Functional responses of pre- and postsynaptic dopamine D_2 receptors in rat brain striatum

Guofen Ma

Autonomous University of Barcelona, Spain

Functional responses of pre- and postsynaptic dopamine D_2 receptors in rat brain striatum

Memory by Guofen Ma, Master of Biochemistry and Molecular Biology, for the degree of Doctor from the Autonomous University of Barcelona.

The work was done under the co-direction of Ph.D. Jordi Ortiz de Pablo and Josefa Sabria Pau at the Autonomous University of Barcelona, Spain

Jordi Ortiz de Pablo

Josefa Sabria Pau

Guofen Ma

Autonomous University of Barcelona, Spain

Abbreviations

AC	adenylyl cyclase
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors
ANOVA	ANalysis Of VAriance
ARI	aripiprazole
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
CaMK	calcium/calmodulin dependent kinase
cAMP	cyclic adenosine 3',5'-monophosphate
CGP	CGP 54626, antagonist of $GABA_B$ receptor
CNS	central nervous system
CRE	cAMP regulated element
CREB	CRE-binding protein
D_1R, D_2R	dopamine D_1 receptor, dopamine D_2 receptor
DAG	diacylglycerol
DARPP-32	dopamine and cAMP-regulated phosphoprotein of 32kDa
DAT	dopamine transporter
DNA	deoxyribonucleic acid
DNS	donkey serum
DTT	dithiothreitol
ERK	extracellular signal-regulated kinase
EPSC	excitatory postsynaptic currents
fEPSP	field excitatory postsynaptic potential
FMR1	fragile X mental retardation 1
FR	fixed ratio

FRET	Förster resonance energy transfer
GABA	γ -aminobutyric acid
GADPH	glyceraldehyde 3-phosphate dehydrogenase
G-protein	guanine protein
GDP	guanosine diphosphate
GEF	guanine exchange factor
GP	glopus pallidus
GPCR	guanine nucleotide binding protein receptors
GRK	GPCR kinase
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
HFS	high frequency stimulation
HRP	horseradish peroxidase
IP 3	inositol (1,3,4) triphosphate
LFS	low frequency stimulation
LTD	long-term depression
LTP	long-term potentiation
MAP	mitogen activated protein
MAPK	MAP kinase
mEPSC	miniature excitatory postsynaptic current
mGluR	metabotropic glutamate receptor
MAO	monoamine oxidase
MEK	MAPK kinase
MSN	medium spiny neurons
OKA	akadaic acid, an PP2A inhibitor

O/N	over night
PAGE	polyacrylamide gel electrophoresis
PAM	positive allostoric modulator
PBS	phosphate buffered saline
PET	positron emission tomography
PIP 2	phosphatidylinositol diphosphate
PI3K	phosphatidylinositol 3-kinases
PKA	protein kinase A
PKC	protein kinase C
PLA 2	phospholipase A 2
PLC	phospholipase C
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PPR	paired pulse ratio
QUIN	quinpirole, agonist of D2-like receptors
RT	room temperature
RACL	raclopride, antagonist of D2 receptors
SDS	sodium dodecyl sulfate
SN	substantia nigra
SP	substance P
STN	subthalamic nucleus
SUL	sulpiride, antagonist of D2-like receptor
TBS	tris buffered saline
TB	tris buffer without saline
TRP	transient receptor potential

TH	tyrosine hydroxylase
VTA	ventral tegmental area

ABSTRACT

The dopaminergic system has been the focus of much research during the past several decades mostly because it is believed to be involved in several pathological conditions such as Parkinson's disease, schizophrenia, Tourette's syndrome, as well as in drug abuse. All the five subtypes of dopamine (DA) receptors belong to the family of the G-protein coupled receptors (GPCRs). Among them, D2 subtype are the primary target for all known antipsychotic drugs (antagonists) and drugs used to treat Parkinson's disease (agonists). Unfortunately, the antagonistic effects on D2 receptors also elicit serious side effects. The low liability of side effects of the newest generation of antipsychotics (aripiprazole) is proposed to be based on selective D2 receptor functions, however further evidence is needed to support this hypothesis. In our study, we observed a variable property of aripiprazole on D2 autoreceptors in response to changes of the extracellular DA concentration, which is consistent with the D2 receptor "selective function" opinion. On the other hand, the concept of the heteromerization between colocalized GPCRs has been widely accepted and is suggested to be a new field for therapeutic improvement. Thus, the studies concerning the identification of new pairs of heteromers related to D2 receptors may broaden the pharmacology of the dopaminergic system. In this thesis, we found functional crosstalk in two pairs of GPCRs: between GABAB and D2R, and between orexin-1 and D2R. Although our results indicate the probability of their heteromerization, further studies are still needed. Dopamine is crucial in the brain reward system, which plays an important role in drug addiction. Chronic cocaine administration is widely reported to alter the dopamine D1 and D2 receptor density or affinity, as well as their interaction with other receptors. In our cocaine self-administration model, we found changes in dopamine system in response to the D2R agonist quinpirole in the long-term and short-term withdrawal rats. In conclusion, our results in cocaine administration emphasize the importance of dopamine system in cocaine addiction. Moreover, the functional crosstalk of D2 receptors with others GPCRs may supply new possibilities for developing innovative treatments in drug abuse as well as in schizophrenia.

RESUMEN

El sistema dopaminérgico ha sido ampliamente estudiado en las últimas décadas, principalmente por su implicación en diversas patologías como la enfermedad de Parkinson, la esquizofrenia o el síndrome de Tourette, así como en el abuso de drogas. Se han descrito cinco subtipos de receptores para la dopamina (DA), todos ellos pertenecientes a la familia de receptores acoplados a proteínas G (GPCRs). De estos cinco subtipos, los receptores D2 son la diana principal de los antipsicóticos (antagonistas) y también de los fármacos utilizados en el tratamiento del Parkinson (agonistas). Desafortunadamente, los efectos antagonistas sobre los receptores D2 conllevan importantes efectos secundarios indeseables. Los antipsicóticos de nueva generación (como el aripiprazole) presentan menos efectos secundarios, hecho que se ha atribuido a diferente selectividad funcional sobre los receptores D2, aunque se requieren más estudios para confirmar esta hipótesis. En nuestro trabajo, hemos observado distinta actividad del aripiprazol sobre los autoreceptores D2 en respuesta a cambios en las concentraciones extracelulares de DA, lo cual es coherente con la hipótesis de que los receptores D2 presenten "selectividad funcional". Por otra parte, actualmente está cada vez más aceptado el concepto de heteromerización entre distintos GPCRs que co-localizan en las mismas muestras tisulares, y representa un nuevo campo para el diseño de mejores terapias. Por ello, los estudios dirigidos a la identificación de nuevos heterómeros que impliquen los receptores D2, permite ampliar la farmacología del sistema dopaminérgico. En esta tesis hemos observado *crosstalk* funcional entre dos pares de GPCRs: entre D2 y GABAB por un lado, y entre D2 y el receptor-1 de Orexina por otro. Aunque nuestros resultados indican que es muy probable que ambos pares de receptores formen heterómeros, se necesitan más datos para corroborarlo. La DA es crucial en el sistema cerebral de la recompensa, que juega un papel primordial en el fenómeno de la adicción. Está descrito que la administración crónica de cocaína altera la densidad y la afinidad de los receptores D1 y D2, así como también su interacción con otros receptores. En nuestro modelo de auto-administración de cocaína en ratas, hemos encontrado cambios en la respuesta del sistema dopaminérgico al agonista D2 quinpirole, después de la abstinencia de corto y también de largo plazo. En suma,

nuestros resultados sobre la administración de cocaína subrayan la importancia del sistema dopaminérgico en la adicción a la cocaína, y además, el *crosstalk* funcional que hemos observado entre los receptores D2 y otros GPCRs abre nuevas posibilidades para el diseño y desarrollo de nuevos tratamientos para el abuso de drogas así como también para la esquizofrenia.

RESUM

El sistema dopaminèrgic has estat molt estudiat en els darrers anys, principalment degut a la seva implicació en diverses patologies com la malaltia de Parkinson, la esquizofrènia o la síndrome de Tourette, així com també en l'abús de drogues. S'han descrit cinc subtipus de receptors per la dopamina (DA), tots els quals pertanyen a la família de receptors acoblats a proteïnes G (GPCRs). D'aquests cinc subtipus, els receptors D2 son la diana principal dels antipsicòtics (antagonistes) i també dels fàrmacs utilitzats en el trastament del Parkinson (agonistes). Malauradament, els efectes antagonistes sobre els receptors D2 comporten importants efectes secundaris indesitjables. Els antipsicòtics de nova generació (com l'aripiprazole) presenten menys efectes secundaris, la qual cosa s'ha atribuït a diferent selectivitat funcional sobre els receptors D2, malgrat que es necessiten més estudis per confirmar aquesta hipòtesi. En el nostre treball, hem observat diferent activitat de l'aripiprazol sobre els auto-receptors D2 en resposta a canvis en les concentracions extracel·lulars de DA, la qual cosa és coherent amb la hipòtesi de que els receptors D2 presenten "selectivitat funcional". Per altra banda, actualment està cada cop més acceptat el concepte de heteromerització entre diferents GPCRs que co-localitzen en les mateixes mostres tissulars, i representa un nou camp per al disseny de millors teràpies. Així doncs, els estudis dirigits a la identificació de nous heteròmers que impliquin els receptors D2, permet ampliar la farmacologia del sistema dopaminèrgic. En aquesta tesis hemos observat *crosstalk* funcional entre dos parells de GPCRs: entre D2 i GABAB por un costat, i entre D2 i el receptor-1 de Orexina per un altre. Malgrat que els nostres resultats indiquen que és molt probable que ambdós parells de receptors formin heteròmers, es necessiten mñes dades per a corroborar-ho. La DA és clau pel sistema cerebral de la recompensa, que juga un paper primordial en el fenòmen de l'addicció. Està descrit que la administració crònica de cocaïna altera la densitat i l'afinitat dels receptors D1 i D2, així com també la seva interacció amb altres receptors. En el nostre model de auto-administració de cocaïna en rates, hem trobat canvis en la resposta del sistema dopaminèrgic a l'agonista D2 quinpirole, després de abstinència de curt i també de llarg termini. En summa, els nostres resultats sobre l'administració de cocaïna destaquen la importància del sistema

dopaminèrgic en l'addicció a la cocaïna, i a més, el *crosstalk* funcional que hem observat entre els receptors D2 i altres GPCRs obre noves possibilitats per al disseny i desenvolupament de nous tractaments per a l'abús de drogues així com també per l'esquizofrènia.

Index

Introduction 1
Neurons 2
The synapse 3
The synaptic transmission 4
Synaptic plasticity 6
Synaptic plasticity and addiction 8
G-protein coupled receptors (GPCRs) 9
Classification of GPCRs 10
G-proteins 10
The intracellular effectors following G protein activation 12
Regulation of GPCR signaling pathways 13
β-Arrestins 13
Regulator of G protein signaling (RGS proteins) 15
GPCR homo- or heteromerization 15
The 'oligomer fingerprint' 16
Other levels of GPCR crosstalk 17
Biological responses of Ligands acting on GPCRs 18
Striatum 20
Anatomy of the striatum 21
Neurophysiology of the striatum 22
Neurological disorders relate to deficit of striatal functions 22
Striatal neuromodulators 23
γ -aminobutyric acid 23
Acetylcholine 23
Dopamine 24
Dopamine system 25

Dopamine synthesis, storage and release in brain 27
Tyrosine hydroxylase 28
Dopamine receptors 28
Dopamine receptors distribution in brain 29
Receptor locations with respect to synapses 30
Postsynaptic receptors 31
Presynaptic autoreceptors 32
Dopamine receptor signaling 32
Dopamine receptor oligomerization 34
The D_1-D_2 and D_2-D_5 heteromers 34
The D_1-D_3 receptor heteromer 36
The D_1-NMDA receptor heteromer 37
The A_2A-D_2 receptor heteromer 37
Pharmacology of dopamine receptors 37
Antipsychotics 38
Drug abuse 39
The brains reward pathway 41
Neuroadaptation of drug addiction 43
Cocaine 43
The development history of cocaine 44
Ways of cocaine ingestion as an abused substance 44
The mechanism of acute cocaine effects 45
Cocaine induced neuronal adaptations 45
Cocaine addiction and CREB phosphorylation 46
Chronic cocaine administration reduces G protein signaling efficacy 48
Morphological changes in dendritic spines 49
Inhibition of neuronal excitability by cocaine administration 50
Cocaine-induced neuroadaptation in dopamine system 50

Cocaine induced changes in D_2 receptor homo- or heterodimerization 51
Approaches in studying cocaine addiction 52
Conditioned place preference (CPP) 52
Cocaine self-administration 52

General objective 55

Methodology 59
Part 1 In vitro dopamine synthesis assessment system 60
Animals 60
Reagents and materials 60
Buffers and mobile phase 60
Puification of L-[3, 5-3H]-tyrosine 61
Preparation of rat brain striatal miniprisms 61
Incubation of samples for [3H]-DA synthesis 62
Determination of protein amount 63
Purification of 3H-DA through HPLC system 65
Calculation of [3H]-DA synthesis 70
Statistics 71

Part 2 Cocaine self-administration 71
Animals 72
Experimental protocol and design 72
Brief procedure of the experimen 73
Cocaine self-administration protocal 76
Preparation of brain slices and incubation in thermomixer 77
16 µm sections cut using cryostat 79
Immunohistochemistry 80

Avidin-Biotin Complex Method for IHC Detection 80
Quench and block of the non-target antigens 81
DAB staining 82
Dehydrated, detergent and mounted of the slides 82
Photograph of the slides 82
Statistics 83
Western blot 83

Results

Result chapter 1: validation of the ex vivo DA synthesis system in rat brain striatum 88
Introduction 88
Objective 88
Results 88
Discussion 91
Conclusion 92
Result chapter 2: Agonist and antagonist effects of aripiprazole on D2-like receptors controlling rat brain dopamine synthesis depend on the dopaminergic tone 93
Abstract 93
Introduction 94
Objectives 96
Results 97
Discussion 102
Conclusion 109
Result chapter 3: Crosstalk of presynaptic D2 receptors with other GPCRs 111
Abstract 111
Introduction 112
Objectives 116

Results..117

Discussion..125

Summary..129

Conclusion 129

Result chapter 4: Chronic cocaine self-administration alters dopamine D2 response into pCREB-positive cells in rat brain striatum 131

Abstract 131

Introduction...132

Objectives 134

Methodology 135

Results..137

Discussion..141

Conclusion 147

General Disccusion 149

Conclusion 157

References 160

Index of figures

Figure 1 Neuron Diagram 2
Figure 2 Chemical synapse 3
Figure 3 Arrangements of various types of synaptic transmission........ 6
Figure 4 homosynaptic and heterosynaptic synaptic plasticity 7
Figure 5 long-term potentiation and long-term depression 8
Figure 6 G-protein activation/deactivation cycle 11
Figure 7 β -Arrestins induced receptor desensitization, endocytosis and trafficking.. 14
Figure 8 Different levels of GPCR crosstalk 18
Figure 9 Biological responses to the ligands which belong to different efficacy classess........ 19
Figure 10 Innervation networks of basal ganglia 20
Figure 11 the chemical structure of dopamine........ 25
Figure 12 DA neurons containing regions and their projection........ 26
Figure 13 Dopamine synthesis, storage, release and re-uptake 27
Figure 14 Characteristics of DA receptors......... 29
Figure 15 D_1-like receptor signaling pathways........ 32
Figure 16 D_2-like receptor signaling pathways........ 33
Figure 17 D_1-D_2 and D_5-D_2 heteromer signaling pathways 36
Figure 18 Diagram of the brain reward circuitry of the mammalian (rat) brain 42
Figure 19 Cocaine........ 45
Figure 20 Cellular events involved in CREB regulation 47
Figure 21 Morphology change of spine caused by chronic cocaine administration... 49
Figure 22 Preparing miniprisms from rat brain striatum........ 62
Figure 23 Experimental designs for the in vitro dopamine synthesis assessment..... 63
Figure 24 Lowry method 64
Figure 25 Scheme of HPLC purification system 65
Figure 26 The mechanism for the molecule retention in the column 66
Figure 27 A typical UV chromatogram pattern of dopamine from a rat striatum sample........ 68
Figure 28 Temporal outline of radioactivity present in the eluates, in comparison with a dopamine chromatogram 69
Figure 29 Calculations of [3H]-DA synthesis, shown in percentage of non-treatment control groups........ 71
Figure 30 Operant chamber (Panlab) for daily cocaine self-administration or sucrose training........ 75
Figure 31 Surgical implantation of catheter 75
Figure 32 vibration slice cutting 78

Figure 33 Procedure of slice incubation in the thermomixer incubator 78

Figure 34 Cryostat slice cutting 79

Figure 35 The principle of the ABC method for IHC detection 81

Figure 36 the whole protocol of IHC using ABC method 82

Figure 37 83

Figure 38. Western blot binding of anti-phospho-CREB (Ser 133) 85

Result chapter 1

Figure 1 Concentration-response curve of Quinpirole on DA synthesis in rat striatal miniprism under 2mM [K^+] 89

Figure 2 Concentration-response curve of okadaic aicd on DA synthesis in rats striatal miniprism under 2mM [K^+] 90

Figure 3 Okadaic acid regulated concentration-response curve of Quinpirole on DA synthesis in rat striatal miniprism under 2mM [K^+] 91

Result chapter 2

Figure 1. Concentration-response curve of Quinpirole (*a*) and Aripiprazole (*b*) on the inhibition of DA synthesis in non-stimulated rat brain striatal miniprisms (basal condition: 2 mM K^+) 97

Figure 2. Inhibition of DA synthesis by 10 nM Aripiprazole is mediated by D2 receptors 99

Figure 3. Concentration-response curve of Quinpirole (*a*) and Aripiprazole (*b*) on the inhibition of DA synthesis in stimulated rat brain striatal miniprisms (high dopaminergic tone condition: 15 mM K^+) 100

Figure 4. 10 nM Aripiprazole blocks quinpirole inhibition of DA synthesis under 15 mM K^+ conditions 102

Result chapter 3

Figure 1 Inhibition effect on DA synthesis by $GABA_B$ receptor selective agonist Baclofen is blocked either by the $GABA_B$ receptor antagonist CGP or the D2R selective antagonist Sulpiride 118

Figure 2 The concentration-response curve of the facilitative effect on DA synthesis elicited by the $GABA_B$ receptor antagonist CGP 54626 (A). The effect of CGP 54626 at 100nM could be abolished either by the selective D2R antagonist Sulpiride (B) or by the selective D2R agonist Quinpirole (C) 120

Figure 3 Inhibition effect on DA synthesis by the orexin-1 receptor agonist orexin is blocked either by the OxR1/2 antagonist SB 334867 (SB33) or the D2R selective antagonist Raclopride 122

Figure 4 The inhibition effect on DA synthesis of D2R agonist could neither be affected by the antagonist (A) nor the agonist (B and C) of OxR1 receptor 123

Figure 5 Both the D1/5 receptor agonist and antagonist have no effect on DA synthesis in rat miniprism (A). In addition, no synergistic effect was observed in D2R and D5R agonist co-administration (B) 125

Result chapter 4

Figure 1 136

Figure 2 .... **138**

INTRODUCTION

Neurons

Brain contains two main groups of cells: the neuron and the glia. A neural network is formed by both of them: the supporting material (glial cells) and the communication material (neurons). In human brains, it is estimated that there are at least 100 billion neurons [Williams et al. 1988]. The neurons could be classified into different types either due to their location or their function or other properties they possess, however, structurally most of them are constituted by three major components: a cell body, an axon and varying numbers of dendrites (Figure 1).

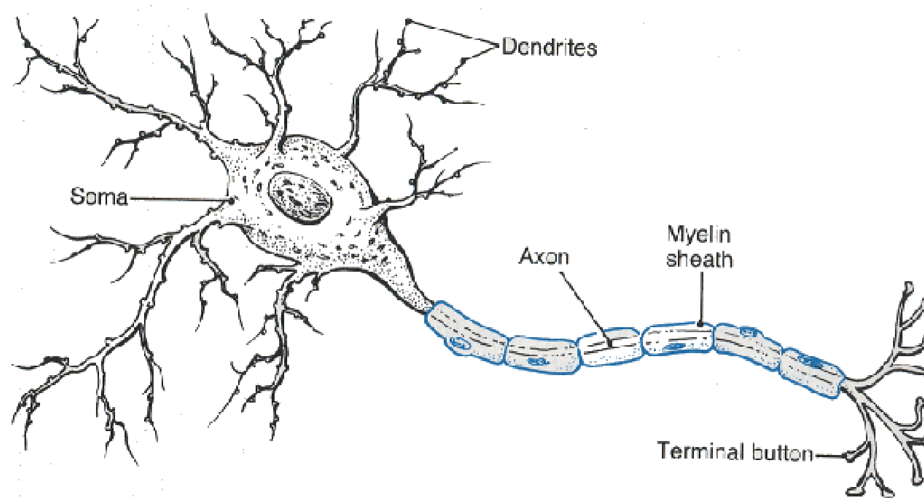


Figure 1 Neuron Diagram

The image is from http://www.mindcreators.com/neuronbasics.htm

The cell body: it contains the nucleus and major cytoplasmic organelles and primarily responsible for synthesizing and processing proteins and forms the postsynaptic component of a synapse on some occasion; it is the smallest part of the neuron.

The axon: it is a fine tubular process that extends from the neuronal cell body and conducts electrical impulses from the cell body to the axon terminals which forms the

presynaptic component of a synapse. The neurons can be subdivided into interneurons and projection neurons depending on whether the axon remains within the CNS regions or extends to another region refers to the cell body.

Dendrites: they are multiple fine branches that extend from the cell body which usually form the postsynaptic component of a synapse; their main functions are reception, processing and integration of the incoming synaptic communications.

The synapse

A typical synapse is composed by the presynaptic element (usually an axon terminal of one neuron), the postsynaptic element (usually located on dendrites of a second neuron) and the synaptic cleft (Figure.2). The synapses permit the presynaptic neurons to pass electrical or chemical signal to the postsynaptic neurons. It is the fundamental unit in accomplishing information transmission which normally needs the participation of numerous synapses. Two types of synapses were observed in CNS:

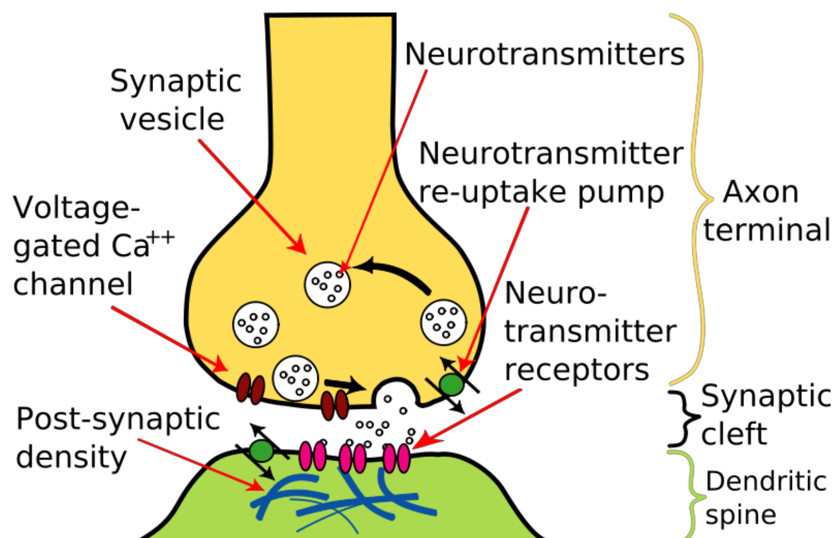


Figure 2 Chemical synapse. The picture shows the basic components of a synapse. In which, the presynaptic part is shown in yellow color and the postsynaptic in green, and the

space between them is the synaptic cleft. Typically, a synapse is formed between the axon terminal and a dendritic spine.

Illustration is adapted from http://www.aokainc.com/chemical-synapse/.

***The chemical synapse**:* the chemical synapse is the main type of synapse in CNS; it is composed by all the three basic elements of a typical synapse. In chemical synapse, the signal transduction process begins with the release of the neurotransmitters which were previously stored in small vesicles in the presynaptic neuron into the synaptic cleft under the action potential effects by means of exocytosis, and then they diffuse and reach to the other side of the synaptic cleft, finally they bind to the receptors located on the postsynaptic neurons and initiate a electrical response or secondary messenger pathway. The chemical synapse can be differentiated by the neurotransmitters released from the presynaptic neuron. There are several categories of neurotransmitters: a) Amino acid transmitters, such as glutamate and γ -aminobutyric acid(GABA); b) Biogenic amines, including dopamine and norepinephrine; c) Peptides; d) Diffusible gases like nitric oxide; and e) Nucleosides, such as adenosine.

The electrical synapse**:** in this type of synapse, the two neurons are connected directly through the so called gap junction. The gap junction is formed by a large number of tightly packed proteins. The electrical synapse permits the electrical currents to flow directly form one neuron to the second neuron.

The synaptic transmission

In this thesis only chemical synaptic transmission will be discussed. What we described above that the postsynaptic dendrite undergoes innervations by a presynaptic nerve terminal represent the classic chemical synaptic arrangement in the CNS (Fig.3A); however, additional types also occur in the brain. We will describe several other types of them which are closely related to our research.

The axoaxonic synapse: an axoaxonic synapse occurs when neurotransmitters released from one nerve terminal act on receptors located on other nearby nerve terminals. Such nerve terminals may or may not be of the same neuron types. Neurotransmitters may act on the autoreceptors if both the two nerve terminals are from the same type of neurons. Although the axoaxonic synapses lack the specializations of the classic synapses, it is likely that axoaxonic synapses contribute an essential dimension to the integration of complex interneuron communication that occurs in functioning neural circuits (Fig.3B). When the neurotransmitters were released and diffused, some of them may act by means of the autoreceptors located on the presynaptic terminals that release them (Fig.3C).

The dendroaxonic synapse: in some cases, the neurotransmitters released from the cell body or the dendrites may act on the nearby axon nerve terminal (Fig.3D).

The mixed types: the neuronal system is too complicated to be only composed by these synapse types who function independently, and it's likely that several of the synapse types mentioned above are coexist in most regions in the brain. As shown in the Figure 3E, in a classic axodendritic synapse, the neurotransmitters released by the axon terminal will not only act on the receptors located on the postsynaptic neuron but also on the presynaptic neuron. In addition, axon terminals extend from other neurons will at the same time release neurotransmitters that act on either both the presynaptic and postsynaptic neurons or only on one of them (Fig. 3E).

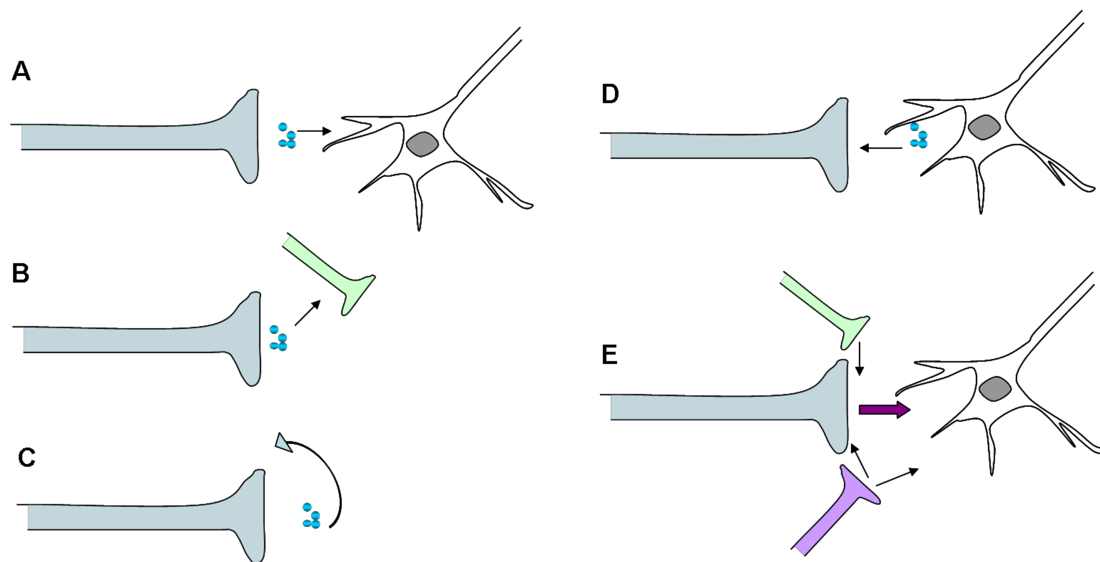


Figure 3 Arrangements of various types of synaptic transmission. See details in the text above.

Synaptic plasticity

To understand what synaptic plasticity is, first we should know what synaptic strength is. Functionally, synaptic strength is defined as the average amount of current or voltage change produced in the postsynaptic neuron by an action potential in the presynaptic neuron. Originally it was supposed that the synapses are merely tools to transfer information from one neuron to another and the synaptic strength is fixed for each kind of synapse. However, this concept was subverted soon enough. The synaptic strength is not a static quantity, it could be modified either by the synapse itself or by other pathways and this is termed as synaptic plasticity. There are normally two types of synaptic plasticity: intrinsic mechanism also known as homosynaptic mechanism which refers to changes in synaptic strength are brought about by its own activity (Fig 4a); and extrinsic synaptic also named heterosynaptic plasticity, the change in synaptic strength are brought about by the activity of another pathway (Fig 4b). As it is shown in the picture, each type of the synaptic plasticity has two possibilities: the inhibition and facilitation of the original synaptic strength. The

long-term potentation (LTP) and the long-term depression (LTD) are the major forms of the long-lasting synaptic plasticity either due to intrinsic activity or extrinsic pathways.

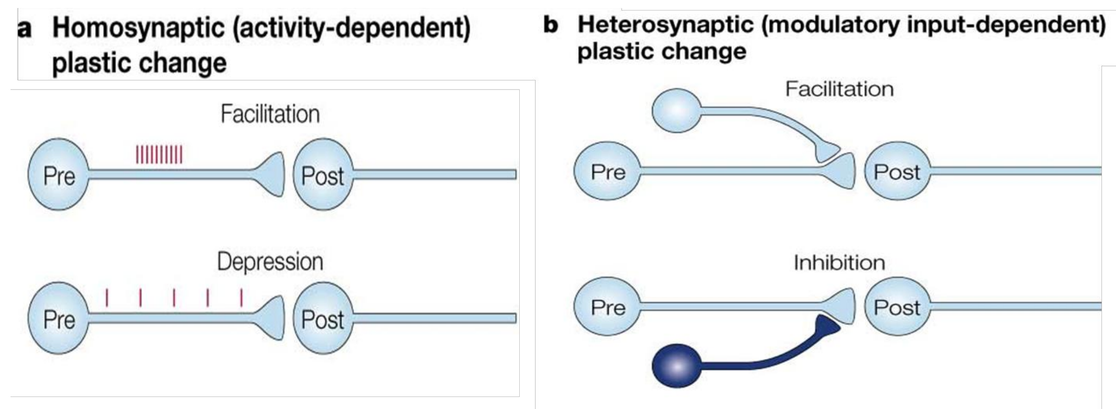


Figure 4 homosynaptic and heterosynaptic synaptic plasticity. a) Synaptic plasticity under homosynaptic rule, that is the synaptic strength elicited by the same synapse is not static, it could be either facilitation or depression. b) Synaptic plasticity under heterosynaptic rule, that is the synaptic strength could be under the modification of an extrinsic synapse, and lead to either facilitation or inhibition of the original synaptic strength.

Illustration is modified from Bailey et al., 2000.

Long-term potentiation (LTP) and long-term depression (LTD)

LTP and LTD, the long-term potentiation and depression of excitatory transmission are widespread phenomena expressed at possibly every excitatory synapse in the mammalian brain. During the past two decades, the mechanisms of them have been widely studied and it is accepted that the mechanisms vary depending on the synapses and circuits in which they operate. However, the usual trigger for both LTP and LTD is calcium influx through NMDA receptor pores. Figure 5 shows an example

of NMDAR dependent LTP and LTD.

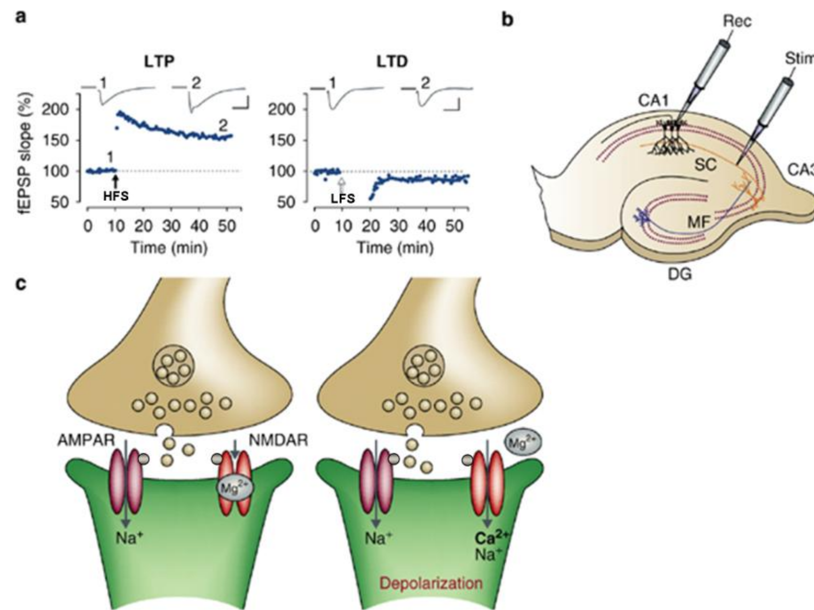


Figure 5 long-term potentiation and long-term depression

a) High-frequency tetanic stimulation (HFS) induced LTP and low frequency stimulation (LFS) induced LDP by recording field excitatory postsynaptic potential (fEPSP) in hippocampal CA1. b) A schematic diagram of the rodent hippocampal slice preparation, typical electrode placements for studying synaptic plasticity, demonstrating the stimulation region CA3 and the fEPSP recording region CA1(SC= Schaffer collateral; MF=mossy fiber; DG=dentate gyrus). c) Under basal synaptic transmission, the transmitter glutamate released from the presynaptic neuron and binds to both NMDAR and AMPAR and lead to slight depolarization because Na+ flows intracellular only through AMPAR channel. However, this slight depolarization remove Mg2+ from NMDAR and finally leads to both Ca2+ and Na+ flow through NMDAR channel. The increased Ca2+ influx is necessary for triggering the subsequent events that drive synaptic plasticity.

Illustration is from Citri and Malenka (2008)

Synaptic plasticity and addiction

The characteristics of drug addiction will be described in details in the cocaine section, and here we only list some documented evidences relating the synaptic

plasticity with drug addiction. The study of the connection between drug addiction and neuronal synaptic plasticity began with the hypotheses that plasticity at these excitatory synapses plays an important role in mediating some of the behavioral consequences of exposure to abused drugs. Since then, there were several lines of *in vivo* and *ex vivo* evidence appeared. For instance, various forms of LTP and LTD can be elicited at excitatory synapses in the VTA and NAc [Gerdeman et al., 2003]; Administration of a single dose of abused drugs cause a significant increase in synaptic strength (LTP) at excitatory synapses on DA cells in the VTA; And also several lines of evidence suggest that drug induced synaptic strength plays a functional role in triggering or mediating some drug-induced behavioral adaptations [Malenka et al., 2004]; In addition, some experiments in chronic *in vivo* cocaine administration found that cocaine could cause a postsynaptic mediated decrease in synaptic strength in medium spiny neurons in the NAc shell, a decrease that appears to share mechanisms with one of the forms of LTD observed in this structure (Thomas et al., 2001); Notably, drugs of abuse can also modify the triggering of LTP and LTD (Malenka et al., 2004). More research focus on digging further connections between drug addiction and synaptic plasticity to understand the pathophysiology and to found a better treatment for this common and devastating neuropsychiatric disorder.

G-protein coupled receptors (GPCRs)

GPCRs are integral membrane proteins that possess seven membrane-spanning domains. They are the largest family of cell-surface (which located on the plasma membrane) receptors. The plasma membrane separates the receptor into the extracellular region (which contains three extracellular loops and a N-terminal tail) and intracellular region (which contains also three intracellular loops and a C-terminal tail) and the seven-transmembrane helices. The basic character of GPCRs is that their extracellular region or the transmembrane domain is responsible for the binding to the specific ligands and the intracellular region for transduction of the signals to specific intracellular effector systems, which usually by means of G proteins coupling.

Classification of GPCRs

GPCRs are the largest class of receptors with more than one thousand being identified so far. The GPCRs superfamily could be divided into 3 major subclasses which share little sequence homology and some functional similarity among each other [Kolakowski et al., 1994]. The subclasses of GPCRs are as follows:

Class A *(rhodopsin receptor family)*: this class contains the largest number of GPCRs including receptors for odorants and small ligands. It is subdivided into 3 groups according the sites of ligands binding: 1) the binding site is located within the seven-TMs, including rhodopsin and β -adrenergic receptors. 2) The binding site includes the N-terminal, the extracellular loops and the superior part of TMs, such as receptors for peptides. 3) The binding site is mostly extracellular, with contact of loop e1 and e3; contains GPCRs for glycoprotein. All types of dopamine receptors are included in this family.

Class B *(secretin receptor family):* the GPCRs in this family have a similar morphology to group 3 GPCRs in Class A in performance of large N-terminal domain. Their ligands include high molecular weight hormones such as glucagon, secretin, orexin A. [Narendra Tuteja, 2009]

Class C *(metabotropic glutamate receptors family):* Class C GPCRs contains metabotropic glutamate receptor (mGluR), Ca^{2+} sensing receptors and GABA-B receptors, and other receptors, in total around two dozens of them. They are characterized by its usually large extracellular amino terminal domain (ATD). Importantly it is well established that all family C GPCRs exist as constitutive dimeric (or oligomeric) complex in brain. [Bräuner-Osborne et al., 2007]

G-proteins

The common feature of GPCRs is they all couple to a specific G-protein when

stimulated as the name originates. G protein is a heterotrimer which is composed of singleα ,β and γ subunits. In their resting state, the three subunits stick together with the GDP bound on the G_{α} subunit. When the receptor is activated by ligands binding, a conformational change of the receptor enable the association of the G_{α} subunit from the receptor. This association alters the conformation of the G_{α} subunit which leads to first the replacement of GDP on G_{α} subunit by GTP; And then the dissociation of the $G_{\beta\gamma}$ complex from G_{α} subunit; Finally the G_{α} subunit leaves the receptor and become an active entity which could regulate the functional activity through many effector proteins in the cell. Simultaneously, another signaling pathways is elicited by the $G_{\beta\gamma}$ complex through other effectors (Fig. 6).

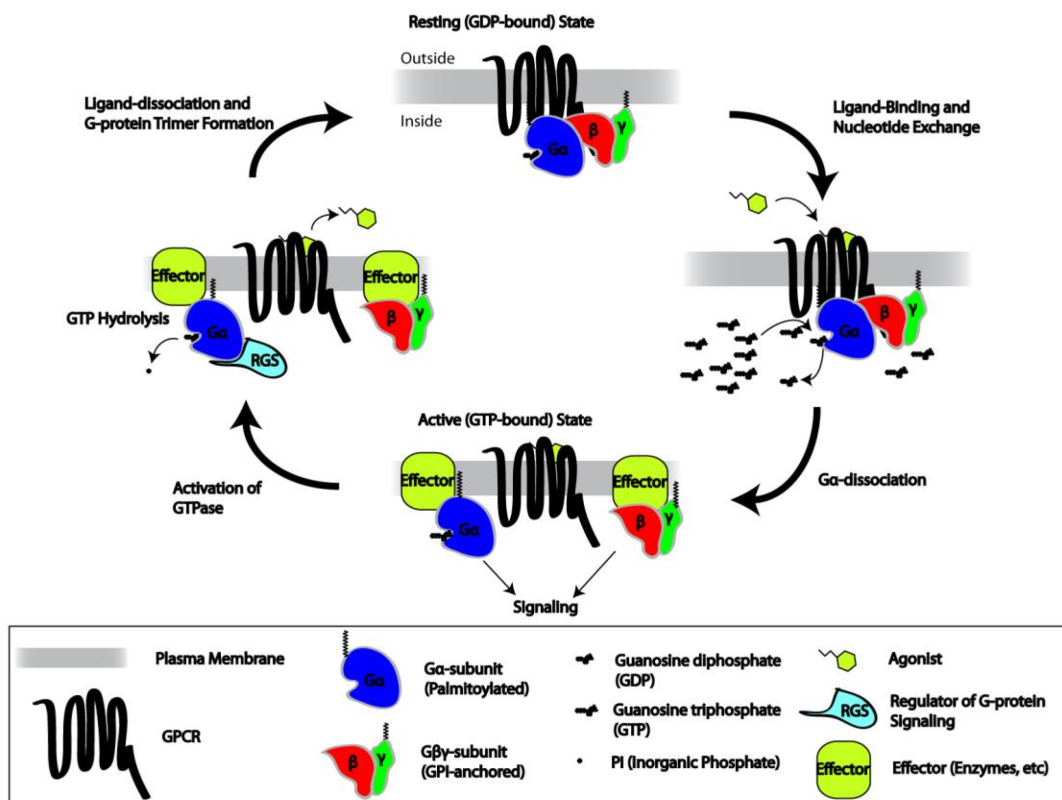


Figure 6 G-protein activation/deactivation cycle.

Abbreviations: GPCR= G-protein coupled receptor; RGS= Regulator of G-protein signalling; GTPase= GTP and GDP hydrolyzing enzyme; GTP= Guanosine triphosphate; GDP= Guanosine diphosphate; Pi= Inorganic phosphate.

Image is from http://upload.wikimedia.org/wikipedia/commons/c/c9/GPCR_cycle.jpg

There are three common types of G protein: Gs, Gi/o and Gq. Each group of receptors could couple to a single or multiple types of G protein. It appears that each type of G proteins have the same β and γ subunits, thus the variants of the α subunit are responsible for the unique G protein signal pathways activated. They are α_s, α_i and α_o, α_q, α_t, α_g, α_{11} and G_{olf}

The intracellular effectors following G protein activation

Two types of effector proteins in the downstream of G protein activation are common in the neurons: ion channels and second messengers.

Ion channels: this regulation process is best established in the receptors that couple to Gi/o subtype, including opioid, D2-dopaminergic, muscarinic cholinergic and 5-HT_{1a}-serotonergic and $GABA_B$ receptors. The common ion channels that participate are K^+channels or Ca^{2+} channels depending on the cell type involved. Usually, when coupled to G proteins, these receptors activate specific inwardly rectifying K^+channels or inhibit Ca^{2+} channels. The regulation of both channels most likely occurs primarily through $G_{\beta\gamma}$ complex and has direct consequence on membrane electrical properties.

Second messengers: neurotransmitters bind to receptors, activate G proteins to release the G_α and $G_{\beta\gamma}$ complex which could both transform the information to downstream events through activating second messengers. The prominent second messenger in the brain include: cyclic adenosine monophosphate(cAMP), cyclic guanosine monophosphate (cGMP), Ca^{2+}, nitric oxide (NO), the major metabolites of phosphatidylinositol-inositol triphosphate (IP3) and diacylglycerol (DAG) – and the major metabolites arachidonic acid, such as prostaglandins.

Most receptor could activate both types of effectors. However, second messengers are much more complex and widespread in the CNS, thus, they draws more attention in my study.

Regulation of GPCR signaling pathways

β -Arrestins

β -Arrestins are ubiquitously expressed in CNS. Their function is to inhibit GPCR/G protein coupling through a process termed as desensitization of the receptors. Normally this process could finally promote GPCR trafficking. Specifically, upon agonist stimulation, GPCRs undergo conformational changes, thus expose the binding sites for heterotrimeric G-proteins in their intracellular domains, and the associating of G-protein from GPCRs initiates the intracellular signaling. However the agonist-occupied receptors immediately become substrates of G-protein coupled receptor kinases (GRKs), and are phosphorylated at their intracellular C-terminals. This phosphorylation, in turn, recruits the binding of β -Arrestins which blocks further G-protein activation and finally abrogates further signal propagation (Fig.7, step 2). In addition, the association of GPCRs with β -Arrestins normally will initiate the receptor internalization (Fig.7 step 3) which may lead to degradation or receptor recycling (Fig.7, step 5a & 5b).

