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Role of Adenylyl Cyclase Type 5 in the Regulation of the Dopamine D3 Receptor Phosphorylation

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Role of Adenylyl Cyclase Type 5 in the Regulation of the Dopamine D3 Receptor Phosphorylation

By

Hassan Gorji

This thesis is submitted as a partial fulfillment of the M. Sc. Program in Neuroscience

Department of Cellular and Molecular Medicine

University of Ottawa

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Abstract

Adenylyl cyclase type 5 (AC5) is expressed in the brain where the highest density of the dopamine D3 receptor (D3R) has been found. The D3R-mediated Gi/o protein activation leads to a specific inhibition of AC5. Therefore, as AC5 is the main signalosome partner of D3R, I hypothesize that D3R phosphorylation is differentially regulated in cells expressing AC5. In HEK293 cells expressing D3R alone, D3R undergo dopamine-induced phosphorylation. Interestingly, in cells co-expressing AC5 and D3R, D3R undergoes a Gαi-dependent dephosphorylation upon dopamine exposure while retaining its ability to be phosphorylated in a Src-dependent manner under basal conditions. In cells co-expressing D3R and AC5, dopamine-induced D3R dephosphorylation and Gi/o mediated inhibition of cAMP production are specifically blocked by pharmacological inhibitors of the serine/threonine phosphatase PP2B and tyrosine phosphatases. Overall, our results suggest a novel paradigm in G protein-coupled receptor signaling whereby AC5 serves as a potential scaffolding complex containing phosphatases regulating the D3R phosphorylation status.

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List of abbreviation

AADC: L-aromatic amino acid decarboxylase

AC5: Adenyly cyclase type 5

AKAP: A-kinase-anchoring proteins

BDNF: Brain derived neurotrophic factor

cAMP: Cyclic adenosine monophosphate

CCV: Clathrin coated vesicle

CNS: Central nervous system

CREB: cAMP response element-binding protein

CT: Cytoplasmic tail

DA: Dihydroxyphenylethylamine

DAG: Diacyl glycerol

DAT: Dopamine transporter

Dn-Src: Dominant negative Src

7-OH-DPAT: (±)-7-hydroxy-N, N-di-n-propyl-2-aminotetralin

D1R: Dopamine receptor type 1

$D2R_{short}$: Dopamine receptor type 2 short form

$D2R_{long}$: Dopamine receptor type 2 long form

D3R: Dopamine receptor type 3

D4R: Dopamine receptor type 4

D5R: Dopamine receptor type 5

ECL: Enhanced chemiluminescence

eLF-1B: Elongation factor-1 beta

FBS: Fetal bovine serum

FSK: Forskolin

GPCR: G protein-coupled receptor

GRK: G protein-related kinase

GSK3: Glycogen synthase kinase-3

HEK293: Human embryonic cells-293

IBMX: 3-Isobutyl-1-methylxanthine

IL3: Third intracellular loop

IP3: Inositol 1,4,5-trisphosphate

L-DOPA: 3, 4 dihydroxyphenylalanine

MEM: Minimal essential medium

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

7MSRs: Seven-membrane-spanning receptors

NAPS: N-(p-amino-phenethy)spiperone

NGF: Nerve growth factor

NMDA: N-methyl-D aspartate

NT-3: Neurotrophin 3

NT-4: Neurotrophin 4

6-OHDA: 6-hydroxydopamine

PIP2: Phosphatidylinositol bisphosphate 2

PKA: Protein kinase-A

PKC: Protein kinase-C

PLC: Phospholipase C

PMSF: Phenylmethylsulfonyl fluoride

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2A

PP2B: Protein phosphatase 2B

PPX: Pramipexole

PTP: Protein tyrosine phosphatase

PTX: Pertussis toxin

PVDF: Polyvinylidene difluoride

RCN: Regulators of calcineurin

RTK: Receptor tyrosine kinase

SAR: Structure activity relationship

SOV: Sodium orthovanadate

STEP: striatal-enriched tyrosine phosphatase

TH: Tyrosine hydroxylase

7TM: Seven transmembrane

TNF: Tumor necrosis factor

TrK: tyrosine kinase receptor

VTA: Ventral tegmental area

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INTRODUCTION

Dopamine

Catecholamines are involved in several neuropsychiatric disorders, and thus they have been the focus of extensive basic and clinical studies. The catecholamines consist of three related neurotransmitter groups that are synthesized sequentially in a subset of neurons in the central and peripheral nervous system. They are organic compounds with a catechol nucleus (a benzene ring with two adjacent hydroxyl groups) and aliphatic chain containing an amine or substituted amine group. The term catecolamine is used to describe dopamine (DA; dihydroxyphenylethylamine) and its two metabolic products norepinephrine and epinephrine. These three neurotransmitters are synthesized by a series of enzymatic modifications of the amino acid tyrosine, which require distinct enzymes.

Dopamine synthesis

The amino acid tyrosine, the precursor used in catecholamine synthesis, is present in high concentrations in the plasma and brain. Tyrosine can be derived from the dietary phenylalanine metabolized by the hepatic enzyme phenylalanine hydroxylase. Then, tyrosine accumulates in catecholamine neurons of the brain. In these neurons, tyrosine is hydroxylated by the enzyme tyrosine hydroxylase (TH) (1) to produce 3,4-dihydroxyphenylalanine (L-DOPA); this intermediary is immediately converted into DA by L-aromatic amino acid decarboxylase (AADC

or also known as DOPA decarboxylase in brain). DOPA decarboxylase has a low substrate specificity as this enzyme mediates decarboxylation of tyrosine and tryptophan. Thus, DOPA decarboxylase is the key enzyme for both DA and serotonin synthesis. In DA-containing neurons, the decarboxylation process is the final step in DA synthesis The entry of tyrosine into the brain depends on an energy-dependent uptake process for large neutral amino acids; tyrosine competes with other large neutral amino acids at this transporter. Under normal conditions, brain tyrosine levels are high enough to saturate TH, and changes in tyrosine availability can not change DA synthesis. This is why TH, and not the tyrosine per se, is the rate limiting factor in DA synthesis. Of course under certain circumstances like uncontrolled diabetes mellitus, in which the size of the large neutral amino acid pool is altered, DA synthesis can be reduced. Catecholamines can inhibit the activity of TH through a competition for the TH enzyme cofactor, pterin. The amount of the chemically-reduced inactive form of pterin (tetrahydrobiopterin) is not saturated under basal conditions, and thus tetrahydrobiopterin plays an important role in regulating the TH activity. Therefore, DA controls its own production by inhibiting TH. Notably, mutations in the gene coding for GTP-cyclohydrolase **1**, the rate limiting enzyme in the synthesis of the pterin cofactor, are responsible for a pathological condition called DOPA-responsive dystonia (2,3).

Dopaminergic pathways

Several neural pathways in the brain convey the neurotransmitter DA from one region of the brain to another (Figure 1). The most important dopaminergic pathways are as follows:

- The mesolimbic pathway
- The mesocortical pathway
- The nigrostriatal pathway
- The tuberoinfundibular pathway

The **mesolimbic pathway** is one of the neural pathways in the brain that links the ventral tegmentum area (VTA) in the midbrain to the nucleus accumbens in the limbic system. The mesolimbic pathway is thought to be involved in producing pleasurable feelings, and is often associated with feelings of reward and desire, particularly because of the connection to the nucleus accumbens, which is also associated with these physiological states. Importantly, because of these observations, the mesolimbic pathway plays a central role in neurobiological theories of addiction. However, recent work suggests that the mesolimbic pathway is involved in reward system rather than euphoric mood states (4).

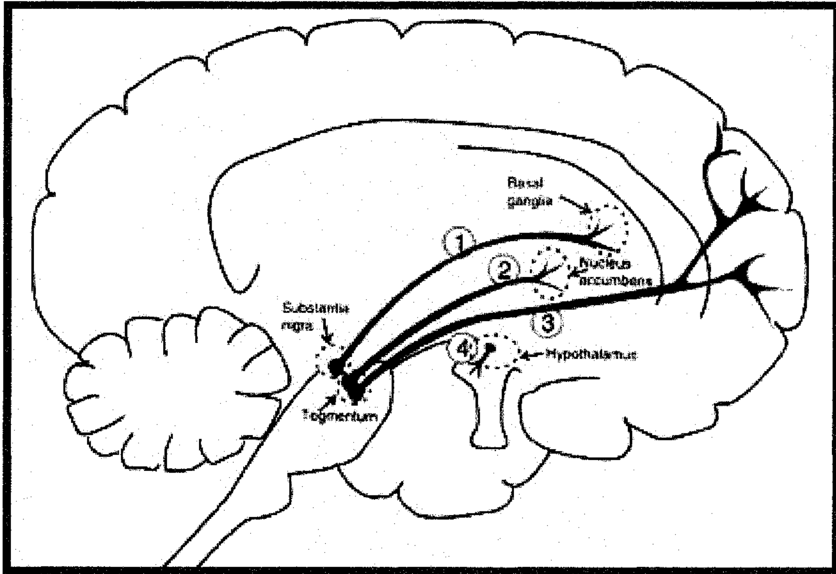
Additionnally, the mesolimbic pathway is one of the major neural pathways targeted by antipsychotic medication. Indeed, although the mechanisms whereby

antipsychotics mediate their therapeutic actions are not fully understood, disruption of DA function (particularly, an excess of this neurotransmitter) in this area has been linked to psychosis and the 'positive symptoms' of schizophrenia particularly delusions and hallucinations (5). Successful antipsychotic medication is therefore thought to have its effect by blocking DA receptors located in the mesolimbic pathway. DA neurons of the mesolimbic pathway are lost in Parkinson's Disease; however, neurons are lost far more quickly in the nigrostriatal pathway. Because severe motor deficits do not become apparent until a reduction of 80-90% in the number of neurons of the nigrostriatal pathway, the loss of mesolimbic neurons is almost asymptomatic.

