studies. Several studies on the effect of antipsychotics on DA synthesis capacity were reviewed. However, there are contradictory results, where one study showed a significant decrease of DA synthesis capacity in schizophrenics with chronic administration of haloperidol (Gründer et al., 2003), while others did not find any significant changes in DA synthesis capacity with ARI (Ito et al., 2012), risperidone (Ito et al., 2009), or between different antipsychotics treatments (Jauhar et al., 2019). Other studies have also found that there is a negative correlation between acute and chronic administrations of haloperidol on DA synthesis capacity: chronic treatment decreases DA synthesis capacity (Gründer et al., 2003), but acute administration of haloperidol increases it (Der-Ghazarian et al., 2010). An increase of DA synthesis capacity with acute treatment was not only seen with haloperidol but also with ARI (Gründer et al., 2003). The inconsistency in the changes of DA synthesis capacity reported between different studies cannot be fully understood, as it might be due to (1) variances in administration dosage, or (2) holistic neural network regulations in addition to its pharmacological action on the D_2 receptor, or (3) receptor desensitization that may differentiate between acute and chronic effects. It is also worth mentioning that in the DA synthesis capacity techniques, DA formation was measured from radiolabeled L-DOPA as the precursor, thus missing the action of TH, the rate-limiting step in DA synthesis.

The similarities and differences in the functional properties and efficacies of D_2-like partial agonist antipsychotics were compared at presynaptic D_2R. Based on the concentration-response curves in fig. 4-14 and fig. 4-18, pharmacological parameters of D_2R partial agonist antipsychotics on [^{3}H]-DA synthesis and on endogenous DA accumulation were analyzed for all the concentrations as well as for those shown to be D_2R-dependent (table 1 and table 2). Referring to

the EC_{50} value, ligand efficacies on D_2R-dependent inhibition of [^{3}H]-DA synthesis and endogenous DA accumulation were compared, with the rank order being ARI > CARI > BREX. In the introduction, I make a comparison of the affinity between partial agonist antipsychotics at D_2R. BREX has the highest affinity for the receptor, followed by ARI and CARI at much less affinity (Kiss et al., 2010; K. Maeda et al., 2014; Shapiro et al., 2003). However, in this study, the efficacy of D_2R partial agonist antipsychotics to inhibit [^{3}H]-DA synthesis and to decrease endogenous DA accumulation is not correlated with their affinity towards the D_2 receptor. Referring to the E_{max} value, as partial agonism, D_2R partial agonist antipsychotics show an intermediate efficacy to inhibit [^{3}H]-DA synthesis and to decrease endogenous DA accumulation. Although this calculation is dependent on the maximal concentrations used in this study, such an intermediate effect would allow fewer DA levels to remain beneath the threshold for the development of positive symptoms induced by excessive DA concentrations (as discussed in the etiology of schizophrenia), but at the same time not to exceed the minimum DA levels, to avoid causing an adverse neurological effect. With the efficacy of partial agonists shown to be intermediate, thus, the important question could arise on the optimal agonist efficacy needed for its therapeutic efficacy. Comparing the efficacy of my results with other studies, my results validate the hypothesis proposed that BREX had lower efficacy at D_2R than ARI, biasing it towards antagonists' effects (Stahl, 2016). In addition, other studies have also found that BREX has less intrinsic activity than ARI at D_2 receptor (K. Maeda et al., 2014; Oosterhof et al., 2014), which is in agreement with my results that BREX does not only have less efficacy but also less potency than CARI and ARI at D_2R in regulating presynaptic D_2R on the inhibition of [^{3}H]-DA synthesis and decreasing spontaneous endogenous DA accumulation (D_2R-dependent).