GRKs is a group of kinases that are important in β -Arrestins regulated GPCR/G-protein signaling pathway. They are a family of protein kinases that regulate the activity of GPCRs by phosphorylation of the intracellular domains of the agonist-occupied GPCRs. They function in both GPCR-dependent and –independent signal pathways. There are 7 known subtypes of GRKs, four among them are expressed ubiquitously (GRK2, 3, 5 and 6) [Yang et al., 2006].

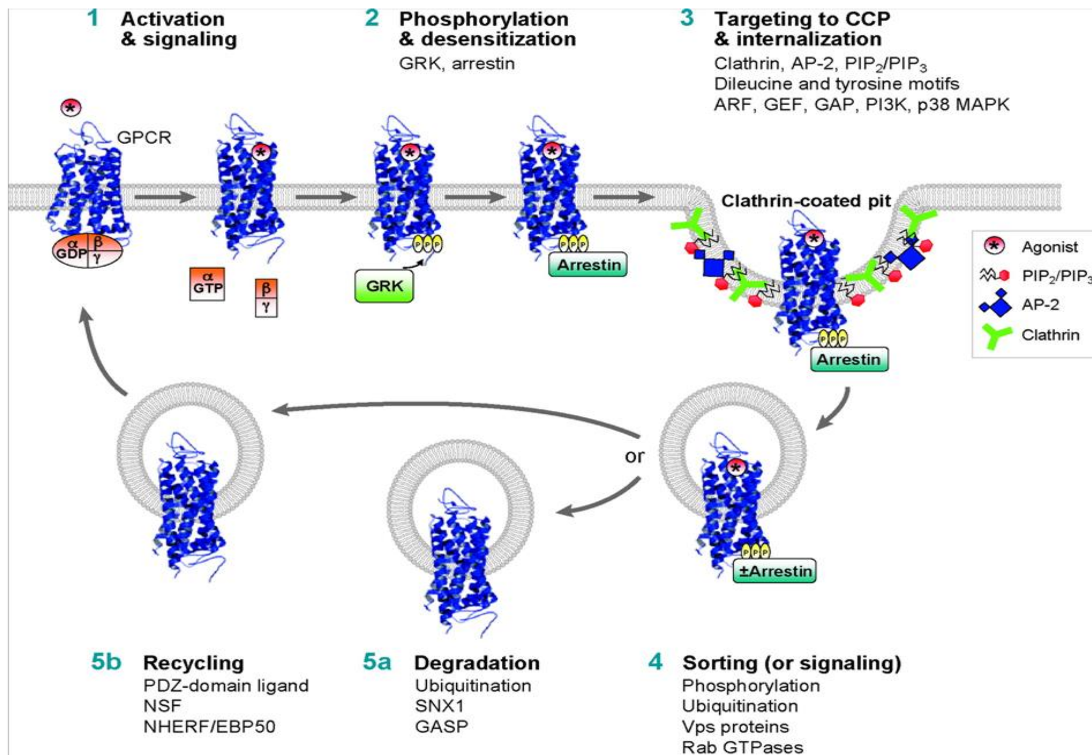


Figure 7β -Arrestins induced receptor desensitization, endocytosis and trafficking.

For details see the text above.

Abbreviations**:** ARF, ADP-ribosylation factor; GAP, GTPase-activating protein; GASP, GPCR-associated sorting protein; GEF, guanine nucleotide exchange factor; NHERF/EBP50, Na+/H+ exchanger regulatory factor/ezrin-radixin-moesin-binding phosphoprotein of 50 kDa; NSF, N-ethylmaleimide-sensitive factor; p38 MAPK, p38 mitogen-activated protein kinase; PDZ, PSD-95/Dlg/ZO-1; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; SNX1, sorting nexin 1; Vps proteins, vacuolar protein–sorting proteins.

The image is from Moore et al., 2007.

Besides the desensitization and endocytosis effects on agonist-stimulated receptors, β -Arrestins could also activate downstream G-protein independent signallings. β -Arrestin acquires an active conformation upon forming a complex with agonist-stimulated 7TMRs and scaffolds MAP kinase, MAP kinase kinase, and MAP kinase kinase kinase, leading to the robust activation of MAP kinase and

subsequently targets to distinct subcellular compartments. This β -Arrestin induced, G-protein independent signalling has been shown to regulate cellular chemotaxis, apoptosis, cancer metastasis and protein translation [Shoney et al., 2011].

Regulator of G protein signaling (RGS proteins)

G protein function can be regulated by several modulator proteins, among them, the RGS proteins are a group of proteins that are characterized by activating the GTPase which promote the hydrolysis of GTP bound on the G_α subunit of G protein which initiate the signal pathway; Thus, rapidly inactivate G-protein and switch off G-protein-coupled receptor signalling. There are more then 20 subtypes of RGS proteins, and so far, all the G proteins except G_s are known to be regulated by those proteins.

GPCR homo- or heteromerization

GPCRs is the largest group of membrane surface-expressing receptors in brain neurons. Various types of GPCRs are co-localized on a single brain neuron. We introduced in the above test the basic signaling pathways when a single receptor is activated, either G-protein dependently or independently; however, more and more evidence suggest that those co-localized receptors tend to form homomers (with identical receptors) or heteromers (with different receptors). The concept of GPCR heteromer is best explained by the two non-functional GPCR monomers $GABA_{B1}$ and $GABA_{B2}$ can assemble into a functional heterodimer on the cell surface [Marshall et al., 1999]. $GABA_B$ receptors belong to the Class C GPCRs. Other typical homomer and heteromer examples are also found in this group, such as the mGlu homodimers and the taste receptor heterodimers (T1R1/T1R3 and T1R2/T1R3) [Nelson et al., 2001, 2002; Johnstone et al., 2012]. Later on, the heteromerization or homomerization was found in various kinds of receptors, for instance: Dopamine receptors [www.gpcr-okb.org.etc], adenosine receptors [Albizu et al., 2010 and the reference within].

For all we know, GPCRs are involved in most physiological responses, and alterations of their function are related to pathological states such as hypertension, diabetes, pain, asthma, immunological and neurological disorders. Around 30-40% of current pharmaceutical drugs target at GPCRs and the regulations of their function are essential for therapeutic strategies [Albizu et al., 2010]. Selection of novel drugs are based on the pharmacological parameters obtained assuming that GPCRs are monomers; As a matter of fact, many GPCRs function as homomers or heteromers, more complicated than we could think. Moreover, assembly of GPCRs into heteromer or homomer could lead to changes in the agonist recognition, signaling and trafficking of participating receptors. Thus, all these concepts would broaden the therapeutic potential of drugs targeting at GPCRs. So evidencing new receptors that could form homo- or heteromers, as well as find out the details of existing homo- or heteromer formed by the multifunctional GPCRs (such as D_2R) could all facilitate the developing of drugs targeting at the specific dimers.

The 'oligomer fingerprint'

Along with the increase of evidence that confirm the existence of receptor oligomers, the specific signaling pathways elicited by those oligomers are also become acknowledged. These specific signaling which usually different compared with the pathways elicited by the single participated receptors are termed as oligomer fingerprint, that is, a specific characteristic that differentiate oligomeric vs monomeric receptors. An oligomer fingerprint could always occur at the pharmacological levels and functional levels.

Pharmacological/biochemical: the affinity of the receptors for their specific ligands can be altered when the receptors forms heterodimers. For example, the fingerprint for A1-A_{2A} receptor heteromer is that A_{2A} receptor activation could reduce the affinity of A1 receptor for its agonists [Ciruela et al., 2006a]. Similar dimer fingerprint is also found in the A2A-D_2 receptor heterodimer [Canals et al., 2003].

***Functional**:* co-activation of the two receptors in a heterodimer may change the signaling pathway triggered by a given neurotransmitter, as well as the traffic of the receptors. An example for this is found in the Oxexin-1-cannabinoid CB1 heterodimer. The dimerization of this two receptors lead to both the localization and function alteration of orexin-1 receptor. Another excellent example for this is found in the D_2 dimerization with other receptors which will be elaborated in the following section. Notably, GPCR antagonism may vary in receptor heteromers. A significant number of prescribed drugs targeting GPCRs are indeed antagonists, and this may also explain why different antagonists for a given receptor do not necessarily have similar in vivo profiles and equally side effects [Franco et al., 2008].

Other levels of GPCR crosstalk

As we have mentioned above, the co-localized GPCRs generally don't operate in isolation, instead they may allosterically interact by forming homo- or heteromers (Fig. 8A). However, homo- or heteromerization is not the only strategy they got for crosstalk between each other. Moreover, the crosstalk between those receptors can occur at various levels besides oligomerization. First, GPCRs can desensitize other GPCRs via activating second messenger-dependent protein kinases (PK) A or C (Fig. 8B). Second, GPCRs may impair other GPCRs by scavenging shared signaling and/or scaffolding proteins (eg. G proteins or β -arrestins) which are essential for receptor signaling (Fig. 8C). Finally, GPCRs can activate distinct signal transduction pathways that may converge and integration at downstream signaling hubs (Fig. 8D). All these three other levels of receptor crosstalk could also bring about functional alterations which are similar to receptor oligomerization, so observation of functional alteration is not enough to clarify the oligomerization property of the participated receptors, and it could only means they could crosstalk on some levels [Vischer et al., 2011].

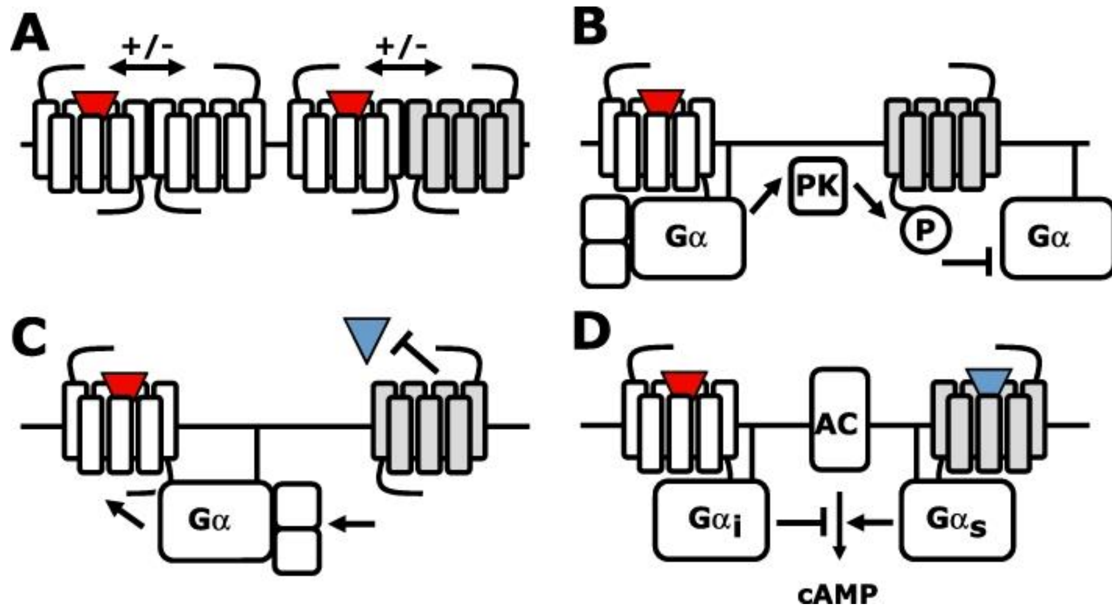


Figure 8 Different levels of GPCR crosstalk. For details see the text above. Image is from Vischer et al., 2011.

Abbreviations: PK, protein kinase; AC, adenylyl cyclase; cAMP, cyclic adenosine 3', 5'-monophosphate.

Biological responses of Ligands acting on GPCRs

Ligands, whether natural or synthetic, act on receptors and perform the effect on the structure and biophysical properties and finally lead to biological response that is known as ligand efficacy. Based on different kinds of biological response, ligands can be subdivided into different efficacy classes (Fig. 9) [Rosenbaum et al., 2009; Arrang et al., 2007]:

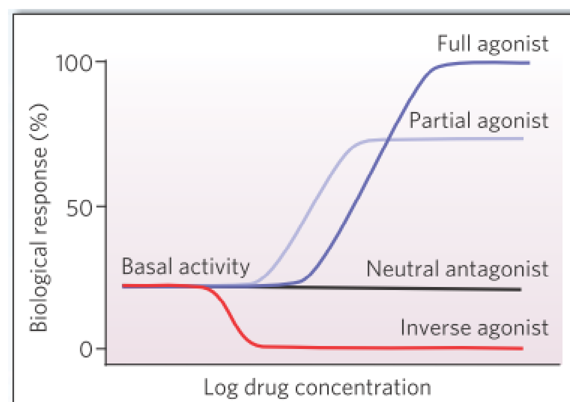


Figure 9 Biological responses to the ligands which belong to different efficacy classess. For details see the following text.

The image is adapted form Rosenbaum et al. (2009).

Full agonist: drugs that stabilize the receptor in an active conformation (state). A full agonist could stimulate the maximum amounts of receptors thus causes the maximal biological response.

Partial agonist: compared with the full agonist which capable to stimulate the maximal amount of receptors of a given system, a partial agonist could only stimulate parts of the receptors and the maximal response it obtained is lower than that of the full agonist.

Neutral antagonist: ligands that have no apparent effect on signaling activity, have no effect on biological response, however, they could antagonize the biological response elicited by other ligands by means of preventing the binding of those ligands to the receptors.

Inverse agonist: inverse agonist stabilizes the receptor in an inactive conformation; they decrease the spontaneous coupling of the receptor to G proteins, thereby suppressing constitutive activity.

Protean agonist: some ligands perform various kinds of efficacies that range from full agonism to full inverse antagonism depending on the levels of constitutive activity in the system, this kind of ligands are termed as protean agonist.

Finally, it is worth to mention that many GPCRs can stimulate multiple signaling systems through binding to different subtypes of G-proteins or signaling independent of G-proteins. And some ligands achieve varying efficacies from different signaling pathways by stabilizing particular sets of conformations that can stimulate specific effectors [Rosenbaum et al., 2009].

Striatum

The striatum is a subcortical part of the forebrain, a main structure of basal ganglia and the main input unit which receives synaptic inputs from cortical, subcortical afferents, the ventral tegmental area, and other midbrain nuclei. The striatum is divided into two main regions: the dorsal striatum and ventral striatum. In primates, the dorsal striatum is subdivided by the internal capsule into the medially located caudate nucleus and the laterally positioned putamen. In rodents, although there is no clearly boundary, yet still the dorsal striatum is subdivided into dorsomedial and dorsolateral striatum. The dorsal striatum receives extensive projections from dorsolateral prefrontal cortex and other frontal regions (eg. premotor cortex, frontal eye fields). The ventral striatum, also known as nucleus accumbens, is constituted by core and shell subregions and receives extensive projections from ventral frontal regions (eg. Orbitofrontal, ventromedial, and ventrolateral cortex). Both dorsal and ventral striatum receive intense dopaminergic inputs from substantia nigra (dorsal striatum) and ventral tegmental area (ventral striatum) [Delgado et al., 2007; and the reference within]. (Fig. 10 and 12).

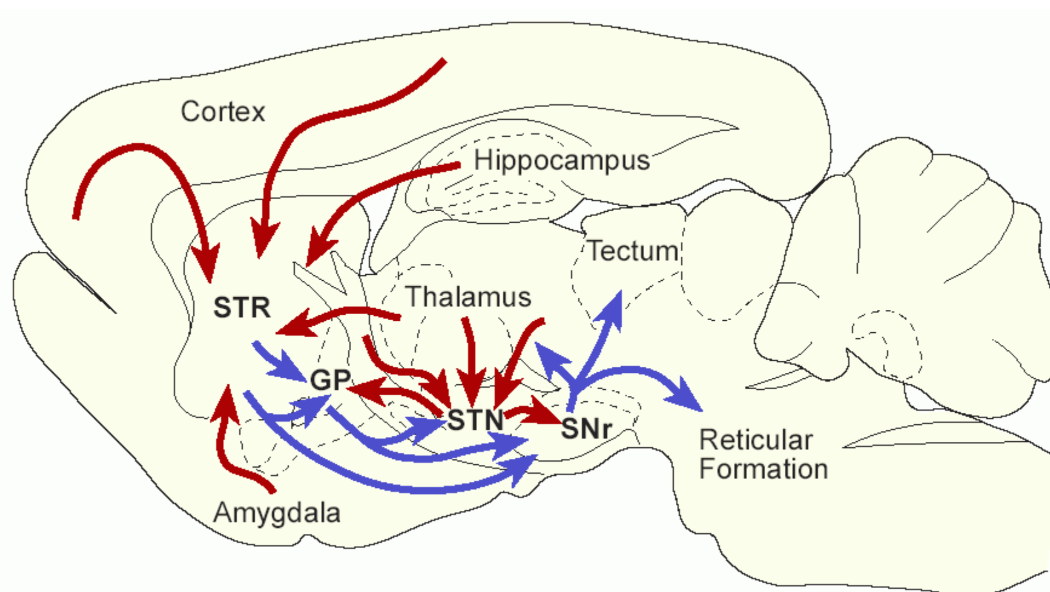


Figure 10 Innervation networks of basal ganglia. The main input nucleus of basal

ganglia is the striatum (STR) and the subthalamic nucleus (STN). Direct connections of the striatum input nuclei are from thalamus, cerebral cortex and limbic structures, the main output nuclei are the substantia nigra pars reticulata (SNr) and the internal globus pallidus/entopeduncular nucleus (not show in the image). The external globus pallidus (GP) is an intrinsic nucleus as most of its connections are with the input and output unclei of the basal ganglia. Red arrows denote excitatory connections while blue arrows respesent inhibitory connections.

Image is from http://images.scholarpedia.org/w/images/5/58/Basal_Ganglia_f1.gif

Anatomy of the striatum

The striatum contains both principal neurons and interneurons. The principal neurons constitute about 90-95% of the total population of striatal neurons and they are called spiny projection neurons (SPNs) which utilize amino acidγ - aminobutyric acid (GABA) as their main neurotransmitter (GABAergic neurons). The SPNs can be subdivided into two approximately equally sized populations on the basis of their projection region, their patterns of axonal collateralization and their neurochemical contents [Surmeier et al., 2011; Bolam et al., 2000]:

Direct pathway SPNs (dSNPs): project preferentially to the output nuclei of the basal ganglia and express the neuropeptides substance P, dynorphin and D_1 subtype of dopamine receptors besides GABA receptors.

Indirect pathways SPNs (iSNPs): project almost exclusively to the globus pallidus and express enkephalin and the D_2 subtype of dopamine receptors besides GABA receptors.

The rest 5-10% of the total striatal neurons are DA receptor-expressing interneuron, these neurons are supposed to play an important role in regulating both types of SPN and lead to indirect response to global DA signal. There are four well-characterized classes of striatal interneuron [Surmeier et al., 2011; Bolam et al., 2000]:

Cholinergic interneurons: coexpress D_2 and D_5 receptors and modulate both SPNs through muscarinic receptors.

Parvalbumin(PV)-expressing GABAergic interneurons: express D_5 receptor and are strongly innervated by globus pallidus neurons that express D_2 receptors, thus creating a microcircuit that is influenced by both D_1- and D_2- class receptors.

Neuropeptide Y (NPY)/nitric oxide-expressing GABAergic interneurons*:* also express D_5 receptor.

Calretinin-expressing GABAergic interneurons: this group of interneurons is indentified as immunoreactive for calretinin.

Neurophysiology of the striatum

The striatum is best known for its role in the planning and modulation of movement pathways, as well as in rewards. Striatal neurons show activity related to the preparation, initiation and execution of movements [Hollerman et al., 2000]. Besides, a large numbers of researches implicate the striatum in reward-related processing [Báez-Mendoza and Schultz, 2013 and reference within]. Traditionally, dorsal striatal neurons are supposed to regulate the motor response whilst studies of nucleus accumbens focused on its role in reward and motivation. However, recent experiments have shown that dorsal striatal neurons also have a role in reward and motivation [Nicola, 2007].

Neurological disorders relate to deficit of striatal functions

It is widely accepted that the importance of the striatum for the basal ganglia is highlighted by the neurological disorders with compromised striatal functions [Graybiel, 2000]. For instance, in Parkinson’s distease, dopaminergic afferents to the striatum are lost and striatal output via the direct and indirect pathways is altered, eventually leading to impaired movement capabilities; in Huntington disease, striatal projection neurons become dysfunctional and degenerate, leading to a disconnection

of the striatum from downstream basal ganglia unclei and severe motor deficits, which affects the dopamine-glutamate balance in the striatum [Albin et al. 1989, DeLong 1990; Mittal and Eddy 2013]; in drug addiction, significant increases in DA release in the striatum have been observed during cocaine self-administration [Ito et al., 2002]; striatal dysfunction is also implicated in other diseases including dystonia, obsessive-compulsive disorder [Breakefield et al. 2008, Graybiel 2008].

Striatal neuromodulators

There are two major neuromodulators in the striatum: Acetylcholine and Dopamine besides GABA.

γ - aminobutyric acid

More than 95% of the striatal neurons are GABAergic neurons which release GABA at the synapse terminals. The major action of GABA on its receptors is inhibitory. The inhibitory GABA effects are believed to involve in numerous neuropsychiatric disorders including epilepsy, Huntington disease, tardive dyskinesia, alcoholism, and sleep disorders. There are two main groups of GABA receptors: the ionotropic GABAA receptors and $GABA_B$ receptors which belong to the typical GPCR super family [Nakayasu et al., 1995].

Acetylcholine

In the striatum, acetylcholine is released into the extracellular spase by tonically avtive cholinergic interneurons [Bolam et al. 1984, Wilsonet al. 1990], although cholinergic interneuros constitute less than 1% of all striatal neurons [Rymar et al. 2004], their dense and extensive axonal arborization ensures the widespread release of acetylcholine. However, they are rapidly degraded by acetylcholinesterase [Kreitzer 2009].

Dopamine

The striatum is densely innervated by dopaminergic fibers where dopamine are released. This feature can be explained with two groups of data obtained by Matsuda and colleges, the first one: a single dopamine neuron that project to the striatum can innervate 2.7% on average and 5.7% at the maximum of striatal neurons; the second one: it is estimated that a single striatal neuron might be under the influence of 95-194 dopaminergic neurons on average [Matsuda et al., 2009]. Moreover, other researchers suggest that dopaminergic boutons represent nearly 10% of all striatal synapses (Groves et al. 1994) and the nearest–neighbor distance between two dopaminergic boutons is only～1.18 μm (Arbuthnott &Wickens 2007). Dopamine receptors are present in every cell type in the striatum, although different cell types express different dopamine receptor subtypes. All the five dopamine receptors are expressed in the striatum as we introduced in the pervious section, among them, D_1R and D_2R subtypes are far more abundant than the rest. It is believed that D_1 and D_2 receptor are separately expressed in dSPNs and iSPNs (Gertler et al., 2008; Valjent et al., 2009). However, recent studies which initially try to evaluate of the proportion of striatal neurons expressing D_1R, D_2R or both obtained a proportion of ～17% of MSNs in NAc shell co-express D_1R and D_2R, while this proportion is ～5-6% in dorsal striatum [Bertran-Gonzalez et al. 2008, 2010; Matamales et al., 2009]. In addition, dSPNs express low levels of D_3 receptor mRNA and iSPNs express low levels of D_5 mRNA, which means that the D_1 and D_2, D_1 and D_3, D_2 and D_5 may co-localize in some neurons in the striatum [Surmeier et al., 1996].

The detailed properties of DA and corresponding receptors will be elucidated in the following sections.

Dopamine system

Dopamine, 3, 4-dihydroxyphenethylamine (Fig.5), a member of catecholamine family, is an important neurotransmitter in brain and has functions in motor control, motivation, arousal, cognition and reward, and in addition plays a role in the lower-level functions including lactation, sexual gratification and nausea. Inside the brain, dopamine-containing neurons (may also termed as dopaminergic neurons, refers to those neurons whose primary neurotransmitters are dopamine) are comparatively few, and the cell bodies are confined to a relatively small brain regions including the substantia nigra (cell group A8 and A9), the ventral tegmental area (VTA, cell group A10), the posterior hypothalamus (cell group A11), the arcuate nucleus (cell group A12) and periventricular nucleus (cell group A14) of the hypothalamus, and also the zona incerta (cell group A13)(Fig. 6). However, these small amount dopaminergic neuronal cell bodies send projections to many brain regions and exert powerful effects on their targets. The most studied dopamine systems in the brain are dopaminergic neurons with their cell bodies located in the substantia nigra and the ventral tegmental area and send projections to the striatum (Fig.12).

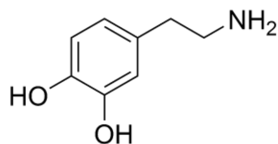


Figure 11 the chemical structure of dopamine.

The substantia nigra. A small midbrain area that forms a component of the basal ganglia. The dopamine neurons are found mainly in a part of this structure called the *pars compacta* (cell group A9) and *retrorubral area* (group A8) [Björklund et al. 2007]. In rodents, their most important projections go to the *striatum*, *globus pallidus*, and *subthalamic nucleus*, which also belong to the basal ganglia, and play important roles in motor control (Fig.9).

The ventral tegmental area **(VTA), another midbrain area.** This cell group (A10) is the largest group of dopaminergic cells in the human brain, though still quite small in absolute terms. They send projections to the *nucleus accumbens* and *the prefrontal cortex* as well as several other areas.[Björklund et al. 2007] These neurons play a central role in reward and other aspects of motivation (Fig.7).

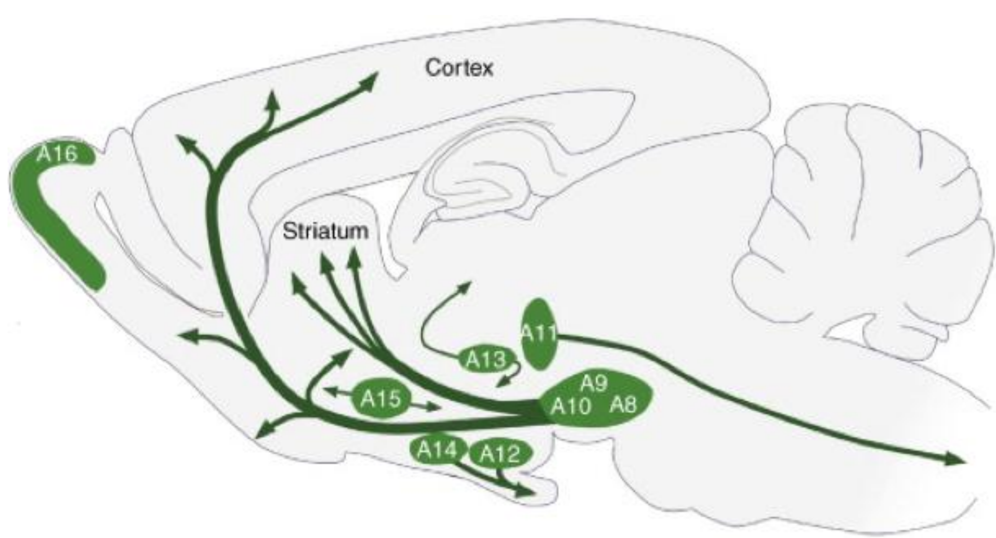


Figure 12 DA neurons containing regions and their projections. The dopaminergic neurons in the mammalian brain are localized in nine distinctive cell groups, from A8 to A16, including the substantia nigra (cell group A8 and A9), the ventral tegmental area (VTA, cell group A10), the posterior hypothalamus (cell group A11), the arcuate nucleus (cell group A12) and periventricular nucleus (cell group A14) of the hypothalamus, and also the zona incerta (cell group A13).
Illustration is from Björklund et al. 2007.

In this thesis, the only brain region we used is the brain striatum, as we could conclude from the above information that the striatum mainly receives the dopaminergic projections from A8, A9 and A10 cell groups that are substantia nigra and ventral tegmental area.

Dopamine synthesis, storage and release in brain

Dopamine synthesis process begins with the hydroxylation of the aminoacid L-tyrosine catalyzed by tyrosine hydroxylase to the intermediate levodopa (L-DOPA), and this intermediate is subsequently decarboxylated by Dopa decarboxylase (DDC) to dopamine (Fig 13). In brain, most dopamine synthesis does not occur in the cell bodies of dopaminergic neurons; rather, this process occurs in the nerve terminal cytoplasm, so the enzymes needed are usually transported from cell bodies to the dopamine synthesis locations (Fig 13). After dopamine was synthesized, it is packaged and stored in the vesicles gathered in the presynaptic nerve terminals of synapses. Dopamine are released to synaptic cleft by means of exocytosis when dopaminergic neuron fires. The dopamine in the synaptic cleft could undergo different paths as follows: 1) dopamine transporter (DAT) mediated reuptake into the presynaptic terminals (most dopamine undergo this path); 2) presynaptic stimulation of autoreceptors; 3) postsynaptic stimulation of the postsynaptic receptors; 4) degradation; 5) diffusion from the synaptic cleft. Presynaptic and postsynaptic stimulation will be illustrated in details in the following sections.

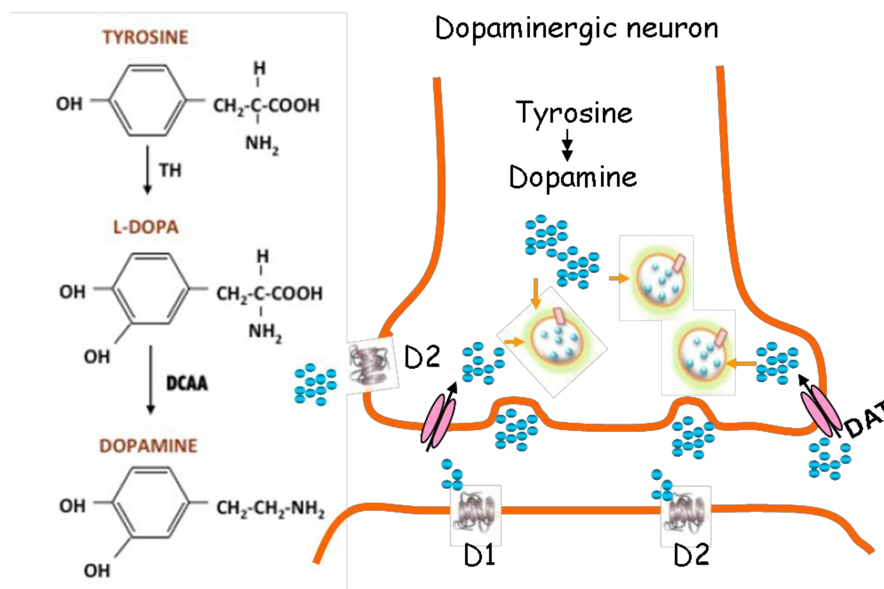


Figure 13 Dopamine synthesis, storage, release and re-uptake. Left: dopamine

synthesis from tyrosine. Right: brief procedure of dopamine synthesis, storage, release and re-uptake at the presynaptic neuronal terminals.

Abbreviations: DCAA, aromatic amino acid decarboxylase; DAT, dopamine transporter, TH, Tyrosine Hydroxylase; D_1, D_1-like dopamine receptor; D_2, D_2-like dopamine receptor.

Tyrosine hydroxylase

Tyrosine hydroxylase (TH; tyrosine 3-monooxygenase; E.C. 1.14.16.2) is the first catalyzed enzyme in the biosynthesis of DA, more important, its activity appears to be rate-limiting in the process. Regulation of the activity of TH is thought to be crucial to maintain dopamine levels. TH activity is modulated by a long-term regulation of gene expression as well as by a short-term regulation of enzyme activity such as catecholamine feedback or phosphorylation state mediated by the kinases or protein phosphatase [Kumer and Vrana, 1996]. Changes in TH phosphorylation state are usually considered as critically involved in the regulation of DA synthesis [Haycock and Haycock, 1991]. Multiple-site phosphorylation of TH has been demonstrated, mainly concentrated at serine residues (Ser), ser8, ser 19, ser 31 and ser 40 [Haycock, 1990; Haycock and Haycock, 1991]. Modulation of TH activity by phosphorylation has been extensively investigated, and bunch of protein kinases (including Ca2+/calmodulin-dependent protein kinase Ⅱ, ERK1/2, cyclic AMP-dependent protein kinases) have been reported to phosphorylate TH, in contrast, the regulation of the TH dephosphorylate involves less kinds of protein phosphatase (so for only one enzyme, protein phosphatase 2A) [Haavik et al., 1989].

Dopamine receptors

The free dopamine in the synaptic clef that were released from the presynaptic neuron which were not directly reuptake by the presynaptic neuron, not degraded or diffused right after their release from the cleft may stimulate the receptors located on the postsynaptic neuron. Receptors specific to dopamine are termed as dopamine receptors. There are two categories of dopamine receptors: D_1-like which includes D_1

and D_5 receptor; and D_2-like which includes D_2, D_3 and D_4 receptor. All of them belong to G-protein coupled receptor super-family (Fig. 14). D_1-like receptors and D_2-like receptors share a high level of homology of their transmembrane domains and have distinct pharmacological properties. Referring to the signaling pathways, D_1-class and D_2-calss receptors possess particular and different properties. Although the typical dopamine receptor functions are associated with the regulation of cAMP and PKA via G-protein mediated signaling, D_1-class receptors are generally couple to $G\alpha_{s/olf}$ and stimulate the second messenger cAMP and activate PKA, in contrast, D_2-class receptors couple to $G\alpha_{i/o}$ and negatively regulate cAMP and inhibit PKA activation [Kebabian et al., 1971]. See details in the following section, Page 28

Receptor Subtype	D_1-like Receptors		D_2-like Receptors		
	D_1	D_5	D_2	D_3	D_4
G protein	G_s	G_s	$G_{i/o}$	$G_{i/o}$	$G_{i/o}$
Transduction Mechanism	↑ AC, ↑ PLC, ↑ L-type Ca^{2+} channels	↑ AC	↓ AC, ↑ $[Ca^{2+}]_i$, K^+ conductance, ↓ Ca^{2+} conductance	↓ AC, activation of MAP kinase	↓ AC, ↓ Ca^{2+} conductance
Localisation	Caudate, putamen, nucleus accumbens, olfactory tubercle	Hippocampus, hypothalamus	Caudate, putamen, nucleus accumbens, olfactory tubercle	Nucleus accumbens, olfactory tubercle, islands of Calleja	Frontal cortex, midbrain, amygdala, cardiovascular system
Likely Physiological Roles	Locomotion, reward, reinforcement, learning and memory, renin secretion	Learning and memory	Locomotion, reward, reinforcement, learning and memory	Locomotion, possible role in cognition and emotion	Mostly unknown - possible role in cognition and emotion, hypertension

Figure 14 Characteristics of DA receptors. The classification, G-protein coupling character, downstream signaling, locations in brain and possible physiological roles of the dopamine receptors.

The illustration is adapted from www.tocris.com

Dopamine receptors distribution in brain

In the brain, dopamine receptors have selective expressions, and each subtype has its unique distribution. D_1 receptors are expressed at a high level of density in the nigrostriatal, mesolimbic and mesocortical area, such as the caudate-putamen

(striatum), nucleus accumbens, substantia nigra, olfactory bulb, amygdala, and frontal cortex, as well as at lower levels in the hippocampus, cerebellum, thalamic areas, and hypothalamic areas. D_5 receptors are expressed at low levels in multiple brain regions such as prefrontal cortex. Especially, a very low level of expression has also been observed in the caudate nucleus and nucleus accumbens. D_2 receptors are found to have the highest expression level in the striatum, the nucleus accumbens, and the olfactory tubercles. The distribution of D_3 receptor in brain is quite limited, the highest level is found in the limbic areas such as in the shell of the nucleus accumbens, the olfactory tubercle, and the islands of Calleja. In the striatum, D_3 receptor is also detected at a significantly lower level. The D_4 receptor has the lowest level of expression compared with other subtypes in the brain, with documented expression in the frontal cortex, amygdala, hippocampus, hypothalamus, globus pallidus, substantia nigra pars reticulata and thalamus (for review Beaulieu and Gainetdinov, 2011, and the reference therein). For the distribution of dopamine receptors in the striatum, see the striatum section, page 21.

Receptor locations with respect to synapses

Postsynaptic receptors

Receptors located on the postsynaptic neurons are termed as postsynaptic receptors. All types of dopamine receptors are observed at postsynaptic neurons. They are stimulated by dopamine released from the presynaptic neurons and conduct the information through activating the downstream pathways in the postsynaptic neurons.

Presynaptic autoreceptors

Autoreceptors are defined as receptors that are sensitive to the neurotransmitters secreted from the neurons on which they are located. Presynaptically localized autoreceptors generally provide an important negative feedback mechanism that

adjusts neuronal firing rate, synthesis and release of the neurotransmitter in response to changes in extracellular neurotransmitter levels [Missale et al., 1998; Sibley, 1999; Beaulieu et al., 2011]. Activation of autoreceptors by released dopamine is thought to be one of the principal mechanisms responsible for auto regulation of dopaminergic neuronal function.

In striatum, D1 receptors are preferentially localized on striatal GABAergic neurons. In contrast to D_1 receptor, in this brain region, D_2 and D_3 receptors both could function through the presynatpic receptors (autoreceptor) or postsynaptic receptors (Missale et al., 1998; Sibley, 1999). We have mentioned above that autoreceptors regulate the extracellular DA concentrations through acting on the synthesis and release. As a matter of fact, previous research obtained from D_3-KO mice suggested that no deficits in autoreceptor functions were apparently observed [Zapata et al., 2001]. And another paper clearly pointed that D_3 receptor having a small but significant role as an autoreceptor that partially regulate secretion, but not on DA synthesis [Joseph et al., 2002]. In contrast, the autoreceptor functions completely lost in D_2-KO mice [Mercuri et al., 1997; L'hirondel et al., 1998]. D_2 receptors seem to be the predominant type of autoreceptors that are involved in the regulation of DA synthesis and other autoreceptor functions. The alternative splicing of an 87-base-pair exon between introns 4 and 5 in the D_2 receptor gene gives rise to the two variants which termed as D_2-short (D_2S) and D_2-long (D_2L). The difference between these two variants lies in the third intracellular loop of the receptor and with additional 29 amino acids in the D_2L subtype [Giros et al., 1989; Monsma et al., 1989]. D_2S has been shown to be mostly expressed presynatptically and to function as autoreceptor, whereas D_2L seems to be predominantly a postsynaptic isoform [Usiello et al., 2000; De Mei et al., 2009]. Plus, it seems that D_2R autoreceptors are generally activated by a lower concentration of dopamine agonists than necessary to activate postsynaptic receptors, so the same dopamine agonist can induce a biphasic effects through acting on the presynaptic and postsynaptic receptors [Beaulieu and Gainetdiow, 2011].

Moreover, there are large amount of dopamine receptors localize on the non-synaptic neuron surfaces, which are neither presynatpically nor postsynaptically. Although the functions and characters of this group of receptors have not been well studied, yet they should not be neglected.

Dopamine receptor signaling

All dopamine receptor subtypes are typical GPCRs. As we have mentioned above, dopamine receptors are subdivided into two classes: D_1-like (D_1, D_5) and D_2-like (D_2, D_3 and D_4). The signaling properties of the two classes of receptors have been widely researched, and the documented literatures suggest that there are both shared and individually different properties between them.

D_1-like receptor signaling is initiated by coupling to the heterotrimeric G protein $G\alpha_{s/olf}$, which subsequently activate adenylate cyclase, cylic AMP-dependent protein kinase, and protein phosphatase-1 inhibitor DARPP-32 (Fig. 15). The increased phosphorylation induced by the activation of cyclic AMP-dependent protein kinase and also by inhibition of the protein phosphatase-1 regulate the activity of numbers of other receptors, enzymes, ion channels and transcription factors [Neve et al.,2004].

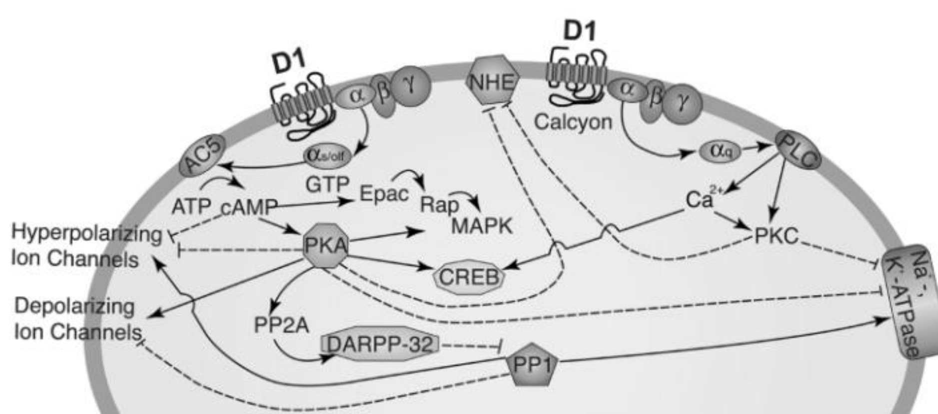


Figure 15 D_1-like receptor signaling pathways. The solid line ending with arrows means stimulatory effects while the dotted lines ending in a bar represent inhibitory effects. Abbreviations: AC5, adenylate cyclase type 5; CREB, cyclic AMP response element

binding protein; DARPP-32, dopamine-related phosphoprotein, 32 kDa; MAPK, mitogen-activated protein kinase; NHE, Naþ/Hþ exchanger; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PP1 or PP2A, protein phosphatase 1 or 2A. Image is adapted from Neve et al. (2004).

D_2-like receptor signaling via the inhibition of adenylate cyclase which is opposite to D_1-like receptor by coupling to the heterotrimeric G$\alpha_{i/o}$ protein, and subsequently decreasing the phosphorylation of PKA substrates such as DARPP-32 at Thr34 [Nishi et al., 1997] and finally leads to inhibition of the Na^+, K^+-ATPase in neostriatal neurons [Nishi et al., 1999]. Besides adenylate cyclase, D_2-like receptor also has other signaling pathways including phospholipases, ion channels, MAP kinases, and the Na^+/H^+ exchanger [Huff et al., 1997], which are regulated by G$\beta\gamma$ complex (Fig. 16).

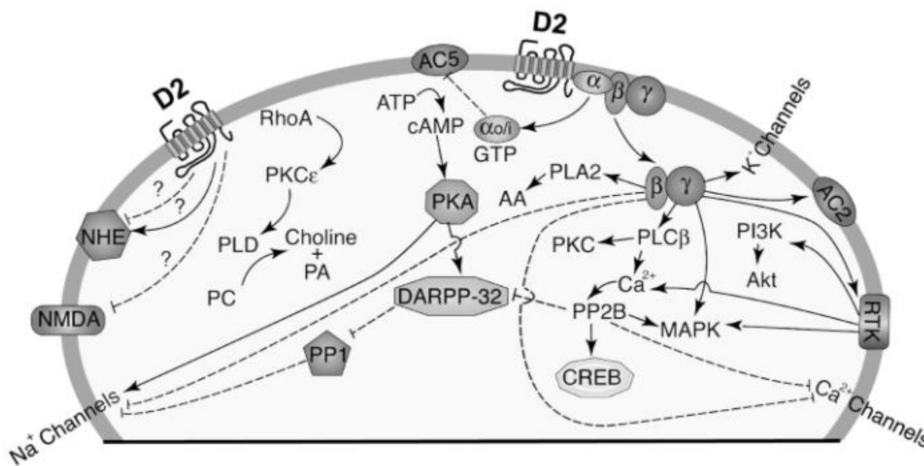


Figure 16 D_2-like receptor signaling pathways. The solid line ending with arrows means stimulatory effects while the dotted lines ending in a bar represent inhibitory effects. Abbreviations: AA, arachidonic acid; AC2 or AC5, adenylate cyclase type 2 or 5; CREB, cyclic AMP response element-binding protein; DARPP-32, dopamine- and cyclic AMP-regulated phosphoprotein, 32 kDa; MAPK, mitogen-activated protein kinase; NHE, Naþ/Hþ exchanger; PA, phosphatidic acid; PC, phosphatidylcholine; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLA2,phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PP1 or PP2A,

protein phosphatase 1 or 2A; RTK, receptor tyrosine kinase.