The **mesocortical pathway** is a neural pathway that connects the VTA to the cortex, particularly the frontal lobes. This dopaminergic pathway is essential to the normal cognitive function of the dorsolateral prefrontal cortex (part of the frontal lobe), and is thought to be involved in motivation and emotional response. Moreover, this pathway is thought to be associated with the negative symptoms of schizophrenia like flat affect (lack of emotional response).

The **nigrostriatal pathway** is a neural pathway that connects the substantia nigra with the striatum. It is one of the four major DA pathways in the brain, and is particularly involved in the production of movement, as part of a system called the basal ganglia motor loop. Loss of the DA neurons in the substantia nigra is one of the main pathological features of Parkinson's disease, leading to a marked

Figure 1:Dopaminergic pathways (http://webs.wofford.edu/)


Basal
ganglia
1
2
Nucleus
accumbens
3
Substantia
nigra
4
Hypothalamus
Tegmentum

reduction of DA function in this pathway (6). Typically, symptoms of the disease do not emerge until 80-90% of DA neurons have been lost.

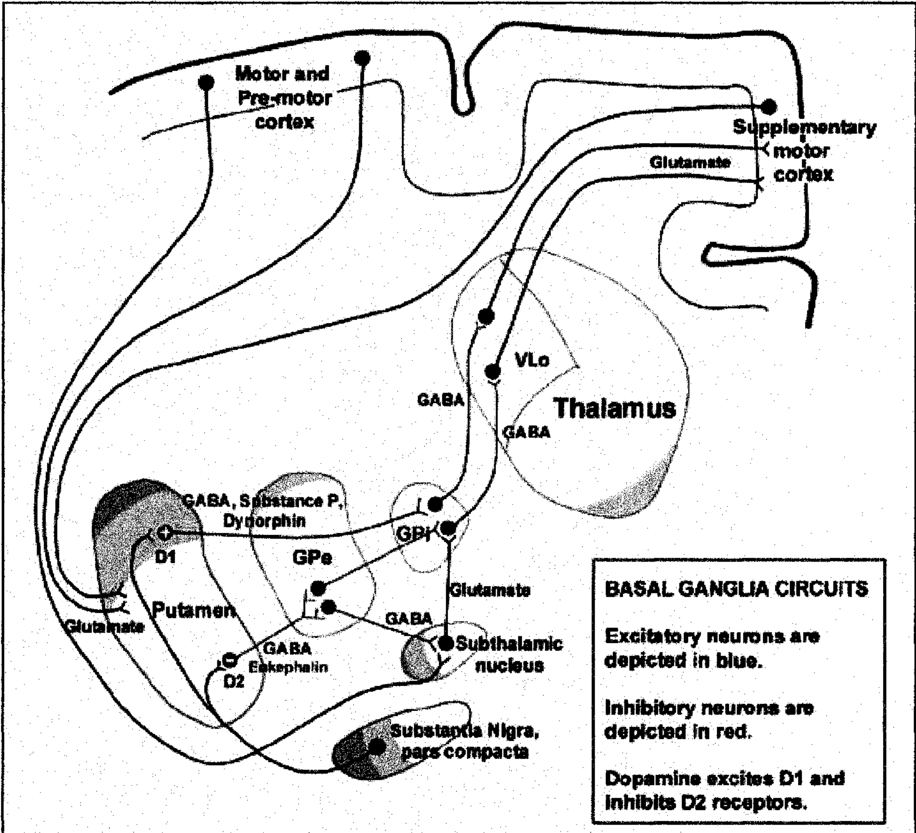
The nigrostriatal pathway is also implicated in the development of tardive dyskinesia, one of the side-effects of antipsychotic drugs. These medications (particularly the older typical antipsychotics like Phenothiazine) block D2-like dopaminergic receptors in multiple pathways in the brain. The desired clinical effect of reducing psychotic symptoms is thought to be associated with blocking DA function in the mesolimbic pathway only. However, as many of these drugs are not selective, they block DA function in all pathways. When this happens in the nigrostriatal pathway, motor problems similar to those found in Parkinson's disease can occur.

The **tuberoinfundibular pathway** is a neural pathway that runs between the hypothalamus and the pituitary gland. Some antipsychotic drugs block DA actions in the tuberoinfundibular pathway, which can cause an increase in blood prolactin levels, leading in some cases to hyperprolactinemia. This can cause abnormal lactation (even in men), disruptions of the menstrual cycle in women, visual problems, headache and sexual dysfunction. These side-effects suggest that the tuberoinfundibular pathway is involved in hormonal regulation, maternal behaviour, pregnancy and sensory processes.

Basal Ganglia

Generally, DA is produced by substantia nigra and VTA, which belong to basal ganglia in the brain. The basal ganglia is a component of the extrapyramidal system, which includes a network of subcortical nuclei of the telencephalon, sub-thalamus, and midbrain, modulating motor and cognitive function of the cerebral cortex. The nuclei in the basal ganglia include dorsal striatum (caudate and putamen), the ventral striatum (nucleus accumbans and anterior perforated substance), the dorsal pallidum (globus pallidus) and the ventral pallidum. Several other subcortical nuclei, the subthalamic nucleus, the substantia nigra, and VTA, reside at the interface between the diencephalon and midbrain. The cortical-basal ganglia-thalamic-cortical loops collect information from multiple forebrain structure, process this information through the basal ganglia, and move it back to the cerebral cortex to control movement and certain cognitive function. At least two loops have been identified: direct and indirect loops (7). In the direct loop, excitatory glutamatergic fibers from several functionally related areas of the isocortex converge on the dorsal striatum. GABAergic neurons in the striatal region, in turn, project to and inhibit neurons in discrete areas of the globus pallidus interna and the substantia nigra pars reticulata. Then these areas provide GABAergic projections to inhibit thalamus. Disinhibition of thalamus, in turn leads to excitation of cerebral cortex (Figuer 2).

Figure 2: Basal ganglia circuit (www.davidson.edu)



Motor and
Pre-motor
cortex
Supplementary
motor
cortex
Glutamate
VLo
Thalamus
GABA
GABA
GABA, Substance P,
Dynorphin
D1
GPe
GPi
Glutamate
Putamen
Glutamate
GABA
GABA
Enkephalin
D2
Subthalamic
nucleus
Substantia Nigra,
pars compacta
BASAL GANGLIA CIRCUITS
Excitatory neurons are
depicted in blue.
Inhibitory neurons are
depicted in red.
Dopamine excites D1 and
inhibits D2 receptors.

The indirect loop can be viewed as a side arm of the direct loop, through which striatal efferents reach the globus pallidus externa and after making synapses, pallidal efferents reach the subthalamic nucleus. Neurons of the subthalamic nucleus project excitatory glutamatergic fibers to globus pallidus interna where they excite GABAergic projection to thalamus and thereby inhibit thalamus. As mentioned above, the cells of the substantia nigra pars compacta and VTA produce DA. DA is able to interact with different types of membrane-bound receptors in the striatum specifically the nucleus accumbens, which belongs to the limbic system and prefrontal region. Dopaminergic projections excite striatal cells of the direct loop through D1-like dopaminergic receptors, while they inhibit striatal cells of the indirect loop through D2-like dopaminergic receptors. The overall effect is an enhancement of cerebral cortical activation through both the direct and the indirect loops. Similar direct and indirect pathways have been identified in the limbic system (8).

Regulation of dopamine synthesis and release

In addition to the rate limiting enzyme (TH), DA synthesis can be regulated by the amount of DA released from neurons. Following release from neurons, DA interacts with membrane-bound autoreceptors located on the presynaptic nerve terminal. Activation of these autoreceptors regulate both the DA release and firing rate of dopaminergic neurons (9). The release- and synthesis-modulating DA autoreceptors are found on axon terminals and somatodendritic regions of

dopamine neurons. The impulse-modulating DA autoreceptors are present on the somatodendritic region of the neurons.. Differences in localization of synthesis- and impulse-modulating autoreceptors to different types of neurons are thought to confer regional specificity on the function of DA neurons. All the DA autoreceptors are members of the D2-like dopaminergic receptor subfamily, which will be discussed in more detail in the next section (10).

Dopamine receptors

The dopaminergic receptors are transmembrane signaling proteins involved in recognition of DA, which mediates numerous physiological actions in the central nervous system (CNS) and periphery. The DA receptors are grouped in two subfamilies: D1-like (D1R and D5R) and D2-like ($D2R_{short}$, $D2R_{long}$, D3R and D4R) receptors. Both subfamilies belong to the large family of heterotrimeric GTP-binding (G) protein-coupled receptors (GPCRs) that are seven-membrane-spanning receptors (7MSRs). While members of D1-like receptor subfamily are primarily linked to stimulatory G proteins ($G\alpha_s$ and $G\alpha_{olf}$) and activation of adenylyl cyclases (AC), the D2R-like subtypes are coupled to AC inhibition through the stimulation of inhibitory G proteins ($G\alpha_{i1-3}$ and $G\alpha_o$) (11). The D1-like and D2-like dopaminergic receptors share the hallmarks of GPCRs i.e. seven hydrophobic spanning domains connected by three extracellular and three intracellular loops with the amino- and carboxyl-terminal ends located extracellularly and intracellularly, respectively. However, D1-like and D2-like receptors share striking structural differences. On one hand, the D1-like

receptors harbor a shorter third intracellular loop (IL3) when compared with their cognate D2-like receptors (~55-62 vs. ~123-155 amino acids). On the other hand, D2-like subtypes display a very short cytoplasmic tail (CT) in comparison to D1-like receptors (~12-14 vs. 114-117 amino acids).