	QUIN	ARI	CARI	BREX
E_{max}, % (total effect)	51	51	52	75
E_{max}, % (D_2R-dependent)	44	42	47	15
EC_{50}, nM (total effect)	4.5	4.8	8.7	51.2 (1st) / 964.8 (2nd)
EC_{50}, nM (D_2R-dependent)	4.2	3.5	7.1	57.5

Table 1 indicates the pharmacology parameters for each concentration-response curve of D_2R agonist and partial agonist antipsychotics on [^{3}H]-DA synthesis at the concentrations mediated by D_2R activation or their total effect which is partly D_2R-independent.

	QUIN	ARI	CARI	BREX
E_{max}, % (total effect)	63	43	44	85
E_{max}, % (D_2R-dependent)	63	43	44	41
EC_{50}, nM (total effect)	76.0	7.6	1.2	1.8 (1st) / 1272.4 (2nd)
EC_{50}, nM (D_2R-dependent)	1.2	0.1	1.3	1.9

Table 2 indicates the pharmacology parameters for each concentration-response curve of D_2R agonist and partial agonist antipsychotics on endogenous DA accumulation at the concentrations mediated by D_2R activation or their total effect which is partly D_2R-independent.

In modulating presynaptic D_2R, I demonstrate that CARI – similarly to ARI – exhibits both agonist and antagonist properties depending on whether the experimental conditions mimic low and high dopaminergic tone (fig. 4-19). These results are consistent with the lower intrinsic activity of D_2R partial agonists as compared to DA and underline the key role of their unique "DA stabilizer" property on dopaminergic neurotransmission. Low dopaminergic tone under our conditions implies that $6.6 \pm 1.7\%$ of newly synthesized [^{3}H]-DA was released to the medium. Increasing K^+ concentrations to 15mM will mimic a high dopaminergic tone (Ma et al., 2015), which prevents both ARI and CARI (fig. 4-19) from inhibiting [^{3}H]-DA synthesis. Therefore, it can be postulated that both ARI and CARI have the same functional profiles and the capacity to stabilize DA synthesis and endogenous DA levels at low dopaminergic tone while blocking DA actions at high dopaminergic tones. Whether "DA stabilization" properties could be demonstrated by both ARI

and CARI during psychosis remains speculative, although such mechanisms were proven in our *ex-vivo* studies under non-depolarized and K^+-induced depolarized conditions.

The discrepancies between D_2R partial agonist antipsychotics in regulating $[^3H]$-DA synthesis and accumulation are dose-dependent. The effect of D_2R partial agonist antipsychotics at high concentrations was not restricted to D_2R activation (D_2R-independent mechanisms). This effect can be seen clearly with the concentration-response curve of BREX. BREX, which had low intrinsic activity at the concentrations related to D_2R activation, appeared to have higher apparent efficacy at high concentrations (D_2R-independent) even in the presence of a D_2R antagonist. This sort of mechanism was shown to disrupt vesicular storage, thus increasing cytosolic DA and facilitating DA efflux from dopaminergic terminals, leading to an increase of both DOPAC and the DOPAC/ DA concentration ratio under non-depolarized conditions. Although the decrement in $[^3H]$-DA synthesis (E_{max} of 75%) and endogenous DA accumulation (E_{max} of 85%) of BREX is far higher than it counts for $[^3H]$-DA concentration quantified in the media (24% of $[^3H]$-DA concentrations) (fig. 4-23), I assumed this was due to the metabolism causing increased oxidation of DA by MAO, as shown by inflation in the DOPAC/ DA concentration ratio. Higher DOPAC and homovanillic acid concentrations were also observed in the *in-vivo* microdialysis study of BREX at high concentrations in the prefrontal cortex and nucleus accumbens (Maeda et al., 2014). This finding is comparable with some of the typical antipsychotics such as haloperidol and chlorpromazine, which have been shown to relatively impair some of the monoamine transport systems at high concentrations (Pletscher, 1977). Haloperidol has also been shown to induce basal DA release, leading to an increased formation of DOPAC and subsequently inhibiting DA synthesis at the concentrations known to be at D_2R-independent (Delanoy & Dunn, 1982). Such mechanisms have also been demonstrated in chronic treatment, which leads to a decrease in DA