Image is adapted from Neve et al. (2004).

Dopamine receptor oligomerization

All of the physiological functions of dopamine, from voluntary movement and reward to hormonal regulation and hypertension, are mediated through dopamine receptors. During the past two decades, the general structural, biochemical and functional properties of the dopamine receptors have been widely studied and remarkable advance have occurred. These remarkable advances led to the development of multiple pharmacologically active compounds that are directly targeted at dopamine receptors. With the development of the GPCRs heteromerization concept, as a typical member of GPCRs, and given its important pharmacological function, the dopamine receptor field is largely focused in heteromerization studies. This is considered as the new field in developing pharmacological compounds. Here in the following text we are going to list some widely documented and functionally important heteromerization data related to dopamine receptors.

The D_1-D_2 and D_2-D_5 heteromers

D_1R and D_2R are the two subtypes in the D_1-like dopamine receptors which with high sequence homology (80%) to each other. Heteromerization of D_1R and D_2R was initially found through co-immuno-precipitation experiment from rat and human striatum [Lee et al., 2004], and were confirmed soon by fluorescence resonance energy transfer (FRET) studies [So et al., 2005]. Due to the high sequence homology of D_5R to D_1R, the heteromerization of D_5R and D_2R was soon observed in cultured cells co-transfected with both receptors [So et al., 2005]. Yet the evidence for their heteromerization in brain is still missing.

Although D_1 and D_5 receptor are closely related, after forming heteromers with D_2R,

they showed different patterns of dopamine-induced calcium regulation (Fig.17). The D_1-D_2 heteromer activation leads to a rapid, transient, intracellular calcium mobilization, this effect is not observed after the activation by either D_1R or D_2R. In contrast, the “fingerprint” of D_5-D_2 heteromer is largely different from the D_1-D_2 heteromer. Stimulate the D_5 component in the D_5-D_2 heteromer seems trigger less calcium influx compared with the activation of D_5 homooligomers; however, co-activation of D_5R and D_2R in the heteromer regain the calcium influx and reach the similar intracellular calcium concentration compared with the D_5 homooligomers [Hasbi et al., 2010]. Although activation of both D_1-D_2 heteromer and D_5-D_2 heteromer increase the intracellular calcium concentration through $G_{q/11}$ protein linked pathways, however, the effect of D_5-D_2 heteromers seems also dependent on the influx of extracellular calcium through store-operated calcium channels [So et al., 2009].

It has been confirmed that in the striatum, around 20% of the neurons co-express D_1 and D_2 receptors [Bertran-Gonzalez et al., 2008; Matamales et al., 2009]. Several lines of evidence have suggested that D_1-D_2 receptor heteromer may have etiological significance in schizophrenia [Melissa et al., 2011], drug abused and depression [Pei et al., 2010]. D_5 receptors are poorly distribute in the striatum, however, immunohistochemistry evidence suggest the colocalization of D_5R with tyrosine hydroxylase (TH) - the marker for dopamine neurons - in the substantia nigra (group A8 and A9) which is a main brain region that send dopaminergic projections to the striatum [Khan et al., 2000]. As we know, D_2 receptors are widely expressed in the dopaminergic neurons especially at the terminals, thus this may give rise to the heteromerization between D_2R and D_5R.

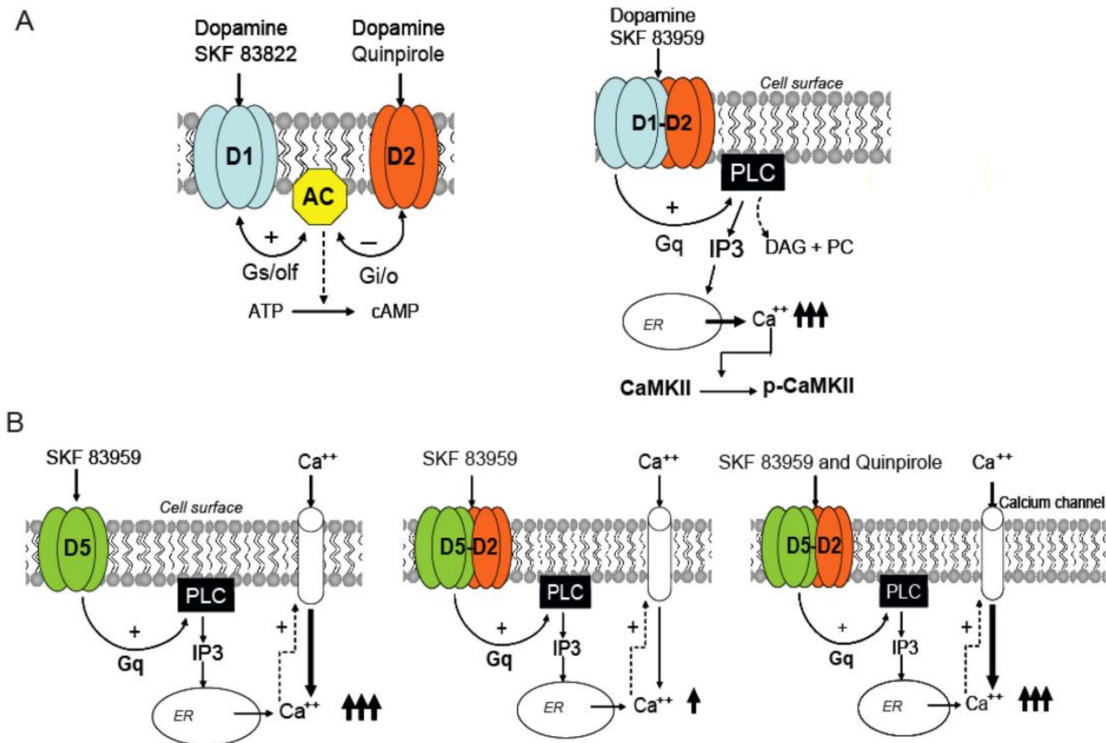


Figure 17 D_1-D_2 and D_5-D_2 heteromer signaling pathways. A. D_1-D_2 heteromer activation leads to a novel signaling pathway through coupling to Gq/11 protein and finally causes a rapid, transient, intracellular calcium mobilization from endoplasmic reticulum. B. For details see text above.

Abbreviations: AC adenylyl cyclase; cAMP cyclic AMP; PLC phospholipase C; IP3 inositol-triphosphate; DAG PC CaMKⅡ calcium metabolize kinase Ⅱ.

Image is adapted from Husbi et al., 2010

The D_1-D_3 receptor heteromer

The D_3R is concentrated in the ventral striatum, co-localized with D_1R in medium spiny neurons. It is reported that D_1R and D_3R form heteromer and the functional properties of the heteromer are different from the homo-oligomers formed individually by D_1R or D_3R. Notably, the D_1R-D_3R dimer couples to Gs protein and elucidate a higher potentiation of AC activation than D1R alone [Marcellino et al., 2008; Fiorentini et al., 2008]. Functionally, the D_1R-D_3R heteromer likely plays a role in the physiological control of the activity of the ventral striatum and may have a pathological implication in the development of motor dysfunction [Missale et al., 2010].

The D_1-NMDA receptor heteromer

In the striatum, D_1R and NMDAR are co-localized in dendritic membranes and post-synaptic density (PSD). A series of different experimental approaches including co-immunoprecipitation, glutathione-S-transferase (GST) –fusion protein pull-down, in vitro binding of recombinant receptor fragments, blot overlay and bioluminescence resonance energy transfer(BRET) have evidenced the existence of D1R/NMDAR heterodimerization involving both NR1 and NR2A [Missale et al., 2010]. In striatal and hippocampal neurons, the interaction of D_1R/NMDAR is responsible for D_1R-mediated inhibition of both NMDA currents and NMDA-mediated excitotoxicity [Lee et al., 2002].

The A_2A-D_2 receptor heteromer

The heteromer formed by A_2A-D_2 receptor is another heteromer involved in schizophrenia. It was first indentified by coimmuno-precipitation in SH-SY5Y neuroblastoma cells [Hillion et al., 2002] and was further confirmed in living cells using FRET [Canals et al., 2003].

Except those heteromers listed above, there are some other types of receptors heteromerization or crosstalk that are participated by dopamine receptors. We are not going to list here anymore, only to mention that there is a good chance for the receptors with co-localization on the membrane to form homo- or heteromers.

Pharmacology of dopamine receptors

Dopamine receptors are largely focused in the field of mental disorders for almost 7 decades. The involvement of dopamine receptors in the human mental disease beginning with the receptor binding techniques which enables the possibility of measuring dopamine receptor binding characteristics in postmortem tissues of the patients (Seeman and Van Tol, 1994). The most unsuspicious hypothesis is the postulated enhanced sensitivity of postsynaptic D2 dopamine receptors in

schizophrenia [seeman et al., 2005]; however, o consistent changes in D1 receptor binding were observed in the patients with schizophrenia. With the development of the techniques and the accumulation of the literatures, dopamine receptors are reported to be relevant in various kinds of human disease. In patients with depression, decreased sensitivity of postsynaptic D2/D3 dopamine receptor in limbic brain area was observed and with the regain of this sensitivity after long-term therapeutic treatment [Willner, 1997]. In bipolar disorders, no alteration in D1 receptor was observed, however, several studies reported the increased density of D2 receptors. In patients with PD, similarly D2 receptor density was altered in basal ganglia, still no changes were observed with D1 receptors [Nikolaus et al., 2009a]. In patients with Huntington's disease, significant reductions in the striatal D1 and D2 receptor binding were observed [Felicio et al., 2009]. In drug abuses, decreased D2 receptor binding was observed in the striatum [Frankle and Laruelle, 2002; Nikolaus et al., 2009b; Volkow et al.,2009] (For details about the drug abuse, see in the drug addiction section, page X). Although dopamine receptors are widely involved in the mental disorders, no conclusive association has been found thus far which results in few therapeutic implications exceptr antipsychotics. However, it seems that D2 dopamine receptors are more involved than D1 dopamine receptors.

Antipsychotics

Antipsychotics are a class of psychiatric medication primarily used to manage psychosis, in particular in schizophrenia and bipolar disorder. Since the first antipsychotic chlorpromazine discovered almost seven decades before, the antipsychotics have been grouped: first generation (also called typical antipsychotics) which in the representative of chlorpromazine and haloperidol; second generation (also called atypical antipsychotics) which in the representative of clozapine; and the third generation which so for only contain one member, aripiperazole. The groups are according to clinical actions as well as mechanism of actions, for instance, the first generation antipsychotics are high affinity dopamine D_2 antagonists, they elicit

antipsychotic actions, at the same time cause many side effects. In contrast, the second generation of antipsychotics is thought to work though "serotonin-dopamine antagonism", which produce equal antipsychotic actions with low liability to induce EPS or TD or other side effects caused by the typical antipsychotics, however, they cause other side effects which results in their abandoned. After the two generations of antipsychotics, the focus is back to dopamine D_2 receptors. Besides the well know concept that antagonism of D_2 receptors could effectively control positive symptoms of schizophrenia, a novel hypothesis was developed related with dopamine autoreceptors (composed by D_2S receptors and D_3 receptors) and receptor functional selectivity. The only member in the tired generation antipsychotic, aripiprazole, is widely studied yet no conclusive opinion about its pharmacological intrinsic activity on D_2 dopamine receptors, thus finally it is termed as has "functional selectivity" to D_2 receptors (For details, see result chapter 2) because aripiprazole seems to elicit D_2-mediated functional effects that encompass the whole range of classic pharmacological intrinsic activity. Nevertheless, aripiprazole, as an effective and widely prescribed antipsychotic which has specific intrinsic affinity on D_2 receptors is a bellwether for the developing of the further antipsychotics [Mailman and Murthy 2010].

Drug abuse

In the human history, so far, there are several drugs and chemical agents which could control human behavior by producing a psychological and physiological drug dependent state called addiction. It is characterized by compulsive drug seeking and consumption despite serious negative consequence such as medical illness and failures in significant life roles. Normally, the addicted individuals lost all the interests that could cheer up the non-addicted ones such as food, sex or promotion, the only interests of them is to consume drugs, or struggle for obtaining drugs despite criminal activities. This drug centered state of addicts usually cost their jobs, personal

relationships, financial standing and finally, in some case, their lives. The drugs that could be addicted and their general information are listed in box 1:

Box 1 Common abused drugs.

Psychomotor stimulant Drugs: include cocaine, amphetamines and methylphenidate; work directly on the monoamine (mainly dopamine) neurotransmitter systems.
Opiates: it's a group of analgesics which include morphine and heroin, act through opiates receptors.
Alcohol: it is widely consumed; act in many ways, and various neurotransmitters such as dopamine, serotonin, GABA, and Glutamate are involved.
Nicotine: act on the receptors for the neurotransmitter acetylcholine.
Cannabis: also known as hashish or marijuana, act on the cannabinoid receptors located in the hippocampus formation, striatum and globus pallidus; this drug is usually inhaled.
LSD--lysergic acid diethylamide: produces vivid hallucinations; it is the one drug that only human could reliably self-administration.
Phencyclidine (PCP): it is described as a "dissociative anaesthetic"; the effects include altered feeling of isolation, cognitive disorganization and drowsiness; it is more noted as a model of human psychosis than as a major drug of abuse.

Drug addiction places enormous burden on society, thus the field focus on the understanding, treating and preventing addiction is heavily invested and a lot of advances have occurred thanks a lot to the good animal model such as drug self-administration (see bellow). To diagnose a person as drug addicted, it is necessary that the addicts have the following categories of symptoms shown in table 1 according to the Diagnostic and Statistical Manual (DSM)-Ⅳ.

Table 1 Common categories and symptoms of drug addiction

Tolerance	1. A need for markedly increased amount of the substance to achieve intoxication or desired effects; Markedly diminished effects with continued use of the same amount of the substance
Withdrawal	2. Criteria for withdrawal from the specific substance;The same as (or closed related to) substance taken to relieve or avoid withdrawal symptoms
Binge	3. The substance often taken in larger amounts or over a longer period than was intended
Frequent relapse	4. A persistent desire or unsuccessful efforts to cut down or control substance use
Drug seeking and taking	5. A great deal of time spent in activities necessary to obtain substance, use the substance, or recover from its effects
Compulsion	6. Continued substance use despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance
social activity disruption	7. Important social, occupational, or recreational activities given up or reduced because of substance use

The brains reward pathway

The rewarding effects of the addicted drugs are the main reason why humans consume the drugs and the laboratory animals self-administer them [Wise, 2009]. The rewarding process of drugs is highly related with the brain reward system. A reward is a stimulus that the brain interprets as intrinsically positive, or as something worth to be approached, while the body acquires enjoyment and arousal. A reinforcing stimulus is one that increases the probability that behaviors paired with reward will be repeated. The neural substrates that underlie the perception of reward and the phenomenon of positive reinforcement are a set of interconnected forebrain structures called brain reward pathways. The reward pathways are mainly consisted by the nucleus accumbens, the basal forebrain components of extended amygdala and the medial prefrontal cortex. All these structures are under dense dopaminergic innervations originated from ventral tegmental area (VTA). It is believed that stimulation of the brain reward system depends on the action of catecholamines, particularly

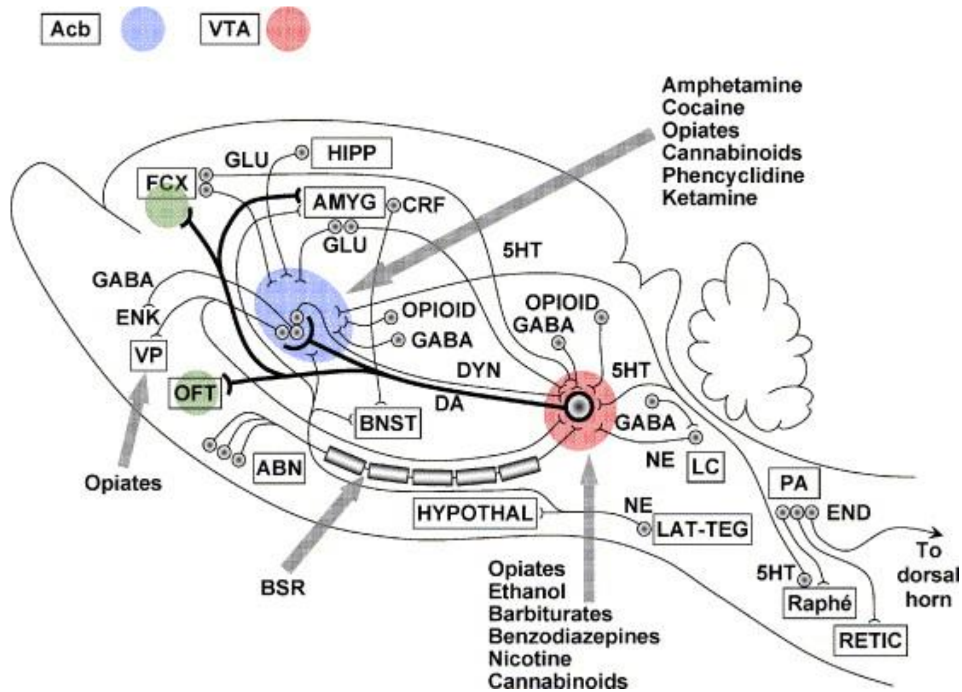


Figure 18 Diagram of the brain reward circuitry of the mammalian (rat) brain. The main action sites of the various addictive drugs to enhance brain reward were highlighted with colors in the diagram. ABN: anterior bed unclei of the medial forebrain bundle; Acb: nucleus accumbens; Amyg: amygdale; BNST: bed nucleus of the stria terminalis; CRF: corticotrophin-releasing factor; DA: subcomponent of the ascending mesocorticolimbic dopaminergic system, believed to be preferentially activated by addictive drugs; DYN: synorphinergic neuronal fiber bundle outflow from the nucleus accumbens; ENK: enkephalinergic neuronal fiber bundle outflow from the Acb; FCX: frontal cortex; GABA: GABAergic inhibitory fiber systems innervations in the ventral tegmental area, the nucleus accumbens, and into the vicinity of the locus ceruleus, as well as the GABA neuronal fiber bundle outflow from the nucleus accumbens; GLU: glutamatergic neural systems originating from the frontal cortex and send projections to both the ventral tegmental area and the nucleus accumbens; 5-HT: serotonergic fibers, originated in the anterior raphe nuclei and project to both the cell body region (VTA) and terminal projection field (Acb) of dopaminergic neuron. BSR: descending, myelinated, moderately fast-conducting component of the brain reward circuitry that is preferentially activated by electrical intracranial self-stimulation (electrical brain stimulation reward). LC: locus ceruleus. NE: noradrenergic fivers, originate in the locus ceruleus and project to the general vicinity of the ventral mesencephalic neuronal cell fields of the VTA; OPIOID: endogenous opioid peptide neural systems which send projections to both VTA and nucleus accumbens. PA: periaqueductal gray matter; Raphe: brain stem serotonergic raphe nuclei; VP: ventral pallidum; VTA: Ventral tegmental areas, the major area in where the reward related

dopaminergic neurons originate.

dopamine (DA). Natural rewards, such as food or sex, as well as most abused drugs, such as alcohol, amphetamine, cocaine, could increase extracellular concentration of mesolimbic dopamine. It appears that addictive drugs are rewarding and reinforcing because they could enhance either dopamine release or the effects of dopamine in the NAc or related structure by means of the rewarding system. The brain reward circuitry is illustrated in figure.18

Neuroadaptation of drug addiction

Essentially, all addictive drugs exert their acute reinforcing properties by stimulating the brain reward system, however, not everyone who consumes drugs becomes addicted, which is thought to be due to the second character of addiction—the neuroadaptations induced by chronic drug use. Repeated exposure to an addictive drug induces profound cellular and molecular changes within neurons of the brain reward circuitry, which in turn are believed to cause the alterations in reinforcement mechanisms that contribute to addiction. Drug-induced neuroadaptations reflect both homeostatic compensations for excessive stimulation by drugs and alterations in multiple memory systems in the brain and serve to remain addiction beyond long periods of time.

Cocaine

Cocaine ($C_{17}H_{21}NO_4$, Fig.18) is a popular drug abused in Europe; survey showed that cocaine has been used at least once in a lifetime by 3.4% of the general population (EMCDDA 2001). Cocaine is a psychomotor stimulant that exerts its effect on both the central and peripheral nervous system. Consumed cocaine could increase alertness, feeling of well-being and euphoria, energy and motor activity, feelings of competence and sexuality. Besides the good feeling induced by cocaine, the negative health and economic consequences of cocaine use are also

considerable, with the drug causing potentially fatal cerebral hemorrhage and cardiovascular events such as arrhythmias, myocardial infarction, and results in approximately US＄580 million in direct health-care costs annually (Caulkins et al, 2002). Large quantities of researches have focused on the various aspects related to this disorder in order to improve the therapeutics of cocaine addiction.

The development history of cocaine

Cocaine is a crystalline tropane alkaloid that is obtained from the leaves of the coca plant. Before its discovery, about more than a thousand years ago, South American indigenous people have chewed the leaves of *Erythroxylon coca* which is declared to give them strength and energy. Later, coca was used in medicine to protect the body from many ailments, reduce the swelling of wounds, to strength broken bones, and etc. However, it took more than 200 years for people to finally achieve the isolation of cocaine – the marrow of coca plant and another 50 years for people to synthesis it via chemical method. In the late nineteen century, cocaine was widely used in medicine as a local anesthetic and consumed as a vice in literature; it was sold in neighborhood drugstores and very popular in drinks containing ethanol and caffeine. As long as the increasing usage of cocaine, the considerable side-effects and its high addiction risk got it prohibited first in medical use in 1914 and then in free trading. In modern society, although there are serious restrictions on cocaine manufactures, it is still prevalent across all socioeconomic strata, including age, demographics, economic, social, political, religious, and livelihood.

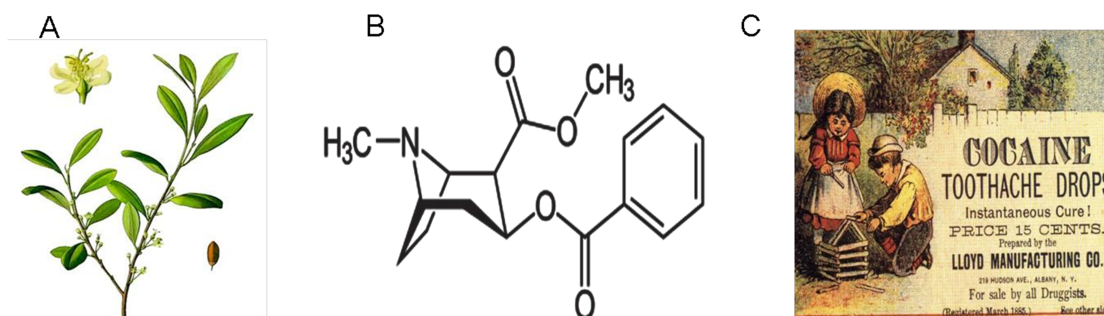


Figure 19 Cocaine

A) The Erytroxylon coca plant contains cocaine in the leaves. B) Molecular structure of cocaine: [1R-(exo,exo)]-3-(benzoyloxy)-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester. C) Advertisement for kids toothache drops with cocaine as its main components (1885).

Ways of cocaine ingestion as an abused substance

Cocaine can be ingested mainly though the following ways [Volkow et al., 2000]:

Oral: chewing coca leaves is the most original way to intake cocaine. Nowadays, coca leaves are typically mixed with an alkaline substance and chewed into a wad that is retained in the mouth between gum and cheek and sucked of its juices. Many users also rub the powder along the gum line, or onto a cigarette filter. Oral administration is quite slow in subjective effects.

Insufflation: it is the most common cocaine powder ingestion ways in the western world and is absorbed through the mucous membranes lining the sinuses. It only takes 14.6 minutes to reach peak subjective effects through this way.

Injection: drug injection provides the highest blood levels of drug in the shortest period of time; the average time to reach peak subjective effects is 3.1 minutes.

Inhalation(smoking): "peak high" of this way is 1.4 ± 0.5 minutes. When smoked, cocaine is sometimes combined with other drugs such as cannabis.

Suppository: this administration approach is not well studied. Generally cocaine is

dissolved in water and withdraw into an oral syringe and then be lubricated and inserted into the anus or vagina.

The mechanism of acute cocaine effects

Although cocaine could be ingested in different ways, it is all absorbed finally into the bloodstream quickly and transported to all over the body, especially the Central Neuronal System (CNS) which is surrounded by the blood brain barrier. Due to its high lipophilic properties, cocaine can easily cross the blood brain barrier and then access to the CNS, where the major subjective effects induced by cocaine occur. Cocaine produces its psychoactive effects by potentiating monoaminergic transmission through actions on dopamine, serotonin and norepinephrine transporters. Briefly, cocaine binds to those transporters to inhibit the reuptake of these neurotransmitters, thus leads to the accumulation of the neurotransmitters and finally results in enhanced and prolonged postsynaptic effects. Action on the dopamine transporter (DAT) is believed to be the most important role for the reinforcing effects of cocaine. In addiction, cocaine is suggested to have effects on sigma receptors, block sodium channels, and others.

Cocaine induced neuronal adaptations

Cocaine addiction induced adaptations occurred at both the cellular and molecular level, including gene and protein expression. These adaptations in turn will promote changes in cell excitability and activity, morphology, and neurotransmission in reward circuit. Most important, the neural adaptations induced by cocaine usually outlast the presence of the drug in the brain and persist in a quite long withdrawal period, and are believed contribute to the reorganization of neural circuits that strength the desire to re-obtain drugs which is termed as relapse.

Cocaine addiction and CREB phosphorylation

Repeated cocaine exposure results in neuronal adaptations, while at the same time, those neuronal adaptations conversely facilitate the oncoming cocaine intake which consolidate cocaine addiction. One of the important cocaine induced neuroadaptation is altered phosphorylation of the transcription factor cyclic AMP (cAMP)-regulated element-binding protein (CREB).

CREB is widely expressed in all cells in the brain; it belongs to a family of proteins that function as transcription factors. Phosphorylation of CREB on Ser 133 activates CREB and increases transcription of a large number of genes that can alter neuronal function and regulate synaptic plasticity. Some common cellular events involved in regulation of CREB are shown in Figure 20.

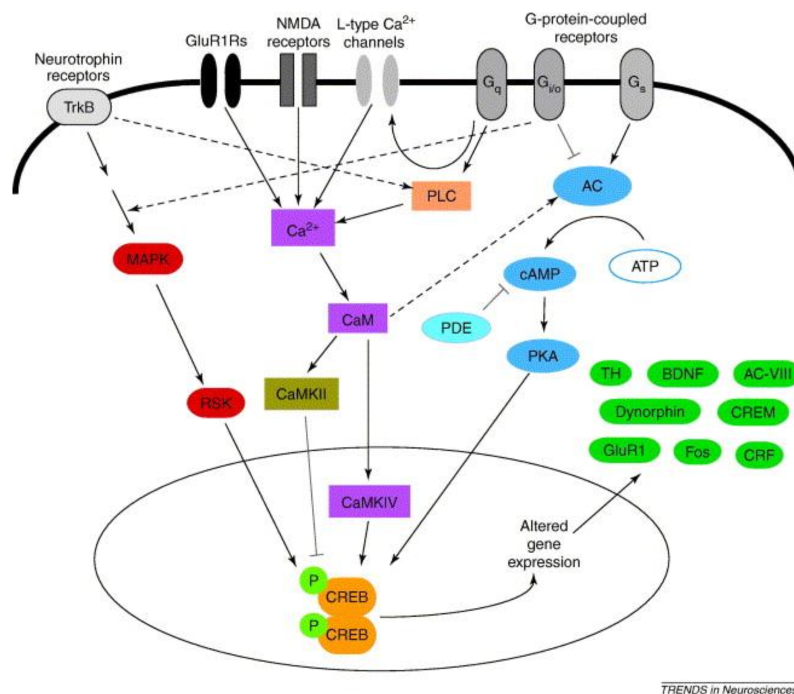


Figure 20 Cellular events involved in CREB regulation. Neurotransmitters or neurotrophins act on the membrane surface receptors (e.g. TrkB, AMPA receptors and GPCRs) will subsequently stimulate the intracellular downstream signal cascades which finally regulate the phosphorylation state of CREB located in the nuclei. Specific

phosphorylation at serine 133 activates various CREB-mediated gene transcription which lead to different protein expression levels (protein products altered by CREB are listed in green). Abbreviations: CaM, calmodulin; GluR1Rs, glutamate receptor subunit GluR1 homomeric AMPA receptors; PDE, phosphodiesterase; PLC, phospholipase C; TrkB, neurotrophin tyrosine kinase receptor type 2.
Image is adapted from William et al. 2005.

The best understanding of the precise role played by CREB in motivational aspects of addiction concerns the nucleus accumbens subregion. The CREB phosphorylation level in striatum is altered either by the acute or chronic cocaine administration. In one study, it is observed that cocaine-induced CREB phosphorylation is enhanced in the nucleus accumbens after challenge injection of cocaine in rats that were repeated exposure to cocaine [Mattson et al., 2005]. And before that, several lines of evidence illustrated that acute expose to the cocaine-related psychostimulant amphetamine could also enhance the CREB activation in striatum [Cole et al. 1995; Simpson et al. 1995; Turgeon et al. 1997]. Notably, the CREB activation levels also play an important role in regulating animals' cocaine consuming habits. It is evidenced that increased CREB activation in the nucleus accumbens decreases an animal's sensitivity to the rewarding effects of cocaine. In addition, increased CREB function in this region also dampens an animal's interest for natural rewards, while decreased CREB function increase this interest [Nestler et al., 2004]. All these results suggest that CREB phosphorylation levels are greatly affected by cocaine addiction, as well as that this transcription factor inversely plays an important role in the process of cocaine addiction and in performing the behavior induced by cocaine administration.

Chronic cocaine administration reduces G protein signaling efficacy.

Besides the modification of gene expression by cocaine addiction through alteration of the transcription factor CREB activation, the neuroadaptation induced by chronic

cocaine administration is also believed on the G protein signaling levels. Neuroimaging experiments in primate addiction models (Morgan et al., 2002) or human addicts (Goldstein and Volkow, 2002) observed reduced signaling through Gi α -coupled receptors. More specifically, reduced Giα function after repeated cocaine adiminstration were also confirmed at the level of protein expression(Nestler et al., 1990; Striplin and Kalivas 1993).

Morphological changes in dendritic spines

Chronic cocaine administration results in marked neuroplasticity of dendritic spines during the reinstatement of drug seeking. When this phenomenon was observed almost 20 years ago, it was suggested that chronic non-contingent amphetamine administration caused dendritic sysmorphisms and increased dendritic spine density in the NAc and prefrontal cortex of rats [Robinson et al., 1997], Later, drug-induced dendritic changes were observed widely in the striatum [Lee et al., 2006; Robinson et al,. 2004; Jedynak et al., 2007]. In addition, figure 21 shows another research reslut which observed this neuroplasticity in chronic non-contingent cocaine administration withdrawal rats, an increase in spine head diameter at 45 min and a reduction in diameter at 120 min after the challenge injection of cocaine [Kalivas et al., 2009].

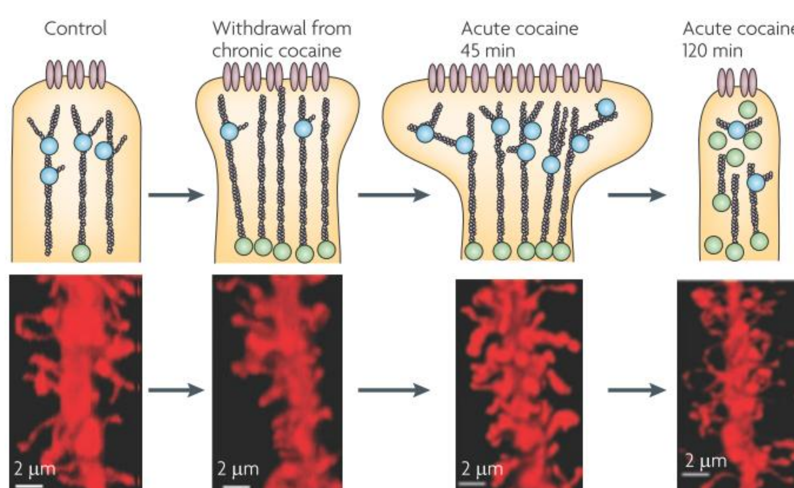


Figure 21 Morphology change of spine caused by chronic cocaine administration.

Withdrawal from chronic non-contingent cocaine administration causes a basal increase in spine head diameter. Acute challenge injection of cocaine enlarge this increase However 120min after the injection of cocaine, the spine head diameter is markedly decreased compared with the basal control level.
Image is adapted from Kalivas et al., 2009.

Inhibition of neuronal excitability by cocaine administration

The down-regulation of nucleus accumbens membrane excitability was observed both after repeated cocaine administration and short withdrawal periods [zhang et al., 1998, 2002; Henry et al., 1995]. Notably,CREB modulates the excitability of nucleus accumbens neurons [Dong et al 2006].

Cocaine-induced neuroadaptation in dopamine system

The mesolimbic dopamine system plays a principal role in forming the cocaine addiction forming process. The direct effect of cocaine on the DA system is to bind to the dopamine transporter (DAT) to block the reuptake of DA from the synaptic cleft to the presynaptic neurons thus markedly elevates extracellular DA levels in the nucleus accumbens (NAcc) and dorsal striatum. The large amount of DA in the synaptic cleft will stimulate the D_1-like and D_2-like receptors both located on the pre- and postsynaptic neurons, as well as diffuse away from the synapse to act on the receptors located on neighboring synaptic or non-synaptic receptors. Prolonged cocaine abuse will influence the DA system in several aspects, like dopamine receptor expression density, extracellular DA levels, receptor functions as well as receptor sensitivities.

The effect of repeated exposure to cocaine on the density of D_1-like and D_2-like dopamine receptors was reported either up-regulated, unchanged or down regulated, seems to depend upon factors such as cocaine dosing regimen, route of administration and length of treatment/withdrawal period [Anderson and Pierce,

2005].

Several lines of evidence confirmed that chronic cocaine administration leads to the decline of extracellular DA levels (down-regulation of DA "tone") after cessation of cocaine administration. In addition, this reduced of DA release seems to persist beyond both short and long withdrawal periods (Rossetti et al., 1992; Weiss et al., 1992b; Wu et al., 1997). However, the natural fluctuation in DA release may mark the extent of this effect.

Cocaine-induced modifications in dopamine receptor function and transduction are well studied and documented in the past 20 years. Prolonged cocaine exposure leads to supersensitivity of D_1 dopamine receptor which mediated the enhanced inhibition of nucleus accumbens neurons to locally applied DA [Henry & White, 1991; Xu et al.,1994; Henry et al., 1998; Beurrier & Malenka, 2002]. In addiction, repeated cocaine treatment causes enhanced cAMP-signaling pathway within the nucleus accumbens and reduced Gia protein levels in this area and in VTA. [Nestler et al., 1990; Terwilliger et al., 1991; Unterwald et al., 1993; Freeman et al., 2001; Lu et al., 2003; but seeCrawford et al., 2004]. In contrast, some researches found increased D_2-receptor sensitivity in coupling to G-proteins [Bailay et al., 2008]. However, to our knowledge none of these studies has been replicated in cocaine self-administrating animals.

Cocaine induced changes in D_2 receptor homo- or heterodimerization

Receptor oligomerization is an important, relatively newer field in the neuropharmacology. As we mentioned before, D_2 receptor could either form homodimer or heterodimers with other receptors. Research found that cocaine treatment significantly reduces the homodimerization of D_2 receptors in HEK 293T cells. In addition, in the same research they also observed a conformational change in A_2A-D_2 receptor heterodimer [Marcellino et al., 2010].

Cocaine induced neuroadaptation in glutamatergic circuits

Besides dopamine , the neuroadaptations in glutamatergic circuits are also believed to play important role in cocaine addiction. Chronic cocaine use is believed to result in long term changes in basal extracellular levels of glutamate (Baker et al., 2003; Wydra et al., 2013) and synaptic strength at glutamatergic synapses in the NAc [Boudreau and Wolf, 2005; Kourrich et al., 2007; Moussawi et al., 2009)].

Approaches in studying cocaine addiction

Most of the advances we mentioned above about the neuronal adaptations induced by cocaine abuse is acquired through animal models. Here we introduce several most used animal models to study cocaine addiction.

Conditioned place preference (CPP)

Place conditioning is widely used to assess the rewarding and aversive properties of various drugs of abuse. Briefly, the procedure consists of two phases: the conditioning phase, where the animal is administrated drugs in a neutral environment, and followed by an expression phase where the animals are tested on their preference for the cocaine-associated environment during a drug-free state. Increased time spent in the cocaine-paired environment is termed conditioned place preference (CPP). CPP is thought to reflect the heightened motivational relevance ascribed to drug-associated stimuli [Mueller & Stewart, 2000].

Cocaine self-administration

Drug self-administration procedures in rodents have proven invaluable for elucidating the neural circuitry of drug reinforcement and reinstatement of drug-seeking behavior. Animal self-administration experiments are typically performed in standard conditioning chambers adapted for the catheters used to deliver a drug intravenously. The catheter is secured to the animal by a harness or

back plate and is tethered to a protective leash that extends upward through a hole in the top of a chamber, where it attaches to rotating swivel on a mechanical arm that allows the subject to move around freely. The chamber is equipped with two identical levers. Pressing only one of them results in the delivery of drug, the other one will deliver no drug instead. Activity on these levers can be used to measure drug administration as well as changes in nonspecific behavior that reflect short- and long-term effects of drugs. Most importantly, the neurochemical research with the post-administration period of animal models provides plenty evidences for neuronal mechanisms that underlie drug reinforcement. The self-administration models provide a rigorous, systematic approach to characterize the reinforcing effects of psychoactive drugs, as they are taken volitionally at the doses that can best produce transitions to addiction. Although this behavioral model best ressembles the essential diagnoses of human addiction, only 1 out of 5 animals may reach a real addiction-like behavior. This percent is similar to the proportion of human drug users that become addicted. Two main factors may regulate the transition to addiction: the exposure to drugs and the vulnerability of the exposed individual [Deroche-Gamonet et al. 2004].

<u>GENERAL OBJECTIVE</u>

In this thesis, the main objective was to increase the knowledge about the D2 receptor properties. Currently this receptor is the main target of antipsychotics and it is involved in the neuroadaptations induced by chronic cocaine abuse. New properties of D2 receptor may arise by heteromerization with other GPCRs. The presentation of the main results in this thesis has been organized in separate chapters with the corresponding objective described as follows:

1, *the partial agonism of the antipsychotic aripiprazole on D2 autoreceptors*

The first and second generations of antipsychotics attenuate the positive symptoms of schizophrenia by acting as D2 receptor antagonists. However, because of their antagonism on D2 receptors, the first two generations of antipsychotics induce a variety of adverse effects. A classic hypothesis postulates that a dopamine autoreceptor agonist may also attenuate the positive symptoms of schizophrenia by inhibiting DA synthesis, release or dopaminergic firing [Neve et al., 1997]. Aripiprazole is a relatively novel antipsychotic which is characterized by having effects on both the positive and negative symptoms of schizophrenia as well as a low liability of adverse effects [Leucht et al 2009; Mailman & Murphy 2010]. One hypothesis related to the mechanism underlying the pharmacological effects of ARI is its partial agonism effect on D2 autoreceptors [Tamminga, 2002]. However, this effect is not easily evidenced. **Thus, one of our major objectives is to monitor the efficacy of ARI on D2 autoreceptors under various experimental conditions designed to facilitate or impair its D2 agonism, to show either full or partial agonism, or even antagonism.**

2, crosstalks between D2 receptor and other receptors.

The oligomerization of GPCRs opens a new field for pharmacology. D2 receptors belong to the typical class A GPCR and could form homomers and heteromers under various conditions [Hillion et al., 2002; So et al., 2005]. The pharmacological effects of some D2 oligomers have also been documented [Melissa et al., 2011]. However,

results are scarce on D2 receptor oligomerizations relevant for neuronal functions. So, to investigate possible heteromerization that may occurred between the co-localized receptors has become a new hot topic for the understanding of D2 receptor's neurophysiologic properties and also in developing new drugs that target specifically on these heteromers. **The other objective of this thesis is to check whether the velocity of DA synthesis which is originally regulated by D2 autoreceptors can be affected by a crosstalk between the following pairs of receptors: GABAB and D2 receptor, Orexin1 receptor and D2 receptor, and D5 and D2 receptors.**

3, *neuroadaptations related to D2R signaling induced by chronic cocaine withdrawal.*

The dopamine system plays an important role in brain reward pathway and drug addiction. Chronic drug administration or addictions induce various neuroadaptations. The fact that cocaine addiction causes profound neuroadaptations in DA system has been well documented so far [Anderson and Pierce, 2005]. A previous work in our lab found a dramatic increase of phospho-CREB level in response to a D2R agonist after 1-day withdrawal from chronic cocaine self-administration in rat nucleus accumbens [Hoffmann et al., 2011]. Neuroadaptations relevant for addiction are believed long lived in the addicted individuals [Rossetti et al., 1992; Weiss et al., 1992b; Wu et al., 1997]. **Thus, the last objective of this thesis is to characterize the alteration of dopamine D2 receptor signaling after both short and long withdrawal periods through measuring changes in phospho-CREB levels induced by the D2 receptor agonist quinpirole in the brain striatum.**

METHODOLOGY

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the decree of the Generalitat of Catalonia (DOGC 2450 7/8/1977). The experimental procedure and protocols for animal handling were approved by the Ethics Committee on Animal Experimentation and Human (CEEAH) of the Universidad Autonoma de Barcelona (number 3336). The *in vitro* DA synthesis assessment system protocol was previously applied by our group [Gonzalez-Sepulveda et al., 2013]. The cocaine self-administration protocol was adapted from the group of David Self, Texas University, and Southwestern Medical Center [Choi et al., 2006].

Part 1 *in vitro* dopamine synthesis assessment system

Animals

All the rats utilized in our study are male Sprague-Dawley rats supplied by the Animal Service Facility of Universidad Autonoma de Barcelona. All the animals used are approximately 8 weeks old with the weights between 250-300g. The rats are housed under stable conditions with the same temperature and humidity, the same length of illumination everyday (12 h cycle, lights on at 7 am), with freely access to food and water before sacrificed. The rats were sacrificed at around 10.00am for all experiments.

Reagents and materials

The L-[3,5-^{3}H]-tyrosine, vials, scintillation fluid Optiphase “HISafe”2 and the scintillation counter Tri-Carb 2810TR were purchased from Perkin Elmer. The HPLC C18 column (Tracer Extrasil: ODS; particle size: 5μm; Fortis Technologies Ltd.) was purchased from Teknokroma. The spectrophotometer plate reader Power Wave XS, the incubator Thermomixer, the Eppendorfs, tubes, the 96-well plates and other materials were purchased from Bio-Tek.

Buffers and mobile phase

Krebs-Ringer Buffers

The Krebs buffer contains: 120mM NaCl, 0.8mM KCl, 2.6mM $CaCl_2$, 0.67mM $MgSO_4$, 1.2mM KH_2PO_4, 27.5mM $NaHCO_3$ and 10mM Glucose. Worth to mention, the $CaCl_2$, $NaHCO_3$ and Glucose must be added into the solution right before the experiment and Krebs buffer should be oxygen-saturated with 95% O2: 5% CO2

bubbling for 15-30s before use.

Mobile phase for L-[3,5-^{3}H]-tyrosine purification

The mobile phase contains: 0.1M NaH_2PO_4, 0.75mM octanesulfonic acid (SOS), 1mM EDTA, and is adjusted to pH 3.4 with H_3PO_4. Mix with methanol (1%) before use. This low pH was chosen to decrease the charge of the carboxyl group in tyrosine, this increasing its retention time to 10.5 minutes.

Mobile phase for [3H]-dopamine purification

The mobile phase contains: 0.1M NaH_2PO_4, 0.75mM SOS, 1mM EDTA, and pH 5.0. Mix with methanol (15%) before use.