The Dopamine D3 receptor

The D3 dopaminergic receptor (D3R) is one of the six known transmembrane signaling proteins involved in the recognition of DA. The human D3R gene is found on choromosome 3, band 3q13.3 (12). It encodes a primary mRNA of more than 53000 bases long with six exons and five introns (13). Similarly the rat D3 gene contains six exons and five introns (14). The translated human protein shows 78% homology with the rat D3 receptor. The difference is a deletion of 46 residues in the third intracellular loop (15). Although spliced variants of the third intacellular loop, such as that seen in the dopamine D2 receptor gene, have not been found in human and rat (except for mouse), several truncated isoforms of the D3 receptor have been reported (16,17). These truncated variants have been shown to lack high affinity agonist binding. It is likely that truncated forms are non-functional but immunoreactivity for some of these shorther variants has been observed in brain (18). These observations suggest that after transcription, some of these truncated forms of D3R are inserted in the plasma membrane. The physiological role of these truncated variants is unclear (19).

D3R distribution

D3 mRNA has been found in the nucleus accumbens and islands of Calleja and relatively low level in anterior caudate, putamen and granular cell layer of dentate gyrus (20,21). The general distribution pattern of the D3R protein is similar to D3R mRNA distribution pattern in human and rat brains (22,23). Overall, the D3R is expressed in striatal, mesolimbic and mesocortical areas of the brain suggesting a role for this receptor in cognition, emotion and motor control.

D3R expression during development

Catecholamines appear to affect a number of developmental processes in primitive organisms, including growth, regeneration and morphogenesis (24). The dopaminergic system appears early in brain development of higher species (25) and a neurodevelopmental role for DA has been suggested (26). By using PCR (27) and the highly selective D2/D3 receptor radioligand [^{125}I] iodosulpride (28), it was shown in rats that the first appearance of D3R mRNA is at embryonic day 14 (E14). D3R mRNA is more abundant during pre- and early post-natal period than in adult: the striatal neuroepithelium expresses strong signals from E14, culminating at E18, and progressively declining after birth (29). In addition, transient expressions in neocortical and medial mammillary body, core part of nucleus accumbens are found in newborns (30). Comparisons between the expression patterns of the D1R, D2R and D3R mRNAs revealed marked differences, suggesting distinct expression, regulation and roles of these DA

receptors. Whereas the D3R mRNA is almost exclusively expressed in the proliferative neuroepithelium during prenatal period, D1R and D2R mRNAs mostly appear in differentiating neurons, before DA innervation. Thus, prenatal D3R mRNA is almost entirely confined in neuroepithelial cells of striatum, amygdala, olfactory bulb and tectum; most of the progeny of which rapidly loses the capacity to transcribe the D3R gene during migration and differentiation. D3R mRNA labeling in the striatal neuroepithelium overlaps the synthetic and mitotic zones (29), which are restricted to a thin layer close to the ventricle at early stages (E14–E16), but spread in the subventricular zone at later stages (31). A specific role of the D3R in neurogenesis can, thus, be inferred from its highest level of expression in the prenatal period as compared to adulthood and its selective localization in the proliferative zone of the neuroepithelium during prenatal period. Such a role is supported by the mitogenic response induced by recombinant D3R stimulation in transfected cells (32). D3R mRNA appears in differentiated neurons of the shell of nucleus accumbens only during the first week of life. It seems to be triggered after the settling of DA innervation, which might apply a trophic influence. This may indicate that, like in the adult brain, the D3R gene expression in the shell of nucleus accumbens is under the positive influence of a trophic factor, synthesized by DA neurons (33). In the human brain, the D3R mRNA is first detected in the neuroepithelium as early as after 6 weeks of gestation, then in differentiated neurons of the putamen and nucleus accumbens at 10 weeks of gestation, whereas the dopaminergic innervation of the area is settled from 9 weeks (34). Hence, a trophic role of dopaminergic

neurons similar to that postulated in rats could also take place in the developing human brain. There is, however, a striking difference between the two species: the cortical neuroepithelium giving rise to the cerebral neocortex is heavily labeled in the human, but not in the rat embryo. The most superficial cortical layers are also labeled, particularly in the frontal cortex, which is consistent with an extended distribution of D3R transcripts in most cortical regions of the adult human brain (35).

Regulation of D3R expression in the brain

In adults, the expression of the D3R in medium sized neurons of the nucleus accumbens, but not in granule cells of the islands of Calleja, is highly dependent upon the dopaminergic innervation: ablation of the afferent neurons by unilateral 6-hydroxydopamine (6-OHDA) results in a dramatic decrease in the D3R density in ipsilateral nucleus accumbens (33). The D3R density is also decreased in a non-human primate model of Parkinson's disease, i.e. in MPTP-treated monkeys (36) or in patients suffering from this disease (37). In striking contrast, the D2R is upregulated under these circumstances. The effect on D3R density was shown to depend on the deprivation of an anterograde factor from dopaminergic neurons, distinct from DA itself and its known peptide co-transmitters, and which is released upon the dopaminergic neuron activation (33). Among the candidate factors for regulating D3R expression, BDNF was particularly attractive, since it is expressed in dopaminergic neurons (38). BDNF immunoreactivity is prominent in

the shell of nucleus accumbens of normal rats (39), and its receptor TrkB co-localizes with the D3R (40). Moreover, BDNF and D3R expression, which are both very low at birth, simultaneously increase during postnatal development. A local infusion of BDNF reversed the 6-OHDA-induced decrease in D3R gene expression indicating that exogenous BDNF compensates for the loss of dopaminergic neurons (40). BDNF belongs to a family of highly conserved polypeptide growth factors including as well nerve growth factor (NGF), neurotrophin NT-3 and NT-4 (41). Similar to many other secreted growth factors, neurotrophins are synthesized as preproproteins, which are subsequently cleaved to smaller, mature forms that homodimerize. At the molecular level, mature neurotrophins exert their effects by interacting with two structurally unrelated receptors: p75NTR, a member of the tumor necrosis factor (TNF) receptor superfamily, and the Trk receptor tyrosine kinases. The two receptors for neurotrophins also differ in terms of ligand binding specificity. While p75NTR is capable of binding to all mature neurotrophins with equivalent affinity but unique kinetics (42, 43), Trk family members exhibit ligand selectivity. Thus NGF is the preferred ligand for Trk A, BDNF and NT-4 for Trk B and NT-3 for Trk C (44). The extracellular motifs of p75NTR and Trk receptors are unrelated, with neurotrophins interacting with the immunoglobulin-like C2 (IgGC2) domains of the Trk receptors, but with the cysteine-rich domains of the p75NTR receptor (45, 46). The intracellular portions of the two receptors also share no homology. Unlike full-length Trk receptors that possess signature tyrosine kinase motifs, the intracellular domain of p75NTR does not exhibit intrinsic ligand-inducible

enzymatic activity (47, 48). The BDNF gene mutation does not impair early development of dopamine neurons (49), nor their later development, since TH, a marker of these neurons, was not significantly affected by the lack of BDNF (40). This suggests that BDNF acts directly on D3R-expressing neurons rather than indirectly via an effect on development of dopaminergic neurons. Moreover, BDNF deprivation selectively reduces the expression of the D3R, and not that of the D1R and D2R (40), which are not, or only marginally down-regulated by 6-OHDA lesions (50). In unilaterally 6-OHDA-lesioned rats, repeated administration of levodopa, leading to extraneuronal DA formation, triggers D3R overexpression not only in the shell of nucleus accumbens, but also in the denervated striatum, a brain structure in which D3R expression is hardly detectable (50). During levodopa treatment of 6-OHDA-lesioned rats, infusion into the denervated striatum of IgG-TrkB, a selective BDNF antagonist, (51), impairs induction of both D3R mRNA and protein expression. This indicates that BDNF is necessary for this process. A D3R overexpression has been shown to be responsible for the development of behavioral sensitization to levodopa, i.e. a progressive enhancement of responsiveness, which appears as an increased number of levodopa-induced rotational movements: the development and extinction of behavioral sensitization parallel D3R expression in the striatum during the treatment with levodopa and after its cessation. Moreover, enhanced rotations are blocked by a preferential D3R antagonist (52) and induced by a selective partial D3R agonist (50). Infusion of IgG-TrkB dose-dependently inhibits behavioral sensitization, indicating that behavioral sensitization is triggered by

BDNF. In fact, striatal BDNF originates mainly from cortical neurons (53). Cortical ablation, however, partially impairs the induction of D3R overexpression in striatum and behavioral sensitization, indicating that both processes require the participation of corticostriatal neurons. Levodopa also induces BDNF mRNA in the frontal cortex in the 6-OHDA-lesioned side, mainly in cortical deeper layer 5 containing pyramidal cell bodies and layer 6, projecting to various subcortical areas, notably including various striatal and accumbal areas (54). This effect critically depends upon the activation of D1R or D5R (40) and is consistent with the presence of D1R on cortical pyramidal cells (55) and with the observation that stimulation of D1R or D5R under similar circumstances phosphorylates CREB (56), a factor activating BDNF gene transcription (57). As mentioned before, D1R and D5R are able to raise cAMP levels in postsynaptic neurons. A postsynaptic rise in cAMP levels can also accelerate the translocation of TrK B into the postsynaptic density (58). So it seems that induction of D3R expression in striatum is triggered by a D1R/D5R stimulation-dependent elevation of BDNF in cortico-striatal neurons, a process prominent in the 6-OHDA-lesioned side as compared to the control side, which accounts for the induction of D3R expression restricted to the lesioned side. Therefore, the regulatory mechanism controlling D3R expression markedly differs from other dopaminergic receptors, in which the level of receptor density and sensitivity is primarily controlled by the endogenous ligand.