synthesis capacity, as shown with schizophrenics who received chronic treatments with haloperidol (Yokoi et al., 2002). It was assumed that the action of these antipsychotics was through an unspecific mechanism that indirectly impairs DA storage inside the synaptic vesicles, unlike TBZ, whose mechanism of action specifically blocks the VMAT-2 activity or AMPH to induce DA release. As interruption of DA storage leads to an increase of cytosolic DA, which can exert a negative-feedback inhibition on TH (as was previously discussed), this mechanism may affect the efficacy and properties of D_2R partial agonist antipsychotics. This can be seen on the BREX effect under K^+-induced depolarized conditions which continuously decreased $[^3H]$-DA synthesis independently of K^+ concentrations (fig. 4-19), thus differentiating the pharmacological properties of BREX from other D_2R partial agonist antipsychotics. Taking into account my results, I propose that the mechanisms of action of ARI and CARI at presynaptic terminals were mostly due to the activation of D_2R (D_2R-dependent), while BREX's were mostly via D_2R-independent mechanisms, leading to the inhibition of DA synthesis/accumulation anyway.

Overall, the significance of my *ex-vivo* results can be evaluated by comparing them with plasma concentration from schizophrenics treated with the same drugs. I found that the plasma concentration was comparable with my data that give the maximal effects on the inhibition of $[^3H]$-DA synthesis and decrease endogenous spontaneous DA accumulation (table 3). In my experiments, the efficacy of ARI and CARI was almost the same on the inhibition of $[^3H]$-DA synthesis and DA accumulation (table 1 and table 2). However, I noted higher plasma concentration for ARI as a result of greater prescription dose in patients due to its pharmacokinetic properties in humans.

	ARI	CARI	BREX
Prescription dose **(mg/ day)**	10 - 30	1.5 - 6	1- 4
Plasma concentration **(µmol/ L)**	334 - 468	6 - 14	21 - 124

Table 3 describes the clinical prescription dose and the plasma concentrations from the clinical studies with ARI, CARI and BREX treatments (Ishigooka et al., 2018; Nakamura et al., 2016; Sparshatt et al., 2010).