Purification of L-[3, 5-^{3}H]-tyrosine

The L-[3, 5-^{3}H]-tyrosine (1 mCi supplied by American Radiolabelled Chemicals) was not used directly; we purified it before use to prove its specific activity. We set this step because some companies sell low quantity radio-chemicals. The specific activity of it we needed in our experiment is 37MBeq/mmol. For the purification, a High performance liquid chromatography (HPLC) was used. The HPLC we use is constituted by an auto sampler, a pump, the C18 Extrasil Tracer column, the appropriate mobile phase which constantly flow through the system, a UV spectrophotometer to measure the absorbance at 285nm, a interface and a computer which control the program and record the results. First, a standard quantity-peak area curve was obtained from the non-radioactive tyrosine solution with the tyrosine quantity as its horizontal axis (X axis) and peak area as its vertical axis (Y axis). Once we have the standard curve, a certain amount of L-[3, 5-^{3}H]-tyrosine (usually 200μL) was injected to the system and the peak with the same retention time as the standard tyrosine was collected into an eppendorf tube. The radioactivity present in 1μL of the solution collected was quantified using a liquid scintillation counter. Based on the peak area and the standard curve, we can calculate the exact total amount of the purified L-[3, 5-^{3}H]-tyrosine, and then divide this quantity by the volume we collected in the eppendorf, this allows to obtain the concentration of the purified L-[3, 5-^{3}H]-tyrosine. The final concentration we need in our experiment is 0.12μM (about 5000 dpm). Through calculation, we could easily figure out the exact volume to add into each sample.

Preparation of rat brain striatal miniprisms

Rats were sacrificed by decapitation, and the brain was quickly removed and preserved in ice-cold Krebs buffer until further processing. The following procedure was performed in the cold room (4 ºC). The brain striatum was dissected and chopped into 300×300μm miniprisms with a McIlwain tissue chopper (Fig. 22). The miniprisms were washed with fresh cold Krebs buffer for three times to get ride of the broken cells and proteases released. Using a centrifuge (288g× 1min) to remove the supernatant three times and finally add about 600μL fresh Krebs buffer to resuspend the miniprisms.

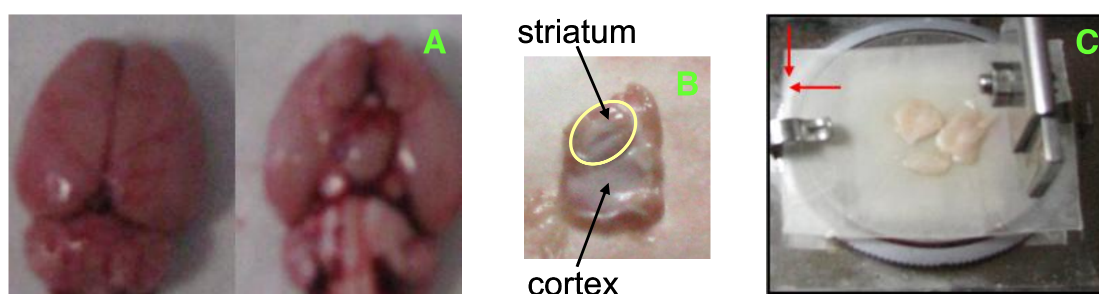


Figure. 22 Preparing miniprisms from rat brain striatum. Rat brain (A) was extracted and the striatum was dissected from the brain (B) in both hemispheres and chop into 300×300μm miniprisms with McIlwain tissue chopper(C). The red arrows in image C indicate the chopping scheme.

Incubation of samples for [3H]-DA synthesis

The step after miniprism preparation is the centre procedure of the whole experiment: the incubation. It was carried out in an epppendorf incubated in an Thermomixer incubator with fixed temperature (37ºC), the discontinuous agitation (450rpm × 15s following with 10s interval) and also with gasification (O2/CO2: 95%/5%). Each Thermomixer incubatior contains 24 wells which suit for 2ml enppendorf tubes. Thus the incubation takes place in the eppendorf which contains 25μl miniprism suspension and 225μl Krebs buffer. For each rat brain, there were 24 samples incubated separately in parallel which usually were divided into four groups according different ligand treatment, so each group still have six paralleled samples. The total period is composed by pre-incubation and incubation period. The ligands applied to stimulate the samples were usually added during the pre-incubation period and [^{3}H]-Tyrosine was added at the end of the pre-incubation period. The incubation period is actually the [^{3}H]-DA synthesis period. The whole incubation time procedure is shown in Figure 23. At the same time, four blank controls are placed on ice through the whole period with the same condition as the others but in the absence of

ligand treatment.

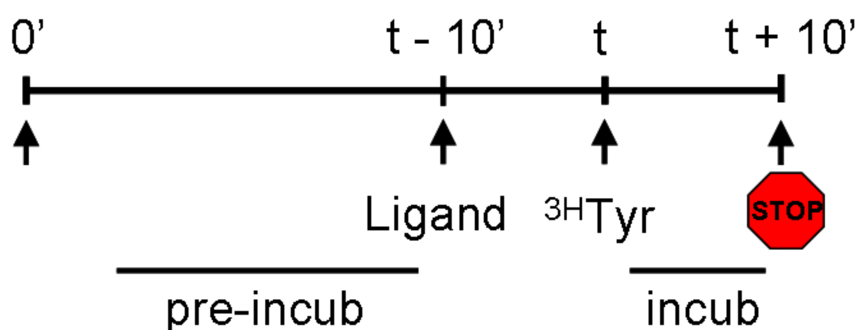


Figure 23 Experimental designs for the in vitro dopamine synthesis assessment. In most of our experiments, the pre-incubation time t equals to 2 hours.

Sample deproteinization preparation

When the incubation period ended, the samples were taken out from the incubator and placed on ice. Added 35µl of the deproteinization solution (which contains 25nmol DA, 32.2nmol ascorbic acid and 7% W/V trichloroacetic acid, in water) into each sample (including the four blank samples). This termination solution could stop reaction by denaturizing the enzymes in the samples (by significantly decreasing the pH level). DA added here is used as internal standard to quantify the recovery efficiency of the extraction of [^{3}H]-DA synthesized during sample incubation as well as to facilitate the correct collection of tritium-labeled dopamine by increasing the peak signal of DA in the HPLC detection system. The samples were then sonicated (~15s) to homogenize the tissues and allow the liberation of the intracellular [^{3}H]-DA synthesized. An aliquot (10µl) of each sample (except the four blank samples) was taken separately into a corresponding eppendorf for determining the amount of protein. Finally the samples were centrifuged 5min at 20817g, the precipitated protein was discarded and the supernatant was recovered into another corresponding tube for HPLC purification.

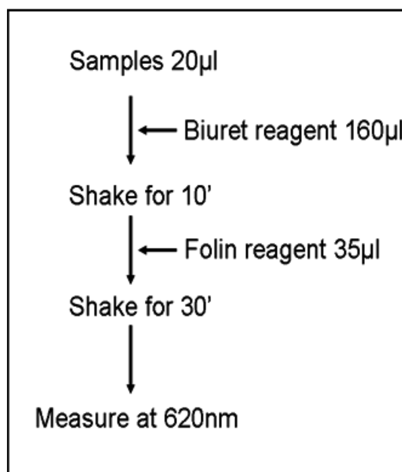
Determination of protein amount

For measuring the protein amount in the samples, we use the Lowry method which is adapted to microplates, as it requires lower volume of sample (20µl) and less reagents (200µl). The principle behind this method lies in the reactivity of the peptide bond nitrogen with the Cu^{2+} under alkaline condition and the subsequent reduction of

the Folin-Ciocalteu phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. During the reaction, the color of the samples turns blue which enable the quantification in a spectrophotometer plate reader at 620nm.

Briefly, in each well of a 96-well plate, we added 20μl of each sample (every sample have three replications), and then we added 160μl biuret reagent (200μl $CuSO_4$ 1%, 200μl tartrate 2% and 19.6ml 0.1N NaOH/2%Na_2CO_3). Leave it for stirring for 10min and then add 35μl Folin regent 1/4 diluted in water and leave it for stirring for another 30min, finally read the plate at 620nm use a Bio-Tek reader. The standard curve is applied with different concentrations of bovine serum albumin from 0 to 0.25 mg/ml which is carried out at the same plate with the samples.

Figure 24 Lowry method



Purification of ^{3}H-DA through HPLC system

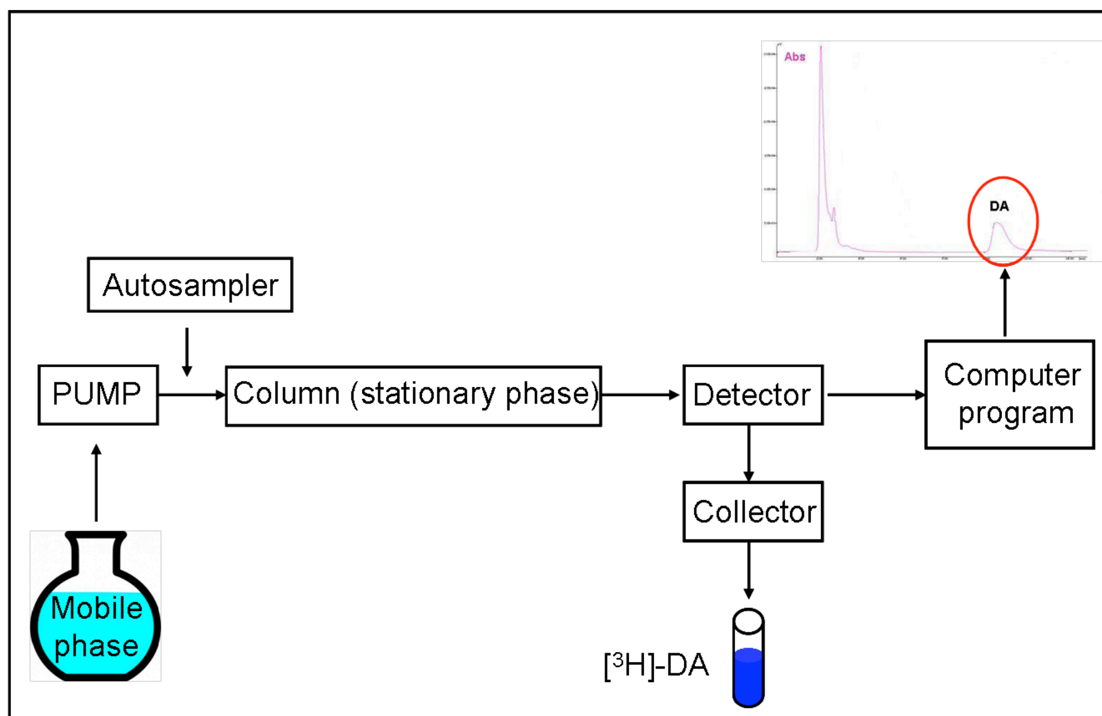


Figure 25 scheme of HPLC purification system. The figure shows the whole DA HPLC purification system. The mobile phase follows through the system constantly under the high pressure that provided by the pump. The C-18 chromatography column serves as the solid phase which acts with the mobile phase and finally separates dopamine in the samples with other contents. The UV detector detects components of the mixture being eluted off the chromatography column and for each component gives a shape of peak on the screen. When the UV detector detect the DA (we primarily inject a DA standard that gives the stable retention time, and for our sample, the peak with the same retention time as the DA standard is our target), a collector connected with the detector by the interface will automatically collect the eluent, in order to collect all the DA in our sample, we collect 2 minutes of the eluent (the peak last around 1 minute). Finally the tritium amount in the form of 3H-DA is recorded using a liquid scintillation counter.

The HPLC purification system contains a pump that supplies high level pressure to make sure that the mobile phase flows constantly through the whole system, an atuo-sampler, a C-18 column that serves as stationary phase, a computer program which includes an interface, and a sample collector (Fig. 25). The separation of different molecules in the sample is based on the different interactions of the components with the stationary phase. In our experiment, we use a C18 column which contains a hydrophobic string with 18 carbons in length. The prolonged carbon string enlarges the interaction area of the molecules with the column. Plus the high concentration of octanesulfonic acid in the mobile phase which also makes it a cation exchange column. These two factors ensured the separation of DA from other molecules in the samples (Fig. 26).

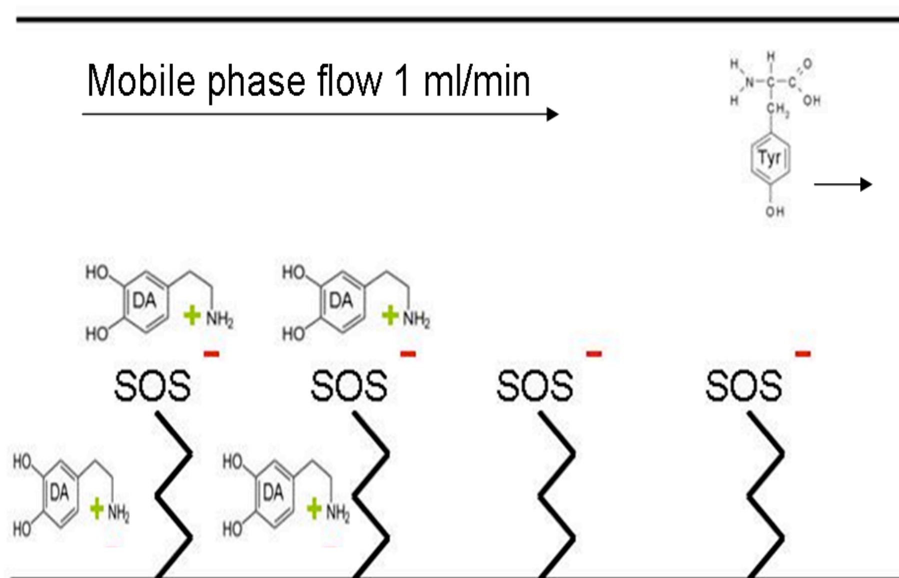


Figure 26 The mechanism for the molecule retention in the column. We use a typical reversed phase chromatography system to separate DA from the sample. The theory about the reversed phase chromatography system is introduced in box 2.

The retention time for dopamine in our HPLC system is around 10.5 min, and it is detected by a UV spectrophotometer at 285nm and collected by a collector automatically into scintillation vials for subsequent determination. The absorbance data were recorded through a computer program Borwin 1.5 software. A sample of chromatogram is shown in figure 27.

Box 2 Theory of reversed phase chromatography.

Theory of reversed phase chromatography

The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, i.e. the stationary phase. Reversed phase chromatography is an adsorptive process by experimental design, which relies on a partitioning mechanism to effect separation. The solute molecules partition (i.e. an equilibrium is established) between the mobile phase and the stationary phase. The distribution of the solute between the two phases depends on the binding properties of the medium, the hydrophobicity of the solute and the composition of the mobile phase. Initially, experimental conditions are designed to favour adsorption of the solute from the mobile phase to the stationary phase. Subsequently, the mobile phase composition is modified to favour desorption of the solute from the stationary phase back into the mobile
phase. In this case, adsorption is considered the extreme equilibrium state wherethe distribution of solute molecules is essentially 100% in the stationary phase.Conversely, desorption is an extreme equilibrium state where the solute isessentially 100% distributed in the mobile phase.

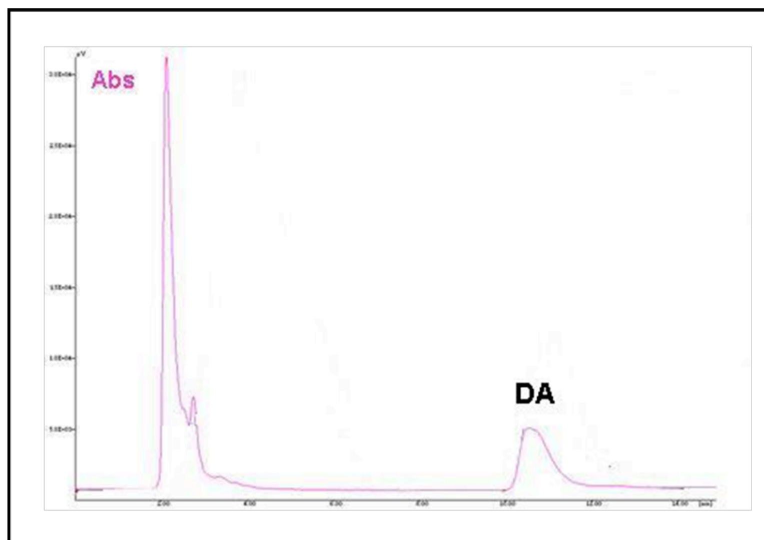


Figure 27 A typical UV chromatogram pattern of dopamine from a rat striatum sample. Most dopamine observed is the internal standard dopamine.

This DA peak detected actually is constituted by three parts of dopamine: the endogenous dopamine, the [^{3}H]-DA which is synthesized from [^{3}H]-Tyr, and the internal standard we added at the end of the incubation. The amounts of the first two parts of DA combined are still too low to be detected by the UV spectrophotometer in our HPLC system, so the adding of the internal standard enables the proper display of the DA peak. In addition, neither the endogenous DA nor internal standard DA are radioactively labeled, so their presence would not affect the final quantification of [^{3}H]-DA. The area of the peak is correspondence with the quantity of the DA contained in the sample. So the other role of the internal DA standard is: through comparing the DA peak area of each sample with that of the external standard (theoretically, the final amount of DA injected from external standard is the same as the internal standard, 25nM), we could get a recovery efficiency that could be used to calculate the total amount of [^{3}H]-DA in the tissue.

In the sample injected to the HPLC, there is excess of [^{3}H]-Tyr besides [^{3}H]-DA (we supply a large amount of tyrosine to make sure the DA synthesis); however, due to the lack of internal standard for tyrosine, the tyrosine peak could not be detected by the UV spectrophotometer. But we have to ensure that the DA sample we collect does not contains the raw material [^{3}H]-tyrosine. To set up the method, we collect the samples coming out of the column from the beginning till the ending of each injection constantly in a scheme of 1 min /tube / minute, and recorded the dpm in each tube. With time and dpm, we make a curve that is shown in Fig. 28 which showed the merged dpm-time and Abs-time curves which confirmed that the tyrosine peak and DA peak do not overlap.

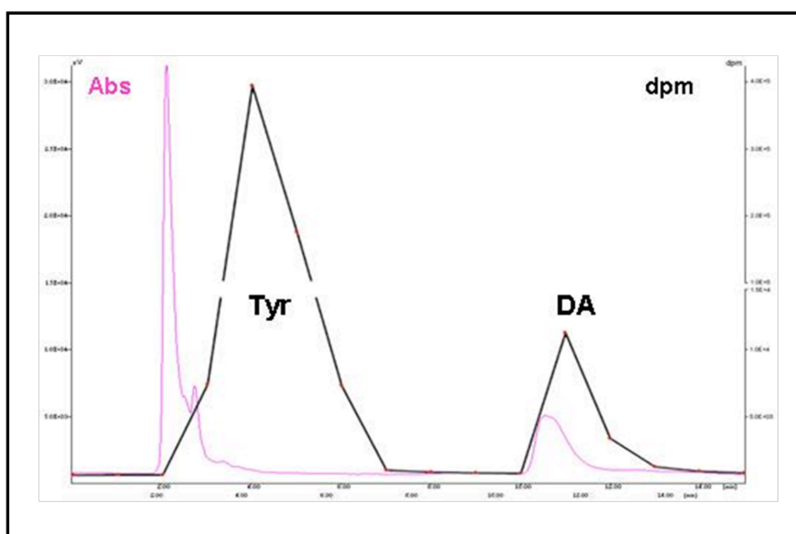


Figure 28 Temporal outline of radioactivity present in the eluates, in comparison with a dopamine chromatogram. The black curve is the dpm value change according to the elution time. The curve in purple is the Abs obtained from the detector.

Through the above experiment, we confirmed that the collected fraction from the automatic sampling only contains [^{3}H]-DA, and the radioactivity of the sample last approximately for 2 minutes. So for each sample, 2 ml eluate was collected. For

recording the radioactivity, we add approximately 6ml scintillation which is capable of converting the kinetic energy of nuclear emissions in light, and finally, the radioactivity is captured by the scintillation counter.

Calculation of [^{3}H]-DA synthesis

To calculate the [^{3}H]-DA synthesized and to normalize the amount of ligand treated groups to the non-treated basal group, the following data were needed:

- The area of the DA peak and the dpm for each sample
- The amount of protein present in each sample
- The radioactivity obtained from the ice-incubated blank samples also should be subtracted.
- External residual of radioactivity in the column. It is due to the successive injection of samples into the system. It is measured every five samples and should be subtracted from the dpm values obtained for each sample.
- Each sample has different recovery efficiency which is calculated from the DA peak area of each sample in comparison to that of external DA standard.
- The incubation time we applied in our experiment is 10min, and finally the value is expressed in total dpm/mg total protein per hour.

Figure 29 shows the detail of the calculation. Briefly, after finishing the purification of [^{3}H]-DA, obtained the peak area and dpm, and also the protein amount for each sample, the data are properly filled into the excel. First, the dpm of the external standard and blank sample was subtracted form each sample. Then, we calculate the total amount of dpm, and divided this value to the protein amount; finally we got the dpm per mg per hour. The results were expressed as percentage normalized to non-treated control group.

	B	C	D	E	F	G	H	I	J	K	L	M
1												
2				average area of standard		average dpm of standard		average dpm of blank		Calculation of [3H]-DA synthesis		
3				AVERAGE(C6:C8)		AVERAGE(D6:D8)		AVERAGE(F9:F10)				
4												
5	demostrates	peak area	DPM	dpm substracted from external	correction dpm from recovery efficiency	corrected dpm subtract the blank value	mg of protein	dpm/mg/h	average	deviation	% of controls	average
6	standard	XX	XX									
7		XX	XX									
8		XX	XX									
9	blank	XX	XX	D9-$G$3	($E$3*E9)/C9							
10		XX	XX	D10-$G$3	($E$3*E10)/C10							
11	control	XX	XX	D11-$G$3	($E$3*E11)/C11	F11-$I$3	XX	G11/(H11*10/60)	AVERAGE (I11:I13)	STDEV (I11:I13)	I11*100/$J$17	100.00
12		XX	XX	D12-$G$3	($E$3*E12)/C12	F12-$I$3	XX	G12/(H12*10/60)			I12*100/$J$17	
13		XX	XX	D13-$G$3	($E$3*E13)/C13	F13-$I$3	XX	G13/(H13*10/60)			I13*100/$J$17	
14	ligands	XX	XX	D14-$G$3	($E$3*E14)/C14	F14-$I$3	XX	G14/(H14*10/60)	AVERAGE (I14:I16)	STDEV (I14:I16)	I14*100/$J$17	AVERAGE (L14:L16)
15		XX	XX	D15-$G$3	($E$3*E15)/C15	F15-$I$3	XX	G15/(H15*10/60)			I15*100/$J$17	
16		XX	XX	D16-$G$3	($E$3*E16)/C16	F16-$I$3	XX	G16/(H16*10/60)			I16*100/$J$17	

Figure 29 calculations of [3H]-DA synthesis, shown in percentage of non-treatment control groups. The incubation period for DA synthesis is 10min in our experiment and "XX" in the image represent data directly obtained from the experiment. The final dpm per mg protein per hour for each incubated sample is calculated through the formula showed in the image.

Statistics

Figures in the result chapter 1, 2 and 3 showed the percentage of [^{3}H]-DA synthesis normalized to the blank control of the identical incubation circumstance. Data were expressed as means (± SEM) of N values which represent the paralleled incubation samples obtained from at least 3 independent incubations. Once normalized to 100% of basal [3H]-dopamine synthesis in each experiment, data were pooled indicating the total number of icubations and rats used to obtain the results. Data were analyzed by Graphpad Prism 5 (Graphpad software Inc) by ANOVA or Student t-test. Confidence level was set at 95% to determine statistical significance.

Part 2 cocaine self-administration

Cocaine self-administration is a widely used and well accepted protocol to study cocaine addiction-like behavior in rodents (as we have introduced in the introduction

cocaine section, page40). Cocaine slef-administration was performed by a technician working for our group (Silvia Fuentes) It is worth to mention that cocaine self-administration pattern and consumption dose can be highly affected by various factors [Choi et al., 2006; Thomsen and Caine, 2007]

- Cocaine infusion time: a faster delivery will increase cocaine self-administration.

- Cocaine dose: affects cocaine reinforcement in a bell shaped manner, with higher cocaine doses producing lower rat of lever press.

- The length of access period to the drug everyday. A long access to cocaine is desirable to produce an addiction-like phenotype.

- The time of drug access (morning/day/night). Self-administration is usually higher during the dark period.

In our experiment, we used a cocaine infusion time of 0.1 mL/10s, and a intermediate dose of 0.5 mg/kg/0.1 ml. Daily access period was only 2 hours due to the high use of equipment in our animal service. Sessions were run daily, 5 days per week. Rats had a minimum of 29 self-administration sessions (range from 29-68)

Animals

The male Sprague-Dawley-OFA rats, obtained from Chanles River and maintained at the animal service of Autonomous University of Barcelona (UAB, Spain) were housed in a circumstance with stable temperature, humidity, and a 12h light/dark cycle for the whole periods until sacrificed. Rats had a mean weight of 567g at the moment of sacrifice (range from 490-647g)

Experimental protocol and design

In the animal service facility, there are 6 operant skinner chambers (Figure 28) which have been adapted for *intravenous* administration of liquid drug solution induced by the active lever press executed by the animal (Figure 28). The animals were individually housed and moved to the operant chamber where they get exposed to the self-administration installation 2 h per experimental day, except during the initial sucrose training periods.

Brief procedure of the experiment

-Individual housing of 15 rats 5-6 weeks old in advance before starting the experiments

-limit their food intake one week before the experiment in order to reduce the rats' body weight to 85% of estimated weight. We use 8mg food/24h, about half of their physiological need.

-Sucrose training: sucrose is a kind of nature reward, and all the rats should undergo sucrose training before in order to facilitate learning of cocaine administration. It is pre-training for the rats to intake cocaine themselves. The cues associated with delivery of the sucrose pellet were the same as that for cocaine delivery during cocaine self-administration (light on over the lever to signal cocaine availability. Chamber light off after delivery to signal a 15 second time-out period). Once the rats had learned to press a lever to obtain 100 sucrose pellets (45mg, Biosarv), they were returned on *ad libitum* food again. Those who had problems with obtaining sucrose pellets were discarded from the study. Sucrose training was usually run in a first overnight session, followed by two or three 2h sessions. All rats had food ad libitum after completion of sucrose training till the end of the procedure.

-11 rats underwent surgery. Among them, 9 rats had a catheter implanted in the jugular vein, and 3 rats underwent a "sham" operation with implantation of a non-functional catheter (Fig. 29).

-All the rats that undergone surgery were allowed to a period for recovery of 3-4 days. The sham rats were handled on a daily basis and never were reintroduced into the operant chamber where they get sucrose again to avoid learning of sucrose extinction.

-The rats with functional catheters were trained to self-administer cocaine in the operant chamber (figure 30, similar as where they self-administer sucrose). Out of 9 rats only 7 of them completed FR5 criteria for cocaine self-administration.

The self-administration chamber was located in the room where is next to the animal housing room in order to reduce the eventual stress induced by moving the animals. Rats were individually housed after the implantation of catheter in flat-top cages to avoid bruising or injuries in the catheter area.

The 15s clue was termed as a “time out period” which means further lever press within the 15s gives no access to the reward (sucrose pellet or cocaine infusion). No consequence was associated with the inactive lever press (Figure 30) or with responding during the 15 seconds time-out period after obtaining cocaine. Responding at the inactive level was recorded, but usually negligible (data not shown).

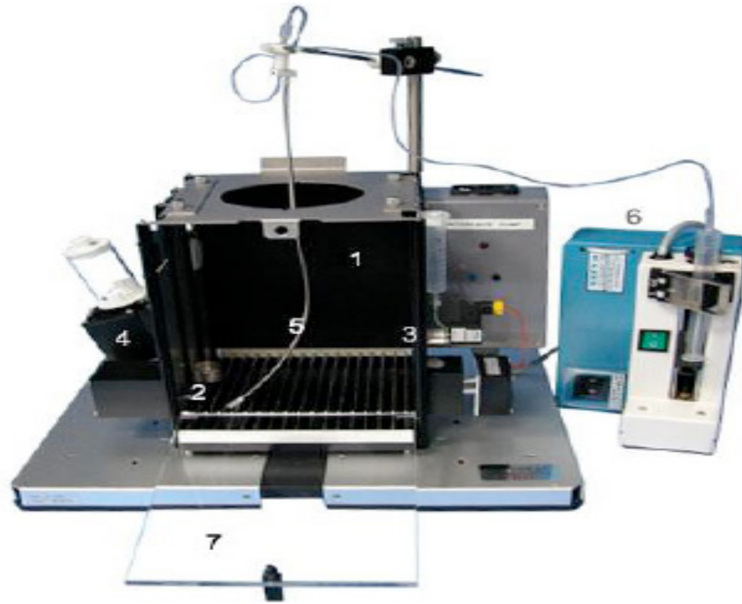


Figure 30 Operant chamber (Panlab) for daily cocaine self-administration or sucrose training. Rats were housed into an operant chamber (1). Rats could press a lever (2) situated on the left as well as on the right side of the chamber. Active lever press gave rise to (4) or an intravenous cocaine injection through a tube (5) which was connected through the back of the rat to its jugular vein. A computer controlled syringe (6) permits injection of the wished volume of cocaine solution. (3) water bottle, (7) chamber door. The operant chamber was placed inside a sound and vibration attenuating box (not shown).The equipment was completed with a computer interface providing data to packwin software

The image is adapted from Panlab (www.panlab.com).

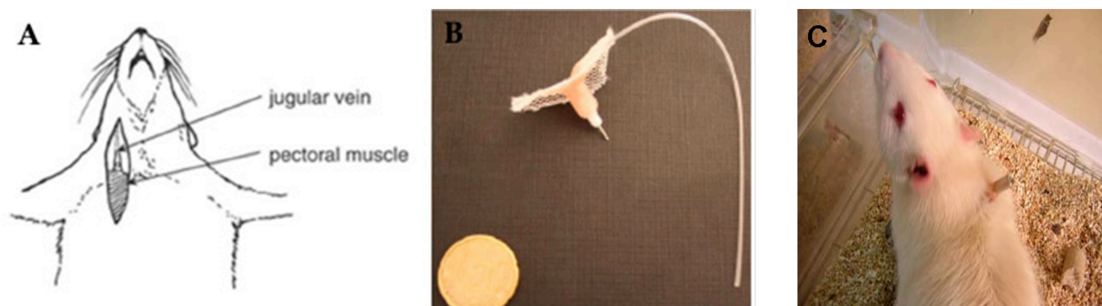


Figure 31 Surgical implantation of catheter. (A) The incision in the right back of the rat,

allowing catheter insertion to the jugular vein. (B) The catheter is bind to a fine mesh which allows attachment on the back of the rat. All the structure is sealed with dental cement. (C) The picture shows a well implanted catheter on a rat back.

Cocaine self-administration

Cocaine self-administration rats

Cocaine self-administration was carried out during the light cycle usually in the afternoon.

Animals acquired cocaine self-administration under a continuous reinforcement schedule. Rats were allowed to self-administer cocaine 5 day/week, and the session terminated either at 2 h of self-administration or when rats received 50 injections of cocaine. In FR1, one press of active lever gives rise to one *intra venous* cocaine delivery. Stable cocaine self-administration was defined as maximum 20% variation in cocaine self-administration in 3 consecutive sessions and minimum 15 doses/2 h. When stable phase was obtained on FR1 (minimum 2 weeks), rats were subjected to FR2 (three active lever presses associate with one cocaine injection) and finally to FR5 (five active lever presses associate with one cocaine injection) which last 3-5 weeks (Figure 29). Only 7 out of 9 reached FR5. Two of these 7 rats that reached FR5 were later put back to FR2 due to some days of poor self-administration. Rats bad a mean of 29 self-administration sessions. (range 29 to 68 sessions) with a mean intake of 11.5mg cocaine/Kg per session. Two groups of rats were than formed in order to sacrifice, differenced by their withdrawal time (Table 1).

Table 1 Average cocaine intake during the whole paradigm.

Group	N	coc wanted		days self adm		days wd		weight sacrif	
		mean mg/kg	SD	mean	SD	mean	SD	mean	SD
1 day wd	3	12.9	1.5	40	9	1	0	534	46
5 wk wd	6	10.9	2.5	50	11	36	2	580	53

Coc: cocaine; adm: administration; wd: withdrawal; sacrif: sacrifice.

Withdrawal from cocaine self-administration

After the last day of cocaine self-administration, rats were housed in their home cage until sacrificed.

- 1-day withdrawal rats were sacrificed 1 day after the last cocaine self-administration (N=3). This group was smaller in number of rats because previous results of our lab observed changes in D2R responses [Haffman et al., 2011]

- 5-weeks withdrawal rats were sacrificed an average of 36 days (range from 33-36) after the last cocaine administration (N=6). This group was the main group to be compared with control rats, in search for long-term changes in D2R responses.

- The control group was composed of 4 sham rats and 2 naïve rats of smaller weight. No difference was found between sham and naïve rats in the results obtained (see below).

Preparation of tissue slices for biochemical analysis

When the cocaine self-administration model was completed, rats were sacrificed after different withdrawal periods and rat brains were used for biochemical analysis. In this thesis, we analyzed the phosphorylation level of CREB stimulated by different treatment during the *ex vivo* incubation of the rat brain by means of the technique immunohistochemistry (IHC).

Preparation of brain slices and incubation in thermomixer

The protocol used was adapted from Nishi 1999 (288). The rat brain was rapidly removed and kept in solid icy/liquid Kreb's buffer with oxygen-saturated (O_2/CO_2:

95%/5%) (124mM NaCl, 4mM KCl, 1.25mM NaH_2PO_4, 1.5mM $MgCl_2$, 1.5mM $CaCl_2$, 10mM glucose and 26mM $NaHCO_3$, pH 7.4). Then the rat brain was fixed on the vibratone plate using agarose gel and sliced into 200μm thick coronal slices using a automatic vibration slicer (Leica; Fig. 32). The whole slicing procedure was taking place with the brain submerged in the icy/liquid Kreb's buffer with bubbled oxygen (O_2/CO_2: 95%/5%). The slices with complete striatum were selected and incubated in a thermomixer incubator using a six-well plate with 3-4 slices in each well. The temperature was risen gently to 23°C in 10 min and slices were further incubated for 50min. Temperature was then gently risen to 32°C, slices were left for 4 h under this temperature with constant oxygenation. We substituted the incubation buffer with 9 ml fresh Kreb's buffer in the middle of the whole incubation procedure. At the end 1 ml of various ligands (quinpirole 10μM, Tocris; or vehicle) were added into the incubation and were given 10 min for stimulation. After that, all the Kreb's buffer were removed and slices were fixed with 4% paraformaldehyde solution (PFA , Sigma), dissolved in TBS, pH 7.6) for 1 hour. The experiment design was shown as a time bar in figure 33. After the fixation, slices were washed with TBS and stored in 30% of sucrose at -20°C until further processed.

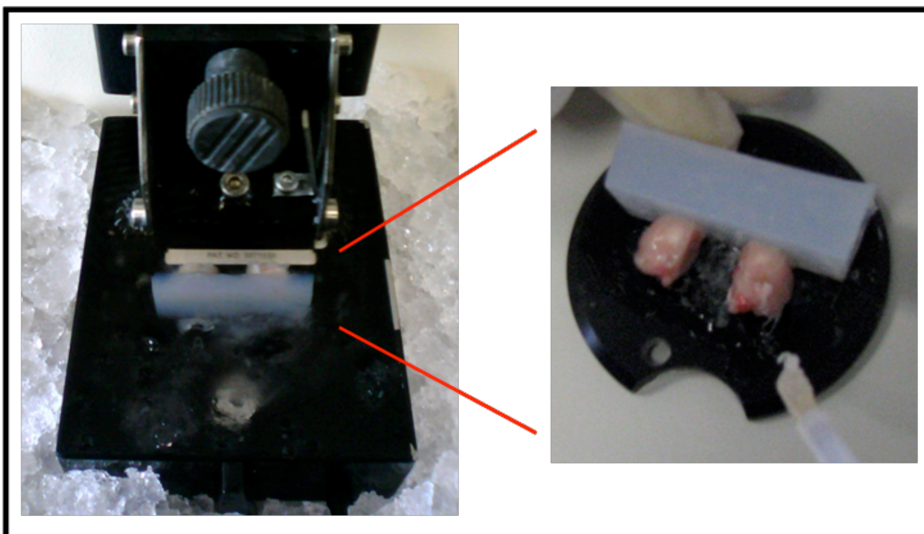


Figure 32 vibration slice cutting. Cutting rat brain into 200μm thick slices, the slices were segregated into several groups for further *ex vivo* incubation. The brain must be submerged in icy-Kreb's buffer.

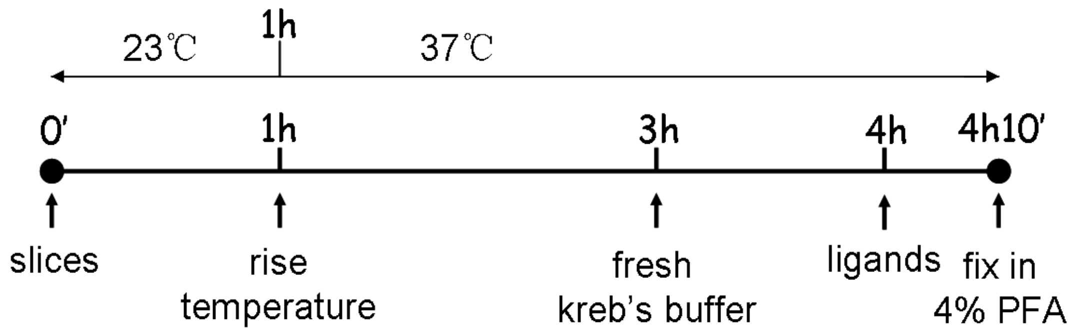


Figure 33 Procedure of slice incubation in the thermomixer incubator. The ligand normally used was quinpirole (10 μM) or vehicle.

16μm sections cut using cryostat.

200μm-thick slices obtained from the vibratone and previously incubated were attached with Tissue-Tek OTC, adhere to a support and place it in the mobile arm (Fig. 34) of the cryostat. After locating the area of the brain corresponding to the slice, sections (16μm thick) were collected directly on slide continuously (not serial) under the help of a anti-roller. We collect three slices on each slide and about 5-7 16μm slides for each incubated slice (200μm-thick). All the slides were well labeled and stored in the freezer at -20°C until further processed.

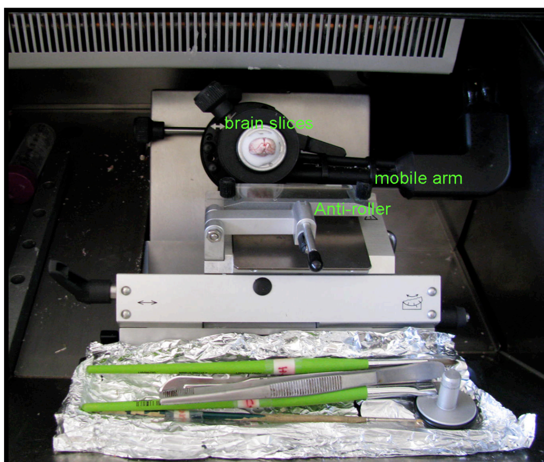


Figure 34 cryostat slice cutting. Cutting rat brain slice (200μm)into 16μm thick slices. The environment temperature is -19 ~ 20℃

Immunohistochemistry

Immunohistochemistry (IHC) is a technique widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue; principally it is based on the specific binding of the antibodies to the antigens in cells of a tissue section. This technique not only enables us to locate the target antigen, but also permits us to calculate the number of positive cells express the target antigen. In this thesis, the IHC was used to detect the phospho-CREB positive nuclei in brain slices after stimulation of the slices with the D2 receptor agonist quinpirole or vehicles.

Avidin-Biotin Complex Method for IHC Detection

Avidin-Biotin Complex (ABC) method is described in 1981 by Hsu and associates [Hsu et al, 1981], which fundamentally by means of the covalent and irreversible binding between biotin and avidin. Biotin, also know as vitamin H, is present in tiny amount in all living cells. In IHC, biotin is conjugated to antibodies or to the enzyme reporters; Avidin is a glycoprotein found in the egg white, it contains four identical subunits, each subunit could bind one molecule of biotin. Avidin-biotin binding is strong, rapid, and once formed, is unaffected by extremes in pH, temperature, organic solvents and other denaturing agents. In the ABC method, a biotinylated secondary antibody would on one side bind to the target antigen specific primary antibody, and on the other side bind to the avidin complex which conjugated to biotinylated enzyme (eg. Horsedish peroxidase).

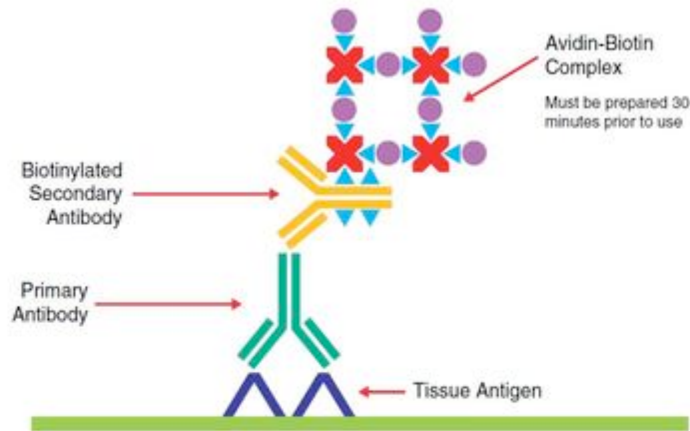


Figure 35 the principle of the ABC method for IHC detection. For details see the text above. The tissue antigen detected in our thesis was the phosphorylated form of the CREB (see text).

The image is adapted from http://ihcgroup2.wikispaces.com/Protocol

Quench and block of the non-target antigens and target antigen detection using ABC method

The glass slides containing tissue slices obtained from the cryostat were thawed at RT for 30min, washed with TBS, and quenched in the solution which contains 3% H_2O_2, 27% TBS and 70% methanol for 12-14mins. After further washing with TBS, the slides were blocked in 5% DNS and 1% BSA diluted in TBS-T 0.5% at 37°C for 1 h. Without washing, the slides were further incubated with the primary antibody phospho Ser^{133} CREB (1:500, Cell Signaling) diluted in blocking solution at 4°C over night(around 20 hours). The morning after, slides were rewarmed at RT for 1 h before further processing. The slides were washed in TBS-T 0.5% and then incubated with the biotinylated secondary antibody (1:1000 in blocking solution, Jackson ImmunoResearch) for 1h at RT. Again they were washed with TBS-T after that and incubated with peroxidase-conjugated streptavidin (1:500 in blocking soulution, Jackson ImmunoResearch) for 1 hour.

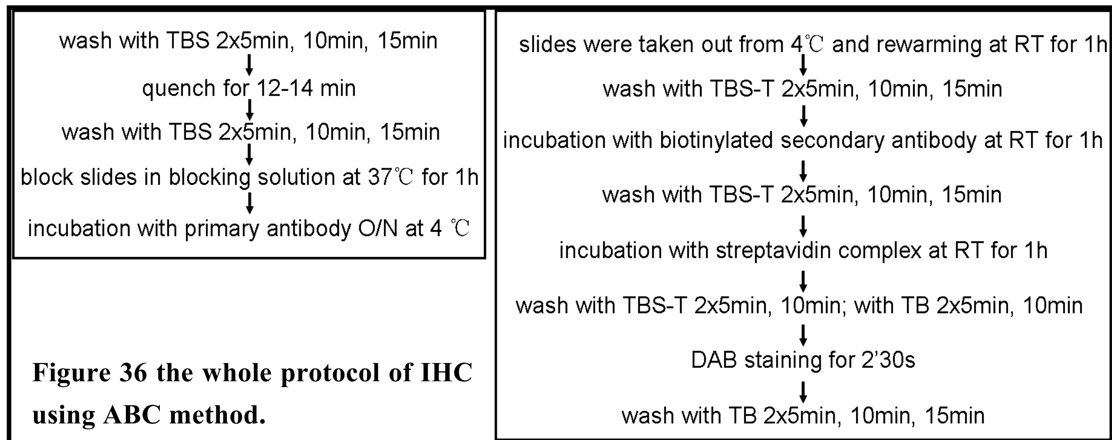


Figure 36 the whole protocol of IHC using ABC method.