Pharmacological properties of D3R

The high degree of homology between the transmembrane regions of D2R and D3R suggests that the pharmacological properties of these two receptors would generally be similar. However, D3R exhibits a significantly higher affinity for dopamine and D2-like agonists such as quinpirole and 7-OH-DPAT (14). The D3R has also a relatively high affinity, albeit significantly lower than D2R, for antipsychotic drugs and D2-preferential antagonists, such as spiperone, chlorpromazine and haloperidol (18). These ligand binding properties suggest that D3R is an important therapeutic target for antipsychotic and antiparkinsonian drugs (59).

Structure-activity relationships of the D3R

The human D3R contains 400 amino acids and is synthesized as a ~44 KDa protein that undergoes post-translational glycosylation (14,19). The seven transmembrane domains are believed to conform to α-helices, with the exception of transmembrane domain 4 in which the Cys-166, Pro-167 bond may introduce a bend in the α-helix (60). The transmembrane regions are likely to play a role in the D3R conformational changes that occur upon agonist binding (61). Based on studies with the prototypical catecholamine β2-adrenergic receptor, one can speculate about the functional importance of some of amino acid residues of the D3R: Ser-193 and Ser-196 in the transmembrane domain 5 which are believed to form a hydrogen-bond with the hydroxyl groups of catechols (62), the amine

group of monoamines may bond with Asp-110 of the transmembrane domain 3. The Cys-103 and Cys-181 may form an extracellular disulfide bond (14) and extracellular Asn residues (Asn-12, Asn-19, Asn-97) represent probable sites of post-translational glycosylation. Studies using chimeric D2/D3 receptors have revealed that the D3R binding conformation displaying high agonist affinity is regulated by sequences found in its third intracellular loop (63). Similar experiments also suggest a role for transmembrane domains 6 and 7 in the determination of antagonist affinity (64).

D3R signaling mechanisms

Studies pertaining to the effect of D3R activation on downstream pathways suggest that D3R-mediated cellular responses are completely dependent on the endogenous signaling molecules expressed in given cells (65). The D3R has been shown to couple to Gi/Go and as well to Gq proteins (66). The D3R can also modulate the activity of K^+ and Ca^{2+} ion channels. D3R activation reduces K^+ outward and Ca^{2+} inward through Gi/Go proteins. In addition, D3R activation can modulate also protein kinase activation. Indeed, D3R can activate the MAPK pathway through Gi/Go proteins (67). Moreover, D3R has been shown to phosphorylate and activate the elongation factor-1B (eLF-1B) through a non G protein-mediated but PKC-dependent pathway (68). A recurring observation with studies (35, 69) investigating the functional properties of D3R is that DA- or agonist-mediated activation of this receptor subtype leads to a substantially reduced or undetectable efficacy for regulating effector activity (e.g. AC or ion

channels) in comparison to the D2R. Structure-activity relationships (SARs) studies have demonstrated that these striking differences in D2R and D3R coupling properties to G proteins could be explained partially by the low degree of identity in the primary sequence found between the intracellular loops of these D2-like subtypes (the most prominent differences being found within IL3). However, co-expression studies of D3R and specific AC isoforms in heterologous cells (HEK293 cells) has elegantly shown that D3R couples robustly to the inhibition of the type 5 AC (AC5) in a Gαi-dependent manner (70).

ACs are membrane-bound enzymes that catalyze the conversion of ATP to cAMP upon activation by G proteins, leading to a cascade of phosphorylation reactions within the cell (70). The membrane-bound ACs have twelve conserved hydrophobic transmembrane segments which are arranged in two sets of six, separated by a large hydrophilic domain. It also has two large cytoplasmic domains (C1 and C2). The most conserved sequences are located in the cytoplasmic domains (C1 and C2) and the overall similarity among the different ACs is roughly 60% (71, 72, 73). There are at least ten isoforms of AC which can be categorized into 5 families; all show distinct biochemical properties and tissue distribution. Group 1 ACs, which are represented by AC1, AC3, and AC8, are stimulated by intracellular calcium in a calmodulin-dependent fashion. Group 2 ACs are characterized by their ability to be conditionally stimulated by Gβy subunits and are represented by AC2, AC4, and AC7. In addition, AC2 and AC7 are stimulated by activators of protein kinase C (PKC) such as phorbol esters. Group 3 ACs include AC5 and AC6, show robust negative regulation by Gαi

subunits and are also inhibited by protein kinase A (PKA) and $Ca2^+$. Group 4 ACs consist of one isoform, AC9, which is insensitive to forskolin stimulation. The last group is a soluble AC, which can act as a bicarbonate sensor (sAC) (74). Importantly, the demonstration of D3R coupling to AC5 remains to be demonstrated *in vivo*. However, in situ hybridization, autoradiography and immunohistochemistry studies have unequivocally shown that AC5 are highly enriched in CNS dopaminergic areas expressing DA receptors including D3R. In fact, the distribution pattern of AC5 generally overlaps with that of D3R in human brain (75). Moreover, AC5 knockout mice display severe deficits in dopaminergic neurotransmission. Overall, the low degree of identity in the primary structure of the cytoplasmic domains of D2-like receptors raise the possibility that homologous (agonist-specific) and heterologous (non agonist-specific) desensitization of D3R signaling is not mediated by the classical paradigm of GPCR regulation.

Classical paradigm of G protein-coupled receptor regulation

The GPCR family is the largest and the most diverse known receptor family comprising more than 1% of the human genome (76). In fact according to the most recent predictions, GPCRs represent the third largest family of genes present in the human genome (77). Based on the identity of GPCR genes, they can be separated into five main subfamilies: the rhodopsin group which includes the majority of the GPCRs (701 members), the glutamate receptor group (15 members), the adhesion receptor family (24 members), frizzled and taste-2

receptor (24 members) and secretin group (15 members) (78-79). GPCRs respond to a diverse array of sensory and chemical stimuli, such as light, odor, taste, pheromones, hormones, and neurotransmitters. GPCRs transduce the information provided by these stimuli into intracellular second messengers that are interpreted as meaningful signals by the cell. GPCRs have been named based on their capability to employ and control the activity of intracellular heterotrimeric G proteins (80). In response to stimulation, the α-helical cylinder movements cause activation of signaling by GPCRs (81). This stimulation is not a simple, one step activation process. Evidences are accumulating for the existence of multiple intermediate activated states of GPCRs (82). It has been shown that most GPCRs display a low, but detectable, basal activity. Agonists promotes the conversion of an inactive to active form of the receptor. This process leads to full receptor activation *via* several intermediate active states (83). Receptors in the basal state can couple with low efficacy to G proteins. Receptor activation by ligand binding causes changes in the relative orientations of transmembrane helices. These changes then affect the conformation of G protein-interacting intracellular loops of the receptor and thus uncover previously masked G protein-binding sites (84, 85). Then G proteins can interact with activated receptor through both its Gα and Gβγ dimer subunits (86). Heterotrimeric G proteins are part of a larger GTPase superfamily. G proteins are composed of three polypeptides (subunits): an α-GDP subunit (having intrinsic GTPase activity), and a βγ dimer that serves as a functional monomer (87). Gα subunits have been divided into 4 groups: Gαs (which includes Gαs and Gαolf),

Gαi (which includes Gαi, Gαt, Gαo, Gαgust, and Gαz), Gαq (which includes Gαq, Gαll, Gα14, Gαl5, and Gα16), and Gαl2 (which includes Gαl2 and Gα13) (88). There are some minor post-translational modifications differences between groups. For example, Gαs is acylated at an amino-terminal glycine whereas Gαi and Gαo are myristoylated at that site (89). Gβγ is a strong intricate dimer which dissociates just under denaturing conditions. The Gβγ dimer serves to augment the affinity of the Gα subunit for its receptor and to control a range of effectors, either directly or in combination with the Gα subunit. Additionally, Gβγ are involved in the translocation and activation of specific G protein coupled receptor kinase isoforms (GRK2 and GRK3) to the plasma membrane (90). The GDP-bound Gα subunit associates with the Gβγ dimer to form an inactive heterotrimeric complex. Following GPCR activation, the inactive heterotrimeric G protein can bind to receptor and the affinity of Gα for GDP decreases. Consequently, GDP dissociates from the Gα subunit to be replaced by GTP. Once GTP is bound, the Gα subunit adopts its activated conformation and dissociates from both the receptor and βγ dimer (following the concomittent GPCR phosphorylation) (91). Both Gα subunit and Gβγ dimer are now able to activate different downstream pathways directly *via* ACs or phospholipases C (PLC) or indirectly (e.g. the regulators of G proteins also known as RGS). Activated G proteins interact with downstream signalling factors to alter the production of second messengers such as inositol phosphates, calcium and cAMP (92). GPCRs activating Gα subunits of the Gi class (e.g. D3R) inhibit cAMP production whereas GPCRs stimulating Gα subunits of the Gs class (e.g.