In the introduction, I have shown different chemical structures between all D_2R partial agonist antipsychotics. It is reported in the literature that the intrinsic efficacy of ARI most likely arises from the extended structures comprising 1,2,3,4-tetrahydroquinoline to bind with the secondary binding pocket at D_2R (Klein Herenbrink et al., 2019). Similarly, and like DA, tetrahydroquinoline showed the ability to inhibit TH by competing with the BH_4 for access to active sites (Scholz et al., 2008). This compound can also be observed in the striatum and substantia nigra from patients with Parkinson's disease. The chemical structures of 1,2,3,4-tetrahydroquinoline are described in fig. 5-3. However, this substituent can only be found with ARI and BREX – but not in CARI or LUMA – as shown in fig. 5-4, and may thus indicate a similar structure-activity relationship between ARI and BREX. In my *ex-vivo* studies, however, ARI's and BREX's similar characteristic of possessing a tetrahydroquinoline substituent in their chemical structures did not show any significant differences that may distinguish them from CARI in regulating presynaptic [^{3}H]-DA synthesis and accumulation. Thus, a direct binding of these antipsychotics to TH seems unlikely as the explanation of my results.
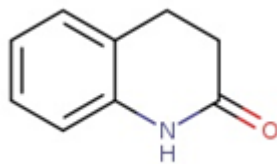
Fig. 5-3. Chemical structure of 1,2,3,4-tetrahydroquinoline.
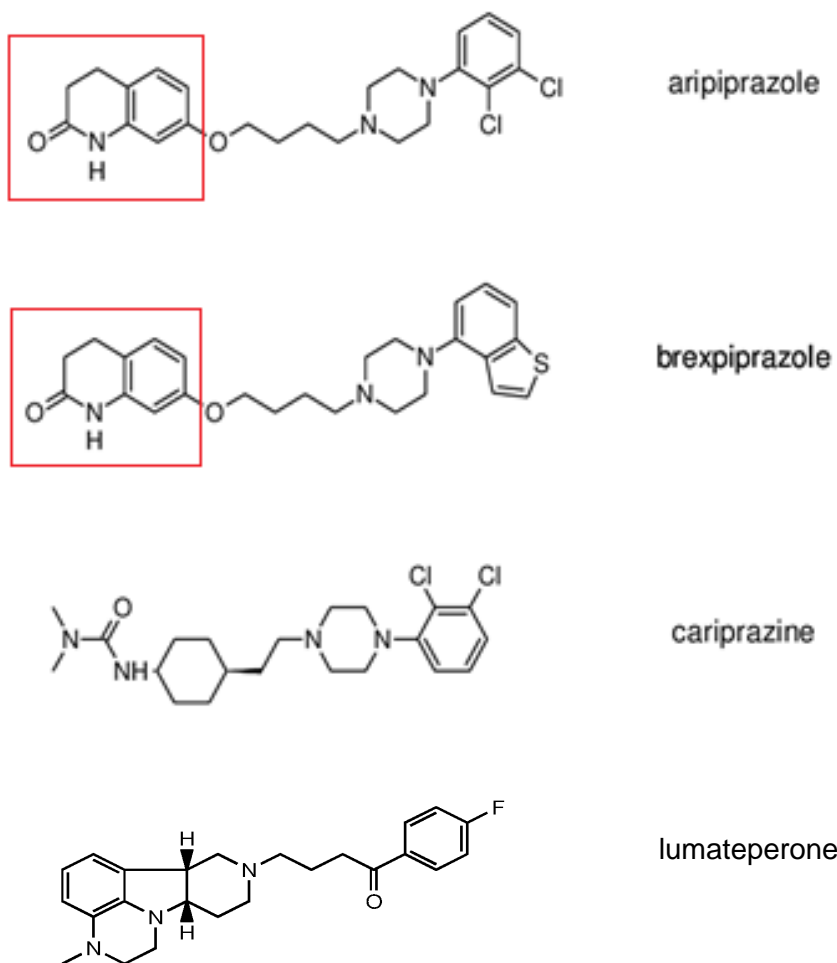
Fig 5-4. Extending structure comprising 1,2,3,4-tetrahydroquinoline substituent (shown with a red box) is in part of the chemical structures in ARI and BREX, but it does not exist in CARI or LUMA.

Discussions

3. **Third objective: Explore the implication of non-D$_2$ receptor components in the properties of D$_2$-like partial agonist antipsychotics on DA dynamics.**

The antipsychotics-mediated decreases of DA accumulation in my *ex-vivo* experiments reflect primarily on the inhibition of DA synthesis by the stimulation of D$_2$R under non-depolarized conditions. Other than that, an AMPH-like interference in vesicular DA storage leading to the inhibition of DA synthesis was reported in fig. 4-23. Nevertheless, there are other types of neuromodulators that may indirectly affect DA levels in the brain. In this study, I explore the possibility that some of the properties of D$_2$R partial agonist antipsychotics (e.g. affinity for 5-HT receptors), may indirectly regulate DA synthesis and accumulation. Even though the limitations of these *ex-vivo* experiments do not demonstrate the full mechanism of the interactions, any effect that we might observe on DA accumulation could be an indicator suggestive of cross-talk between DA and other receptors or neuromodulators.