DAB staining

After the last incubation with streptavidin complex, the slides were washed first with TBS-T 0.5% and then with TB. The staining solution was prepared containing 0.5mg/ml of DAB (Sigma) and 0.03% of H_2O_2 (sigma) in TB while the slides washing. Finally the slides were stained with DAB solution for 2mins and 30seconds. The slides were well washed with TB after DAB staining (Fig. 36).

Dehydrated, detergent and mounted of the slides

The slides were hydrated with increased concentration of ethanol and detergent in pure xylene and in the end, mounted with resin. The slides were kept in slice boxes at RT after they dried out.

Photograph of the slides and calculation of the positive nuclei

All the slides were imaged using a Nikon Eclipse 90i with objective x20. For each brain striatal slice, four sub-regions (Fig.37A) were divided and for every sub-region, three nearby images were taken to obtain an average number of positive nuclei (Fig.37B). Each image taken from brain slices was used to calculate pCREB positive nuclei with a software named ImageJ and under through a manually verification.

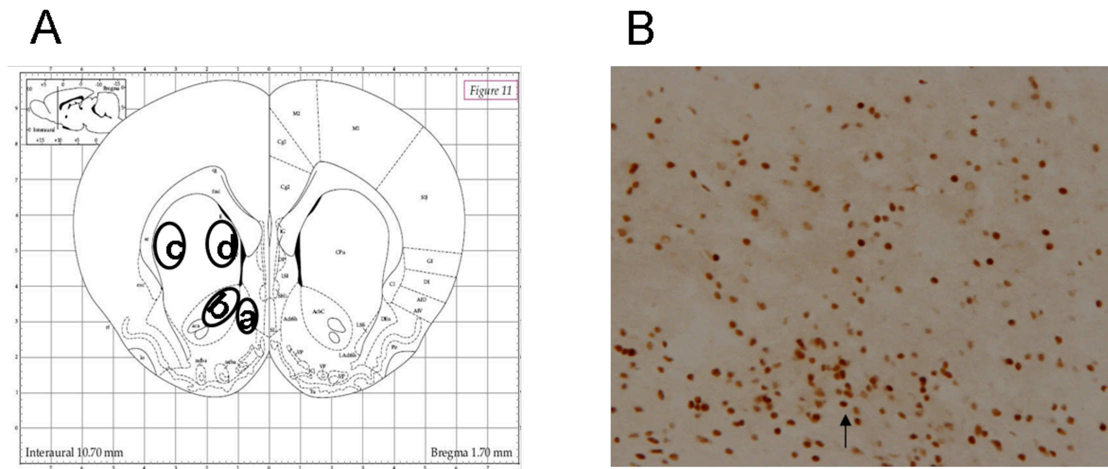


Figure 37 Graph A is schematic representation of the regions of nucleus accumbens (NAc) shell (a), NAc core (b), dorsolateral (c) and dorsomedial (d) where the photographs were obtained. Only the rostral face (+ 1.7 mm from Bregma) of the coronal section is shown. The circles depict approximations of the regions where the photos took for counting the numbers of phosphorylated CREB immunoreactivity nuclei neurons. **Graph B is an example of Photomicrographs (20×) of phosphorylated CREB immunoreactivity nuclei neurons obtained from sham rat brain striatum, the nucleus accumbens (NAc) shell.** The real area for each photomicrograph is 5.95μm^2.

Statistics

Immunohistochemistry results either show the mean of phospho-CREB-immunoreactive (pCREB-IR) nuclei neurons per 5.95μm^2 or they were normalized to the group that is going to compared with. The number of data, N, refers to independent rats. Each rat yield various brain slices, where conditions in general were done in duplicate. The slices were treated as independent samples and averaged with slices from the same rat and region. Data was analyzed by ANOVA. Confidence level was set at 95% to determine statistical significance.

Western blot

The aim of western blot experiment in my study is to validate the pCREB antibody used in the immunohistochemistry. For the western blot experiment, the same *ex vivo* incubation slice in thermomixer as done for immunohistochemistry until half hour before the terminating of slice incubation.Then the total volume of buffer was replaced by 400μl of fresh Krebs buffer at 30-32℃. Incubation was terminated by removing the complete volume of liquid and quickly transferring tubes onto dry ice. Depending on the tissue size, each sample was added 50-150μl of ice-cold lysis buffer made of 1mM orthovanadate, 50mM Tris-HCl (pH 7.5), 25mM sodium pyrophosphate, 50mM NaCl, 1% Triton X100, 50mM sodium fluoride, 5μM zinc chloride, 2mM dithiothreitol, phosphatase inhibitor cocktail 1 (Sigma-Aldrich, St Louis, MO, USA) and protease inhibitor cocktail 1(Sigma-Aldrich). Samples were sonicated on ice for 15 seconds and centrifuged (17949g X 20 minutes at 4℃). Supernatant protein concentration was determined by a modified by Lowry's method. Proteins were dissolved in denaturing electrophoresis loading buffer and boiled at 99℃ for 5 minutes. Equal amounts of protein (10μg) were separated by 10% sodium dodecyl sulfate-polyacrilamide gel electrophoresis(SD-PAGE). Proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), washed in phosphate buffered saline-0.05% Tween 20 or Trizma-buffered saline-0.05% Tween 20 (TBS-T) followed by blocking in 5% donkey serum (DNS, sigma) dissolved in 1% of bovine serum albumin (BSA) in TBST for 1 hour. The primary antibody were prepared in blocking reagent and incubated overnight at 4℃ with gentle agitation after washing. Horseradish peroxidase-conjugated secondary antibodies were incubated at room temperature for 1 hour. Antibody binding was detected by enhanced chemiluminecence (Millipore). Figure 38 shows the antibody is specific bind to Ser 133-phosphorylated CREB, as shown by the molecular weight of the only band appearing.

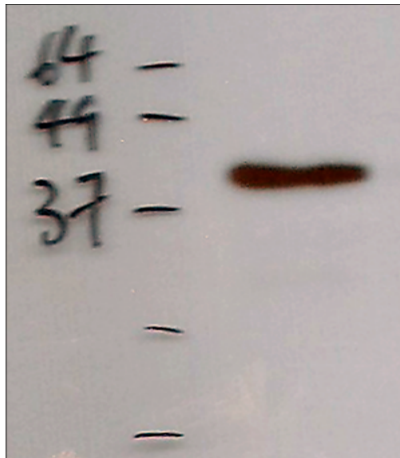


Figure 38. Western blot binding of anti-phospho-CREB (Ser 133). The concentration of pCREB antibody we use is 1:500 as they recommended. No additional bands were observed with this antibody. On the right is molecular weight standard used.

RESULTS

Result chapter 1: validation of the ex vivo DA synthesis system in rat brain striatum

Introduction

Brain striatum is the main output region of dopamine neurons originated from VTA. Previous studies confirmed that the striatum neurons are under dense DA innervations, and it is estimated that a single striatal neuron might be affected by 95-194 dopaminergic neurons terminals on average [Matsuda et al., 2009 introduction]. Dopamine terminals are the major location where DA is synthesized from L-tyrosine. DA synthesis process is regulated by several enzymes, among which the tyrosine-hydroxylase (TH) is the rate-limiting enzyme. The activity of TH is stimulated by phosphorylation of this enzyme at Ser^{19}, Ser^{31} and Ser^{40} by various protein kinases including Ca2+/calmodulin-dependent protein kinase Ⅱ (CaMKⅡ Haycock and Haycock, 1991), ERK1/2 (Haycock et al., 1992) and cyclic AMP-dependent protein kinases (Haycock and Haycock, 1991). Although the phosphorylation of TH involves large number of protein kinase, the dephosphorylation of this enzyme seems to be regulated by a single protein phosphatase that is protein phosphatase 2A (PP2A, Haavik et al., 1989). Short-term regulation of dopamine biosynthesis is modulated by the phosphorylation state of TH which could be affected by both the protein kinases and the protein phosphatase.

Objective

The objective of this part is to determine whether our *ex vivo* DA synthsis mesearment system is sensitive enough to reflect the affect of the exogenous stimulus which should influence short-term DA synthesis through finally acting on the TH phosphorylation.

Results

Previous studies in our lab suggested an incubation-time-dependent decrease of DA synthesis in our system until 2 hours of pre-incubation (data not show). So the incubation time we applied in my study is 2 hours. The experiment design is shown under each graph.

Dopamine D2R activation lead to the downregulation of PKA, thus finally inhibit the DA synthesis

In the striatum, dopamine synthesis from the dopaminergic terminals is tightly controlled via several mechanisms involving dopamine autoreceptors (Kehr et al., 1972; Westerink and De Vries, 1989). So as the most important theoretical basis of our study, this opinion is primarily tested in our system. Dopamine autoreceptors are composed mainly by the D2 subtype. The graph in Fig. 1A shows that the selective D2 receptor agonist could inhibit DA synthesis concentration dependently with the following parameter: Emax= 47.8 ± 0.42 %; EC50= 11.4 nM. The selective D2R antagonist sulpiride at 100nM could block the inhibition effect of quinpirole at 100nM.

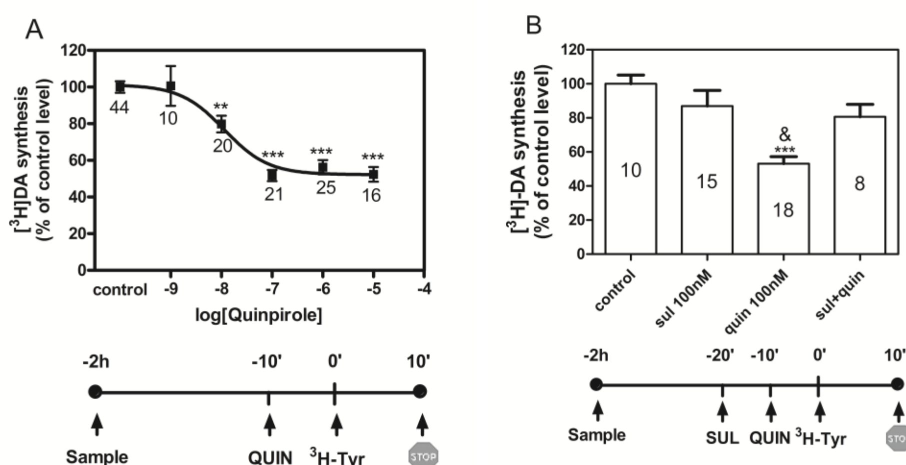


Figure 1 Concentration-response curve of Quinpirole on DA synthesis in rat striatal miniprism under 2mM [K$^+$] (A). The inhibition effect of QUIN on DA synthesis was abolished by the selective D2R antagonist sulpiride(B). Experimental design is shown in the time bar under the graph. Data represent the means ± SEM of N incubations indicated over the symbols. $^{**}p<0.01$ and $^{***}p<0.001$ vs. control; $^{\&}p<0.05$ vs. group treated with both agonist and antagonist in graph B. One-way ANOVA followed by Bonferroni's

test.

The PP2A inhibitor facilitate DA synthesis

Okadaic acid is a potent inhibition of the protein phosphatase PP1 and PP2A/2B. We have mentioned above that the dephosphorylation of TH is believed to under the single regulation of PP2A. Fig.2 shows the PP2A inhibitor okadaic acid could facilitate DA synthesis concentration dependently with the following parameter: Emax= 236.1 ± 21.8 %; EC50= 90nM.

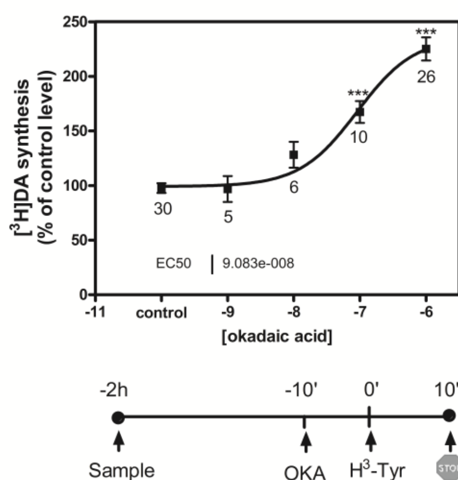


Figure 2 Concentration-response curve of okadaic aicd on DA synthesis in rats striatal miniprism under 2mM [K^+]. Experimental design is shown in the time bar under the graph. Data represent the means ± SEM of N incubations indicated over the symbols. $^{***}p$<0.001 vs. control. One-way ANOVA followed by Bonferroni's test.

A loss of the affinity of QUIN to D2R under the co-treatment of QUIN and OKA

D2R activation by its selective agonist down-regulate the PKA accumulation and finally inhibit DA synthese, while okadaic acid inhibit PP2A and finally facilitate the DA synthesis. So quinpirole treatment and okadaic acid treatment oppositely regulate DA synthesis. The concentration dependent curve of quipirole under the presence of okadaic acid is shown in figure 3. We observed a right-shift of the concentration curve with an IC50 being nearly 2000 times higher (figure 3).

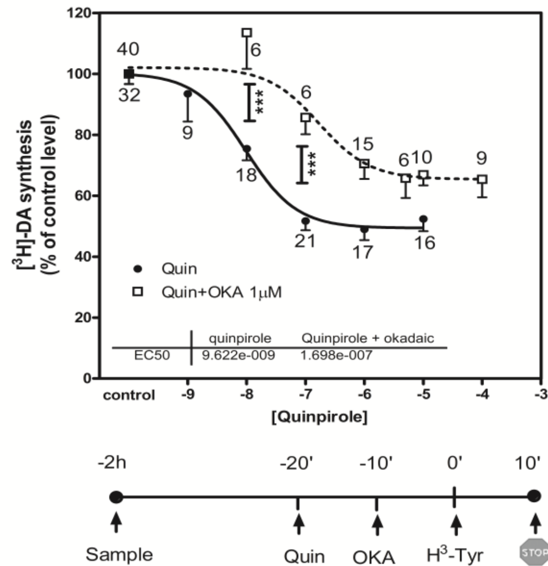


Figure 3 Okadaic acid regulated concentration-response curve of Quinpirole on DA synthesis in rat striatal miniprism under 2mM [K^+]. Okadaic acid was added into the incubation system 10 min after quinpirole and the concentration for okadaic acid is 1μ M**.** Experimental design is shown in the time bar under the graph. Data represent the means ± SEM of N incubations indicated over the symbols. $^{**}p$<0.01 and $^{***}p$<0.001 vs. the connected groups. One-way ANOVA followed by Bonferroni's test.

Discussion

As we mentioned in the introduction that the short-term DA synthesis is modulated by the phosphorylation state of TH, which could be regulated either by the protein kinases or by the protein phosphatase. The dose-dependently inhibition effect of the D2R agonist QUIN and the similar dose-dependently facilitation effect of the PP2A inhibitor okadaic acid on DA synthesis measured in our *ex vivo* DA synthesis assessment system provide firm evidence that our assessment system could reflect the alteration of TH phosphorylation state stimulated by different exogenous stimulus. For example, the stimulation of D2R by its agonist could eventually inhibit PKA activation level which could phosphorylate TH, thus this down-regulation of TH phosphorylation state is transformed to the down-regulation of short-term DA synthesis. Similarly, okadaic aicd inhibit the PP2A which facilitate the TH

phosphorylation and finally up-regulate the short-term DA synthesis. To our knowledge, the dopamine D2 receptor signaling are composed by the early phase and late phase. In the late phase of D2R signaling, the D2 receptor stimulate the formation of a protein complex of β -arrestin 2, PP2A and Akt [Beaulieu and Gainetdinow, 2011 and references within] So the loss of the D2R affinity elicited by the okadaic acid may due to the inhibition effect of it to the PP2A which block the late phase of the D2R signaling. In other words, GPCRs lost their affinities under the desensitization state. Thus, another explaination for the affinity lost may be that okadaic acid desensitizes a portion of the D2 receptors.

Conclusion

In summary, the ex vivo experiment could get ride of the un-specific influence that is not elicited by the treatment and the alteration of the amount of DA synthesized is under the directly regulation of the stimulus we applied during the ex vivo incubation. Our synthesis assessment system could perfectly reflect the stimulation state to the miniprisms through comparing the alteration of DA synthesized amount which is regulated by the phosphorylation state of TH.

Result chapter 2: Agonist and antagonist effects of aripiprazole on D2-like receptors controlling rat brain dopamine synthesis depend on the dopaminergic tone

Abstract

The atypical antipsychotic drug aripiprazole binds with high affinity to a number of G protein coupled receptors including dopamine D2 receptors. However, the efficacy of aripiprazole as a partial agonist at D2 receptors remains controversial. In the present work, we examined the properties of aripiprazole at D2-like autoreceptors by monitoring the changes of dopamine synthesis in adult rat brain striatal miniprisms incubated *ex vivo*. As it has been reported that dopaminergic tone could influence this process, we designed a basal condition (2 mM K^+) which represents a low dopaminergic tone, and a stimulated condition (15 mM K^+) which evokes dopamine release and thus mimics a relatively higher dopaminergic tone. Under both basal and stimulated conditions quinpirole showed a clear agonistic activity. Under the basal condition, aripiprazole acted as an agonist at D2-like autoreceptors and fully activated them at about 10 nM, inhibiting dopamine synthesis similarly to quinpirole. The effect of aripiprazole at higher concentrations appeared not restricted to D2-like autoreceptor activation. Dopaminergic tone influenced the efficacy of aripiprazole at stimulating D2 receptors, because under the 15 mM K^+ condition aripiprazole failed to decrease dopamine synthesis at nanomolar concentrations. Moreover, under 15 mM K^+, 10 nM aripiprazole could totally block the effect of quinpirole. Thus under high dopaminergic tone, aripiprazole acts as a

D2-like autoreceptor antagonist rather than an agonist. These data are clear evidence that aripiprazole influences D2-like autoreceptor mediated signaling in completely opposite manners depending on the level of dopaminergic tone in brain tissue.

Introduction

Aripiprazole (OPC-14597, ARI), a derivative of a dopamine (DA) autoreceptor agonist OPC-4392, is considered as an effective antipsychotic drug with a controversial mechanism of action (Mailman & Murphy, 2010). As compared to other antipsychotics, ARI has a safe and tolerable side-effect profile which includes low potential for extrapyramidal symptoms, weight gain, prolactin elevation, and sedation [Leucht et al 2009]. Due to its clinical efficacy and little chance to provoke adverse events during the therapeutic process, a lot of research has been addressed to explain the unique properties of ARI as well as to supply solid bases for developing a new generation of antipsychotics. According to these observations, ARI seems to be a DA D2 receptor partial agonist with also high affinity binding to many G protein coupled receptors, such as serotonergic, adrenergic, and histaminergic receptors [Shapiro et al 2003].

It is believed that the partial agonism of ARI at D2 receptor would be the main explanation for its unique clinical profile. However, increasing evidence makes hard to consider ARI as a simple D2 partial agonist. Thus, the functional action of ARI at DA D2 receptors may vary among agonist, partial agonist or antagonist depending upon the

assessment system, the cell-type selected, the function examined, the receptor density, the receptor reserve levels [Shapiro et al 2003, Tadori et al 2009], D2-D3 heteromerization [Maggio & Millan, 2009] or the surrounding environment which likely differs between brain regions [Koener et al 2012]. The concept of "functional selectivity" proposed by Lawler et al. [Lawler et al 1999] to interpret the unique behavior of ARI at D2 receptor seems more appropriate than simply consider it as a partial agonist.

In the early stage, Kikuchi et al. demonstrated the apparent inconsistency of the behavior of ARI at D2 presynaptic autoreceptors and D2 postsynaptic receptors, as it seems to perform as an agonist at D2 autoreceptors while as an antagonist at D2 postsynaptic receptors [Kikuchi et al 1995]. The antagonism at postsynaptic D2 receptors has been accepted as a way of controlling symptoms of schizophrenia, based on the therapeutic effect of conventional antipsychotics in treating positive symptoms [Mailman & Murphy 2010]. Additionally, activation of D2 autoreceptors might also be beneficial for the treatment of some symptoms of schizophrenia [Tamminga, 2002]. However this agonistic effect of ARI is controversial as it appears to be quite dependent upon the surrounding conditions like receptor density or receptor reserve [Tadori et al 2011a, 2011b]. However most of these reports were done in cell-lines transfected with high density of D2S receptor, which is the main subtype of the D2 autoreceptors [Usiello et al 2000). Also, the dopaminergic tone in the surrounding milieu is proved to be very important in evidencing the agonistic profile of ARI [Iñiguez et al 2008]. Shapiro et al (2003) illustrated that the functional actions of ARI at cloned human D2-dopamine receptors are cell-type selective, and that a range of actions (agonism, partial agonism,

antagonism) at cloned D2-dopamine receptors could be observed depending upon the cell type and function examined [Shapiro et al 2003]. Thus, more reliable evidence from non-cloned, native receptor systems is needed to shed light on ARI's actions *in vivo*.

In our lab, we have developed a method which allows measuring the DA synthesized in dopaminergic neuron terminals in a relatively short period of time [Gonzalez-Sepulveda et al 2013]. Being this a presynaptic response it is regulated by the D2-like autoreceptors located on dopaminergic terminals. Thus, the stimulation or blockade of D2 autoreceptors will be reflected as changes in DA synthesis. By using this method in the present study we monitored the direct functional effect of ARI at D2-like autoreceptors under low or a relatively higher dopaminergic tone.

Objective

In this paper, we intend to

1. Test the effect of aripiprazole on D2 autoreceptor regulated DA synthesis under low extracellular DA concentration and to define its property on D2 autoreceptor in the comparison with the typical D2 receptor agonist quinpirole.

2. Test the effect of aripiprazole on D2 autoreceptor regulated DA synthesis under relatively high extracellular DA concentration and to define its property on D2 autoreceptor in the comparison with the typical D2 receptor agonist quinpirole.

Result

10 nM ARI inhibits DA synthesis through D2-like receptors under 2 mM [K^+] (basal conditions).

Activation of D2 receptors can inhibit DA synthesis in dopaminergic neurons in rat brain striatum. To confirm this, the well-known D2 receptor agonist Quinpirole (QUIN) was used as a standard reference. Under 2 mM [K^+] (basal condition), QUIN decreased DA synthesis in a dose-dependent manner. As shown in Fig. 1A, QUIN effects followed a one-site model with the following parameters: Emax= 47.8 ± 0.42 %; EC50= 11.4 nM. This effect was totally blocked by the selective D2 receptor antagonist sulpiride (data not shown).

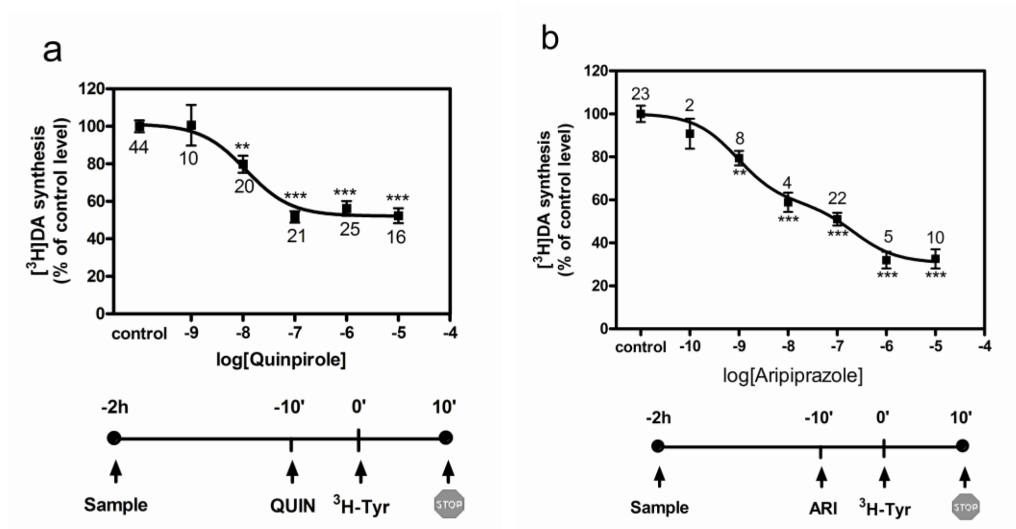


Figure 1. Concentration-response curve of Quinpirole (*a*) and Aripiprazole (*b*) on the inhibition of DA synthesis in non-stimulated rat brain striatal miniprisms (basal

condition: 2 mM K^+). Experimental design is shown in the time bar under the graph. Data represent the means ± SEM of N incubations indicated over the symbols. $^{*}p < 0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. control. One-way ANOVA followed by Bonferroni's test.

Under the same experimental conditions, we obtained the concentration-response curve of ARI on the inhibition of DA synthesis shown in Fig.1B. Unlike the dose-response curve of QUIN, this curve best fitted with a two-site model with the following parameters：Emax= 69 ± 0.43 %; $EC50_1$= 0.93 nM and $EC50_2$= 0.21 μM (Table 1). By comparison with the QUIN dose curve, we made the hypothesis that only the first part of the ARI dose-response curve was due to its effects on D2 autoreceptors. To test this hypothesis, the typical selective D2 receptor antagonist sulpiride was applied to block D2 receptors. Sulpiride at 1 M fully eliminated the inhibitory effect of ARI at 10 nM on DA synthesis, which confirmed our hypothesis (Fig. 2A). In addition, QUIN failed to make a concentration-related response when added to the miniprisms 10 min after ARI 10 nM, as shown in Fig. 2B. The comparisons between the two dose-response curves of QUIN (one in the absence and the other in the presence of ARI 10 nM, Fig. 2B) indicate that the D2 autoreceptors have already been activated by ARI 10 nM. This also confirmed our hypothesis that low doses (≤100 nM) of ARI inhibit DA synthesis due to its action on D2-like receptors.

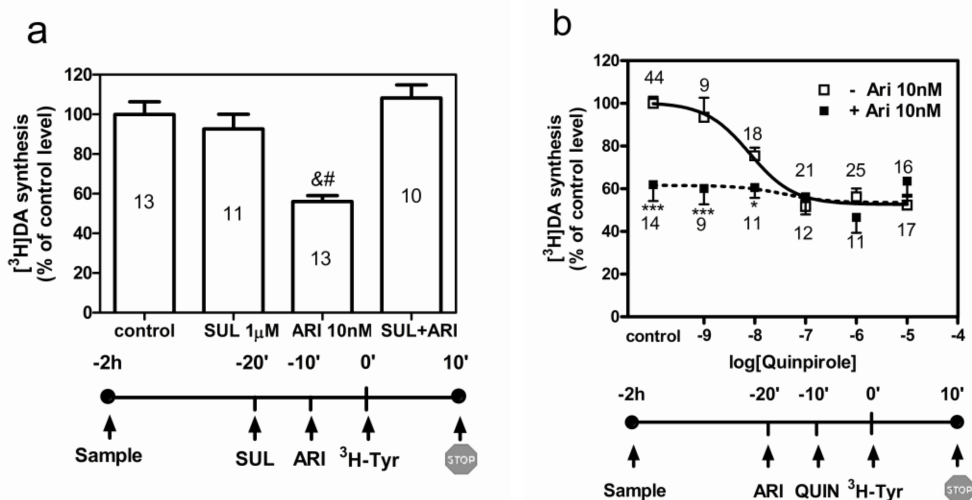


Figure 2. **Inhibition of DA synthesis by 10 nM Aripiprazole is mediated by D2 receptors. a: Striatal miniprisms were incubated with sulpiride (1 μM) and Aripiprazole (10 nM) either alone or together under 2 mM K+ condition**. Sulpiride 1 μM could totally eliminate the effect of Aripiprazole at 10 nM. b：Concentration-response curve of Quinpirole in the presence of 10 nM Aripiprazole under 2 mM K+ condition. Experimental design is shown in a time bar under each graph. Data represent the means ± SEM of N incubations indicated by the symbols or inside bars. &p< 0.001 vs. control, #p < 0.001 vs. group treated with both Aripiprazole 10 nM and sulpiride 1 μM; *p<0.05 and ***p<0.001 vs. group treated in the absence of Aripiprazole at the same dose; One-way ANOVA followed by Bonferroni's test.

ARI failed to activate D2 autoreceptors under 15 mM [K^+] (stimulated conditions).

Increasing the potassium concentration in Krebs buffer from 2 mM to 15 mM increased the amount of DA released as expected. In preliminary experiments, the

percent of newly synthesized [^{3}H]-DA released to the medium under 2 mM K^+ was 1.13 ± 0.95 % (mean ± S.D., N=23 incubations) while under 15 mM K^+ was 7.5 ± 3.0 % (N=38 incubations). This led to a 6.6 fold increase in DA released. The increased concentrations of extracellular DA may facilitate its interaction with D2 autoreceptors. Previous reports have shown that the dopaminergic tone played a very important role in evidencing the agonist property of ARI [Iñiguez et al, 2008]. Similarly, in our present study we also got different effects of ARI under different extracellular DA concentrations, which could also represent differences in the dopaminergic tone (Table 1 and Fig. 3). Under 15 mM potassium conditions, ARI failed to inhibit DA synthesis at doses lower than 10   M (Fig. 3B). We also measured the effect of QUIN under the same situation.

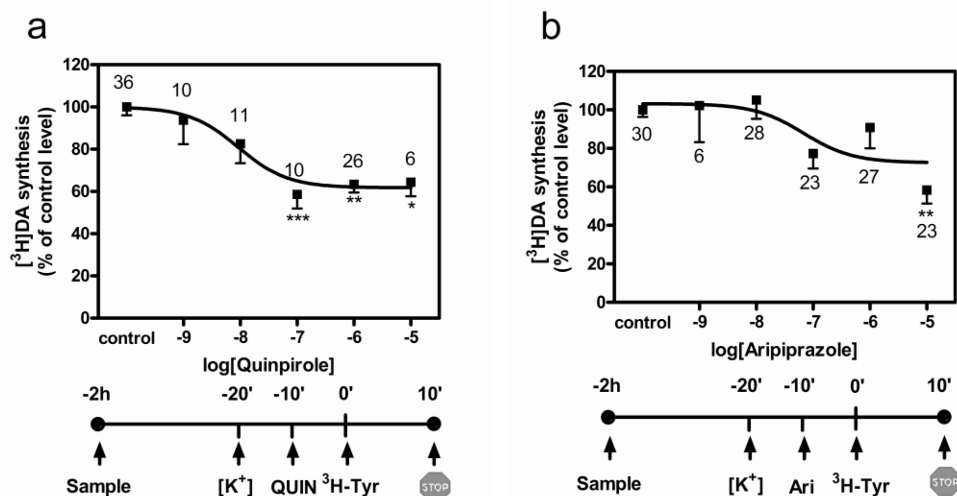


Figure 3. Concentration-response curve of Quinpirole (a) and Aripiprazole (b) on the inhibition of DA synthesis in stimulated rat brain striatal miniprisms (high dopaminergic tone condition: 15 mM K+). Experimental design is shown in the time bar

under each graph. Data represent the means ± SEM of N incubations indicated by the symbols. *p < 0.05, **p< 0.01 and ***p< 0.001 vs. control, one-way ANOVA followed by Bonferroni's test.

As a typical D2 agonist, QUIN acted as a stable agonist under both conditions, as the dose-response curve of QUIN under 15 mM [K^+] was quite similar with the curve under 2 mM [K^+](Fig. 1A and Fig. 3A), and the parameters for the two curves did not differ much either (see Table 1). The reason why ARI failed to stimulate D2 autoreceptors in the depolarizing conditions could be either because ARI failed to bind to D2 receptors in the presence of the high concentration of DA, or because ARI managed to bind but failed to stimulate D2 receptors more efficiently than dopamine, behaving like an antagonist. To figure out the reason, we treated the miniprisms with both ARI and QUIN, and we observed that 10 nM ARI could totally eliminate the effects of QUIN at either 10 nM or 100 nM (Fig. 4). These results prove that ARI binds D2 receptors under these conditions but behaves as an antagonist under high dopaminergic tone. This result is fully consistent with a high affinity and low efficacy of ARI for the stimulation of D2 receptors. Moreover, in the presence of 15 mM K^+, 10 nM ARI tended to increase DA synthesis (Fig. 4), as it would be expected for an antagonist of K^+-released DA.

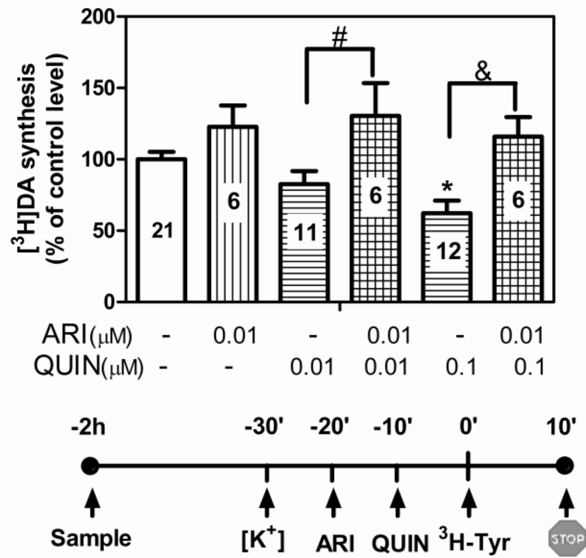


Figure 4. 10 nM Aripiprazole blocks quinpirole inhibition of DA synthesis under 15 mM K+ conditions. Miniprisms were treated with Aripiprazole (10 nM) and Quinpirole (10 nM and 100 nM) either alone or together. Experimental design is shown in the time bar under the graph. Data represent the means ± SEM of N incubations indicated inside the bars. *p<0.05 vs. blank control. #p<0.05 and &p<0.05 in the two groups of connected bars, one-way ANOVA followed by Bonferroni's test.

Discussion

In the present paper we show that aripiprazole (ARI) actions on native D2-like autoreceptors in rat brain presynaptic dopaminergic terminals can be those of an agonist or an antagonist depending on whether the experimental conditions mimic low or high dopaminergic tone. These results are consistent with the well-known low efficacy of ARI to stimulate D2 receptors, that is characteristic of a partial agonist when

compared to DA. We must note that this work uses an approach that can be considered more physiologically relevant than previous articles in receptor-transfected cells. First, D2 receptors involved were neuronal. Second, DA competing with ARI was released by K^+-stimulation of brain tissue *ex vivo*. And finally, the response measured is [^{3}H]-DA synthesis, a specific process of catecholaminergic neurons, mostly dopaminergic in the striatum.

Presynaptic D2 receptors are thought to be the D2S subtype, as compared to the full-length D2L subtype (Usiello et al., 2000). Previous work by Kikuchi et al. (1995) suggested that the agonist or antagonist properties of ARI were dependent on the presynaptic versus postsynaptic localization of D2 receptors, respectively. However, here we show that ARI can show both agonist and antagonist properties at the same presynaptic location controlling DA synthesis. This indicates that the subtype of D2 receptor involved does not explain the switch in ARI's behavior. In agreement, later work by Kikuchi's group (Tadori et al. 2009, 2011a, 2011b) found that ARI can be agonist and antagonist on recombinant human D2S receptors depending on the level of receptor expression and competing DA. Thus, our results validate in brain tissue the later hypothesis. Presynaptic D2-like receptors can be fully stimulated by ARI, provided that competing DA is maintained at low levels. In contrast, during nerve terminal release of DA, ARI behaves as an antagonist due to its lower efficacy to stabilize the active conformation of D2 receptors as compared to DA. The subtype of D2-like receptors controlling DA synthesis likely is the D2S, although a minor contribution of D3 receptors or postsynaptic D2 receptors can not be completely ruled out (Usiello et a., 2000;

Anzalone et al., 2012).

	2 mM K^+	15 mM K^+
Quinpirole		
IC_{50}	11 nM	9.3 nM
Emax	49 %	39 %
Aripiprazole		
IC_{50} (1st / 2nd component)	0.93 nM / 0.21 μM	68 nM
Emax (1st / 2nd component)	41 % / 69 %	17 %

Table 1. Parameters of Quinpirole and Aripiprazole inhibition of rat brain [3H]-dopamine synthesis under basal (2 mM K+) or stimulated (15 mM K+) conditions representing low and high dopaminergic tone respectively.

Previous work also showed that the intrinsic efficacy of D2 partial agonists seems to depend on receptor reserve (Meller et al., 1987; Burris et al., 2002). A partial agonist like ARI might stabilize D2 receptors in a conformation that activate G proteins only for short periods. As a consequence it may need to occupy significantly more receptors than the full agonist quinpirole (QUIN) to reach a similar maximal agonist effect. These "extra" receptors available for ARI's agonist properties can come from the so-called receptor reserve. A higher receptor reserve has been hypothesized for presynaptic D2 receptors as compared to postsynaptic D2 receptors (Meller et al., 1987). Therefore a partial

agonist like ARI will appear to have higher intrinsic efficacy at presynaptic D2 receptors versus postsynaptic D2 receptors due to receptor reserve. This assumption fits experimental data (Kikuchi et al., 1995; Burris et al., 2002). Nevertheless a high receptor reserve is necessary, but not sufficient for ARI to show its agonist properties: a low dopaminergic tone is also required. Previous papers showing ARI's agonist effects on DA synthesis required a low dopaminergic tone, achieved by reserpine or gamma-butyrolactone treatment to animals (Kikuchi et al., 1995; Iñiguez et al., 2008). Reserpine depletes DA stores and gamma-butyrolactone is considered a nerve impulse inhibitor preventing DA release. In our work, an interruption of nerve impulses takes place at slicing striatal tissue, by cutting off nerve endings from dopaminergic cells bodies. Therefore our basal conditions (2 mM K^+) may well be considered relatively similar to gamma-butyrolactone in that they produce a low dopaminergic tone, where only 1 % of newly synthesized [^{3}H]-DA is released to the medium. Accordingly, we may wonder whether ARI is actually able to act as an agonist *in vivo*, where dopaminergic tone might be fluctuating within a range difficult to define. Indeed, human results suggest that ARI has actions consistent with agonism on D2 receptors controlling prolactin release, in contrast with most antipsychotics (Safer et al., 2013). Whether ARI acts as a D2 agonist on nerve terminals involved in psychosis remains speculative, but it is worth considering as it led to ARI's discovery (Kikuchi et al., 1995). Beneficial effects of selective dopaminergic autoreceptor agonists have been postulated for the treatment of psychosis (Tamminga, 2002). However, if a high dopaminergic tone is expected during psychosis, the antagonistic action of ARI on both pre- and postsynaptic D2

receptors will more likely prevail and correlate with its antipsychotic effects. On the other hand, the postulated "dopamine stabilization" elicited by ARI would only make sense on presynaptic D2 receptors, which have sufficient receptor reserve to account for ARI's agonist and antagonist properties. The lower receptor reserve of postsynaptic D2 receptors would only allow antagonist-like effects of ARI, based on the lack of sufficient G protein activation. Therefore postsynaptic "dopamine stabilization" would not be possible unless alterations in receptor sensitivity or functional selectivity add new levels of complexity to ARI's postsynaptic actions (Meller et al., 1987; Mailman & Murphy 2010).

When DA release was stimulated by 15 mM K^+, nanomolar concentrations of ARI did not decrease [^{3}H]-DA synthesis like QUIN, but instead they clearly blocked QUIN action as an antagonist. In addition, in the absence of QUIN we observed a slight tendency to increase [^{3}H]-DA synthesis in ARI's treated samples. This could reveal ARI's antagonism of K^+-released endogenous DA stimulating D2 receptors. However, we do not know how much of endogenous DA was released by 15 mM K^+ or the concentrations of extracellular DA achieved. These values would be hard to obtain within the same experiment in our conditions. We know from preliminary experiments that the percent of newly synthesized [^{3}H]-DA released by 15 mM K^+ increases by 6.6 fold in average as compared to 2 mM K^+. We hypothesized that this K^+ stimulation would model a higher dopaminergic tone in rat brain miniprisms *ex vivo*, although any comparison of our results with dopaminergic tone in human schizophrenics would be speculative. Nevertheless we observed a remarkable switch of ARI's behavior from

agonist to antagonist by increasing K^+. Therefore our method could be useful for future pharmacological research of newer D2/3 based antipsychotics with low liability of side effects based on ARI's clinical experience. New compounds like brexpiprazole or cariprazine are being tested clinically. Additional D2 partial agonists already exist, but ARI has been the most clinically successful so far. An important question to consider on this issue is the degree of intrinsic efficacy of a partial agonist that is optimal for best antipsychotic effects (Tamminga 2002). ARI is considered to have low intrinsic efficacy, that is, its activity is closer to that of an antagonist than to that of an agonist. Previous D2 partial agonists tested may have failed to display the adequate intrinsic efficacy for antipsychotic clinical effects (Natesan et al., 2010). An important additional question is whether there is a clear relationship between D2 partial agonism and low liability of extrapyramidal side effects. Two main hypothesis are currently being considered: First, D2 occupancy by antagonists might have a threshold over which extrapyramidal effects would appear. And second, transient D2 blockade would elicit less extrapyramidal side effects than continuous blockade (Seeman 2002). Surprisingly, ARI occupancy of D2 receptors at clinically relevant doses has been shown to be well above the threshold considered for other antipsychotics, and ARI is not relatively fast at dissociating from D2L receptors (Kuroki et al., 2002; Seeman 2005; Maggio & Millan, 2010). This suggests that ARI's D2 partial agonism might contribute to low liability of extrapyramidal effects. Because the conformation of D2 receptors stabilized by ARI's binding might be in between that of an agonist and an antagonist, we could speculate that ARI's stabilized D2 receptor conformation might allow for G protein activation during a short

percent of time. In contrast the rest of antipsychotics would stabilize D2 receptor conformations where G protein binding is still shorter or can not happen at all. ARI's short time of D2 - G protein activation might not elicit significant postsynaptic responses, but it may allow a higher D2 receptor occupancy than other antipsychotics without producing extrapyramidal side effects.

Our determination of $[^3H]$-DA synthesis in brain tissue *ex vivo* is methodologically faster than classical DOPA accumulation *in vivo*. We obtained 24 brain miniprism incubations from the striata of a single rat. QUIN and ARI IC_{50} values obtained were very similar to reported literature (Table 1). QUIN reached its maximal effect (48 % decrease) at 100 nM. In contrast, the maximal effect of ARI was 68 % at the concentration 1 μM, with a curve best adjusted to two-sites. ARI not only has affinity for D2 receptors, it also has significant affinity for a large number of other G-protein coupled receptors, such as serotonergic, adrenergic, and histaminergic receptors (Shapiro et al. 2003). We did not attempt to block the low-affinity effect of ARI on $[^3H]$-DA synthesis, which could be related to actions on serotonin or other receptors. The selective 5-HT1A/7 receptor agonist R(+)-8-OH-DPAT attenuates amphetamine-induced DA synthesis in rat striatum [Kuroki et al. 2000], and also other agonists of 5-HT1A receptors inhibit tyrosine hydroxylase in rat striatum [Johnson et al. 1996]. However 5-HT1A receptors are considered as somatodendritic and unlikely in dopaminergic terminals. The serotonergic actions of ARI deserve further investigation as they could contribute to its unique antipsychotic effects (Jordan et al 2002; Shapiro et al., 2003). Here in our study, the non-D2 receptor effect of ARI on the inhibition of DA synthesis might be related with

serotonin 5-HT1A/7, 5-HT2C receptors or receptors for additional neurotransmitters (DiMateo et al., 2001; Shapiro et al., 2003), but further studies are needed to confirm these hypotheses. This effect does not disappear under 15 mM K^+ condition, and its higher IC_{50} suggests it is not D2-mediated.

In summary, we observed different properties of ARI under different levels of dopaminergic tone in the surrounding milieu: agonist under low dopaminergic tone and antagonist under a relatively higher dopaminergic tone. This is likely based on ARI's low efficacy as agonist and the high receptor reserve of presynaptic D2 autoreceptors. In contrast, the D2 receptor full agonist QUIN acted as a clear agonist under both conditions. We also observed an effect of ARI at higher concentrations that is not apparently D2 mediated. These results indicate that ARI's effects on presynaptic D2-like receptors in brain tissue have unique properties which clearly differentiate it from other traditional antipsychotics.