D1R) activate cAMP production. cAMP in turn activates the cAMP-dependent protein kinase or PKA (93).

PKA is a tetramer composed of two catalytic subunits and two regulatory subunits. Regulatory subunits repress the activity of catalytic subunits when they are bound together. The regulatory subunits bind cAMP when it is formed by AC and free the catalytic subunits, hence releasing their inhibition. The active PKA catalytic subunits, when they are released, phosphorylate target substrate proteins on serine and threonine residues, altering the activity of the modified protein and creating a cellular response to the extracellular stimulus acting on the GPCR. The PKA pathway is an example of a signal transduction cascade that tie several signalling events together to amplify the original signal in the cell. For each GPCR molecule that is activated, several G proteins can be activated, and each active G protein can synthesize many cAMP molecules promoting PKA and further downstream intracellular signaling (e.g. transcription factors).

GPCRs can also transduce signals by activating PKC isoforms (94). PKCs are a ubiquitous family of serine/threonine protein kinases. The pathway leading to PKC activation starts with a class of GPCRs that interact with and activate Gq proteins. Activated GTP-bound Gq stimulates its downstream target PLC to hydrolyze the membrane lipid PIP2, producing IP3 and diacylglycerol (DAG). IP3 is water-soluble and diffuses through the cytoplasm to the ER, where it binds to and opens a calcium channel, releasing calcium stores from inside the ER into the cytoplasm. Calcium alters many cellular processes, in part by binding to regulatory proteins such as calmodulin and calcineurin. The interaction of both

DAG and calcium with PKC activates its kinase activity and the phosphorylation of many different protein targets alters their activity.

In addition to the classical AC/PKA and PLC/PKC pathways, Gαt or transducin (the retinal Gα subunit) activates a distinctive signaling pathway, the photoreceptor cGMP phosphodiesterase (95). Gβγ interacts with its effectors through the Gα binding site on Gβ subunit. There are numerous signaling pathways regulated by Gβγ. For instance, Gβγ has well defined effects on some isoforms of the classical second messenger enzymes, phospholipase Cβ2 (PLCβ2) and PLCβ3 (96) and AC. Gβγ potentiates Gαs-mediated activation of AC2, AC4, and AC7 while inhibiting AC1. The Gβγ dimer also binds to the phosphoprotein phosducin. It has been demonstrated that phosducin is able to sequester Gβγ and thereby regulate its availability through a PKA-regulated mechanism (97).

The GPCR-mediated G protein-linked responses are rapidly attenuated. The attenuation or desensitization is very important; any deficit in this physiological process may cause uncontrolled stimulation and lead to diseases. The GPCR desensitization is defined as an increase in the refractoriness of a receptor to mediate intracellular signaling following repeated or sustained exposure to an agonist. The classical paradigm of GPCR regulation includes two types of desensitization: homologous and heterologous. Homologous desensitization is caused by agonist-dependent activation of the same GPCR. This is a two-step process involving the activity of specific receptor kinases called G protein-coupled receptor kinases (seven isoforms: GRK1-7) and cytosolic proteins

termed arrestins (four isoforms: rod arrestin, cone arrestin, β-arrestin 1 and β-arrestin 2) (98). Agonist-induced desensitization can also implicate second messenger-dependent kinases (protein kinase A or PKA; protein kinase C or PKC). Heterologous or "non agonist-specific" desensitization is exclusively mediated by the activation of a different GPCR that involves the activity of a second-messenger kinases only. Classical desensitization of GPCRs (e.g. β2-adrenergic receptor) has been associated with **three temporally distinct** cellular events. **First**, short-term exposure to agonists (seconds to minutes) leads to a rapid uncoupling of the receptor from its G protein due to receptor phosphorylation. **Second**, upon agonist occupancy, rapid endocytosis of the receptor follows GPCR phosphorylation. **Third**, long-term exposure to agonist (hours to days) leads to a profound reduction of the total receptor number (downregulation) mediated by cell surface proteolysis and/or targeting to lysosomes. Rapid agonist-mediated desensitization involves covalent phosphorylation of receptors by PKA, PKC and GRKs. For the β2-adrenergic receptors and most of GPCRs, PKA and PKC-mediated receptor phosphorylation directly uncouples the receptor from G proteins. In contrast, phosphorylation of the agonist-occupied or activated form of the receptor by a GRK promotes the binding of arrestins to the phosphorylated GPCR, which disrupts the receptor/G protein interaction leading to desensitization. It is worth mentioning that β-arrestins are found in several neuronal pathways and immunoelectron microscopy studies revealed that β-arrestins are concentrated at synapses along with GRKs (99, 100). Importantly, β-arrestins preferentially bind to agonist-

activated and GRK-phosphorylated GPCRs as opposed to non-phosphorylated or PKA- and PKC-phosphorylated GPCRs. Moreover, β-arrestins are adaptors that can bind clathrin and α2-adaptin of the AP2 heterotetrameric adaptor complex to regulate dynamin-dependent agonist-promoted GPCR internalization through clathrin-coated vesicles (CCV); a process leading to receptor dephosphorylation by phosphatases. Indeed, after desensitization and internalization, GPCRs can be resensitized by dephosphorylation in phosphatase-enriched endosomes. The dephosphorylated GPCR can then recyled back to the cell surface in the preligand exposed state. However, not all GPCRs exhibit the same desensitization and internalization features. Some are desensitized by GRK in a receptor phosphorylation-independent manner and some are GRK phosphorylated and bind arrestins but do not internalize or do so by non-CCV mechanisms.

Regulation of D2-like receptors.

Studies have shown that the D2R undergoes homologous and heterologous regulation by second-messenger kinases, GRKs and arrestins. Agonist-induced internalization of D2R involves the recruitment of arrestins at the plasma membrane. It is currently unknown whether regulation of D3R responsiveness and signaling works through classical mechanisms. In fact, a study by Caron's group has shown a differential regulation of D2R and D3R by GRKs and β-arrestins using the heterologous system HEK293 cells. In striking contrast to

D2R, Kim *et al* (101) reported that D3R is not phosphorylated in the absence of agonist and undergo a subtle (barely detectable) agonist-induced receptor phosphorylation. Moreover, the study indicated a lack of DA-induced D3R internalization and plasma membrane translocation of arrestins. This phenotype was reversed following the exchange of the IL2 and IL3 of D2R and D3R.

Rationale, hypothesis and objectives.

We reasoned that the lack of DA-induced phosphorylation and internalization may require a cellular component that is not expressed in HEK293 cells. We hypothesize that one potential partner for D3R regulation could be a specific type of adenylyl cyclases, namely AC5. As stated above, HEK293 cells co-expressing D3R and AC5 display a robust DA-induced inhibition of AC activity. The specific objective of my Masters research project is to investigate the functional importance of AC5 in the regulation of phosphorylation of the D3R.

METHODS

Cell Culture and Transfection

Human embryonic kidney 293 (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C and 5% CO2 in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin (10 µg/ml) (Invitrogen, Burlington, Ontario, Canada). Cells were seeded into 100-mm dishes ($2.5X10^6$ cells/dish) and transiently transfected with a total of 7.5 µg of DNA/dish using a modified calcium phosphate precipitation method as described previously (102). Specifically, HEK293 cells were transfected with the HA-tagged human D3R (Ser9 variant) alone (5 µg of receptor expression construct:2.5 µg of empty pCMV5) or with canine AC5 (5 µg of receptor expression construct:2.5 µg of AC5 expression construct) unless indicated otherwise. Experiments were performed 60 hours following DNA transfection.

Membrane Preparation and Radioligand Binding

For binding studies, on the day of experiment, transfected cells were washed with PBS, scraped in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4; 5 mM EDTA), and centrifuged twice at 40,000 g for 20 min at 4°C. The pellet was resuspended in binding buffer (50 mM Tris-HCl, pH 7.4; 120 mM NaCl; 5 mM KCl; 4 mM MgCl2; 1.5 mM CaCl2; 1 mM EDTA) using a Brinkman Polytran (17,000 r.p.m for 15 s). Binding assays were carried out with 100 µl of membranes in a total volume of 500 µl using [^{3}H]-spiperone as radioligand. To determine non-specific

and specific binding, membranes were incubated with a saturating concentration of [^{3}H]-spiperone (~ 3 nM) in the presence and absence of 10 µM (+)-butaclamol for 60 min at 25°C. The binding assays were terminated using rapid filtration through glass fiber filters (GF/C, Whatman) and the filters were washed three times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4; 120 mM NaCl). The bound radioactivity was calculated by liquid scintillation counting using a Beckman Counter (LS 6500). Protein concentrations were measured using the Bio-Rad assay kit with bovine serum albumin (BSA) as standard. For all studies described below, the maximal receptor expression (Bmax) was between 1-3 pmol/mg membrane proteins.