In the introduction (section 13.4), I have mentioned the differences between all three D$_2$R partial agonist antipsychotics with regard to their binding affinity between DA D$_2$ and DA D$_3$ on DA receptors. CARI has been shown to have higher affinities (Kiss et al., 2010), and rapid binding kinetics (A. Frank et al., 2018) towards the D$_3$ receptor than ARI and BREX. At low concentrations, CARI likely binds to the D$_3$ receptor (Girgis et al., 2016). However, there are no clear results for the role of the D$_3$ receptor on the autoregulation of DA terminals, as only a few studies have reported D$_3$ regulation of extracellular DA levels (Koeltzow et al., 1998), but not DA synthesis (Joseph et al., 2002). Since DA itself has a higher affinity towards the D$_3$ than the D$_2$

receptor (Sokoloff et al., 1990), it can be hypothesized that the D_3 receptor may play an important role in regulating DA feedback inhibition at presynaptic terminals. In this *ex-vivo* assessment (fig. 4-25), I have showed a higher affinity of CARI towards the D_3 receptor was not involved in its regulation on DA synthesis. My result was also in agreement with another *ex-vivo* study showing that the effect of CARI on dopaminergic firing was not affected by D_3 blockade (Delcourte et al., 2018).

It is noteworthy that 5-HT accumulates spontaneously *ex-vivo*, like DA (fig. 4-26). To my knowledge, this Ph.D. thesis is the first description of such spontaneous 5-HT accumulation, now reported by us in González-Sepúlveda et al., submitted. Other than the differences between receptor subtypes within the D_2-like family, D_2R partial agonist antipsychotics also showed a high affinity for 5-HT receptors. Therefore, in this study, I have compared the effect of D_2R partial agonist antipsychotics on 5-HT with DA concentrations in rat brain striatum incubated *ex-vivo*. I have demonstrated (fig. 4-27) that D_2R-independent mechanisms of D_2R partial agonist antipsychotics decrease 5-HT accumulation with different efficacies. Interestingly, the differences between antipsychotics were consistent with their affinity towards 5-HT receptors: BREX has a higher affinity towards all 5-HT receptor subtypes compared with ARI and CARI, and shows the best statistically significant inhibition on 5-HT concentration and higher 5-HIAA/ 5-HT concentration ratio. The increase of the 5-HIAA/ 5-HT ratio is interpreted by me as showing that partial agonist antipsychotics may have similar effects to disrupt DA and 5-HT storage and to induce basal 5-HT release. This mechanism increases cytosolic 5-HT, leading to an increased formation of 5-HIAA and to an increase of the 5-HIAA/ 5-HT ratio. By comparing the effect of D_2R partial agonist antipsychotics on 5-HT with DA concentration, 5-HIAA with DOPAC levels and 5-HIAA/ 5-HT with DOPAC/ DA concentration ratio (fig. 4-27), it can be concluded that

similar effects of D_2R-independent mechanisms by D_2R partial agonist antipsychotics on 5-HT, but to a lesser extent on DA, were observed.

Some neurotransmitters have been known to indirectly modulate dopaminergic neurotransmission. Among them are glutamate (Kulagina et al., 2001), GABA (Pitman et al., 2014), acetylcholine (Shin et al., 2017), endocannabinoids (Covey et al., 2017), opioids (Schlösser et al., 1995), adrenergic (O'Neill et al., 2007), histamine (Aquino-Miranda et al., 2016), and 5-HT (Olijslagers et al., 2006). In fact, ARI has been shown to regulate most of these neurotransmitters levels, including 5-HT (Choi et al., 2017), adrenergic (Threlfell et al., 2012), GABA (Pan et al., 2016), endocannabinoids (Cheng et al., 2008), opioids (Ferreira et al., 2017), and glutamate (Choi et al., 2017) other than DA. CARI also showed the same with glutamate (Choi et al., 2017; J. Kehr et al., 2018), noradrenaline (J. Kehr et al., 2018) and 5-HT (Choi et al., 2017; J. Kehr et al., 2018), while BREX has been reported to regulate glutamate (Björkholm et al., 2017), 5-HT (Oosterhof et al., 2014, 2016), noradrenaline (Oosterhof et al., 2014), and adrenergic (Munkhof et al., 2017; Oosterhof et al., 2014), other than DA levels. Even though for this *ex-vivo* experimental protocol I had minced the rat brain striatum interrupting neuronal firing, receptors located on the same dopaminergic terminals or neighboring cells allow the possibility of being blocked and releasing modulators different from DA, which could trigger actions on DA terminals. For example, 5-HT receptors were expressed on dopaminergic neurons (Olijslagers et al., 2006). Together with the effect of D_2R partial agonist antipsychotics on 5-HT levels as discussed above, I hypothesized that 5-HT probably acts as a neuromodulator and indirectly modulates dopaminergic dynamics. This is supported by several studies on the possibility of cross-talk between 5-HT and DA, as I discussed in the introduction. Such mechanisms may enhance the efficacy of partial agonist antipsychotics on its inhibition of DA synthesis in my *ex-vivo* assessments. However, my experiment with