Conclusion

Aripiperizole, instead of being a D2 receptor partial agonist, regulate the the D2 autoreceptors controlled DA synthesis as a full agonist at low extracellular DA concentration, however, regulate the D2 autoreceptor controlled DA synthesis as an antagonist when increased the extracellular DA concentration.

Result chapter 3: Crosstalk of presynaptic D2 receptors with other GPCRs

Abstract

In the central nervous system (CNS), dopamine is involved in motor control, endocrine function, reward, cognition and emotion. Pharmacological agents targeting directly or indirectly at dopamine receptors have been clinically used in the managing of several neurological and psychiatric disorders including Parkinson's disease, schizophrenia and Attention deficit hyperactivity disorder (ADHD), as well as cocaine addiction. Dopamine receptors belong to the Class A G-protein coupled receptor super-family (GPCRs). Many GPCRs can form dimers or higher order oligomers which may generate crosstalk signaling with different properties than monomeric GPCRs. One of the future directions in managing dopamine-related pathologic conditions may involve discovering the heteromers formed among dopamine receptors or among dopamine receptor and other GPCRs. In our study, we observed that both the orexin receptor agonist orexin and $GABA_B$ receptor agonist baclofen could inhibit presynaptic dopamine synthesis mediated by D2 autoreceptors. Given the overlapping of distribution of these two receptors compared with D2 receptor, and based on the blockade of these effects with D2R antagonists, we suggest there's strong probability that these two receptors may form heteromers with presynaptic D2 receptors. D5R is a subtype that belongs to D1-like subclass of dopamine receptor, the expression of D5 receptors at the dopaminergic terminals enables them to

co-localize with D2 autoreceptors, which are believed to be the main type of dopamine autoreceptors. Although D2-D5 heteromers have been reported in culture cells, in our study, we failed to observe any crosstalk between these two subtypes in regulating DA synthesis.

Introduction

Dopamine dysfunctions in the central nervous system (CNS) are involved in various neurological and psychotic disorders including schizophrenia [Rao et al., 2013], Parkson's disease [Wright et al., 2013] and Huntington's disease [Jakel and Maragos, 2000; Cyr et al., 2006]. In addition, drug addiction is a serious neuronal disorder that is believed to relate with dopamine circuits. Among the treatments of these neuropsychiatric diseases, several clinically target directly at dopamine receptors because agonist and antagonist of these receptors are the traditional focus of drug development. All dopamine receptors belong to Class A G-protein coupled receptor super-family (GPCRs) and consists of five structurally distinct subtypes which can be subdivided into two subgroups - D1-like dopamine receptor (which contains D1R and D5R); D2-like dopamine receptor (which contains D2R, D3R and D5R) - on the basis of their structure and pharmacological as well as the difference in the downstream signal pathways [Oak et al., 2000].

GPCRs, also known as seven-transmembrane domain receptor (7TM), include a large number of receptors of the extracellular messengers, which activate the intracellular G-protein and eventually lead to physiological responses. Around 50% of

the prescription drugs on the market regulate GPCR function, among them nearly 60% directly target at GPCRs [Jacoby et al., 2006]. Originally, it was believed that GPCR act as monomers, while along with the accumulation of evidence, this conception has been replaced by the idea that GPCRs usually form homomers or heteromers on the neuronal membrane [Bouvier 2001; Rios et al., 2001; George et al., 2002; Agnati et al., 2003; Prinster et al., 2005; Maurel et al., 2008]. As a consequence of physical oligomerization, a functional crosstalk between GPCRs is often observed in the cultured cells which express different kinds of them [Rives et al., 2009]. As either oligomerization or functional crosstalk between receptors could both obtain unique pharmacological profile, in each case, their existence adds new level of the complexity to cell signaling. So it is generally suggested that the interaction among GPCRs opens a new field for the understanding of neurophysiology and neuropathology, and at the same time supplies novel targets for therapeutics. Further consideration is derived from the fact that drugs (agonists or antagonists) may display different affinities at their receptors depending on whether the targeted receptors were in the form of monomers or oligomers [Franco et al., 2008]. Taken together, oligomers of GPCR may bring about breakthrough treatments for the various diseases mentioned above. In the past decade, the properties in the oligomerization or down-stream signal interactions participated by the dopamine receptor especially by D2 receptor subtype have been widely researched. The achievements are inspiring as the oligomerization is evidenced to occur widely: 1) within the dopamine receptor group: D1-D2 heteromer [Lee et al., 2004], D1-D3 heteromer [Marcellino et al., 2008],

D1-D5 heteromer [So et al., 2009]; D2-D3 heteromer [Scarselli et al., 2001], D2-D4 heteromer [Borroto-Escuela et al., 2011], and D2-D5 heteromer [So et al., 2009], besides, each subtype also could form homomers; 2) beyond dopamine receptor group: between the adenosine receptors and dopamine receptors, A_1R-D_1R heteromer [O'Neill et al., 2007] and $A_{2A}R$-D_2R heteromer [Hillion et al., 2002], between dopamine D2 receptor and histamine H3 receptor [Ferrada et al., 2008], D2R-mGluR5 heteromer [Popoli et al., 2001], A2A-D2-mGluR 5 heterotrimer [Cabello et al., 2009], 5-HT_{2A}-D_2 heteromer [Albizu et al., 2011]. The co-localization of these different receptors in the same brain areas led to the study of their possible heteromerization. However most of the results obtained involved receptor-transfected cells and evidence of actual heteromerization in brain tissue is scarce.

The striatum is the main input structure of the basal ganglia, which are subcortical structures involved in processing of information related to the performance and learning of the complex motor acts. Dopamine receptors are predominantly distributed in this brain area [Muly et al., 2010; Fisher et al.,1994], more importantly, dopaminergic projections terminals, which contain presynaptic D2 autoreceptors, densely innervate striatal structures. There are various different kinds of other receptors localized on the neuron membrane in the striatum as well, including $GABA_B$ receptors and orexin receptor. Functional $GABA_B$ receptors are prototypical of dimeric class C GPCRs, expressed as obligate heterodimers of the two subtypes, $GABA_{B1}$ and $GABA_{B2}$ [Jones et al., 1998]. Previous studies reported that $GABA_B$ receptor agonists could be able to influence the activity of dopaminergic neurons in the

substantia nigra [Muller et al., 1989] and ventral tegmental area [Enberg et al., 1993]. These effects elicited by the stimulation of $GABA_B$ receptors are explained at the anatomical level that a large proportion of dopaminergic neurons in these brain regions express $GABA_B$ receptors [Wirtshafter and Sheppard, 2001]. Immunohistochemical evidence showed that $GABA_B$ receptors are widely expressed, almost in all the neurons in the striatum where contains large amount of dopmainergic terminals that originated from VTA and substantia nigra [Lu et al., 1999; Waldvogel et al., 2004]. Thus in the striatum, we expect that the D2 receptor mediated DA synthesis in the dopaminergic terminals may also under the influence of $GABA_B$ receptors.

Similarly, documented literatures suggested a functional interplay between orexin and mesolimbic dopamine neurons in VTA [Korotkova et al., 2003]. And further evidence using immunohistochemical approach confirmed both orexin-1 and orexin-2 receptors are expressed in the dopaminergic neurons in this brain area [Narita et al., 2006]. Importantly, the same literature also proposed that the hypocretin-orexin system may directly implicate in the rewarding effect through activation of mesolimbic dopamine pathway in the VTA. Later on, the study in the regulation of cocaine addiction by orexin system also suggested that this effect may through the modulation of the mesolimbic dopamine system [España et al., 2010; España et al., 2011]. The striatum is a centre region of the mesolimbic dopamine system which receives dense dopaminergic projections originated from VTA and play a critical role in the rewarding processing through dopamine pathways. We hypothesize that the interaction of hypocretin-orexin system and dopamine system may also occur in this brain region.

The heteromerization between D2R and D5R was observed in cultured cells transfected with both receptors [So et al., 2009]. However, in real tissues, the co-localization between these two subtypes is scarce. In the striatum, D2 receptors are widely expressed in either presynpatic or postsynaptic neurons [Bouthenet et al., 1991; reviewed in Ref: Missale et al., 1998], in contract, D5 receptors are poorly expressed in this brain region [Ciliax et al., 2000; reviewed in Ref: Missale et al., 1998]. Nevertheless, some suggested that D5 subtype may also express presynaptically in the striatal dopaminergic neurons where D2R is widely expressed [Khan et al., 2000]. So in our study, we mean to check the effect of D5R in the regulation of DA synthesis controlled by D2 autoreceptors.

In our lab, we have developed a method which allows measuring the DA synthesized in dopaminergic neuron terminals in a relatively short period of time [Gonzalez-Sepulveda et al 2013]. Being this a presynaptic response it is regulated by the D2-like autoreceptors located on dopaminergic terminals. Thus, the stimulation or blockade of D2 autoreceptors will be reflected as changes in DA synthesis. By applying the agonist or antagonist of the other two receptors and monitoring the changes in DA synthesis velocity, we could clearly see the influence of these receptors on D2 receptor' effect, thus the influence may reflect the crosstalk between the receptors and D2R.

Objectives

In this chapter, we propose

1. To determine if there is crosstalk between D2R and $GABA_B$ receptor by assessing the DA synthesis regulation in brain dopaminergic terminals.

2. To determine if there is crosstalk between D2R and Orexin-1 receptor on the by assessing DA synthesis regulation in brain dopaminergic terminals.

3. To evident the D2R and D5R heteromerization in brain striatum dopaminergic neuronal terminals on the effect in DA synthesis regulation.

Results

Crosstalk between D2 receptor and $GABA_B$ receptor

In our study, striatal miniprisms derived from adult rats were incubated *ex vivo*, and the effects on DA synthesis elicited by different ligands were recorded and compared. As shown in the Fig.1A, we observed that the selective agonist of $GABA_B$ receptor Baclofen (BACL) could inhibit dopamine (DA) synthesis with statistical significance at both concentrations of 100nM and 1μ M, however no stronger effect was observed when at higher concentrations of this agonist. As we have shown in the result chapter 1 that the stimulation of D2R typically inhibits DA synthesis (Fig.1, result chapter 1), so we asked whether BACL inhibits DA synthesis through $GABA_B$-D2R oligomers or through $GABA_B$ receptor independently of D2R. Further experiments confirmed this inhibition elicited by BACL could be blocked either by the potent selective $GABA_B$ receptor antagonist CGP54626 (CGP) (Fig.2B) or by the D2R selective antagonist sulpiride (SUL, Fig.2C), which suggests that $GABA_B$ receptors are involved in the

regulation of DA synthesis through D2 receptors. These results do not prove D2-$GABA_B$ receptor oligomerization but could be explained by such interaction of these participated receptors.

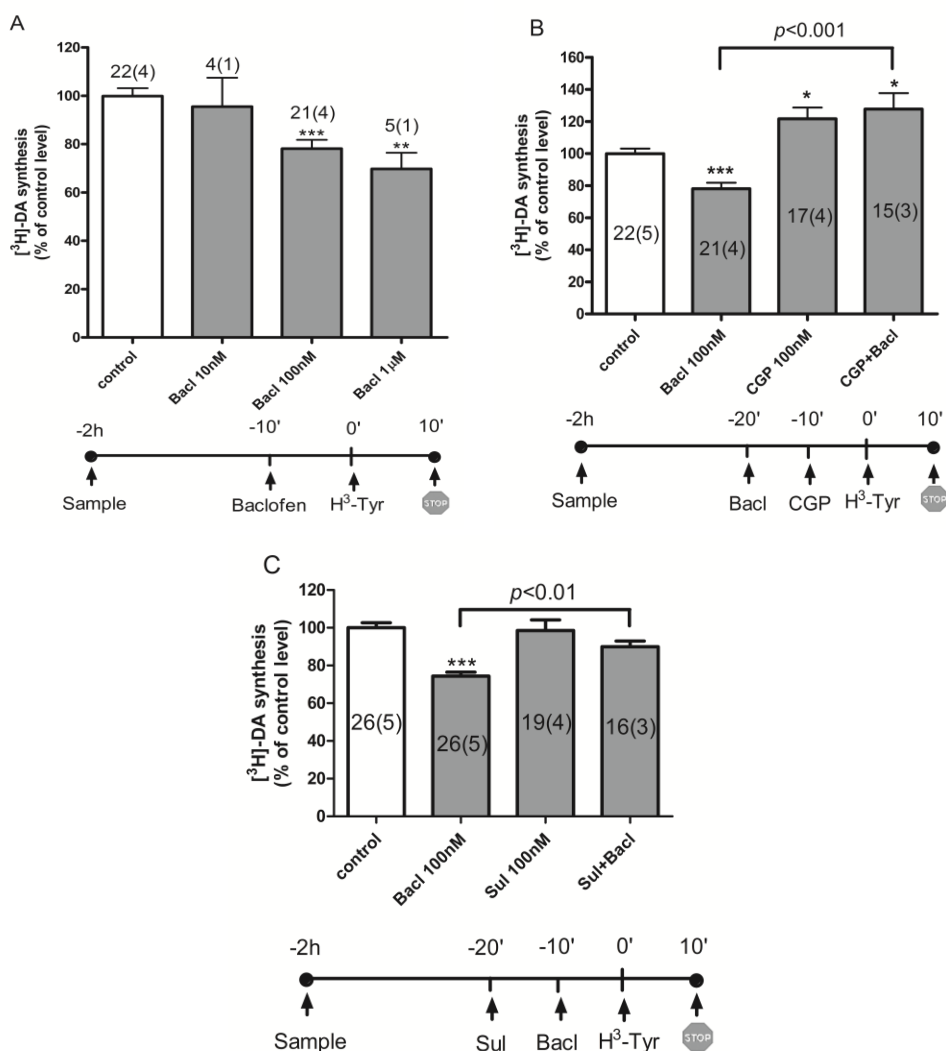


Figure 1 Inhibition effect on DA synthesis by $GABA_B$ receptor selective agonist Baclofen is blocked either by the $GABA_B$ receptor antagonist CGP or the D2R selective antagonist Sulpiride. A): The inhibition effect of Baclofen on DA synthesis. B): the selective antagonist of $GABA_B$ receptor CGP could totally block Baclofen's effect at 100nM, in addition, CGP itself significantly facilitate DA synthesis. C): the inhibition effect of Baclofen at 100nM on DA synthesis could also be blocked by the D2R antagonist Sulpiride at 100nM. Experimental design is shown in the time bar under each graph. Data

represent the means ± SEM of N incubations indicated over the symbols outside the parentheses; the symbols in the parentheses indicated the rat number used. $^{*}p < 0.05$, $^{**}p<0.01$ and $^{***}p < 0.001$ vs. control. p values between the connected groups are shown in the graph. One-way ANOVA followed by Bonferroni's test.

In addition, we observed that the $GABA_B$ receptors antagonist (CGP) could facilitate DA synthesis significantly (Fig.1B) and this facilitative effect is concentration dependent (Fig.2A, related parameters: Emax=200.4±1.6%; EC50=196nM). The facilitative effect of CGP on DA synthesis was unexpected. Moreover, this facilitation effect is under the control of D2R because it could be counteracted by both the antagonist (Fig.2B) and the agonist (Fig.2C) of D2R. Since to our knowledge neither CGP nor BACL were documented have any affinity to D2 receptor, our results supplied solid evidence that $GABA_B$ receptor could regulate DA synthesis in dopaminergic neuron teriminals. D2R is largely involved in this process which suggests that $GABA_B$ receptors and D2 receptors may have formed heteromers in that account for the crosstalk observed.

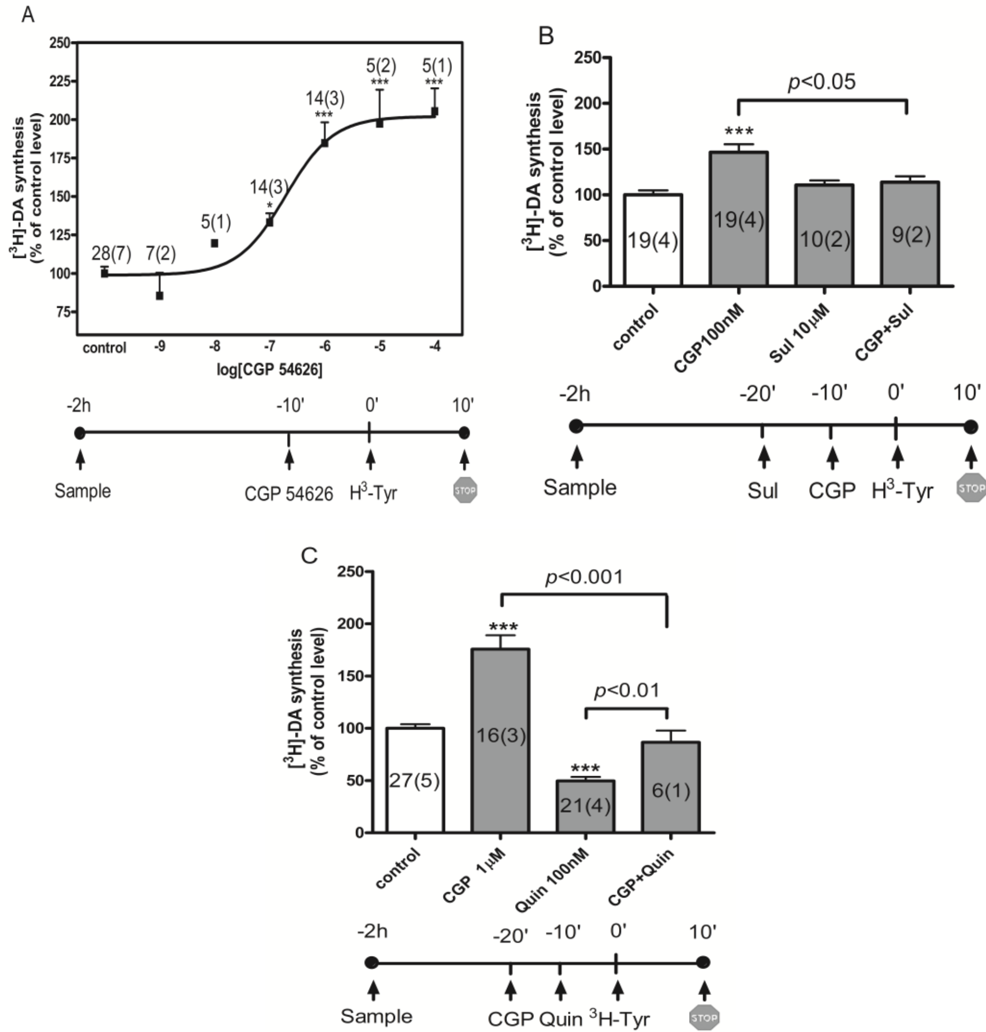
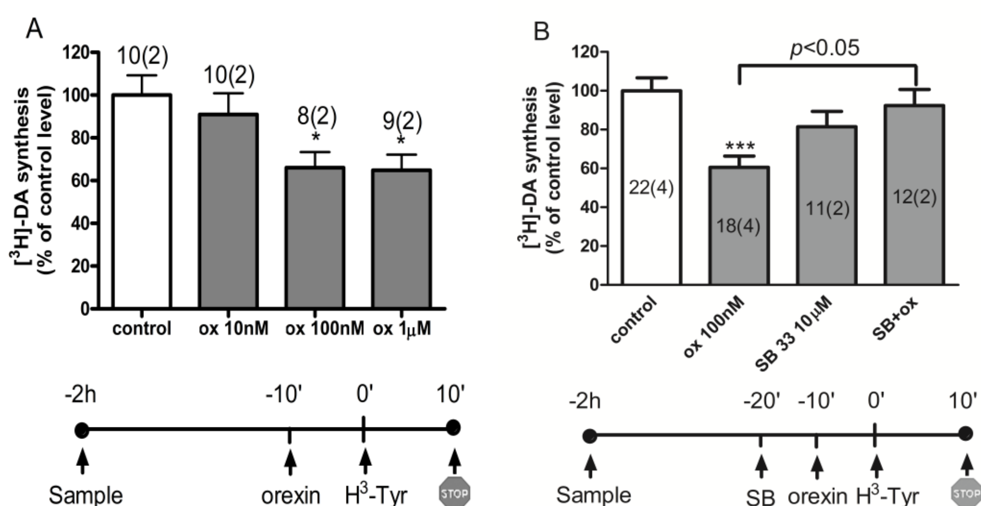


Figure 2 The concentration-response curve of the facilitative effect on DA synthesis elicited by the $GABA_B$ receptor antagonist CGP 54626 (A). The effect of CGP 54626 at 100nM could be abolished either by the selective D2R antagonist Sulpiride (B) or by the selective D2R agonist Quinpirole (C). Experimental design is shown in the time bar under each graph. Data represent the means ± SEM of N incubations indicated over the symbols outside the parentheses; the symbols in the parentheses indicated the rat number used. $^{*}p < 0.05$, $^{**}p<0.01$ and $^{***}p < 0.001$ vs. basal control. p values between the connected groups are shown in the graph. One-way ANOVA followed by Bonferroni's test.

Crosstalk between D2 receptor and orexin receptor

In the VTA, both OxR1 and OxR2 were found in dopamine neurons [Narita et al.,

2006]. We presume that orexin receptor may participate in the regulation on DA synthesis mediated by D2 autoreceptors in the striatum which is the major dopaminergic projection area of VTA. Primarily, we determined the effect of the agonist of OxR1/2 orexin A on DA synthesis, and we observed that orexin A inhibited DA synthesis significantly at both 100nM and 1 μ M (Fig. 3A). Further experiments showed that this inhibition effect could be blocked by both the selective OxR1 antagonist (SB334867, Fig.3B) and D2R antagonist (raclopride Fig.3C) which suggests that the OxR1 participate in the regulation effect on DA synthesis that are mediated by D2R.



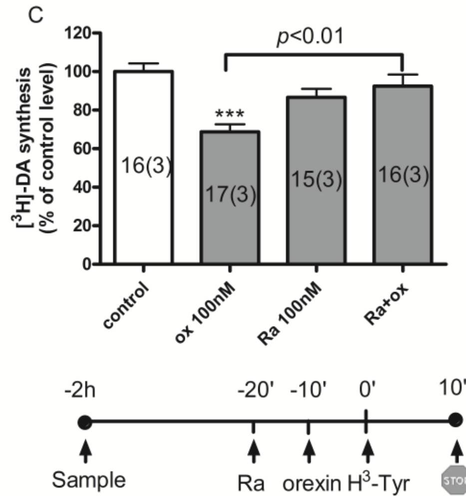


Figure 3 Inhibition effect on DA synthesis by the orexin-1 receptor agonist orexin is blocked either by the OxR1/2 antagonist SB 334867 (SB33) or the D2R selective antagonist Raclopride A): The inhibition effect of orexin-A on DA synthesis. B): the selective antagonist of orexin-1 receptor SB33 could totally block orexin A's effect at 100nM. C): the inhibition effect of orexin at 100nM on DA synthesis could also be blocked by the D2R antagonist Racopride at 100nM. Experimental design is shown in the time bar under each graph. Data represent the means ± SEM of N incubations indicated over the symbols outside the parentheses; the symbols in the parentheses indicated the rat number used. $^{*}p < 0.05$ and $^{***}p<0.001$ vs. basal control; *p* values between the connected groups are shown in the graph. One-way ANOVA followed by Bonferroni's test.

However, the selective antagonist of OxR1 SB 334867 (SB, 10μ M) failed to affect the inhibition effect elicited by the D2R agonist QUIN (Fig.4A); No synergism was observed when quinpirole and orexin A were applied simultaneously at sub-maximal concentration (10 nM both, Fig.4B). Our results provide solid evidence that orexin receptors need D2R in order to regulate DA synthesis through D2R in the dopaminergic neuron terminals. The one way influence we observed in our study

between these two receptors may suggest that the inhibition effect on DA synthesis elicited by OxR1/2 is dependent on the D2R signaling pathway. However, it seems D2R do not appear to need the activation of OxRs to regulate DA synthesis.

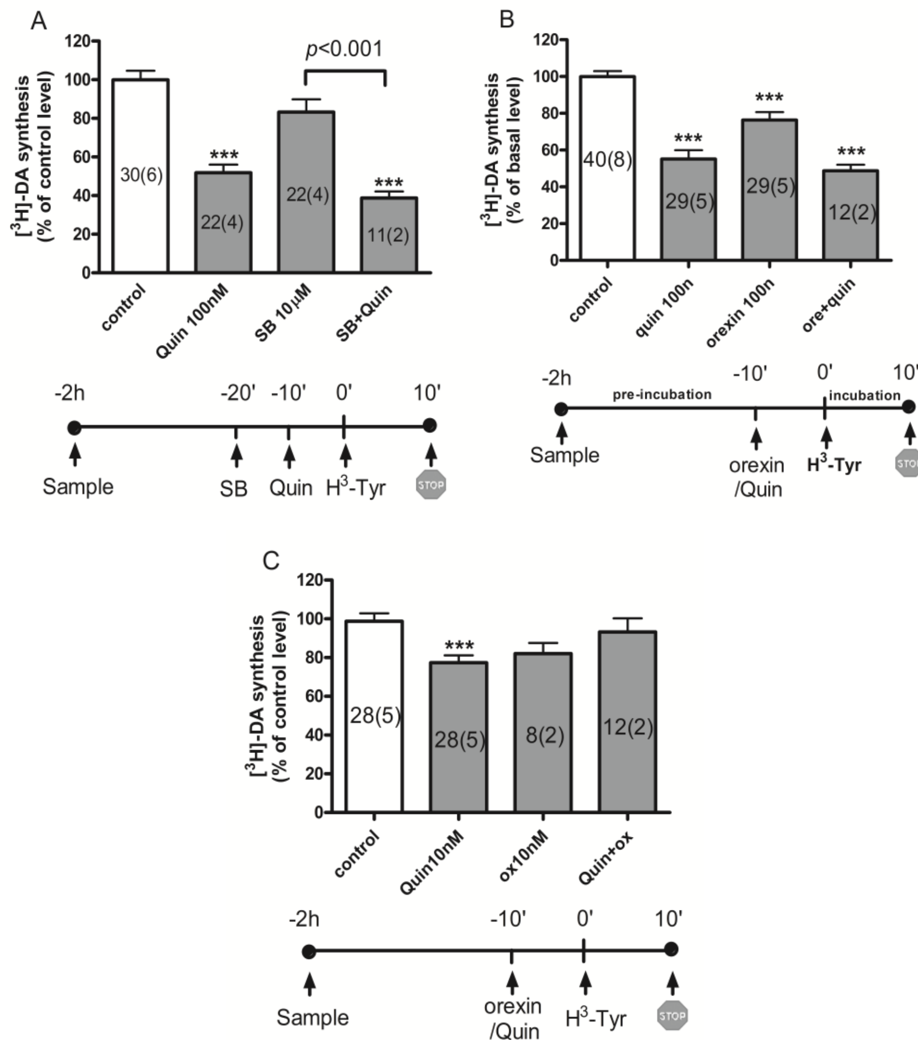


Figure 4 The inhibition effect on DA synthesis of D2R agonist could neither be affected by the antagonist (A) nor the agonist (B and C) of OxR1 receptor. Experimental design is shown in the time bar under each graph. Data represent the means ± SEM of N incubations indicated over the symbols outside the parentheses; the symbols in the parentheses indicated the rat number used. $^{***}p<0.001$ vs. basal control; *p* values between the connected groups are shown in the graph. One-way ANOVA followed by Bonferroni's test.

No crosstalk between D2R and D5R was observed in the regulation of DA synthesis

Both D1 and D5 receptors are localized in striatal neurons, however only D5 receptors were found in the dopaminergic neuron that co-localize with the dopaminergic neuron marker TH [Khan et al., 2000]. D5 receptors belong to the D1-like subclass of DA receptor, but their expression levels in this area appear to be low, however, D5-D2 heteromer was previously documented in the cultured cells [So et al., 2009]. So in our study, we hypothesized that D5R subtype may also regulate DA synthesis controled by D2 autoreceptors. To our knowledge, there still no D5R selective agonist or antagonist available in the market, so the ligands we applied in our study: the agonist SKF 38393 and the antagonist SCH 23390 both have similar affinity to D1R and D5R. However, as D1R is not expressed in the dopaminergic terminals, so the effect elicited by these ligands may only due to D5R stimulation. We found that neither SKF38393 (SKF) nor SCH23390 (SCH) have any effect on DA synthesis (Fig. 5A). In addition, miniprisms that were treated with agonists of both receptor showed no significant different compared with that were treated only with D2R agonist which means no significant synergism was observed on DA synthesis was observed in our study (Fig. 5B).

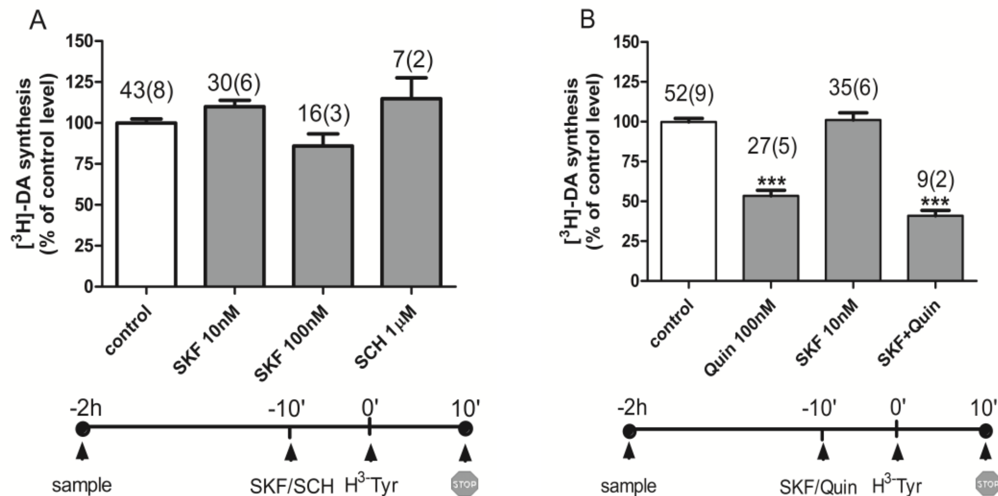


Figure 5 Both the D1/5 receptor agonist and antagonist have no effect on DA synthesis in rat miniprism (A). In addition, no synergistic effect was observed in D2R and D5R agonist co-administration (B). Experimental design is shown in the time bar under each graph. Data represent the means ± SEM of N incubations indicated over the symbols outside the parentheses; the symbols in the parentheses indicated the rat number used. $^{**}p < 0.01$ and $^{***}p<0.001$ vs. basal control. One-way ANOVA followed by Bonferroni's test

Discussion

The alteration of DA synthesized in a certain short-period elicited by different stimulus is mediated by the phosphorylation state of TH which is under the regulation of D2 autoreceptor. Thus, in our study, we monitor the effect of various ligands on D2 autoreceptor controlled DA synthesized in a fixed period of time (10min), this method could directly reflect the interaction between the corresponding receptors to their ligands and their allosteric communication with D2 autoreceptor in brain tissue. However, our method does not demonstrate such allosteric interactions; it only shows crosstalk that could be suggestive of heteromerization.

The crosstalk between GABAB receptor and D2R

GABA$_B$ receptors belong to the Class C GPCRs which is the first class of GPCRs evidenced to require dimeric organization for their functioning [Pin et al., 2003]. GABA$_B$ receptors are obligatory dimeric complexes consisted of GABA$_{B1}$ and GABA$_{B2}$, GABA$_{B1}$ subunit is responsible for the natural ligand binding whereas GABA$_{B2}$ subunit is necessary for the G-protein coupling and cell surface expression of the heteromeric complex [Jones et al., 1998; White et al., 1998]. In our study, we observed that the activation of GABA$_B$ receptors regulate DA synthesis mediated by D2R, which suggests there is a physical interaction between these GABA$_B$ dimmers and D2R or between GABA$_B$1 and D2R. However such interactions have not been described in any literature to our knowledge. The GABA$_B$ receptor agonist (BACL) and antagonist (CGP) could individually inhibit or facilitate DA synthesis, and both effects were abolished by the D2R antagonist. Of note, the antagonist CGP is more potent (200%, the maximal facilitation effect normalized to basal level) compare with the slight effect of the agonist BACL (36%, the maximal inhibition effect compared with basal level). This may be indicative of basal GABA release in our brain miniprisms, which could partly activate GABA$_B$ receptors in control conditions. In addition, co-incubation CGP with D2R agonist QUIN caused a counteracting effect of each other. However, co-incubation CGP with GABA$_B$ receptor agonist BACL, all we could observe is the facilitative effect induced by CGP; BACL lost its inhibition effect (Fig. 1B). It is widely accepted that GPCRs may function differently when forming heteromers, leading for instance to shifting in G-protein coupling [George & O'Dowd, 2007; Rashid et al., 2007;

Fan et al., 2005]. It's possible that in our study D2R and $GABA_B$ receptor had formed heteromers that control a simple downstream signal which in both cases is mediated by Gi. Heteromerization would add a new level of complexity to Gi controlled by both D2 and $GABA_B$ receptors. In addition, a important “dimer figureprint” for the GPCR heteromers is that the natural antagonist of its components may display different affinity before and after the heteromerizaiton [Franco et al., 2008]. In our study, we also observed an abnmoral efficacy of the $GABA_B$ antagonist CGP. This phenomenon could be explained either by the reason mentioned above that there exist basal GABA release in our brain miniprisms or by the heteromerzation constituteed by these GPCRs. The latter explanation shows a very important character of heteromers because lots of the prescribed drugs targeting at GPCRs are antagonists and which also explained why different antagonist for the same receptor may have different in vivo profile and induce different side-effects.

In our study we observed a crosstalk between D2R and $GABA_B$ receptor, and based on our result, there is a large possibility that these two receptors had formed heteromer in striatal dopaminergic terminals. Further evidence is needed to confirm this hypothesis.

Crosstalk between D2R and orexin receptor

Orexin receptor is another potential heterodimerization partner of D2R in the striatum. This hypothesis is based on the wide expression of the OxR1/2 receptors in the dopaminergic neurons in VTA, which send the dopaminergic projections to the

striatum [España et al., 2011]. Furthermore, a parallel study in a collaborated lab observed the heteromerization between these two receptors in cultured cells may also support this hypothesis (data not shown). In our study, we showed an interaction between these two receptor signaling pathways. In detail, the orexin receptor agonist peptide Orexin A inhibited DA synthesis and this effect could be blocked either by its own antagonist SB or by the D2R antagonist RACL. Following experiments showed that the selective antagonist of orexin-1 receptor SB could not eliminate the inhibition effect induced by the D2R agonist QUIN, and this effect either could be blocked (when both of the agonist were at low concentration 10nM) or affected (when both of the agonist were at high concentration 100nM) by the orexin receptor agonist Orexin A in the co-incubation experiment. These results may suggest that orexin receptors' effect is finally dependent on D2R signaling. The interaction between these two receptors may happened in the downstream of signal pathways elicited by each receptor although this conclusion would be speculative. Finally it seems that activation of orexin receptor may dampen the D2R signaling instead of potentiating it.

No crosstalk was observed between D2R and D5R

GPCRs can form heteromers or homomers when co-localized in the same membrane surface and they maybe more likely to interact when closely related than divergent receptor subtypes [Milligan et al., 2008, 2009]. So as we showed in the introduction there are several of interactions between different subtypes of dopamine

receptors. However when we try to find the evidence for the D2R and D5R crosstalk both expressed on the dopaminergic neurons, surprisingly we failed. Previously the interaction between these two receptor subtypes was reported in cultured cells co-transfected D2R with D5R [So et al., 2009]. In addition, Nimitvilai and Brodi showed that D5 receptor may desensitize D2 receptors when studying electrophysiological inhibitor of DA neurons. [Nimitvilai and Brodie, 2010]. The reason that we didn't observe the interaction may due to that the receptor density is too low so that D5 effection downstream signals controlling DA synthesis are too weak for our assessing system to detect.

Summary

We used an *in vitro* approach to study the GPCR crosstalk in the fresh brain tissue. This approach enables us to determine the interaction of two distinct receptors at the downstream signaling levels which is elicited by the activation of receptors and it maybe modified by the receptor heteromerization.

Conclusion

1 we provide new evidence for the crosstalk between D2R and $GABA_B$ receptor in regulating DA synthesis in dopaminergic neuron terminals, and based on our results, we suggest that this crosstalk may occur at the receptor level.

2 we observed an interaction between OxR1/2 receptor and D2R in regulating DA synthesis in dopaminergic neuron terminals; however we believe that this interaction

maybe due to at least in part shared signals between orexin receptor and D2R in regulating DA synthesis.

3 although previous studies have reported the D2-D5 receptor hetermerization, we didn’t observe any crosstalk between these two subtypes in regulating DA synthesis in dopaminergic neuron terminals.

Result chapter 4: Chronic cocaine self-administration alters dopamine D2 response into pCREB-positive cells in rat brain striatum

Abstract

This study was conducted to determine the influence of the short-term or long-term withdrawal from chronic cocaine self-administration on D2-like receptor response in different brain striatal sub-regions. It is widely accepted that alterations in dopamine neurotransmission contribute to addiction, and the neuroadaptation induced by cocaine addiction seems to be maintained for a long period. Stimulation of D2-like receptor by the agonists reduces cAMP accumulation and eventually leads to the inhibition of PKA and regulation of other kinases which could influence the phosphorylation of CREB. Previous research proposed that CREB activation may participate in drug reward and drug aversion. A former study in our lab found chornic cocaine administration would potentiate D2R activation elicited CREB phosphorylation. In the present study, male Sprague-Dawley rats were exposed to cocaine self-administration for 6-11 weeks. Brain from sham/naïve controls or 1-day or 5-weeks cocaine withdraw rats were extracted and sliced; slices with the striatum area were incubated *in vitro* with or without the D2R agonist quinpirole. The number of positive neurons that express phosphorylated CREB (pCREB) was measured using an immunohistochemistry technique. Unfortunately, we failed to observe the potentiating effect of cocaine addiction on D2R activation induced alteration of CREB activation, however, we found that cocaine administration seems to alter this effect differently in nucleus accumbens and dorsal striatum compare to the sham/naive rats; in addition, we found a regional difference of the basal pCREB level in the striatum from both sham/naive and cocaine withdrawal (1-day/5-weeks) rats. Our results provide novel information about the neuroadaptation in dopamine signaling elicited by withdrawing from repeated cocaine administration.

Introduction

Chronic cocaine self-administration model serves as a good animal behavior model for researching human cocaine addiction, which is a serious health problem characterized by compulsive drug use despite series of adverse consequence including medical illness and failures in significant life roles. The addicted individuals (including experimental animals and humans) still have a high risk of relapse even after months or years of abstinence, which adds much difficulty to fully recover from drug addiction. This high risk of relapse is thought to be related with the multifaceted neuroadaptations induced by cocaine abuse in the mesocorticolimbic dopamine system which may act as a gateway to alter other brain functions. This is because the midbrain dopamine system plays critical roles not only in the reward and motor systems but also in higher order functions, including cognition and memory [Grant et al 1996]. It is widely accepted that the alteration in dopamine neurotransmission contribute to addiction [Volkow et al., 2009]. The major dopaminergic neuron projection region: the *striatum*, is a heterogeneous structure which could be subdivided into several regions. The region at the base of the striatum is the nucleus accumbens (NAc), the key zone for mediating the rewarding effect of the abused drugs which act directly through increasing the extracellular dopamine levels. The nucleus accumbens can be further divided into nucleus accumbens core and shell. Previous studies suggest that the shell subregion may have a predominate role in regulating drug taking (reward), whereas the NAc core may regulate drug seeking (incentive motivation) [Ito et al., 2000; Alderson et al., 2001; Di Ciano and Everitt, 2001; Hutcheson et al., 2001; Rodd-Henricks et al., 2002; Choi et al., 2006]. Besides the NAc, the dorsal striatum also plays an important role in drug addiction, as plenty of observations suggest that the dorsal striatum mediates the habitual nature of well established drug seeking [Tiffany, 1990; Robbins and Everitt, 1999; Everitt et al., 2001].

The major neuronal population of the striatum, the medium spiny neurons consist of

two approximately equal amount of neurons which separately express dopamine D1-like (D1 and D5 receptors, named D1R for simplicity) and D2-like (D2, D3 and D4 receptors, named D2R for simplicity) receptors [Gerfen et al., 1990]. D1R and D2R act oppositely on adenylyl cyclases (AC) through coupling to different G-protein subunits: D1R couples to the $G\alpha_{s/olf}$ subunit and positively regulates AC while D2R couples to the $G\alpha_{i/o}$ subunit, which exerts a negative regulation on AC pathway [Ron and Jurd, 2005]. Both of the two groups of receptors provide inhibitory feedback regulation on cocaine intake during cocaine self-administration behavior [Edwards et al., 2007]. However in addicted individuals that are abstinent of cocaine and experience a withdrawal period, D1R and D2R perform differently in reinstating cocaine seeking behavior: D2R facilitates while D1R may attenuate this behavior. [Wise et al, 1990; Self et al, 1996; De Vries et al,1999; Alleweireldt et al, 2002; Dias et al, 2004]. Previous studies also suggest that D2R play a major role in eliciting relapse to cocaine seeking when environmental stimuli activate the mesolimbic dopamine system [Phillips et al, 2003; Pruessner et al, 2004]. In addition, repeated administration of drugs may increase the sensitivity of D2 receptor to the ligands while some studies have observed that the availability of D2R was consistently decreased in the striatum of addicted individuals [Edwards et al., 2007; Bailey et al., 2008; Volkow et al., 2007].

cAMP-response-element-binding protein (CREB) is a well-known transcription factor which is crucial for the transmission of events that occurred at the cytoplasmic membranes to the nucleus where alters gene expression. Several lines of documented evidence suggested that CREB plays an important role in cocaine addiction. CREB in NAc appears to regulate the rewarding and aversive effect of cocaine (Nestler et al., 2004). Specifically, CREB activation in the NAc counteracts drug reward and increase drug aversion using the conditioned place preference (CPP) behavior test. However, recent studies of CREB involvement in cocaine self-administration – the gold standard in this field – indicated that CREB activity in the NAc shell increase the motivation of cocaine [Larson et al., 2011]. Stimulation of

dopamine receptors is believed to regulate the CREB activation through regulating the PKA levels as well as other kinases [William et al., 2005].

When the addicted experimental animals or human cocaine users are under abstinence of cocaine, the propensity to relapse often increases after the prolonged drug withdrawal stage [Neisewander et al., 2000; Grimm et al., 2001; Lu et al., 2004a,b; Kosten et al., 2005]. These observations revealed that cue-induced cocaine seeking increase between day 1 and day 90, and it could finally return to basal level by 6 months. These facts evidenced that the neuroadaptations induced by cocaine addiction do not disappear with abstinence of cocaine; instead, it seems that the neuroadaptations exacerbate or at least persist during the withdrawal period.

A previously study in our lab observed that the D2R selective agonist induced enhance of CREB phosphorylation in the nucleus accumbens is strongly potentiated by the chronic cocaine self-administration [Haffmann et al., 2011]. As the withdrawal period in the previous study applied was 1 day, plus it didn't differentiate the NAc shell and core subregions, in addition, it didn't check this phenomenon in the other subregions of the striatum. Thus the aim of the present study is to expand the previous study by extending the withdrawal period and expanding the brain regions that will be checked.

Objectives

In this part, we propose:

1 To compare the number of pCREB positive cells in rat brain striatal subregions in control and chronic cocaine self-administration rats;

2 To estimate the influence of short-term and long-term withdrawal from chronic cocaine self-administration on the basal number of pCREB positive cells;

3 To assess the response to the D2R agonist quinpirole applied *ex vivo* through measuring the changes in pCREB positive cells in both control and cocaine withdraw

rats;

4 To determine whether the neuoradaptations induced by short-term withdrawal period persist beyond a long-term withdrawal period.