D3R Electrophoretic Mobility: Cold Immunoprecipitation Studies

On the day of assay, HEK293 cells transfected with HA-tagged D3R (Ser9 variant) alone or with AC5 were incubated with ascorbic acid (100 µM, control) or DA (10 µM, treated) for 15 min at 37ºC. The cells were put on ice and washed 2 times with ice-cold PBS and solubilized in 800 µl of radioimmunoprecipitation assay plus (RIPA+) buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM NaF and 10 mM disodium pyrophosphate) containing protease inhibitors (20 µg/ml phenylmethylsulfonyl fluoride (PMSF), 0.2 µg/ml aprotinin, 0.1 µg/ml pepstatin A, 10 µg/ml benzamidine, leupeptine and soybean trypsin inhibitor) for 1 h at 4ºC. At the end of solubilization period, the cell extracts were clarified by

centrifugation at 15000 g for 15 min at 4°C. Two aliquots (25 μl) of the supernatants were taken for protein concentrations determined using Bio-Rad DC protein assay kit and BSA as standard. Supernatant fractions were then transferred to 1.5 ml conical tubes containing 50 μl of 10% (v/v) protein A-Sepharose beads in 2% BSA and precleared on a rotating wheel for 1 h at 4°C. Protein A-Sepharose beads were pelleted and 50 μl of rat monoclonal anti-HA affinity matrix was added to supernatants in new tubes. Following overnight rotation at 4°C, anti-HA affinity matrix was pelleted and the supernatant discarded. Subsequently, the beads were washed five times with 1 ml of ice-cold RIPA+ buffer and dried. Then 60 μl of SDS sample buffer (25 mM Tris-HCl (pH 6.5), 8% (v/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol) was added to each tube and immunocomplexes were dissociated at room temperature for 2 hours. Samples were then resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using 10% gels (v/v). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer apparatus at 18 V for 15 min and probed with mouse anti-HA biotin antibody (1: 1000; Covance, USA). Horseradish peroxidase-conjugated anti-mouse antibody (1:7500) was used as the secondary antibody and proteins were visualized by enhanced chemiluminescence using ECL western blotting detection reagents (Amersham Biosciences).

Whole Cell Phosphorylation Assays

On the day of experiment, HEK293 cells transfected with HA-tagged D3R alone or with AC5 were labeled in 20 mM HEPES-buffered phosphate free Dulbecco's modified Eagle's medium (pH 7.4) containing gentamicin (10 μg/ml) and 0.15 mCi/ml [^{32}P]orthophosphate for 90 min at 37°C. After labeling period, whole cell phosphorylation experiments were carried out essentially as described for cold immunoprecipitation studies (see previous section). SDS-PAGE gels containing resolved HA-tagged D3R immunocomplexes were fixed (10% (v/v) acetic acid and 10% (v/v) methanol) and dried. The extent of D3R phosphorylation was assessed with Typhoon PhosphorImager 8600 (Amersham Pharmacia Biotech) and values normalized for lane background and receptor number. Dried gels were then exposed to Kodak films at -80°C overnight. Similar experiments were also conducted in the presence of Gαi (pertussis toxin or PTX) or phosphatase inhibitors as described in the next section.

Whole Cell cAMP Assays

HA-tagged D3R was transfected alone or with canine AC5 in HEK293 cells to assess the regulation of endogenous ACs and transfected AC5 by D3R under inhibitor pretreatments. Assays were carried out in the absence or presence of phosphatase inhibitors or pertussis toxin (PTX). Whole cell cAMP assays were performed as described previously (103, 104). Briefly, 18-24 hours following transfection, HEK293 cells were reseeded in 12-well dishes and grown for an

additional 24 hours. The next day, cells were labeled with $[^3H]$-adenine (1 μCi/ml) in fresh minimum essential medium containing 5% (v/v) fetal bovine serum, gentamicin (10 μg/ml) in the absence or presence of PTX (20 ng/ml) for 18 hours at 37˚C in a 5% CO2 environment. On the day of assay, the labeling medium was removed and replaced with 20 mM HEPES-buffered MEM (containing 1 mM isobutylxanthine (IBMX)) and cells incubated with ascorbic acid (100 μM), forskolin (5 μM (FSK); AC activator), DA (100 μM) or FSK+DA for 15 min at 37˚C. To assess the role of phosphatases in the D3R-mediated inhibition of AC, cells were pretreated with specific phosphatase inhibitors (microcystin-LR (PP1/PP2A), 5nM; cyclosporine-A (PP2B/calcineurin), 5 μM; sodium ortho-vanadate (tyrosine phosphatases), 10 μM) for 15 min prior to FSK and DA stimulation (15 min) at 37˚C. At the end of the FSK and DA incubation period, the medium was aspirated, and each well filled with 1 ml of lysis solution containing 2.5% (v/v) perchloric acid, 1 mM cAMP, and $[^{14}C]$-cAMP (3.75 nCi, ~ 7500 cpm) for 30 min at 4˚C. The lysates were then transferred to tubes containing 0.1 ml of 4.2 M KOH (neutralizing solution) and precipitates were pelleted by a low-speed centrifugation (~ 500 g) at 4˚C. Intracellular $[^3H]$-cAMP in supernatants was purified by a sequential column chromatography method using Dowex (AG 50W-X4) and alumina columns as described previously (105). The amount of intracellular $[^3H]$-cAMP (CA) over the total amount of intracellular $[^3H]$-adenine (TU) was calculated to determine the relative AC activity (CA/TU X 1000).

Statistics

In the present study, values are reported as arithmetic means ± S.E. Two-way ANOVA with Bonferroni posttests and unpaired t test (two-tailed p value) were performed using GraphPad Prism version 4.03 for Windows, GraphPad Software (San Diego, CA, www.graphpad.com). The level of significance was established at $p < 0.05$.

Results and Discussion

4.1 The D3R displays two electromobility bands in HEK293 cells.

We perform immunoprecipitation and immunoblotting experiments to assess the electrophoretic mobility of the HA-tagged D3R in HEK293 cells. Bioinformatic analyses using Scansite 2.0 (http://scansite.mit.edu/) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) servers predict a theoretical molecular weight of 46 kDa for HA-tagged D3R and four potential N-glycosylation sites located on extracellular domains (Asn12, Asn19, Asn97) and exofacial end of TM4 (Asn173) of the receptor (Figure 3). Our results show that D3R displays two electrophoretic mobility bands (~46 and ~65 kDa) in HEK293 cells, which likely corresponds to unglycosylated (or partially / co-glycosylated) and fully glycosylated forms of the receptor (Figure 4).

Figure 3. Schematic representation of the secondary structure of the human HA-tagged D3R. Amino acid sequence of HA epitope is depicted using black circles. The potential N-glycosylation and phosphorylation sites are indicated using arrowheads and asterisks, respectively. The double arrowhead represents the serine-to-glycine substitution observed in the Bal I polymorphism.

HA epitope

pka

pkc

pka

pka

pkc

pkc

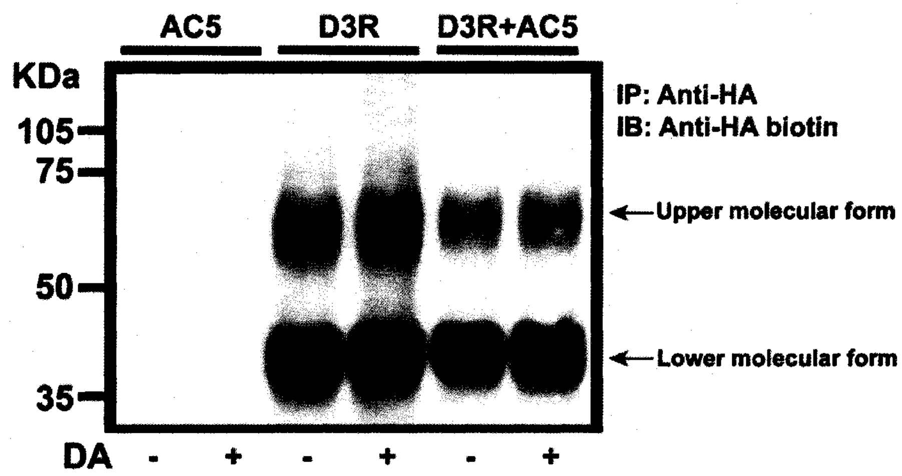
pka

pka

pka

pka

Figure 4. Electrophoretic mobility of the human HA-tagged D3R in HEK293 cells. A representative example of co-immunoprecipitation and immunoblotting studies (n=3) using HEK293 cells transfected with the adenylyl cyclase type 5 (AC5) alone, the human HA-tagged D3 receptor (D3R) alone or with AC5 (D3R+AC5) is shown. Transfected cells were solubilized in RIPA+ buffer and clarified supernatants incubated with rat monoclonal anti-HA agarose. Immunoprecipitated proteins were resolved on 10% (v/v) SDS-PAGE gels, transferred on PVDF membranes and subjected to immunoblotting procedures using mouse primary anti-HA biotin and secondary horseradish-conjugated anti-mouse antibodies. D3R has two electrophoretic mobility bands in HEK 293 cells, referred to as the upper (~65 KDa) and lower (~46 KDa) molecular forms. Molecular weight markers are shown and expressed in kilodaltons (KDa). DA, dopamine.