selective ligands, which showed similar properties with D_2R partial agonist antipsychotics including 8-OH-DPAT ($5\text{-}HT_{1A}$ and $5\text{-}HT_7$ receptors agonist), MDL 100907 ($5\text{-}HT_{2A}$ receptor antagonist) and SB 258719 ($5\text{-}HT_7$ receptor antagonist), showed a lack of interaction between these 5-HT receptor subtypes and DA accumulation. Two possibilities may explain these results: (1) there is no cross-talk between those 5-HT receptor subtypes and the D_2 receptor, and a higher affinity of D_2R partial agonist antipsychotics on these selected 5-HT receptor subtypes did not have any effects that may alter their efficacy to decrease DA accumulation in rat brain striatum incubated *ex-vivo*; (2) the 5-HT receptor density in the brain striatum is much lower than the D_2 receptor, so any regulation leading to slight changes would be difficult to quantify or to give a significant decrease on DA accumulation in our assessment system. This opens the possibility of involvement from other neurotransmitters or neuromodulators, e.g. glutamate receptor, which is also expressed on dopaminergic neurons (Engblom et al., 2008), together with the existence of metabotropic DA with glutamate receptors (Hu et al., 1999).

Limitations

Some limitations of my ex-vivo experiments have been observed, as follows:

➢ In terms of the methodology, I have found the limitation of the total number of sample incubations for each experiment. This was due to the maximum sample tubes that can be placed in the incubator during incubations. This limitation increased the variability of the number of replicates for each drug concentration used, which was accumulated from more than one brain incubation.

➢ For the determination of endogenous DA and DOPAC concentrations, I also found there is variability on the baseline between different rats used. Such variations affect the average values when pooling the data from more than one brain incubation. Recent results from our group show that not only do both DA and DOPAC concentrations vary between animals, but brain slicing also increases DOPAC levels while reducing DA concentrations (Anna Galán, unpublished, 2020). Thus, the DA accumulation reflects a DA replenishment of functional dopaminergic nerve endings, reaching higher levels than previously to slicing, and representing maximal storage capacity.

➢ Since the study aimed to measure the changes in presynaptic regulation on DA synthesis/accumulation, the effects of the activation of D_2L receptor at postsynaptic dopaminergic terminals that may retrogradely be affecting presynaptic DA synthesis were not studied by our experimental set-up, and they may likely contribute to their clinical profile. This may be important since QUIN (Lindgren et al., 2003), ARI (Kikuchi et al., 1995), and CARI (Kiss et al., 2010) have been shown to activate D_2L receptor at the same time. Also, a Master's work performed in our

group with my co-supervision (Nayadoleni Nieves Rivera, 2019) has shown that under depolarizing conditions, the release of postsynaptic messengers may alter D_2R responses.

> ➢ The effect of DA levels in *ex-vivo* studies does not represent the pharmacological effect of the activation of the D_2 receptor alone, while several regulations might be involved that may indirectly modulate DA levels. However, the challenging complexity of rat striatal tissue may offer an attractive model of study of drug action on brain synapses, with future applications to live animal models and human studies.