Methodology

The procedure for building chronic cocaine self-administration model and the *ex vivo* brain slices incubation have been introduced in the section of experimental in the theis and in the previous studies in our lab [Haffmann et al., 2011]. To expand the previous result obtained in our lab, we divided the cocaine addition rats into two groups: rats that experience 1 day withdraw period and rats that experience 5 weeks withdraw period. To check the D2R agonist induced effect on CREB phosphorylation, instead of using western blot technique which is difficult to differentiate the brain regions, we use the immunohistochemistry technique. Briefly, rat brains were sliced into 0.5mm thick slices and incubated *ex vivo*. Quinpirole or vehicle was added to the ex vivo slice incubation system at the end of the 4-hour-long period of pre-incubation and left co-incubated with the slices for 10 minutes before terminating the incubation. After the fixation, all the slices were sectioned into thinner slices (16 μ m) which are fit for immunohistochemistry. The phospho-CREB was stained and the slices were imaged under microscope. Finally the number of immunoreactive neurons in different striatal sub-regions were recorded and compared. The comparisons involved different sub-regions, among the sham rats and cocaine addiction withdrawal rats, as well as between quinpirole (QUIN, at 10μ M) treated and vehicle-treated slices. Through comparison, we find the number of pCREB positive neurons in sham rats and naïve rats in each brain region are statistically equal to each other, so we combine the data obtained in sham and naïve rats as the blank control for our comparison.

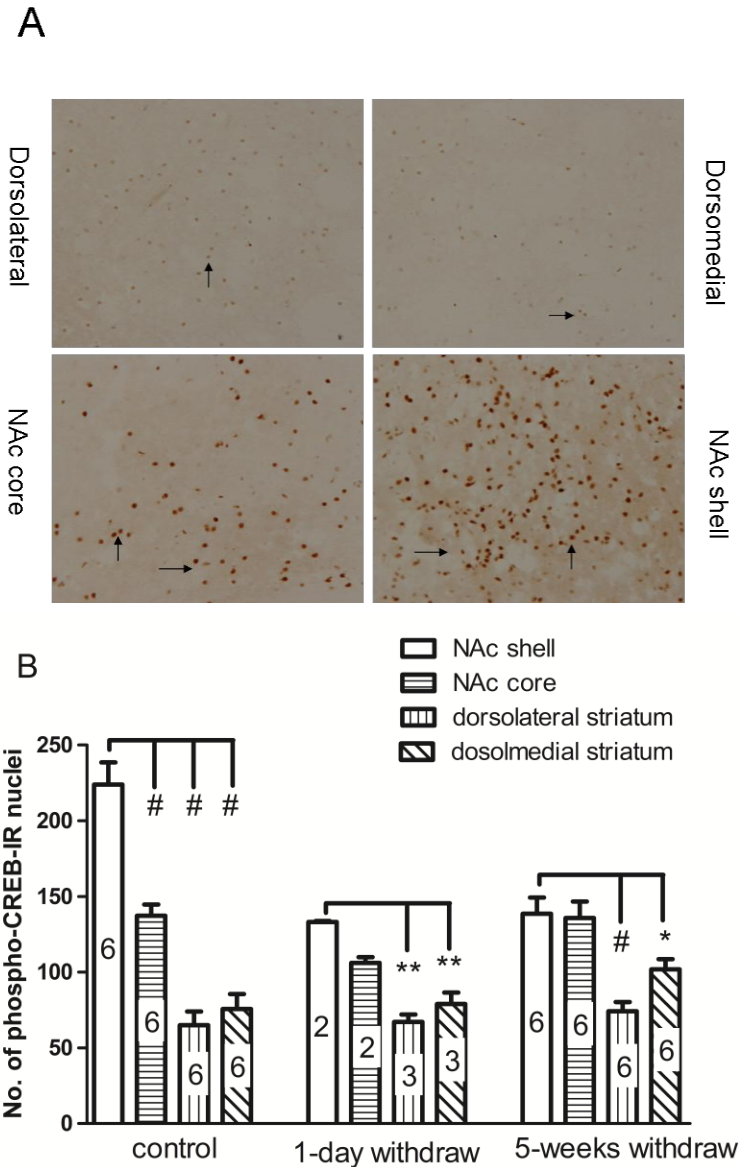


Figure 1 A): Photomicrographs (20×) of phosphorylated CREB immunoreactivity nuclei neurons in the nucleus accumbens (NAc) shell, NAc core, dorsolateral and dorsomedial striatum at +1.8 mm from Bregma. All photos are taken from the same slice. Arrows point to the phospho-CREB-IR nuclei. The real area for each photomicrograph is 5.95μ m^2. **B):** Numbers of pCREB-IR nuclei neurons in different sub-regions of rat brain striatum obtained from control and cocaine withdrawal rats. The three separate groups of bars represent the sham rats, rats that are 1-day withdrawal and 5 weeks withdrawal from the chronic cocaine self-administration (6-11 months of cocaine self-administration). Values in each bar was expressed as means± SEM of the average number of pCREB-IR nuclei neurons from the four striatal sub-regions showed in figure 35A. Statistic analysis of data

was done by the one-way ANOVA. Symbols in each bar represent the experiment rats number used. *p <0.05, **p <0.01 and $^{\#}p$ <0.001 compared between the connected two groups.

Results

Regional differences in the number of the phospho-CREB-immunoreactive neurons were observed both in sham controls and cocaine self-administration withdrawal rats, and cocaine self-administration seems to bridge this difference.

Striatum is a heterogeneous structure which could be subdivided into several different regions. The dorsal striatum and the ventral striatum (also called nucleus accumbens, NAc) are the main subregions of striatum. In drug addiction process, both regions are identified to perform different but important roles. In our study, we found that in the non-quinpirole treated groups, the number of pCREB positive neurons in nucleus accumbens shell was significantly higher than that in dorsal striatum either in sham controls or in cocaine withdrawal rats (Figure 1 A and B). Focusing on the nucleus accumbens shell, we observed a sharp descend in the number of pCREB-IR nuclei neurons after chronic cocaine self-administration. Moreover, this downwards remained for the whole withdrawal period (1-day withdrawal: SEM=40.5±0.3% and 5-weeks withdraw: SEM=38.1±4.8% *vs* sham rats. Figure 2A and B).

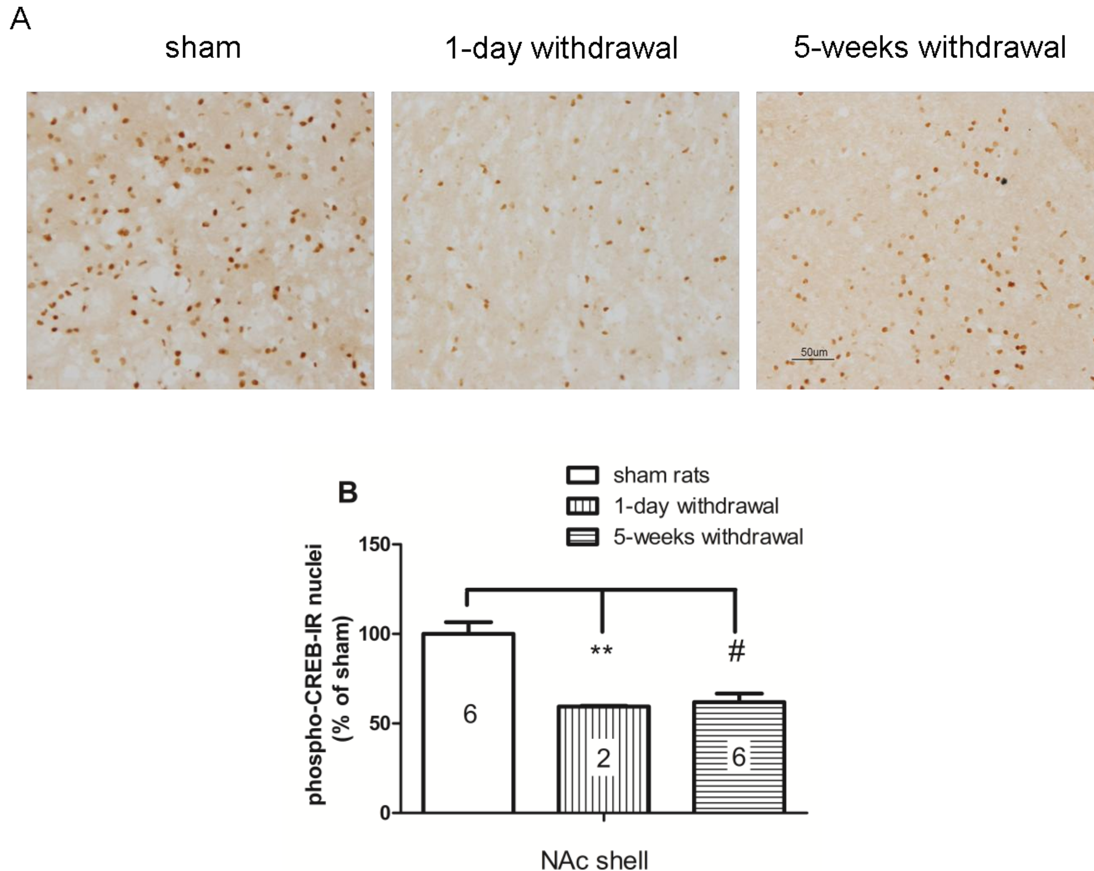


Figure 2 A) Example of Photomicrographs (20×) of pCREB-IR nuclei neurons in the nucleus accumbens (NAc) shell obtained from sham rats, 1-day withdrawal cocaine rats and 5-weeks withdrawal cocaine rats. B) Comparison among the number of pCREB-IR nuclei neurons in sham and cocaine withdrawal rats in the NAc shell. Values in each bar was expressed as means± SEM of the average number of pCREB-IR nuclei neurons from the four striatal sub-regions showed in figure 35A. Statistical analysis of data was done by one-way ANOVA. Symbols in each bar represent the experiment rat number used. ***p* <0.01 and *#p* <0.001 compared between the connected two groups.

Withdrawal from chronic cocaine self-administration alters the response to a dopamine D2 receptor agonist in a regional-selective fashion

Previous results obtained by Self's group have suggested that drug-induced up-regulation in cyclic AMP (cAMP)-protein kinases A (PKA) signaling in the nucleus accumbens contributes to the escalating drug intake and a propensity for relapse by

differentially altering the sensitivity of D1 and D2 dopamine receptors [Self et al. 2004]. The CREB phosphorylation level could be regulated by the cAMP-PKA signaling system, thus, we wanted to know whether chronic cocaine administration alters the regulation of pCREB levels by a D2R agonist. Our results are organized by sub-regions as defined above.

Table 1 Effects of quinpirole treatment on CREB phosphorylation in NAc shell in sham rats, and rats that are 1-day or 5-weeks withdrawn from chronic cocaine self-administration.

NAc shell	sham	1-day withdrawal	5-weeks withdrawal
control	223.8±14.6 6(13)	133.1±0.6 2(4)	138.5±10.7 6(16)
quinpirole	142.3±22.4 6(17)	157.0±7.3 2(6)	162.9±14.1 6(23)
effect trend	↓	↑	↑
statistic	*P*<0.05 of *spss* analyse for two factors interaction		

Data are expressed as mean of every photomicrograph from the same brain region of the same cocaine and ligand treatment; total numbers of photos range from 21-120 for all groups.

NAc shell

In the NAc shell, QUIN-treated slices tended to decrease pCREB-immunoreactive nuclei neurons compared with vehicle-treated slices in control rats (Table 1). However, when rats experienced cocaine self-administration, the response to QUIN changed: in the slices obtained from the 1-day and 5-weeks cocaine withdrawal rats, QUIN increased the number of pCREB-IR nuclei neurons. When we analyzed the statistics of the influence of these two factors (cocaine administration and QUIN activation) with SPSS, we got a statistical significance in the interaction of these two factors, which means quinpirole elicits a different and significant effect on CREB phosphorylation that depends on history of cocaine self-administration (Table 1).

Table 2 Lack of effects of quinpirole treatment on CREB phosphorylation in NAc core in sham rats, and rats that are 1-day or 5-weeks withdrawn from chronic cocaine self-administration.

NAc core	sham	1-day withdrawal	5-weeks withdrawal
control	137.3±7.3 6(23)	106.1±3.9 2(5)	135.7±10.8 6(20)
quinpirole	108.0±11 6(22)	132.7±18.7 2(7)	117.6±6.7 6(23)
effect trend	=	=	=
statistic	*P*>0.05 of *spss* analyse for two factors interaction		

Data are expressed as mean of every photomicrograph from the same brain region of the same cocaine and ligand treatment; total numbers of photos range from 21-120 for all groups.

NAc core

Unexpectedly, no statistically significant effect of quinpirole were observed within this area (Table 2).

Dorsal striatum

The dorsal striatum includes the dorsomedial and dorsolateral striatum. In these two subregions, we observed a consistent alteration of dopamine receptor signaling elicited by cocaine self-administration. QUIN tended to increase the number of pCREB-IR nuclei neurons in control rats, however, it tended to decrease this number in rats either after 1-day or 5-weeks withdrawal from the chronic cocaine administration. The statistically analyses yielded significance of the interaction between quinpirole and cocaine withdrawal factors (Table 3 and 4). Of note, this interaction is opposite to that previously observed in the NAc shell.

Table 3 Effects of quinpirole treatment on CREB phosphorylation in dorsolateral in sham rats, and rats that are 1-day or 5-weeks withdrawn from chronic cocaine self-administration.

dorsolateral	sham	1-day withdrawal	5-weeks withdrawal
control	64.9±9.1 6(23)	67.1±9.1 3(9)	74.2±6.1 6(24)
quinpirole	77.4±9.1 6(22)	52.7±6.6 3(10)	64.6±3.5 6(29)
effect trend	↑	↓	↓
statistic	*P*<0.05 of *spss* analyse for two factors interaction		

Data are expressed as mean of every photomicrograph from the same brain region of the same cocaine and ligand treatment; total numbers of photos range from 21-120 for all groups.

Table 4 Effects of quinpirole treatment on CREB phosphorylation in dorsomedial in sham rats, and rats that are 1-day or 5-weeks withdrawn from chronic cocaine self-administration.

dorsomedial	sham	1-day withdrawal	5-weeks withdrawal
control	75.6±9.8 6(18)	79.0±7.4 3(9)	101.8±6.8 6(24)
quinpirole	92.8±8.4 6(21)	60.0±2.8 3(10)	77.5±6.8 6(29)
effect trend	↑	↓	↓
statistic	*P*<0.05 of *spss* analyse for two factors interaction		

Data are expressed as mean of every photomicrograph from the same brain region of the same cocaine and ligand treatment; total numbers of photos range from 21-120 for all groups.

Discussion

Our study is methodologically novel, as it uses *ex vivo* incubation of the brain slices from sham and cocaine withdrawal rats and finally use the immunohistochemistry technique to label the phosphorylated CREB-immunoreactive nuclei *in situ*. First, it is important to point out that our cocaine withdrawal groups are composed of rats that are well motivated by the drug. Our rats had a mean drug intake of 11.5 mg cocaine per Kg of body weight during 46 sessions of self-administration (Table 1 in the experimental) and 7 out of the 9 rats reached FR5. Second, at the time to sacrifice of experimental rats, our withdrawal period (minimal 24 hours) ensures that cocaine was completely metabolized (Azar et al. 1998, Telang et al., 1999), so no effects should come from cocaine itself. Finally, we perform a very long incubation time (>4 hours) to stabilize the brain slices, and to discard any possible non-specific effects due to the brain processing. We evidenced that the levels of positive neurons are comparable between naïve rats (younger, untrained rats) and sham rats (of similar age compared with cocaine withdrawal rats, subjected during late adolescence to handling, sucrose-training, partial food restriction and surgery). Taken together, the amounts of neurons with phospho-CREB immunoreactivity nuclei should reflect either basal levels or response to quinpirole.

The regional differences between NAc shell and dorsal striatum in the amount of neurons with phospho-CREB immunoreactivity nuclei we observed was once showed in the paper authored by Mattson BJ between brain striatum Caudate-putamen and nucleus accumbens [Mattson et al., 2005]. However, in that paper Mattson didn't apply the comparison between these two sub-regions, neither showed the statistically difference between them, moreover, they didn't differentiate the core and shell subregions in the nucleus accumbens. As a supplement to Mattson's result, later research that carried out by Culin and colleagues did not find statistically difference between shell and core in the number of pCREB positive cells [Culm et al., 2004]. Thus our results supply firm evidence for the regional-difference of CREB activation

state on statistically level. The NAc shell receives more limbic imputs from the cortex than the other striatal subregions, and sends outputs to amygdela and related limbic structures. However most of there connections may have been cut during the slice preparation. This fact, together with the 4 h stabilization period after slicing, makes unlikely that the higher number of basal pCREB positive nuclei in the NAc shell is related to precious behavior. It is more likely that such higher basal number of pCREB positive cells is related to local characteristics or short connections within the slice that may trigger cascades controlling CREB phosphorylation. The fact that a similar result was previously observed by Mattson and colleagues [Mattson et al., 2005] using classical immunohistochemistry after paraformaldehyde perfusion indicates that basal pCREB activation in the NAc is not controlled by long distance neuronal circuits. Considering the important role of CREB activation in learning and memory, and moreover, in depression and anxiety [Carlezon et al., 2005], our result may be relevant for the functional differences of striatal subregions we have mentioned in the introduction.

Literature evidence has confirmed that cocaine administration could enhance the cocaine dependent activation of the transcription factor CREB in the nucleus accumbens [Mattson et al., 2005] and striatum as well [Hollander et al., 2010]. On the other hand, CREB also plays a very important role in regulating the motivational aspects of cocaine addiction. As mentioned in the introduction, using the behavioral place preference test it has been shown that enhanced CREB activation in the NAc decrease animals' sensitivity to the rewarding effects of morphine and cocaine, whereas down-regulation of CREB function has the opposite effect [Carlezon et al., 1998; Barrot et al., 2002]. In contrast, result observed with cocaine self-administration model point to a potentiating role of NAc CREB (Larson et al., 2011). In our study, we observed that after 1-day or 5-weeks of withdrawal from chronic cocaine administration, the numbers of neurons with phospho-CREB immunoreactivity nuclei in the NAc shell are largely decreased (nearly 50%) compared with the control rats. The precise role of CREB in drug addiction needs further results. However the

persistence of pCREB changes through both short and long withdrawal periods may correlate with long-lasting neuroadaptations in individuals with a special state after abstinence of cocaine intake. The characteristic of addicted individuals under abstinence is that they are much more sensitive to the drugs than naïve individuals. This state usually lasts a long period and in our opinion it may contribute to the liability of the serious relapse experienced by the drug addicted individuals.

In contrast to the previous study in our lab, we failed to observe the potentiating effect of cocaine on D2R activation elicited up-regulation of CREB activation appeared in 1 day withdraw rats, however, we observed a cocaine induced alteration of CREB activation in response to the D2R selective agoinst, moreover, we found this effect of cocaine is in regional-selective fashion. Although we use the same cocaine self-administration model and the same *ex vivo* slice incubation system, however, in order to differentiate the striatal sub-region, instead of using Western blot technique, we use immunohistochemistry. The inconsistent result may due to that Western blot measure the mount of the phosphorylated CREB protein, however, the immunohistochemistry enables to count the pCREB positive cells. In addition, the phospho-CREB antibody used in western blot and immunohistochemistry experiment also recognizes the phosphorylated form of the CREB-related protein activatin transcription factor-1 (AFT-1), and in western blot, this protein could be separated from pCREB protein, however, immunohistochemistry experiments can not distinguish them, thus the pCREB positive cells we count may partly be the phosphorylated AFT-1 positive cells.

In striatum, D1R and D2R are widely expressed and segregated into the two main subtypes of brain striatum principal neurons: D1-enriched direct pathways (dSNPs) and D2-enriched indirect pathways (iSNPs) [Le and Bloch, 1995]. However, about 17% of the MSNs in NAc shell as well as 5-6% of MSNs in dorsal striatum have been observed to co-express both D1R and D2R [Bertran-Gonzalez et al. 2008, 2010; Matamales et al., 2009]. The co-expression of both dopamine receptors provide a

good chance for their heteromerization, moreover, the presence of the D1-D2 receptor heteromer was demonstrated by different techniques in both brain striatum and transfected cells that co-express D1R and D2R [Lee et al., 2004; Hasbi et al., 2009]. In our work, we do not know wheter pCREB is activated by quinpirole in D2 positive/D1 negative cells or in D2 positeve/D1 positive cells. Neverthe less, we assume that the action of quinpirole occurs in D2 positive cells, as the simpler explanation of a response to a D2 agonist.

CREB is activated through phosphorylation at Ser133 which stimulate the recruitment of CBP and thereby lead to activation of gene transcription. CREB can be phosphorylated through several different kinases including cyclic AMP-dependent protein kinase (PKA) [Montminy and Bilezikjian 1987], and calcium/calmodulin-dependent kinase (CaMKs Ⅰ, Ⅱ and Ⅳ) which is activated by increased calcium influx [Sheng et al. 1991; Matthews et al. 1994], as well as Erk 1/2, Akt/PKB and PKC (Brami-Cherrier et al., 2002; Yan et al 1999). Notably, CAMKⅡ induce the phosphorylation of CREB at serine 142, but in our study we only measure the phosphorylation at serine 133 [Matthews et al. 1994; Wu et al., 2001]. Previous study suggested that the up-regulation of CREB activation induced by acute cocaine administration is acting through the PKA pathways stimulated by extracellular DA [Ron and Jurd, 2005]. In the dorsal striatum, a microRNA was found to amplify cAMP/CREB signaling after cocaine self-administration [Hollander et al., 2010]. The two main dopamine receptor subtypes expressed in striatum, D1R and D2R, oppositely regulate the accumulation of cAMP. D1R facilitate the cAMP accumulation thus increase the activation of PKA, while D2R inhibit cAMP accumulation then lead to the decline of PKA activation level. The D1-D2 heteromer activation induces distinct signal pathways through coupling to Gq/11 and finally causes the phospholipase C (PLC) stimulation and intracellular calcium release. The later one could increase the levels of both the CaMKⅡ and Ⅳ[Lee et al., 2004]. At this point, the simplest explanation for our results is that quinpirole stimulates $D1^{+}/D2^{+}$ cells to increase calcium levels and subsequently CREB is phosphorylated. Alternatively, quinpirole

may act on D1-/D2+ cells to decrease cAMP/PKA this decreasing CREB phosphorylation signal transduction associated with these pathways may suffer adaptations by chronic cocaine consumption.

In summary of the last section, it seems that CREB phosphorylation could be regulated by dopamine system through the following pathways: a) facilitated through stimulating the D1R; b) inhibited through stimulating the D2R; c) facilitated through activation of the D1-D2 heteromer. QUIN is a typical agonist of D2R, thus theoretically treatment with QUIN should inhibit the CREB phosphorylation through activating D2R. However, a previous study once observed that repeated QUIN treatment significantly increase the CREB phosphorylation levels in nucleus accumbens [Culm et al., 2004]. So we hypothesize that there exist a balance in regulating the phosphorylation of CREB among these pathways and the infusion of the exogenous QUIN breaks this balance and consequently changes the CREB phosphorylation level. The results that we obtained in this study fit well with this hypothesis, that is: in control rats, we observed an inhibitory effect of QUIN on CREB phosphorylation in nucleus accumbens while a stimulatory effect in dorsal striatum, this is difficult to explain simply by only one signal pathway and even more when chronic coaine self-administration reverses this tendency.

Cocaine addiction has been reported to have various influences on dopamine systems including alterations in the extracellular DA concentration, the D1R and D2R density and G-protein binding sensitivity in striatum [Anderson and Perice, 2005]. All these alterations are definitely going to change the previous balance in regulating CREB phosphorylation. For instance, the decreased extracellular DA concentration that may follow withdrawal of chronic cocaine administration would reduce the activation of all three pathways. However, two of them (D1R and D1-D2 heteromer) facilitate CREB phosphorylation, so this may contribute the decline in the numbers of the phospho-CREB-IR nuclei neurons in cocaine withdrawal rats (Fig. 2B). However, remember that our slices were preincubated for 4 h ex vivo before pCREB

immunohistochemical dyeing, during this time, extracellular dopamine should be in function of the state of high affinity dopamine removal, no dopamine neuron activity may have participated as afferents had been sliced. So far, there is still no consistent opinions about the D1R and D2R density alteration after chronic cocaine administration, up-regulation, down-regulation and no change have all been obtained related to the density of these two receptors. Nevertheless, the proportion of the two receptors plays a critical role in the balance we hypothesized. The alteration of the density of either receptor will change the balance into another phase (we can call it balance 2). It's possible to that this balance 2 could react differently to the infusion of exogenous D2R agonist QUIN (Tables1-4). This new balance seems to persist beyond the long-term withdrawal period in all the striatum except in the NAc core subregion.

In summary, we observed a regional difference in the basal levels of CREB phosphorylation either in sham rats or in chronic cocaine administration withdrawal rats. In addition, we found that after chronic cocaine self-administration, the CREB phosphorylation levels tended to declined in NAc shell compared with control rats. More importantly, we found a different effect of QUIN treatment on CREB phosphorylation levels in withdrawal rats compared with sham rats in all the sub regions of brain striatum. We hypothesize that this difference may occur because: primarily there exists a balance of the dopamine receptors D1R, D2R and D1-D2 heteromer which could maintain the levels of CREB phosphorylation. Secondly, the infusion of any exogenous DA receptor ligands lead to the alteration of the CREB phosphorylation level. Lastly, repeated cocaine administration seems to change the pervious balance and a new balance (which is named as balance 2) that could react differently to the exogenous compounds (QUIN) may form under the regulation of cocaine. This alteration caused by chronic cocaine is long lasting in all striatum except the NAc core where our results did not reach statistical significance.

Conclusion

1 We observed both in sham rats and cocaine withdrawal rats that the basal number of pCREB positive cells is much higher in the NAc shell than in dorsal striatum;

2 The basal number of pCREB positive cells in NAc shell is significantly higher than in the NAc core in sham rats but not in cocaine withdrawal rats.

3 In the NAc shell subregion, QUIN tends to inhibit the activation of the transcription factor CREB. However, in short-term and long-term cocaine withdrawal rats, QUIN turns to facilitate the CREB activation.

4 In dorsal striatum, QUIN tend to facilitate the activation of the transcription factor CREB. However, in short-term and long-term cocaine withdrawal rats, QUIN revised to inhibit the CREB activation. This pattern is opposite to that observed in the NAc shell.

GENERAL DISCUSSION

General discussion

The efficacy of the atypical antipsychotic aripiprazole on D2 autoreceptor

For both the first and second generations of antipsychotics, their treatment has always been connected to D2 receptors, mostly perform full antagonism or partly antagonism effects on D2 receptors. The newer antipsychotic aripiprazole, instead of being a D2R antagonist, is most believed function as a D2R partial agonist. However, increasing evidence replace this “partial agonist” opinion with a “functional selectivity” hypothesis proposed by Lawer et al [Lawler et al 1999]. “Functional selectivity” describes the characteristic of drugs which cause markedly different signaling trough a single receptor [Mailman and Murthy, 2010], so in the case of aripiprazole, it explains the efficacy variation of aripiprazole on D2R from agonism, partial agonism to antagonism depending on the surrounding milieu as well as the test function chosen [Shapiro et al., 2003].

In our study we observed an efficacy switch of aripiprazole on D2 autoreceptors from agonist to antagonist when increased the potassium concentration in the milieu. We believed the especially low extracellular DA concentration under the low potassium condition and the increased DA concentration triggered by potassium are account for the efficacy switch. The antagonist effect on D2R is thought to control the positive symptom of schizophrenia; in addition, the agonist effect on the D2 autoreceptors could also be benefit for the positive symptom due to its inhibition on DA release and on dopaminergic neuron firing [Neve et al., 1997]. The good aspect for the ligands which are D2 autorecptors agonists on controlling the positive symptom is that they are less liability to cause the adverse effects induced by the prolonged antagonism of D2 receptors. This may account for the low liability to adverse effects of aripiprazole. On the other hand, however, the variability of aripiprazole’s efficacy may give rise to individual differences occurred in the therapy.

Identification of crosstalk between D2R and $GABA_B$ receptor, between D2R and

orexin-1 receptor, but not between D2R and D5R in rat brain striatum.

Based on the technique we applied in our study, actually we are testing the interaction between D2 autoreceptors which control the DA synthesis and the other three receptors. There is no clear evidence that reveal the localization of both $GABA_B$ receptors and orexin receptors on dopaminergic terminals in the striatum, however solid evidence showed both $GABA_B$ receptors and orexin receptors are expressed in dopaminergic neurons in VTA, a major brain region which send dopaminergic projection to the striatum. In addition, the distribution of them in striatum enhanced the probability of their localization on dopaminergic terminals in the striatum. In our study, we observed effects on the DA synthesis elicited by the two types of receptors.

The functional interaction of $GABA_B$ receptors and D2 autoreceptors at the dopaminergic terminals first further confirmed the localization of $GABA_B$ receptors in the dopaminergic neurons in the striatum; and second, this result may suggest that $GABA_B$ receptors participated in the various neuropsysiologies and neuropharmacologies that are traditionally D2 dopamine receptor involved, for instance, the reward pathway, the schizophrenia or drug abuse. Previous studies proposed that the $GABA_B$ agonist baclofen could decrease cocaine self-administration across a wide range of schedules of reinforcement and access conditions [Roberts and Brebner, 2000]. The effect of baclofen is qualitatively different from that produced by DA drugs, neither due to a uniform change in self-administration rat. One explanation for its effect is that baclofen may attenuate the reinforcing effects of cocaine without influencing the interoceptive cues used to titrate intake [Roberts and Zito, 1987]. Our results suggest that this effect may also function partly through the interaction of $GABA_B$ receptors with D2 autoreceptors. Thus far, most of the studies which relate $GABA_B$ receptors with drug abuses are about the receptor agonist, however, in our study, we found that the receptor antagonist CGP 54626 enhanced the DA synthesis through D2 autoreceptors, our result may open a new window for the further study in the drug addiction field.

Similarly, the possibly localization of orexin receptors was the major force for our study in their heteromerization. However, respect to orexin receptors, there is one more reason that is the hypocrietin-orexin system seems to participate in the regulation of cocaine addiction by means of acting on the mesolimbic dopamine system [Espana et al., 2010, 2011]. The inhibition effect of the orexin-1 receptor agonist on DA synthesis we observed is quite new in the orexin field. However based on the result we obtained, the only conclusion we could make is that the downstream signal pathway elicited by D2R may be affected by the orexin-1 receptor stimulation induced signals. Nevertheless, our results suggest in the striatum the crosstalk between these two kinds of receptors occurs; further more, our results are consistent with the previous result which may explained that the regulation effect of the hypocrietin-orexin system on cocaine addiction may happen at the molecular level that the stimulation of orexin receptors influences the downstream signaling of D2 dopamine system.

The D2-D5 receptor heteromer has been observed by the previous research [So et al., 2009] and based on the principal that GPCRs of the same type or belong to the types that are highly homologus are easy to form oligomers [Milligan et al., 2008, 2009]. However we didn't observe any clue that suggests there are interactions between these two subtypes of dopamine receptors let alone the heteromerization between them. We could explain our results in two aspects: 1) the D5R density in the striatum is quite low, and even lower on the dopaminergic terminals, so the signal elicited by the activation of D2-D5 heteromer may too low to detect in our assessment system; 2) the heteromerization may not exist on the dopaminergic neurons.

The in vivo balance constituted by dopamine receptors in the striatum is affected by chronic cocaine self-administration

Dopamine system and CREB activation are both believed to play important roles in cocaine addiction, at the same time, chronic cocaine consume would lead to changes in the dopamine system or state of CREB activation. CREB is best known for its roles

in learning and memory which are believed to play important roles in aspects of addiction. The mesocorticolimbic dopamine system is a key mechanism involved in drug reward and reinforcement, a system that involves dopaminergic projections from mesencephalon that synapse onto the nucleus accumbens, striatum, aymgdala and prefrontal cortex, which are brain-regions with widely CREB expression [Mcpherson and Lawrence, 2007; and references within].

In our study, we observed a regional difference of basal CREB phosphorylation levels in the brain striatum. Specifically, the CREB phosphorylation level in the nucleus accumbens especially in NAc shell is much higher than that in the dorsal striatum. Our results provide a basic knowledge that may explain the functional differences CREB played in different striatum sub-regions. Focus on the nucleus accumbens, we observed the basal CREB phosphorylation level was reduced largely (by nearly 50%) after long-term cocaine administration and was not recovered at the end of 5-weeks withdrawal period (Figure 2, Result chapter 4). The decline of CREB activation level could enhance the sensitivity of the addicted individuals to the drugs they used to consume [Carlezon et al., 1998; Barrotet al., 2002]. Thus the low levels of CREB phosphorylation appeared after abstinence of cocaine may exactly reflect the strong desire of the addicted individuals to cocaine. Besides, the increased sensitivity of rats to cocaine may also induce severe relapse when cocaine is back to available to them.

Last but most important, we found the exogenous D2R agonist quinpirole treatment leads to different effects on CREB phosphorylation levels in sham and chronic cocaine self-administration rats. We proposed a hypothesis of dopamine receptor constituted balance to understand our result.

The hypothesis of the dopamine receptor balance in brain striatum

In the striatum, the two main subtypes of dopamine receptor D1R and D2R are widely but most separately distributed in the proximately equally sized iSNPs and

dSNPs and [Surmeier et al., 2011; Le and Bloch, 1995]. Several studies tried to evaluation the proportions of striatal neurons expressing D1R, D2R or both have all failed however they observed a fraction of MSNs (17%) co-express both receptors in NAc shell, as well as 5-6% in dorsal striatum [Bertran-Gonzalez et al. 2008, 2010; Matamales et al., 2009]. Moreover, presence of the D1-D2 receptor heteromer was demonstrated by different techniques in both brain striatum and transfection cells co-expressed D1R and D2R [Lee et al., 2004; Hasbi et al., 2009]. In addition, another group of data revealed that the striatum neurons are under dense innervations of dopaminergic neurons [Matsuda et al., 2009], as well as up to 10% of the striatal synapses are participated by dopaminergic terminals where DA are released [Groves et al. 1994]. The extracellular DA could stimulate all the receptors including D1R, D2R and even D1-D2 heteromers (there may also exist D1 homomer and D2 homomer, which are classified into D1R and D2R), and finally regulate the CREB phosphorylation. Summary from the above knowledge, although we don't know the exact proportion of each component, we believed that in the striatum, a balance of the dopamine receptor composed signaling may exist. The infusion of exogenous QUIN could stimulate D2R, displace DA in D2R binding, activate D1-D2 heteromer together with DA, and lead to activation of reserved D1R and D2R. So the QUIN administration would break this balance and then change the CREB phosphorylation. Cocaine addiction has a profound influence on dopamine system. Cocaine could block the dopamine transporter (DAT), inhibit the DA uptake thus increase the extracellular DA concentration. Long-term cocaine administrations then have profound influence on the dopamine receptors and finally will adjust the balance to reach another stable phase that we call it balance 2. Balance 2 may have different proportion of each component, and may also under different stimulation of the extracellular DA concentrations. We suppose it is not difficult to understand that balance 2 reacted differently to the exogenous compound quinpirole, as we observed in our study. Researches related to each of the component be affected by chronic cocaine administration have been reported, yet no consistent opinions about that have been obtained. In our study, we didn't monitor the exact change of each component, but

instead we observed a macroscopic change that may caused by any of the component or combine.

CONCLUSION

Conclusion

1, the mechanism of antipsychotics related to D2 autoreceptors: results about the atypical antipsychotic, aripiprazole

- We validated an *ex vivo* assessment system to monitor the DA synthesis velocity regulated by D2 autoreceptors, and by confirming the capacity of a traditional D2R agonist, quinpirole, to induce a concentration dependent inhibition of DA synthesis velocity in rat brain striatal miniprisms.

- By means of this assessment system, we observed a reversal of the efficacy of the atypical antipsychotic, aripiprazole. Aripiprazole acts as a full D2R agonist like quinpirole under low dopaminergic tone while as an antagonist under increased dopaminergic tone provoked by DA release which is triggered by increasing the potassium concentration in the incubation buffer.

- We also observed a non-D2 inhibitory effect elicited by aripiprazole on DA synthesis.

2, crosstalks between D2 receptor and other receptors.

- Using the same *ex vivo* DA synthesis assessment system, we observed an inhibitory effect and elicited by a $GABA_B$ receptor agonist and a facilitation effect elicited by a $GABA_B$ receptor antagonist on DA synthesis, which were both counteracted by the D2R antagonist sulpiride. This observation could be attributed to a crosstalk or even a heteromer formed between GABAB receptor and D2 receptor.

- The agonist of orexin-1 receptor inhibited DA synthesis, and this effect was blocked by both the orexin receptor antagonist and the D2R antagonist. However, the orexin antagonist could not counteract the effect elicited by the D2R agonist. All these data suggest that there exists a crosstalk between these two receptors,

yet this evidence is insufficient to suggest the heteromerization between these two receptors.

- We didn't observe any effect of the agonist of D5R (SKF 38393) or any synergism between this compound and the D2R agonist quinpirole on DA synthesis in our assessment system. Two possibility may explain this result: 1) there is no crosstalk between these two receptor in regulating DA synthesis; 2) the D5R density in brain striatum is much lower than that of the D2R, even if there is a crosstalk between these two receptors, it still would be difficult to detect the downstream signal elicited by the crosstalk.

3, neuroadaptations of D2R signaling induced by withdrawal from chronic cocaine self-administration.

- We found a regional difference in the basal number of phospho-CREB immunoreactive nuclei in striatum in both sham and chronic cocaine withdrawal rats. Specifically the pCREB level in nucleus accumbens shell is significantly higher than that in dorsal striatum. However the difference is less pronounced after chronic cocaine self-administration.

- In the nucleus accumbens shell, the D2R agonist quinpirole induced different response in sham rats compared with cocaine withdrawal rats. Specifically it decreased the number of phospho-CREB immunoreactive nuclei in sham rats while increased this number in both acute and long-term withdrawal rats. However, the exact opposite effect is found in the dorsal striatum. In this brain region, quinpirole increased the number of phospho-CREB immunoreactive nuclei while decreased this number in both acute and long-term withdrawal rats.

References:

Abdolmaleky HM, Cheng K-h, Faraone SV, Wilcox M, Glatt SJ, Gao F, Smith CL, Shafa R, Aeali B, Carnevale J, Pan H, Papageorgis P, Ponte JF, Sivaraman V, Tsuang MT, Thiagalingam S (2006) Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. Hum Mol Genet 15:3132-3145.

Agnati LF, FerréS, Lluis C, Franco R, Fuxe K (2003) Molecular Mechanisms and Therapeutical Implications of Intramembrane Receptor/Receptor Interactions among Heptahelical Receptors with Examples from the Striatopallidal GABA Neurons. Pharmacol Rev 55:509-550.

Albin RL, Young AB, Penney JB (1989) The functional anatomy of basal ganglia disorders. Trends Neurosci 12:366-375.

Albizu L (2010) Heteromerization of G protein-coupled receptors: relevance to neurological disorders and neurotherapeutics. CNS & neurological disorders drug targets 9:636.

Albizu L, Holloway T, González-Maeso J, Sealfon SC (2011) Functional crosstalk and heteromerization of serotonin 5-HT2A and dopamine D2 receptors. Neuropharmacology 61:770-777.

Alderson HL, Parkinson JA, Robbins TW, Everitt BJ (2001) The effects of excitotoxic lesions of the nucleus accumbens core or shell regions on intravenous heroin self-administration in rats. Psychopharmacology 153:455-463.

Alleweireldt AT, Kirschner KF, Blake CB, Neisewander JL (2003) D1-receptor drugs and cocaine-seeking behavior: investigation of receptor mediation and behavioral disruption in rats. Psychopharmacology 168:109-117.

Anderson S, Pierce R (2005) Cocaine-induced alterations in dopamine receptor signaling: implications for reinforcement and reinstatement. Pharmacol Therapeut 106:389-403.

Anzalone A, Lizardi-Ortiz JE, Ramos M, De Mei C, Hopf FW, Iaccarino C, Halbout B, Jacobsen J, Kinoshita C, Welter M (2012) Dual control of dopamine synthesis and release by presynaptic and postsynaptic dopamine D2 receptors. The Journal of Neuroscience 32:9023-9034.

Arbuthnott GW, Wickens J (2007) Space, time and dopamine. Trends Neurosci 30:62-69.

Arrang J-M, Morisset Sv, Gbahou F (2007) Constitutive activity of the histamine H3 receptor. Trends Pharmacol Sci 28:350-357.

Aston-Jones G, Smith RJ, Moorman DE, Richardson KA (2009) Role of lateral hypothalamic orexin neurons in reward processing and addiction. Neuropharmacology 56, Supplement 1:112-121.

Azar MR, Acar N, Erwin V, Barbato GF, Morse AC, Heist CL, Jones BC (1998) Distribution and clearance of cocaine in brain is influenced by genetics. Pharmacol Biochem Be 59:637-640.

Báez-Mendoza R, Schultz W (2013) The role of the striatum in social behaviour. Frontiers in neuroscience 7.

Bailey A, Metaxas A, Yoo J, McGee T, Kitchen I (2008) Decrease of D2 receptor binding but increase in D2-stimulated G-protein activation, dopamine transporter binding and behavioural sensitization in brains of mice

treated with a chronic escalating dose 'binge' cocaine administration paradigm. Eur J Neurosci 28:759-770.

Bailey CH, Giustetto M, Huang Y-Y, Hawkins RD, Kandel ER (2000) Is Heterosynaptic modulation essential for stabilizing hebbian plasiticity and memory. Nat Rev Neurosci 1:11-20.

Baker DA, McFarland K, Lake RW, Shen H, Tang X-C, Toda S, Kalivas PW (2003) Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse. Nat Neurosci 6:743-749.

Barrot M, Olivier JD, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, Impey S, Storm DR, Neve RL, Yin JC (2002) CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proceedings of the National Academy of Sciences 99:11435-11440.

Beaulieu J-M, Gainetdinov RR (2011) The physiology, signaling, and pharmacology of dopamine receptors. Pharmacol Rev 63:182-217.

Bertran-Gonzalez J, Bosch Cm, Maroteaux M, Matamales M, Hervé D, Valjent E, Girault J-A (2008) Opposing patterns of signaling activation in dopamine D1 and D2 receptor-expressing striatal neurons in response to cocaine and haloperidol. The Journal of neuroscience 28:5671-5685.

Bertran-Gonzalez J, HervéD, Girault J-A, Valjent E (2010) What is the degree of segregation between striatonigral and striatopallidal projections? Frontiers in neuroanatomy 4.

Beurrier C, Malenka RC (2002) Enhanced inhibition of synaptic transmission by dopamine in the nucleus accumbens during behavioral sensitization to cocaine. The Journal of neuroscience 22:5817-5822.

Björklund A, Dunnett SB (2007) Dopamine neuron systems in the brain: an update. Trends Neurosci 30:194-202.

Bolam J, Hanley J, Booth P, Bevan M (2000) Synaptic organisation of the basal ganglia. J Anat 196:527-542.

Bolam J, Wainer B, Smith A (1984) Characterization of cholinergic neurons in the rat neostriatum. A combination of choline acetyltransferase immunocytochemistry, Golgi-impregnation and electron microscopy. Neuroscience 12:711-718.

Borroto-Escuela DO, Craenenbroeck KV, Romero-Fernandez W, Guidolin D, Woods AS, Rivera A, Haegeman G, Agnati LF, Tarakanov AO, Fuxe K (2011) Dopamine D2 and D4 receptor heteromerization and its allosteric receptor-receptor interactions. Biochem Bioph Res Co 404:928-934.

Boudreau AC, Wolf ME (2005) Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. The Journal of neuroscience 25:9144-9151.

Bouthenet M-L, Souil E, Martres M-P, Sokoloff P, Giros B, Schwartz J-C (1991) Localization of dopamine D3 receptor mRNA in the rat brain using in situ hybridization histochemistry: comparison with dopamine D2 receptor mRNA. Brain Res 564:203-219.

Bouvier M (2001) Oligomerization of G-protein-coupled transmitter receptors. Nat Rev Neurosci 2:274-286.

Brauner-Osborne H, Wellendorph P, Jensen AA (2007) Structure, pharmacology and therapeutic prospects of family C G-protein coupled receptors. Current drug targets 8:169-184.