AC5
D3R
D3R+AC5
KDa
105
75
50
35
IP: Anti-HA
IB: Anti-HA biotin
Upper molecular form
Lower molecular form
DA
-
+
-
+
-
+

No HA-tagged D3R immunoreactive bands could be detected in HEK293 cells transfected with AC5 only. In addition, our results show that the electrophoretic mobility of HA-tagged D3R is not altered in cells expressing AC5 (Figure 4). These results suggests that the intracellular sorting of AC5 (a twelve transmembrane protein) does not interfere with D3R maturation process. Meanwhile, it remains to be established whether these two D3R forms have distinct signaling properties. This issue will be discussed in more detail in a section to follow. Interestingly, a previous photoaffinity labeling study using [^{125}I]-NAPS (N-(p-amino-phenethy)spiperone) showed that the FLAG-tagged D3R displays three electrophoretic mobility bands in HEK293 cells. A major broad band was detected at 60-80 kDa (106). Additionally, 40-50 kDa and 25 kDa bands were also observed (106). Kim and collaborators (106) suggested that the 25 kDa band represents an unprocessed form of the FLAG-tagged receptor. The lack of detection of a 25 KDa in our studies suggest that processing of HA- and FLAG-tagged forms of D3R are potentially different. However, another study using FLAG-tagged D3R has reported a receptor electrophoretic mobility similar to our HA-tagged D3R (107). Importantly, preliminary studies from our lab indicate that the ligand binding properties of the wild-type and HA-tagged forms of D3R are not significantly different (data not shown).

4.2 D3R undergoes constitutive and dopamine-induced phosphorylation in HEK293 cells.

Whole cell phosphorylation experiments performed in HEK293 cells expressing the receptor alone demonstrate that the 46 KDa (lower band) and 65 KDa (upper band) forms of HA-tagged D3R are constitutively phosphorylated. The extent of D3R phosphorylation is further increased following dopamine exposure (15 min) suggesting that the agonist-occupied form of D3R in HEK293 cells serves as a potential substrate for endogenous kinases that remain to be identified (Figure 5).

Interestingly, both forms of HA-tagged D3R are phosphorylated following DA stimulation (Figure 5). These results suggest that the 46 KDa band corresponds to a functional form of D3R expressed at the plasma membrane. Our results are in striking contrast to previous studies reporting that D3R is not significantly phosphorylated in HEK293 cells expressing the receptor alone or with GRK2 (101, 106). The underlying reasons for this discrepancy are unclear. However, as reported in other GPCR signaling studies using HEK293 cells, different cell isolates and/or cell culture conditions may explain this discrepancy (108)

Figure 5. Regulation of the D3R phosphorylation status by adenylyl cyclase type 5 (AC5) in HEK293 cells. A, Representative example (n=3) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with the receptor in the absence or presence of AC5. **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R. Results are expressed percent relative to control (basal of D3R alone). The extent of phosphorylation of the upper and lower molecular forms of the receptor upon DA exposure was significantly different in cells expressing D3R alone in comparison with cells co-expressing D3R and AC5 ($p<0.05$, two-way ANOVA). The basal phosphorylation was not significantly altered following co-expression of AC5 ($p>0.05$). KDa, kilodaltons; DA, dopamine.

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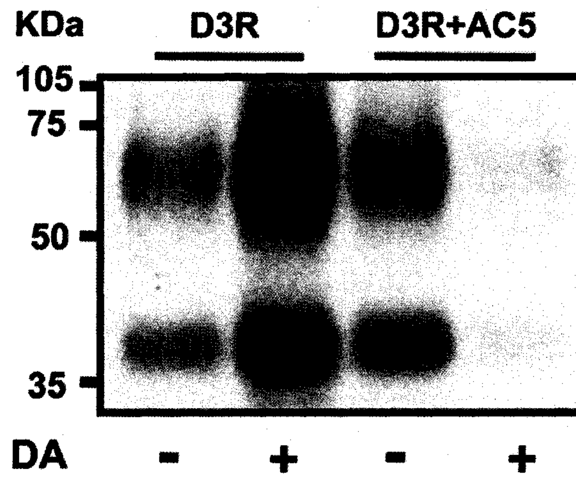

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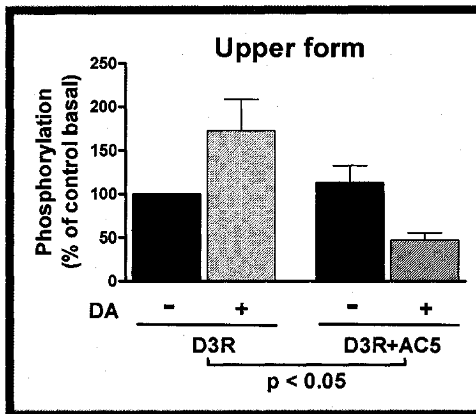

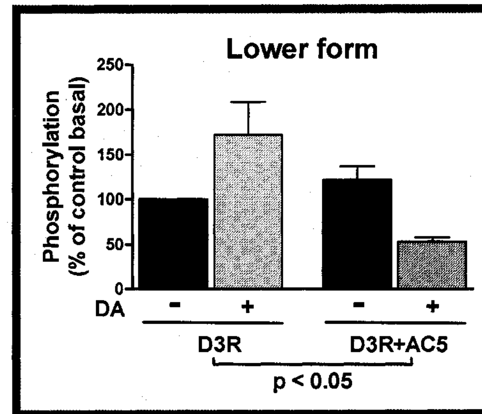

4.3 AC5 expression mediates dopamine-induced dephosphorylation of D3R in HEK293 cells.

Whole cell phosphorylation studies were performed in HEK293 cells transfected with HA-tagged D3R and AC5. Under these experimental conditions, the constitutive phosphorylation of D3R was not significantly changed (Figure 5). Intringuingly, our results clearly showed that both molecular forms of HA-tagged D3R undergo a robust agonist-induced dephosphorylation following a 15 min-exposure to DA (Figure 5). These results suggest a novel GPCR paradigm whereby agonist binding/activation promotes receptor dephosphorylation in an AC isoform-specific manner. Notably, HEK293 cells endogenously express AC1, AC2, AC3, AC6 and AC7 but not AC5 (109, 110). Therefore, our studies suggest that AC5 is an important signaling partner in the regulation of the phosphorylation status of D3R. Meanwhile, it remains to be established whether AC5-mediated effects on D3R phosphorylation status following DA exposure are dependent on a direct physical interaction between these two molecular entities. Notwithstanding this issue, our studies imply a central role for phosphatases in the regulation of D3R phosphorylation status by AC5. We hypothesize that the formation of a D3R-AC5 signaling complex or signalosome containing protein phosphatases promote the dephosphorylation of constitutively phosphorylated D3R following DA exposure. This view is supported by a recent study (111). Indeed, while this thesis was in preparation, Crossthwaite *et al.* (111) have shown that the cytoplasmic amino terminus of AC8 interacts directly with the catalytic subunit of serine/threonine protein phosphatase 2A (PP2A). Additionally,

studies performed using dopamine transporter (DAT) and β-arrestin 2 (βarr2) knockout mice have shown that DA-dependent behaviors controlled by the D2-like receptors (D2R, D3R and D4R) involve the formation of signalosomes containing βarr2 associated with phosphatase (PP2A) and kinase (Akt) activities (112). As mentioned before, the classical paradigm for GPCR desensitization postulates that agonist-promoted receptor phosphorylation initiates the desensitization process, which is rapidly followed by receptor internalization, dephosphorylation and recycling to the cell surface (113). Therefore, the AC5-mediated DA-induced D3R dephosphorylation may represent a novel mechanism whereby a constitutively phosphorylated GPCR (e.g D3R) is desensitized by dephosphorylation following agonist binding or activation in a AC isoform-dependent manner. However, an alternative and more attractive explanation is that the constitutively phosphorylated D3R is desensitized and unable to couple to inhibition of endogenous ACs. We would like to propose that the DA-induced dephosphorylation of D3R mediates its resensitization and thus enable the functional coupling of D3R with G proteins. In fact, we believe that this process underlies the robust coupling to Gi-like proteins and DA-mediated inhibition of AC activity previously observed in cells co-expressing D3R and AC5 but not in cells expressing D3R alone (70). In a series of experiments described in the following sections, we have addressed the potential role of serine/threonine protein phosphatases in the regulation of D3R phosphorylation status in HEK293 cells expressing the receptor alone or with AC5. Notably, we performed whole cell phosphorylation assays in the presence of classical pharmacological blockers of

serine/threonine protein phosphatases (PP1/PP2A, microcystin-LR; PP2B/calcineurin, cyclosporine A) and protein tyrosine phosphatases (PTP, sodium orthovanadate) to assess the role of different protein phosphatase subfamilies and their potential interplay in regulating the phosphorylation/dephosphorylation of D3R and D3R-mediated inhibition of AC5. To do so, HEK293 cells transfected with HA-tagged D3R alone or with AC5 were pretreated with pharmacological inhibitors of phosphatases for 15 min prior to DA and FSK stimulation (15 min). Results obtained from these studies are described and discussed below.

4.4 The serine/threonine phosphatases PP1 and PP2A do not play a role in the AC5-dependent dopamine-induced D3R dephosphorylation.

HEK293 cells transfected with D3R alone or with AC5 were pretreated with microcystin-LR (5nM), a PP1/PP2A blocker. Microcystin-LR pretreatment had no significant effect on the phosphorylation and dephosphorylation status of the higher and lower molecular forms of HA-tagged D3R in the absence or presence of DA (Figures 6 and 8A-B). Our data suggest that the serine/threonine phosphatases PP1 and PP2A are not involved in the control of constitutive and DA-induced D3R phosphorylation in cells lacking expression of AC5. Similarly, constitutive phosphorylation and DA-induced dephosphorylation of D3R in AC5-expressing HEK293 cells is not regulated by PP1/PP2A phosphatase activities.