Future studies

Several experiments were planned and proposed during this study. However, with the time-limit of the thesis together with the unexpected pandemic of Covid-19 that brought about a new regulation for mobility to the University, these experiments were delayed, and can be followed up in the future. These include:

➢ **LUMA.**

As mentioned in the introduction, LUMA was recently approved by the U.S. F.D.A. in December 2019 as an antipsychotic. The unique description of LUMA as compared to other partial agonist antipsychotics is its ability to act differently on different D_2 receptors (partial agonist at D_2S and an antagonist at D_2L); whether such characteristics mark a real difference with ARI, CARI and BREX should be studied. As the second objective of this thesis is to compare and examine the efficacy and properties of partial agonist antipsychotics in modulating presynaptic D_2R, inclusive data from LUMA would be useful for comparison. The short time span after the U.S. F.D.A.'s approval of LUMA made it impossible to include it in the present work.

➢ **Combination of PAOPA with partial agonist antipsychotics.**

I have shown that PAOPA significantly enhances the QUIN effect to decrease DA accumulation. PAOPA has also been shown to prevent deficits in social interaction induced by NMDA receptor antagonists (Dyck et al., 2011); thus the combination of PAOPA with partial agonist antipsychotics may have beneficial implications on their efficacy for the treatments of both positive and negative symptoms in schizophrenia.

➢ **Do D$_2$R independent mechanisms of partial agonist antipsychotics that mediate DA release can be observed with other antipsychotics?**

I have demonstrated that at high concentrations, D$_2$R partial agonist antipsychotics interfere with DA storage and induce DA release from synaptic vesicles. These mechanisms increase cytosolic DA, which tends to modulate a negative-feedback inhibition on TH. Although it has been shown that haloperidol may have a similar mechanism at high concentrations (Delanoy & Dunn, 1982), it will be interesting if these experiments are extended to other typical or atypical antipsychotics, if they exhibit similar mechanisms that may affect their properties.

➢ **D$_2$ receptor occupancy.**

I have shown the differences in the efficacy of partial agonist antipsychotics at presynaptic D$_2$R. The efficacy of these ligands is also highly associated with their ability to bind at the receptor. Therefore, a comparison between receptor occupancy and the ligands' effect may be beneficial to compare my *ex-vivo* data with others from *in-vivo* or clinical studies. Since partial agonist antipsychotics may bind to both D$_2$S and D$_2$L receptors and unless we can carefully isolate between these two membranes, it will be difficult to correlate between the total and selected receptor occupancy at D$_2$R.

➢ **Postsynaptic "retrograde" messenger regulation of DA accumulation.**

In addition to D$_2$R, there are other neurotransmitter receptors on DA cell bodies that may regulate DA synthesis at presynaptic terminals. These include GABA$_B$, opioid, muscarinic acetylcholine and ionotropic metabotropic glutamate receptors, which have been shown to inhibit DA release (Dewey et al., 1993; Schlösser et al., 1995; Schmitz et al., 2002; Zhang & Sulzer, 2003). Such interactions may affect the efficacy and property of D$_2$R partial agonist antipsychotics in regulating DA synthesis at presynaptic terminals.

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VI. CONCLUSIONS

Conclusions

1. **First objective: Increase our understanding of the overall homeostatic mechanisms toward regulation of DA accumulation at presynaptic neurons in the striatum.**

    ➤ D_2R exerts a negative-feedback inhibition on TH. The effect was observed with a D_2-like agonist, QUIN, and a partial agonist antipsychotic, ARI. Both compounds decreased DA accumulation and DOPAC concentration *ex-vivo* but with different efficacy corresponding to their full agonist and partial agonist characteristics. While QUIN decreased the DOPAC/ DA concentration ratio, ARI did not significantly change it.

    ➤ Blockade of D_2R by D_2 antagonist, SUL, prevented the effects of QUIN but not those of DA application to the incubation, suggesting additional effects of DA to modulate a negative-feedback inhibition on TH other than stimulating D_2R, probably exerting direct TH blockade by reuptaken cytosolic DA.