Bravo SA, Rangel-Barajas C, Garduño BnFn (2014) Pathophysiology of L-Dopa Induced Dyskinesia–Changes in

D1/D3 Receptors and Their Signaling Pathway.

Breakefield XO, Blood AJ, Li Y, Hallett M, Hanson PI, Standaert DG (2008) The pathophysiological basis of dystonias. Nat Rev Neurosci 9:222-234.

Burris KD, Molski TF, Xu C, Ryan E, Tottori K, Kikuchi T, Yocca FD, Molinoff PB (2002) Aripiprazole, a novel antipsychotic, is a high-affinity partial agonist at human dopamine D2 receptors. J Pharmacol Exp Ther 302:381-389.

Cabello N, Gandía J, Bertarelli DCG, Watanabe M, Lluís C, Franco R, Ferré S, Luján R, Ciruela F (2009) Metabotropic glutamate type 5, dopamine D2 and adenosine A2a receptors form higher-order oligomers in living cells. J Neurochem 109:1497-1507.

Canals M, Marcellino D, Fanelli F, Ciruela F, de Benedetti P, Goldberg SR, Neve K, Fuxe K, Agnati LF, Woods AS (2003) Adenosine A2A-dopamine D2 receptor-receptor heteromerization qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. J Biol Chem 278:46741-46749.

Carlezon Jr WA, Duman RS, Nestler EJ (2005) The many faces of CREB. Trends Neurosci 28:436-445.

Carlezon WA, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ (1998) Regulation of cocaine reward by CREB. Science 282:2272-2275.

Caulkins JP, Pacula RL, Paddock SM, Chiesa J (2002) School-based drug prevention: What kind of drug use does it prevent?: RAND corporation.

Choi K-H, Whisler K, Graham D, Self D (2006) Antisense-induced reduction in nucleus accumbens cyclic AMP response element binding protein attenuates cocaine reinforcement. Neuroscience 137:373-383.

Ciliax BJ, Nash N, Heilman C, Sunahara R, Hartney A, Tiberi M, Rye DB, Caron MG, Niznik HB, Levey AI (2000) Dopamine D 5 receptor immunolocalization in rat and monkey brain. Synapse 37:125-145.

Ciruela F, Casadó V, Rodrigues RJ, Luján R, Burgueño J, Canals M, Borycz J, Rebola N, Goldberg SR, Mallol J (2006) Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. The Journal of neuroscience 26:2080-2087.

Citri A, Malenka RC (2007) Synaptic plasticity: multiple forms, functions, and mechanisms. Neuropsychopharmacol 33:18-41.

Cole RL, Konradi C, Douglass J, Hyman SE (1995) Neuronal adaptation to amphetamine and dopamine: molecular mechanisms of prodynorphin gene regulation in rat striatum. Neuron 14:813-823.

Crawford CA, Choi FY, Kohutek JL, Yoshida ST, Mcdougall SA (2004) Changes in PKA activity and Gs ALFA and Golf ALFA levels after amphetamine-and cocaine-induced behavioral sensitization. Synapse 51:241-248.

Culm KE, Lugo-Escobar N, Hope BT, Hammer Jr RP (2004) Repeated quinpirole treatment increases cAMP-dependent protein kinase activity and CREB phosphorylation in nucleus accumbens and reverses quinpirole-induced sensorimotor gating deficits in rats. Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology 29:1823-1830.

Cyr M, Sotnikova TD, Gainetdinov RR, Caron MG (2006) Dopamine enhances motor and neuropathological consequences of polyglutamine expanded huntingtin. The FASEB Journal 20:2541-2543.

Daskalakis ZJ, George TP (2009) Clozapine, GABAB, and the Treatment of Resistant Schizophrenia. Clin Pharmacol Ther 86:442-446.

De Mei C, Ramos M, Iitaka C, Borrelli E (2009) Getting specialized: presynaptic and postsynaptic dopamine D2 receptors. Current opinion in pharmacology 9:53-58.

De Vries TJ, Schoffelmeer AN, Binnekade R, Vanderschuren LJ (1999) Dopaminergic mechanisms mediating the incentive to seek cocaine and heroin following long-term withdrawal of IV drug self-administration. Psychopharmacology 143:254-260.

Delgado MR (2007) Reward-Related Responses in the Human Striatum. Ann Ny Acad Sci 1104:70-88.

DeLong MR (1990) Primate models of movement disorders of basal ganglia origin. Trends Neurosci 13:281-285.

Deroche-Gamonet Vr, Belin D, Piazza PV (2004) Evidence for addiction-like behavior in the rat. Science 305:1014-1017.

Di Ciano P, Everitt BJ (2001) Dissociable effects of antagonism of NMDA and AMPA/KA receptors in the nucleus accumbens core and shell on cocaine-seeking behavior. Neuropsychopharmacol 25:341-360.

Di Matteo V, De Blasi A, Di Giulio C, Esposito E (2001) Role of 5-HT2C receptors in the control of central dopamine function. Trends Pharmacol Sci 22:229-232.

Dias C, Lachize S, Boilet V, Huitelec E, Cador M (2004) Differential effects of dopaminergic agents on locomotor sensitisation and on the reinstatement of cocaine-seeking and food-seeking behaviour. Psychopharmacology 175:414-427.

Dong Y, Green T, Saal D, Marie H, Neve R, Nestler EJ, Malenka RC (2006) CREB modulates excitability of nucleus accumbens neurons. Nat Neurosci 9:475-477.

Edwards S, Whisler KN, Fuller DC, Orsulak PJ, Self DW (2006) Addiction-related alterations in D1 and D2 dopamine receptor behavioral responses following chronic cocaine self-administration. Neuropsychopharmacol 32:354-366.

Engberg G, Kling-Petersen T, Nissbrandt H (1993) GABAB-Receptor activation alters the firing pattern of dopamine neurons in the rat substantia nigra. Synapse 15:229-238.

España R, Melchior J, Roberts DS, Jones S (2011) Hypocretin 1/orexin A in the ventral tegmental area enhances dopamine responses to cocaine and promotes cocaine self-administration. Psychopharmacology 214:415-426.

España RA, Oleson EB, Locke JL, Brookshire BR, Roberts DCS, Jones SR (2010) The hypocretin–orexin system regulates cocaine self-administration via actions on the mesolimbic dopamine system. Eur J Neurosci 31:336-348.

Everitt BJ, Dickinson A, Robbins TW (2001) The neuropsychological basis of addictive behaviour. Brain Res Rev 36:129-138.

Fan T, Varghese G, Nguyen T, Tse R, O'Dowd BF, George SR (2005) A Role for the Distal Carboxyl Tails in Generating the Novel Pharmacology and G Protein Activation Profile of mu and delta Opioid Receptor Hetero-oligomers. J

Biol Chem 280:38478-38488.

Felicio AC, Shih MC, Godeiro-Junior C, Andrade LA, Bressan RA, Ferraz HB (2009) Molecular imaging studies in Parkinson disease: reducing diagnostic uncertainty. The neurologist 15:6-16.

Ferrada C, FerréS, Casadó V, Cortés A, Justinova Z, Barnes C, Canela EI, Goldberg SR, Leurs R, Lluis C, Franco R (2008) Interactions between histamine H3 and dopamine D2 receptors and the implications for striatal function. Neuropharmacology 55:190-197.

Fiorentini C, Busi C, Gorruso E, Gotti C, Spano P, Missale C (2008) Reciprocal regulation of dopamine D1 and D3 receptor function and trafficking by heterodimerization. Mol Pharmacol 74:59-69.

Fisher RS, Levine MS, Sibley DR, Ariano MA (1994) D2 dopamine receptor protein location: Golgi impregnation-gold toned and ultrastructural analysis of the rat neostriatum. J Neurosci Res 38:551-564.

Franco R, Casadó V, Cortés A, Mallol J, Ciruela F, FerréS, Lluis C, Canela E (2008) G-protein-coupled receptor heteromers: function and ligand pharmacology. Brit J Pharmacol 153:S90-S98.
Franco R, Casadó V, Cortés A, Mallol J, Ciruela F, Ferré S, Lluis C, Canela EI (2008) G-protein-coupled receptor heteromers: function and ligand pharmacology. Brit J Pharmacol 153:S90-S98.
Frankle WG, Laruelle M (2002) Neuroreceptor imaging in psychiatric disorders. Ann Nucl Med 16:437-446.

Freeman WM, Nader MA, Nader SH, Robertson DJ, Gioia L, Mitchell SM, Daunais JB, Porrino LJ, Friedman DP, Vrana KE (2001) Chronic cocaine-mediated changes in non-human primate nucleus accumbens gene expression. J Neurochem 77:542-549.

Gardner EL (2011) Addiction and brain reward and antireward pathways.

George SR, O'Dowd BF (2007) A novel dopamine receptor signaling unit in brain: heterooligomers of D1 and D2 dopamine receptors. The Scientific World Journal 7:58-63.

George SR, O'Dowd BF, Lee SP (2002) G-Protein-coupled receptor oligomerization and its potential for drug discovery. Nat Rev Drug Discov 1:808-820.

Gerdeman GL, Partridge JG, Lupica CR, Lovinger DM (2003) It could be habit forming: drugs of abuse and striatal synaptic plasticity. Trends Neurosci 26:184-192.

Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma F, Sibley DR (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. Science 250:1429-1432.

Gertler TS, Chan CS, Surmeier DJ (2008) Dichotomous anatomical properties of adult striatal medium spiny neurons. The Journal of Neuroscience 28:10814-10824.

Giros B, Sokoloff P, Martres M-P, Riou J-Fo, Emorine LJ, Schwartz J-C (1989) Alternative splicing directs the expression of two D2 dopamine receptor isoforms.

Goldstein RZ, Volkow ND (2002) Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex. Am J Psychiat 159:1642-1652.

González-Sepúlveda M, Rosell S, Hoffmann HM, Castillo-Ruiz M, Mignon V, Moreno-Delgado D, Vignes M, Díaz J, Sabriá J, Ortiz J (2013) Cellular distribution of the histamine H3 receptor in the basal ganglia: Functional modulation of dopamine and glutamate neurotransmission. Basal Ganglia 3:109-121.

Gouldson PR, Higgs C, Smith RE, Dean MK, Gkoutos GV, Reynolds CA (2000) Dimerization and domain swapping in G-protein-coupled receptors: a computational study. Neuropsychopharmacol 23:S60-S77.

Grant S, London ED, Newlin DB, Villemagne VL, Liu X, Contoreggi C, Phillips RL, Kimes AS, Margolin A (1996) Activation of memory circuits during cue-elicited cocaine craving. Proceedings of the National Academy of Sciences 93:12040-12045.

Graybiel AM (2000) The basal ganglia. Curr Biol 10:R509-R511.

Graybiel AM (2008) Habits, rituals, and the evaluative brain. Annu Rev Neurosci 31:359-387.

Grimm JW, Hope BT, Wise RA, Shaham Y (2001) Neuroadaptation: incubation of cocaine craving after withdrawal. Nature 412:141-142.

Groves P, Linder J, Young S (1994) 5-hydroxydopamine-labeled dopaminergic axns: Three-dimensional reconstructions of axons, synapses and postsynaptic targets in rat neostriatum. Neuroscience 58:593-604.

Haavik J, Schelling DL, Campbell DG, Andersson KK, Flatmark T, Cohen P (1989) Identification of protein phosphatase 2A as the major tyrosine hydroxylase phosphatase in adrenal medulla and corpus striatum: evidence from the effects of okadaic acid. Febs Lett 251:36-42.

Hasbi A, Fan T, Alijaniaram M, Nguyen T, Perreault ML, O'Dowd BF, George SR (2009) Calcium signaling cascade links dopamine D1-D2 receptor heteromer to striatal BDNF production and neuronal growth. Proceedings of the National Academy of Sciences 106:21377-21382.

Hasbi A, O'Dowd BF, George SR (2010) Heteromerization of dopamine D2 receptors with dopamine D1 or D5 receptors generates intracellular calcium signaling by different mechanisms. Current opinion in pharmacology 10:93-99.

Haycock JW (1990) Phosphorylation of tyrosine hydroxylase in situ at serine 8, 19, 31, and 40. J Biol Chem 265:11682-11691.

Haycock JW, Ahn NG, Cobb MH, Krebs EG (1992) ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. Proceedings of the National Academy of Sciences 89:2365-2369.

Haycock JW, Haycock D (1991) Tyrosine hydroxylase in rat brain dopaminergic nerve terminals. Multiple-site phosphorylation in vivo and in synaptosomes. J Biol Chem 266:5650-5657.

Henry DJ, Hu X-T, White FJ (1998) Adaptations in the mesoaccumbens dopamine system resulting from repeated administration of dopamine D1 and D2 receptor-selective agonists: relevance to cocaine sensitization. Psychopharmacology 140:233-242.

Henry DJ, White FJ (1991) Repeated cocaine administration causes persistent enhancement of D1 dopamine receptor sensitivity within the rat nucleus accumbens. J Pharmacol Exp Ther 258:882-890.

Hillion Jl, Canals M, Torvinen M, Casadó V, Scott R, Terasmaa A, Hansson A, Watson S, Olah ME, Mallol J (2002) Coaggregation, Cointemalization, and Codesensitization of Adenosine A2A Receptors and Dopamine D2Receptors. J Biol Chem 277:18091-18097.

Hillion Jl, Canals M, Torvinen M, Casadó V, Scott R, Terasmaa A, Hansson A, Watson S, Olah ME, Mallol J, Canela EI, Zoli M, Agnati LF, Ibáñez CF, Lluis C, Franco R, FerréS, Fuxe K (2002) Coaggregation, Cointemalization, and Codesensitization of Adenosine A2A Receptors and Dopamine D2Receptors. J Biol Chem 277:18091-18097.

Hoffmann HM, Nadal R, Vignes M, Ortiz J (2012) Chronic cocaine self-administration modulates ERK1/2 and CREB responses to dopamine receptor agonists in striatal slices. Addict Biol 17:565-575.

Hollerman JR, Tremblay L, Schultz W (2000) Involvement of basal ganglia and orbitofrontal cortex in goal-directed behavior. Prog Brain Res 126:193-215.

Hsu S-M, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577-580.

Huff RM (1997) Signaling pathways modulated by dopamine receptors. The Dopamine Receptors 167-192.

Hutcheson D, Parkinson JA, Robbins TW, Everitt BJ (2001) The effects of nucleus accumbens core and shell lesions on intravenous heroin self-administration and the acquisition of drug-seeking behaviour under a second-order schedule of heroin reinforcement. Psychopharmacology 153:464-472.

Iñiguez S, Cortez A, Crawford C, McDougall S (2008) Effects of aripiprazole and terguride on dopamine synthesis in the dorsal striatum and medial prefrontal cortex of preweanling rats. J Neural Transm 115:97-106.

Ito R, Dalley JW, Howes SR, Robbins TW, Everitt BJ (2000) Dissociation in conditioned dopamine release in the nucleus accumbens core and shell in response to cocaine cues and during cocaine-seeking behavior in rats. The Journal of Neuroscience 20:7489-7495.

Ito R, Dalley JW, Robbins TW, Everitt BJ (2002) Dopamine release in the dorsal striatum during cocaine-seeking behavior under the control of a drug-associated cue. The Journal of neuroscience 22:6247-6253.

Jacoby E, Bouhelal R, Gerspacher M, Seuwen K (2006) The 7 TM G-Protein-Coupled Receptor Target Family. ChemMedChem 1:760-782.

Jakel RJ, Maragos WF (2000) Neuronal cell death in Huntington's disease: a potential role for dopamine. Trends Neurosci 23:239-245.

Jedynak JP, Uslaner JM, Esteban JA, Robinson TE (2007) Methamphetamine-induced structural plasticity in the dorsal striatum. Eur J Neurosci 25:847-853.

Johnson EA, Fox JL, Azzaro AJ (1995) The anxiolytic serotonin 5-HT1A receptor agonists buspirone, ipsapirone and gepirone are inhibitors of tyrosine hydroxylation in rat striatum. Behav Brain Res 73:331-335.

Johnstone EK, Pfleger KD (2012) Receptor-Heteromer Investigation Technology and its application using BRET. Frontiers in endocrinology 3.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao W-J, Johnson M, Gunwaldsen C, Huang L-Y, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C (1998) GABAB receptors function as a heteromeric assembly of the subunits GABABR1 and GABABR2. Nature 396:674-679.

Jordan S, Koprivica V, Chen R, Tottori K, Kikuchi T, Altar CA (2002) The antipsychotic aripiprazole is a potent, partial agonist at the human 5-HT1A receptor. Eur J Pharmacol 441:137-140.

Joseph J, Wang Y-M, Miles P, Budygin E, Picetti R, Gainetdinov R, Caron M, Wightman R (2002) Dopamine autoreceptor regulation of release and uptake in mouse brain slices in the absence of D3 receptors. Neuroscience 112:39-49.

Kalivas PW (2009) The glutamate homeostasis hypothesis of addiction. Nat Rev Neurosci 10:561-572.

Kebabian JW, Greengard P (1971) Dopamine-sensitive adenyl cyclase: possible role in synaptic transmission. Science 174:1346-1349.

Kehr W, Carlsson A, Lindqvist M, Magnusson T, Atack C (1972) Evidence for a receptor-mediated feedback control of striatal tyrosine hydroxylase activity. J Pharm Pharmacol 24:744-747.

Khan Z, Gutierrez A, Martín R, Penafiel A, Rivera A, De La Calle A (2000) Dopamine D5 receptors of rat and human brain. Neuroscience 100:689-699.

Kikuchi T, Tottori K, Uwahodo Y, Hirose T, Miwa T, Oshiro Y, Morita S (1995) 7-(4-[4-(2, 3-Dichlorophenyl)-1-piperazinyl] butyloxy)-3, 4-dihydro-2 (1H)-quinolinone (OPC-14597), a new putative antipsychotic drug with both presynaptic dopamine autoreceptor agonistic activity and postsynaptic D2 receptor antagonistic activity. J Pharmacol Exp Ther 274:329-336.

Koener B, Focant MnC, Bosier B, Maloteaux J-M, Hermans E (2012) Increasing the density of the D2L receptor and manipulating the receptor environment are required to evidence the partial agonist properties of aripiprazole. Progress in Neuro-Psychopharmacology and Biological Psychiatry 36:60-70.

Kolakowski Jr LF (1994) GCRDb: a G-protein-coupled receptor database. Receptor Channel 2:1.

Kourrich S, Rothwell PE, Klug JR, Thomas MJ (2007) Cocaine experience controls bidirectional synaptic plasticity in the nucleus accumbens. The Journal of neuroscience 27:7921-7928.

Kreitzer AC (2009) Physiology and pharmacology of striatal neurons. Annu Rev Neurosci 32:127-147.

Kumer SC, Vrana KE (1996) Intricate regulation of tyrosine hydroxylase activity and gene expression. J Neurochem 67:443-462.

Kuroki T, Dai J, Meltzer HY, Ichikawa J (2000) R(+)-8-OH-DPAT, a selective 5-HT1A receptor agonist, attenuated amphetamine-induced dopamine synthesis in rat striatum, but not nucleus accumbens or medial prefrontal cortex. Brain Res 872:204-207.

Lawler CP, Prioleau C, Lewis MM, Mak C, Jiang D, Schetz JA, Gonzalez AM, Sibley DR, Mailman RB (1999) Interactions of the novel antipsychotic aripiprazole (OPC-14597) with dopamine and serotonin receptor subtypes. Neuropsychopharmacol 20:612-627.

Le Moine C, Bloch B (1995) D1 and D2 dopamine receptor gene expression in the rat striatum: sensitive cRNA probes demonstrate prominent segregation of D1 and D2 mRNAs in distinct neuronal populations of the dorsal and ventral striatum. J Comp Neurol 355:418-426.

Lee FJ, Xue S, Pei L, Vukusic B, Chéry N, Wang Y, Wang YT, Niznik HB, Yu X-m, Liu F (2002) Dual regulation of NMDA receptor functions by direct protein-protein interactions with the dopamine D1 receptor. Cell 111:219-230.

Lee K-W, Kim Y, Kim AM, Helmin K, Nairn AC, Greengard P (2006) Cocaine-induced dendritic spine formation in D1 and D2 dopamine receptor-containing medium spiny neurons in nucleus accumbens. Proceedings of the National Academy of Sciences of the United States of America 103:3399-3404.

Lee SP, So CH, Rashid AJ, Varghese G, Cheng R, Lan√ßa AJ, O'Dowd BF, George SR (2004) Dopamine D1 and D2 Receptor Co-activation Generates a Novel Phospholipase C-mediated Calcium Signal. J Biol Chem 279:35671-35678.

Leucht S, Corves C, Arbter D, Engel RR, Li C, Davis JM (2009) Second-generation versus first-generation antipsychotic drugs for schizophrenia: a meta-analysis. The Lancet 373:31-41.

L'hirondel M, Cheramy A, Godeheu G, Artaud F, Saiardi A, Borrelli E, Glowinski J (1998) Lack of autoreceptor-mediated inhibitory control of dopamine release in striatal synaptosomes of D2 receptor-deficient mice. Brain Res 792:253-262.

Lu L, Grimm JW, Dempsey J, Shaham Y (2004) Cocaine seeking over extended withdrawal periods in rats: different time courses of responding induced by cocaine cues versus cocaine priming over the first 6 months. Psychopharmacology 176:101-108.

Lu L, Grimm JW, Shaham Y, Hope BT (2003) Molecular neuroadaptations in the accumbens and ventral tegmental area during the first 90 days of forced abstinence from cocaine self-administration in rats. J Neurochem 85:1604-1613.

Maggio R, Millan MJ (2010) Dopamine D2-D3 receptor heteromers: pharmacological properties and therapeutic significance. Current opinion in pharmacology 10:100-107.

Mailman RB, Murthy V (2010) Third generation antipsychotic drugs: partial agonism or receptor functional selectivity? Curr Pharm Design 16:488.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Marcellino D, FerréS, Casadó V, Cortés A, Le Foll B, Mazzola C, Drago F, Saur O, Stark H, Soriano A (2008) Identification of dopamine D1-D3 receptor heteromers indications for a role of synergistic D1-D3 receptor interactions in the striatum. J Biol Chem 283:26016-26025.

Marcellino D, FerréS, Casadó V, Cortés A, Le Foll B, Mazzola C, Drago F, Saur O, Stark H, Soriano A, Barnes C, Goldberg SR, Lluis C, Fuxe K, Franco R (2008) Identification of Dopamine D1-D3 Receptor Heteromers: INDICATIONS FOR A ROLE OF SYNERGISTIC D1-D3 RECEPTOR INTERACTIONS IN THE STRIATUM. J Biol Chem 283:26016-26025.

Marcellino D, Navarro G, Sahlholm K, Nilsson J, Agnati LF, Canela EI, Lluís C, Århem P, Franco R, Fuxe K (2010)

Cocaine produces D2 R-mediated conformational changes in the adenosine A2A R-dopamine D2 R heteromer. Biochem Bioph Res Co 394:988-992.

Marshall F, White J, Main M, Green A, Wise A (1999) GABA (B) receptors function as heterodimers. Biochem Soc T 27:530-535.

Matamales M, Bertran-Gonzalez J, Salomon L, Degos B, Deniau J-M, Valjent E, HervéD, Girault J-A (2009) Striatal medium-sized spiny neurons: identification by nuclear staining and study of neuronal subpopulations in BAC transgenic mice. PloS one 4:e4770.

Matsuda W, Furuta T, Nakamura KC, Hioki H, Fujiyama F, Arai R, Kaneko T (2009) Single nigrostriatal dopaminergic neurons form widely spread and highly dense axonal arborizations in the neostriatum. The Journal of Neuroscience 29:444-453.

Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR, McKnight GS (1994) Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Mol Cell Biol 14:6107-6116.

Mattson BJ, Bossert JM, Simmons DE, Nozaki N, Nagarkar D, Kreuter JD, Hope BT (2005) Cocaine-induced CREB phosphorylation in nucleus accumbens of cocaine-sensitized rats is enabled by enhanced activation of extracellular signal-related kinase, but not protein kinase A. J Neurochem 95:1481-1494.

Maurel D, Comps-Agrar L, Brock C, Rives M-L, Bourrier E, Ayoub MA, Bazin H, Tinel N, Durroux T, Prezeau L, Trinquet E, Pin J-P (2008) Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization. Nat Meth 5:561-567.

Meller E, Bohmaker K, Namba Y, Friedhoff AJ, Goldstein M (1987) Relationship between receptor occupancy and response at striatal dopamine autoreceptors. Mol Pharmacol 31:592-598.

Mercuri N, Saiardi A, Bonci A, Picetti R, Calabresi P, Bernardi G, Borrelli E (1997) Loss of autoreceptor function in dopaminergic neurons from dopamine D2 receptor deficient mice. Neuroscience 79:323-327.

Milligan G (2008) A day in the life of a G protein-coupled receptor: the contribution to function of G protein-coupled receptor dimerization. Brit J Pharmacol 153:S216-S229.

Milligan G (2009) G protein-coupled receptor hetero-dimerization: contribution to pharmacology and function. Brit J Pharmacol 158:5-14.

Missale C, Fiorentini C, Collo G, Spano P (2010) The neurobiology of dopamine receptors: evolution from the dual concept to heterodimer complexes. Journal of Receptors and Signal Transduction 30:347-354.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors: from structure to function. Physiol Rev 78:189-225.

Mittal SK, Eddy C (2013) The role of dopamine and glutamate modulation in Huntington disease. Behav Neurol 26:255-263.

Monsma FJ, McVittie LD, Gerfen CR, Mahan LC, Sibley DR (1989) Multiple D2 dopamine receptors produced by alternative RNA splicing.

Montminy MR, Bilezikjian LM (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328:175-178.

Moore CA, Milano SK, Benovic JL (2007) Regulation of receptor trafficking by GRKs and arrestins. Annu Rev Physiol 69:451-482.

Morgan D, Grant KA, Gage HD, Mach RH, Kaplan JR, Prioleau O, Nader SH, Buchheimer N, Ehrenkaufer RL, Nader MA (2002) Social dominance in monkeys: dopamine D2 receptors and cocaine self-administration. Nat Neurosci 5:169-174.

Moussawi K, Pacchioni A, Moran M, Olive MF, Gass JT, Lavin A, Kalivas PW (2009) N-Acetylcysteine reverses cocaine-induced metaplasticity. Nat Neurosci 12:182-189.

Mueller AL, Brodie MS (1989) Intracellular recording from putative dopamine-containing neurons in the ventral tegmental area of Tsai in a brain slice preparation. J Neurosci Meth 28:15-22.

Mueller D, Stewart J (2000) Cocaine-induced conditioned place preference: reinstatement by priming injections of cocaine after extinction. Behav Brain Res 115:39-47.

Muly EC, Maddox M, Khan ZU (2010) Distribution of D1 and D5 dopamine receptors in the primate nucleus accumbens. Neuroscience 169:1557-1566.

Nakayasu H, Kimuka H, Kuriyama K (1995) Cerebral GABAA and GABAB Receptors. Ann Ny Acad Sci 757:516-527.

Natesan S, Reckless GE, Barlow KB, Nobrega JN, Kapur S (2011) Partial agonists in schizophrenia-why some work and others do not: insights from preclinical animal models. The International Journal of Neuropsychopharmacology 14:1165-1178.

Neisewander JL, Baker DA, Fuchs RA, Tran-Nguyen LT, Palmer A, Marshall JF (2000) Fos protein expression and cocaine-seeking behavior in rats after exposure to a cocaine self-administration environment. The Journal of Neuroscience 20:798-805.

Nelson G, Chandrashekar J, Hoon MA, Feng L, Zhao G, Ryba NJ, Zuker CS (2002) An amino-acid taste receptor. Nature 416:199-202.

Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS (2001) Mammalian sweet taste receptors. Cell 106:381-390.

Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci 2:119-128.

Nestler EJ (2004) Molecular mechanisms of drug addiction. Neuropharmacology 47:24-32.

Nestler EJ, Terwilliger RZ, Walker JR, Sevarino KA, Duman RS (1990) Chronic cocaine treatment decreases levels of the G protein subunits Gi ALFA and Go ALFA in discrete regions of rat brain. J Neurochem 55:1079-1082.

Neve KA, Seamans JK, Trantham-Davidson H (2004) Dopamine receptor signaling. Journal of receptors and signal transduction 24:165-205.

Nicola SM (2007) The nucleus accumbens as part of a basal ganglia action selection circuit. Psychopharmacology

191:521-550.

Nikolaus S, Antke C, Müller H-W (2009) In vivo imaging of synaptic function in the central nervous system: I. Movement disorders and dementia. Behav Brain Res 204:1-31.

Nikolaus S, Antke C, Müller H-W (2009) In vivo imaging of synaptic function in the central nervous system: II. Mental and affective disorders. Behav Brain Res 204:32-66.

Nimitvilai S, Brodie MS (2010) Reversal of Prolonged Dopamine Inhibition of Dopaminergic Neurons of the Ventral Tegmental Area. J Pharmacol Exp Ther 333:555-563.

Nishi A, Snyder GL, Fienberg AA, Fisone G, Aperia A, Nairn AC, Greengard P (1999) Requirement for DARPP-32 in mediating effect of dopamine D2 receptor activation. Eur J Neurosci 11:2589-2592.

Nishi A, Snyder GL, Greengard P (1997) Bidirectional regulation of DARPP-32 phosphorylation by dopamine. The Journal of neuroscience 17:8147-8155.

Nohesara S, Ghadirivasfi M, Mostafavi S, Eskandari M-R, Ahmadkhaniha H, Thiagalingam S, Abdolmaleky HM (2011) DNA hypomethylation of MB-COMT promoter in the DNA derived from saliva in schizophrenia and bipolar disorder. J Psychiat Res 45:1432-1438.

Oak JN, Oldenhof J, Van Tol HHM (2000) The dopamine D4 receptor: one decade of research. Eur J Pharmacol 405:303-327.

O'Neill C, Nolan BJ, Macari A, O'Boyle KM, O'Connor JJ (2007) Adenosine A1 receptor-mediated inhibition of dopamine release from rat striatal slices is modulated by D1 dopamine receptors. Eur J Neurosci 26:3421-3428.

Pei L, Li S, Wang M, Diwan M, Anisman H, Fletcher PJ, Nobrega JN, Liu F (2010) Uncoupling the dopamine D1-D2 receptor complex exerts antidepressant-like effects. Nat Med 16:1393-1395.

Phillips PE, Stuber GD, Heien ML, Wightman RM, Carelli RM (2003) Subsecond dopamine release promotes cocaine seeking. Nature 422:614-618.

Pin J-P, Galvez T, Prézeau L (2003) Evolution, structure, and activation mechanism of family 3/C G-protein-coupled receptors. Pharmacol Therapeut 98:325-354.

Popoli P, Pezzola A, Torvinen M, Reggio R, Pintor A, Scarchilli L, Fuxe K, Ferre S (2001) The Selective mGlu5 Receptor Agonist CHPG Inhibits Quinpirole-Induced Turning in 6-Hydroxydopamine-Lesioned Rats and Modulates the Binding Characteristics of Dopamine D2 Receptors in the Rat Striatum Interactions with Adenosine A2a Receptors. Neuropsychopharmacol 25:505-513.

Prinster SC, Hague C, Hall RA (2005) Heterodimerization of G Protein-Coupled Receptors: Specificity and Functional Significance. Pharmacol Rev 57:289-298.

Pruessner JC, Champagne F, Meaney MJ, Dagher A (2004) Dopamine release in response to a psychological stress in humans and its relationship to early life maternal care: a positron emission tomography study using [11C] raclopride. The Journal of Neuroscience 24:2825-2831.

Rao NP, Remington G (2013) Investigational drugs for schizophrenia targeting the dopamine receptor: Phase II trials. Expert Opinion on Investigational Drugs 22:881-894.

Rashid AJ, So CH, Kong MM, Furtak T, El-Ghundi M, Cheng R, O'Dowd BF, George SR (2007) D1-D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. Proceedings of the National Academy of Sciences 104:654-659.

Rios CD, Jordan BA, Gomes I, Devi LA (2001) G-protein-coupled receptor dimerization: modulation of receptor function. Pharmacol Therapeut 92:71-87.

Rives M-L, Vol C, Fukazawa Y, Tinel N, Trinquet E, Ayoub MA, Shigemoto R, Pin J-P, Prézeau L (2009) Crosstalk between GABAB and mGlu1a receptors reveals new insight into GPCR signal integration. The EMBO Journal 28:2195-2208.

Robbins T, Giardini V, Jones G, Reading P, Sahakian B (1990) Effects of dopamine depletion from the caudate-putamen and nucleus accumbens septi on the acquisition and performance of a conditional discrimination task. Behav Brain Res 38:243-261.

Roberts D, Brebner K (2000) GABA Modulation of Cocaine Self-Administration. Ann Ny Acad Sci 909:145-158.

Roberts D, Morgan D, Liu Y (2007) How to make a rat addicted to cocaine. Progress in Neuro-Psychopharmacology and Biological Psychiatry 31:1614-1624.

Roberts D, Zito K (1987) Interpretation of lesion effects on stimulant self-administration. In: Methods of assessing the reinforcing properties of abused drugs, pp 87-103: Springer.

Robinson TE, Kolb B (1997) Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. The Journal of neuroscience 17:8491-8497.

Robinson TE, Kolb B (2004) Structural plasticity associated with exposure to drugs of abuse. Neuropharmacology 47:33-46.

Rodd-Henricks ZA, McKinzie DL, Li T-K, Murphy JM, McBride WJ (2002) Cocaine is self-administered into the shell but not the core of the nucleus accumbens of Wistar rats. J Pharmacol Exp Ther 303:1216-1226.

Ron D, Jurd R (2005) The" ups and downs" of signaling cascades in addiction. Science Signaling 2005:re14.

Rosenbaum DM, Rasmussen SrG, Kobilka BK (2009) The structure and function of G-protein-coupled receptors. Nature 459:356-363.

Rossetti ZL, Hmaidan Y, Gessa GL (1992) Marked inhibition of mesolimbic dopamine release: a common feature of ethanol, morphine, cocaine and amphetamine abstinence in rats. Eur J Pharmacol 221:227-234.

Rymar VV, Sasseville R, Luk KC, Sadikot AF (2004) Neurogenesis and stereological morphometry of calretinin-immunoreactive GABAergic interneurons of the neostriatum. J Comp Neurol 469:325-339.

Safer DJ, Calarge CA, Safer AM (2013) Prolactin serum concentrations during aripiprazole treatment in youth. J Child Adol Psychop 23:282-289.

Scarselli M, Novi F, Schallmach E, Lin R, Baragli A, Colzi A, Griffon N, Corsini GU, Sokoloff P, Levenson R, Vogel Z, Maggio R (2001) D2/D3 Dopamine Receptor Heterodimers Exhibit Unique Functional Properties. J Biol Chem 276:30308-30314.

Seeman P (2002) Atypical antipsychotics: mechanism of action. Canadian journal of psychiatry Revue canadienne de psychiatrie 47:27-38.

Seeman P (2005) An update of fast-off dopamine D2 atypical antipsychotics. Am J Psychiat 162:1984-a-1985.

Seeman P, Van Tol HH (1994) Dopamine receptor pharmacology. Trends Pharmacol Sci 15:264-270.

Seeman P, Weinshenker D, Quirion R, Srivastava LK, Bhardwaj SK, Grandy DK, Premont RT, Sotnikova TD, Boksa P, El-Ghundi M (2005) Dopamine supersensitivity correlates with D2High states, implying many paths to psychosis. Proceedings of the National Academy of Sciences of the United States of America 102:3513-3518.

Self DW (2004) Regulation of drug-taking and -seeking behaviors by neuroadaptations in the mesolimbic dopamine system. Neuropharmacology 47, Supplement 1:242-255.

Shapiro DA, Renock S, Arrington E, Chiodo LA, Liu L-X, Sibley DR, Roth BL, Mailman R (2003) Aripiprazole, a novel atypical antipsychotic drug with a unique and robust pharmacology. Neuropsychopharmacol.

Sheng M, Thompson MA, Greenberg ME (1991) CREB: a Ca (2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 252:1427-1430.

Shenoy SK, Lefkowitz RJ (2011) Œ≤-Arrestin-mediated receptor trafficking and signal transduction. Trends Pharmacol Sci 32:521-533.

Sibley DR (1999) NEW INSIGHTS INTO DOPAMINERGIC RECEPTOR FUNCTION USING ANTISENSE AND GENETICALLY ALTERED ANIMALS 1. Annu Rev Pharmacol 39:313-341.

Simpson J, Wang J, McGinty J (1995) Repeated amphetamine administration induces a prolonged augmentation of phosphorylated cyclase response element-binding protein and Fos-related antigen immunoreactivity in rat striatum. Neuroscience 69:441-457.

So CH, Varghese G, Curley KJ, Kong MM, Alijaniaram M, Ji X, Nguyen T, O'Dowd BF, George SR (2005) D1 and D2 dopamine receptors form heterooligomers and cointernalize after selective activation of either receptor. Mol Pharmacol 68:568-578.

So CH, Verma V, Alijaniaram M, Cheng R, Rashid AJ, O'Dowd BF, George SR (2009) Calcium Signaling by Dopamine D5 Receptor and D5-D2 Receptor Hetero-Oligomers Occurs by a Mechanism Distinct from That for Dopamine D1-D2 Receptor Hetero-Oligomers. Mol Pharmacol 75:843-854.

Striplin CD, Kalivas PW (1993) Robustness of G protein changes in cocaine sensitization shown with immunoblotting. Synapse 14:10-15.

Surmeier DJ, Carrillo-Reid L, Bargas J (2011) Dopaminergic modulation of striatal neurons, circuits, and assemblies. Neuroscience 198:3-18.

Surmeier DJ, Song W-J, Yan Z (1996) Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. The Journal of neuroscience 16:6579-6591.

Tadori Y, Forbes RA, McQuade RD, Kikuchi T (2009) Receptor reserve-dependent properties of antipsychotics at human dopamine D2 receptors. Eur J Pharmacol 607:35-40.

Tadori Y, Forbes RA, McQuade RD, Kikuchi T (2011) Functional potencies of dopamine agonists and antagonists at human dopamine D2 and D3 receptors. Eur J Pharmacol 666:43-52.

Tadori Y, Forbes RA, McQuade RD, Kikuchi T (2011) In vitro pharmacology of aripiprazole, its metabolite and experimental dopamine partial agonists at human dopamine D2 and D3 receptors. Eur J Pharmacol 668:355-365.

Tamminga C (2002) Partial dopamine agonists in the treatment of psychosis. J Neural Transm 109:411-420.

Telang FW, Volkow ND, Levy A, Logan J, Fowler JS, Felder C, Wong C, Wang GÄ (1999) Distribution of tracer levels of cocaine in the human brain as assessed with averaged [11C] cocaine images. Synapse 31:290-296.

Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM, Nestler EJ (1991) A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res 548:100-110.

Thomas MJ, Beurrier C, Bonci A, Malenka RC (2001) Long-term depression in the nucleus accumbens: a neural correlate of behavioral sensitization to cocaine. Nat Neurosci 4:1217-1223.

Thomsen M, Caine SB (2007) Intravenous drug self-administration in mice: practical considerations. Behav Genet 37:101-118.

Tiffany ST (1990) A cognitive model of drug urges and drug-use behavior: role of automatic and nonautomatic processes. Psychol Rev 97:147.

Turgeon SM, Pollack AE, Fink JS (1997) Enhanced CREB phosphorylation and changes in c-Fos and FRA expression in striatum accompany amphetamine sensitization. Brain Res 749:120-126.

Tuteja N (2009) Signaling through G protein coupled receptors. Plant Signal Behav 4:942-947.

Unterwald EM, Cox BM, Kreek MJ, Cote TE, Izenwasser S (1993) Chronic repeated cocaine administration alters basal and opioid-regulated adenylyl cyclase activity. Synapse 15:33-38.

Usiello A, Baik J-H, Rougé-Pont Fo, Picetti R, Dierich Ae, LeMeur M, Piazza PV, Borrelli E (2000) Distinct functions of the two isoforms of dopamine D2 receptors. Nature 408:199-203.

Valjent E, Bertran-Gonzalez J, HervéD, Fisone G, Girault J-A (2009) Looking BAC at striatal signaling: cell-specific analysis in new transgenic mice. Trends Neurosci 32:538-547.

Vischer HF, Watts AO, Nijmeijer S, Leurs R (2011) G protein-coupled receptors: walking hand-in-hand, talking hand-in-hand? Brit J Pharmacol 163:246-260.

Volkow N, Fowler J, Wang G, Baler R, Telang F (2009) Imaging dopamine's role in drug abuse and addiction. Neuropharmacology 56:3-8.

Volkow ND, Wang G-J, Fischman MW, Foltin R, Fowler JS, Franceschi D, Franceschi M, Logan J, Gatley SJ, Wong C (2000) Effects of route of administration on cocaine induced dopamine transporter blockade in the human brain. Life Sci 67:1507-1515.

Waldvogel HJ, Billinton A, White JH, Emson PC, Faull RLM (2004) Comparative cellular distribution of GABAA and

GABAB receptors in the human basal ganglia: Immunohistochemical colocalization of the α1 subunit of the GABAA receptor, and the GABABR1 and GABABR2 receptor subunits. The Journal of Comparative Neurology 470:339-356.

Weiss F, Markou A, Lorang MT, Koob GF (1992) Basal extracellular dopamine levels in the nucleus accumbens are decreased during cocaine withdrawal after unlimited-access self-administration. Brain Res 593:314-318.

Westerink B, De Vries J (1989) On the mechanism of neuroleptic induced increase in striatal dopamine release: brain dialysis provides direct evidence for mediation by autoreceptors localized on nerve terminals. Neurosci Lett 99:197-202.

White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH (1998) Heterodimerization is required for the formation of a functional GABAB receptor. Nature 396:679-682.

Williams RW, Herrup K (1988) The control of neuron number. Annu Rev Neurosci 11:423-453.

Willner P (1997) The mesolimbic dopamine system as a target for rapid antidepressant action. Int Clin Psychopharm 12:S7-14.

Wilson C, Chang H, Kitai S (1990) Firing patterns and synaptic potentials of identified giant aspiny interneurons in the rat neostriatum. The Journal of Neuroscience 10:508-519.

Wirtshafter D, Sheppard AC (2001) Localization of GABAB receptors in midbrain monoamine containing neurons in the rat. Brain Res Bull 56:1-5.

Wise R, Murray A, Bozarth MA (1990) Bromocriptine self-administration and bromocriptine-reinstatement of cocaine-trained and heroin-trained lever pressing in rats. Psychopharmacology 100:355-360.

Wise RA (2009) Roles for nigrostriatal--not just mesocorticolimbic--dopamine in reward and addiction. Trends Neurosci 32:517-524.

Wright BA, Waters CH (2013) Continuous dopaminergic delivery to minimize motor complications in Parkinson's disease. Expert Review of Neurotherapeutics 13:719-729.

Wu JC, Bell K, Najafi A, Widmark C, Keator D, Tang C, Klein E, Bunney BG, Fallon J, Bunney WE (1997) Decreasing striatal 6-FDOPA uptake with increasing duration of cocaine withdrawal. Neuropsychopharmacol 17:402-409.

Wu X, McMurray CT (2001) Calmodulin kinase II attenuation of gene transcription by preventing cAMP response element-binding protein (CREB) dimerization and binding of the CREB-binding protein. J Biol Chem 276:1735-1741.

Wydra K, Golembiowska K, Zaniewska M, Kamińska K, Ferraro L, Fuxe K, Filip MÇ (2013) Accumbal and pallidal dopamine, glutamate and GABA overflow during cocaine self-administration and its extinction in rats. Addict Biol 18:307-324.

Xu M, Hu X-T, Cooper DC, Moratalla R, Graybiel AM, White FJ, Tonegawa S (1994) Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. Cell 79:945-955.

Yang W, Xia S-H (2006) Mechanisms of regulation and function of G-protein-coupled receptor kinases. World J

Gastroentero 12:7753.

Yokoi F, Gründer G, Biziere K, Stephane M, Dogan AS, Dannals RF, Ravert H, Suri A, Bramer S, Wong DF (2002) Dopamine D2 and D3 receptor occupancy in normal humans treated with the antipsychotic drug aripiprazole (OPC 14597): a study using positron emission tomography and [11C] raclopride. Neuropsychopharmacol 27:248-259.