    ➤ The phosphatase inhibitor OKA does not only increase DA accumulation but simultaneously appears to increase its metabolite levels more than DA accumulated.

    ➤ The efficacy of QUIN to decrease DA accumulation increased in the presence of the D_2 positive allosteric modulator PAOPA.

    ➤ Higher cytosolic DA due to interruption of DA storage via inhibition of VMAT-2 activity by TBZ exerts a negative-feedback inhibition on TH. This effect decreased DA accumulation but increased DOPAC levels, consistently with the higher metabolization of cytosolic DA.

- Simultaneous incubation with TBZ and ARI did not show synergistic effects on DA accumulation. This suggests that the prominent regulation by cytosolic DA on TH overcomes D_2R activation effects mediated by ARI.

- Depolarization induced by increasing K^+ concentrations decreases DA accumulation, consistently with DA inhibition of its own synthesis and potentiation of metabolism after being released.

- AMPH has similar effects as those produced by higher dopaminergic tone induced by higher K^+ concentrations. This effect decreased DA accumulation, probably through D_2R stimulation by released DA, but it did not increase DOPAC levels as the effect with TBZ, suggesting less cytosolic DA was available to be metabolized due to D_2R activation.

- The effect of MPH is dependent on membrane potential-induced DA release by 15mM K^+. Under this condition, MPH attenuates effects induced by 15mM K^+ on DA accumulation, DOPAC levels and the DOPAC/ DA concentration ratio.

2. **Second objective: Examine and compare the mechanism of actions of D_2-like partial agonist antipsychotics at presynaptic D_2R.**

The properties of QUIN and ARI previously documented at presynaptic D_2R were used as references in this study (Ma et al., 2015). Thus, the same [^{3}H]-DA synthesis method and endogenous DA accumulation (González-Sepúlveda et al., submitted) were used in rat striatal brain samples incubated *ex-vivo*. I found that:

- All D_2-like partial agonist antipsychotics activated D_2R to inhibit presynaptic [^{3}H]-DA synthesis and decreased the spontaneous endogenous DA accumulation – similarly to QUIN –. Ligands' efficacies on D_2-dependent (blocked by SUL) inhibition of [^{3}H]-DA synthesis and endogenous DA accumulation were compared, with the rank order being ARI > CARI > BREX.

- Both CARI and ARI showed similar agonist properties at D_2 receptor under non-depolarized conditions, but lost this property to become antagonists under K^+-stimulated conditions, differently to BREX that maintained D_2R-independent inhibition of [^{3}H]-DA synthesis under both conditions.

- The effect of D_2-like partial agonist antipsychotics at high concentrations was not restricted to D_2R activation (D_2-independent mechanisms). This mechanism increased cytosolic DA, as shown by the increase both in DOPAC levels and in the DOPAC/ DA concentration ratio. This enhanced their efficacy to inhibit [^{3}H]-DA synthesis and decreased endogenous DA accumulation through disrupted DA storage. They likely induced DA release from dopaminergic terminals (partially similar to AMPH) as they increased DA metabolism.

3. Third objective: Explore the implication of non-D$_2$ receptor components in the properties of D$_2$-like partial agonist antipsychotics on DA dynamics.

The non-D$_2$ receptor components of D$_2$-like partial agonist antipsychotics were studied to investigate whether they contribute to the D$_2$-independent mechanisms described above. Under the same experimental conditions, I observed that:

➢ The higher affinity of CARI towards the D$_3$ receptor than ARI and BREX is not involved in CARI effects on DA accumulation.

➢ A spontaneous increase in the 5-HT accumulation during tissue samples incubation was also observed, a similar phenomenon as occurred on DA.

➢ D$_2$R-independent activation mechanisms of partial agonist antipsychotics influence both 5-HT and DA dynamics but affect 5-HT to a lesser extent than DA accumulation.

➢ The high affinity of ARI, CARI and BREX towards certain 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_7$ receptor subtypes does not affect either the decrease of DA accumulation.

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VIII. APPENDIX