Figure 6. Effect of microcystin-LR (MIC) on the regulation of receptor phosphorylation in HEK293 cells expressing D3R alone.

A, Representative example (n=3) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with the D3R alone pretreated with microcystin-LR (MIC, a PP1/PP2A inhibitor). **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells pretreated with microcystin-LR (MIC). Results are expressed as percent relative to control basal (no DA, no MIC). The MIC pretreatment had no significant effect on the extent of D3R phosphorylation under basal conditions or following DA exposure when compared with control ($p>0.05$; two-way ANOVA). KDa, kilodaltons; DA, dopamine.

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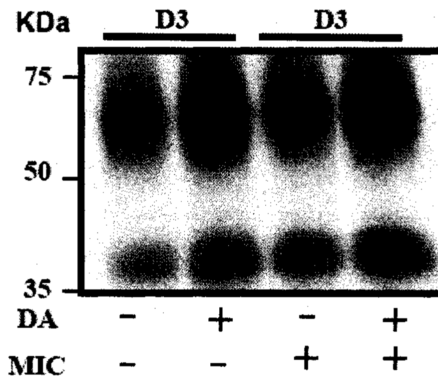

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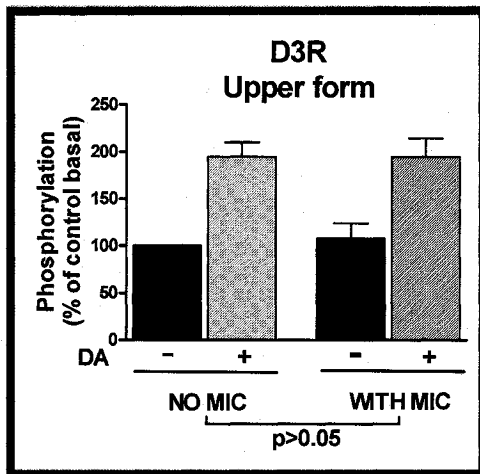

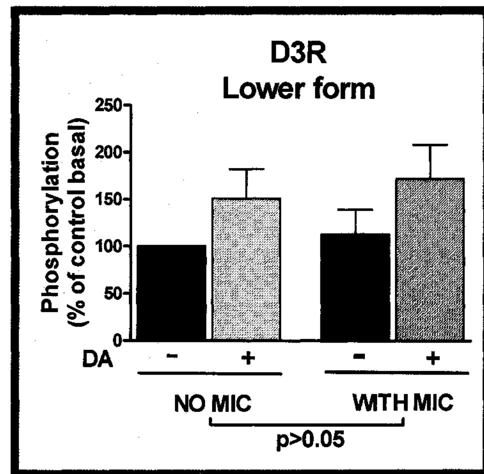

4.5 Blockade of the serine/threonine protein phosphatase 2B (PP2B) inhibits AC5-dependent dopamine-induced D3R dephosphorylation.

Whole cell phosphorylation studies were conducted in cells pretreated with cyclosporine A (5µM) to inhibit PP2B (also called calcineurin). Our data show that PP2B inhibition in cells expressing HA-tagged D3R alone leads to an increase in constitutive and DA-induced phosphorylation of the two molecular forms of D3R (Figure 7). These results suggest that PP2B tonically inhibits the basal and DA-induced phosphorylation of D3R in cells not expressing AC5. This is the first evidence for a role of phosphatase activity in regulating the phosphorylation properties of D3R. Interestingly, a recent co-immunoprecipitation study has shown that the D1R and PP2B can form a complex in mouse neocortex tissues (114). This complex seems to be required for efficient D1R coupling to G proteins (114). Furthermore, inhibition of L-type calcium currents and excitability of striatal neurons is mediated by D2R through a signaling cascade involving PLCβ1, IP3 and PP2B (115). Importantly, it remains to be established whether a D3R/PP2B signalosome can be detected in HEK293 cells and *in vivo*.

Additionally, in light of the recent study showing the formation of a complex between AC8 and PP2A (111), we cannot rule out that the D3R phosphorylation status in HEK293 cells is regulated by PP2B in a AC isoform-specific manner. In fact, our studies using cells co-expressing D3R and AC5 pretreated with cyclosporine A suggest that the phenotypic regulation of constitutive phosphorylation and agonist-promoted phosphorylation/ dephosphorylation of GPCRs by PP2B is dependent on the formation of AC-specific signalosomes.

Figure 7. Effect of cyclosporine A (CYC-A) on the regulation of receptor phosphorylation in HEK293 cells expressing D3R alone.

A, Representative example (n=3) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with the D3R alone pretreated with cyclosporine A (CYC-A, a PP2B inhibitor). **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells pretreated with cyclosporine A (CYC-A). Results are expressed as percent relative to control basal (no DA, no CYC-A). DA exposure leads to a significant increase in the extent of D3R phosphorylation in comparison to basal conditions ($p<0.05$; two-way ANOVA). CYC-A had a significant effect on the extent of D3R phosphorylation under basal conditions and following DA exposure when compared with no pretreatment ($p<0.05$; two-way ANOVA). KDa, kilodaltons; DA, dopamine.

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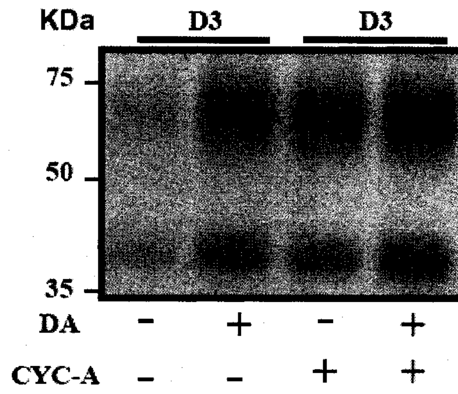

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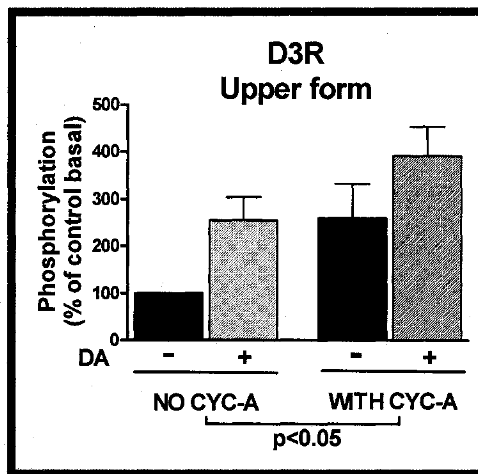

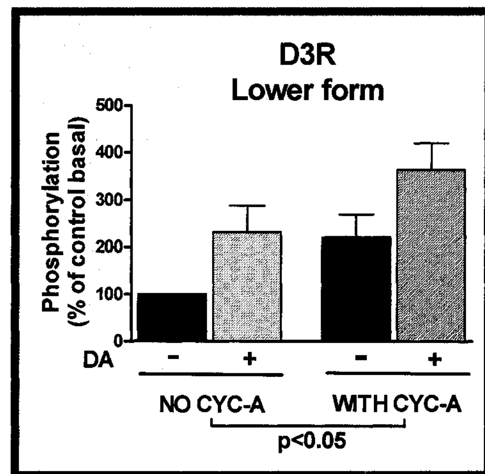

In support of this assertion, our studies performed in cells expressing AC5 demontrate that the constitutive phosphorylation of D3R is significantly reduced following a blockade of PP2B (Figures 8 A & C). These results are in striking contrast to those obtained in cells expressing D3R alone (Figure 7). While the extent of constitutive phosphorylation of D3R is unchanged by expression of AC5 (Figure 5), our data suggest that the kinase/phosphatase signaling pathways regulating the constitutive phosphorylation of D3R in cells expressing the receptor alone or with AC5 are different. This is further supported by our results showing that inhibition of PP2B by cyclosporine A in cells expressing AC5 not only blocks DA-induced D3R dephosphorylation but promotes DA-induced receptor phosphorylation (Figures 8 A & C). These results are not easily explained. However, studies have shown that PP2B can promote indirect effects on receptor-mediated intracellular signaling through a multifaceted interplay between serine/threonine and tyrosine kinases and phosphatases (116-121). Based on our results, we would like to propose that the AC5/PP2B signalosome controlling D3R phosphorylation status in the absence or presence of DA is a multifaceted process involving downstream AC5/PP2B-dependent kinase and phosphatase activities. In particular, our results obtained with cyclosporine A suggest a potential role of tyrosine phosphorylation/dephosphorylation in the AC5- and PP2B-dependent regulation of D3R phosphorylation status. In fact, two

Figure 8. Effect of microcystin-LR (MIC) or cyclosporine A (CYC-A) on the regulation of receptor dephosphorylation in HEK293 cells expressing D3R and adenylyl cyclase type 5 (AC5).

A, Representative example (n=2) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with D3R and adenylyl cyclase type 5 (AC5) pretreated with microcystin-LR (MIC) or cyclosporine A (CYC-A, a PP2B inhibitor). **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells pretreated with microcystin-LR (MIC). Results are expressed as percent relative to control basal (no DA, no MIC). DA exposure leads to a significant decrease in the extent of D3R phosphorylation in comparison to basal conditions ($p<0.05$; two-way ANOVA). MIC had no significant effect on the extent of D3R dephosphorylation when compared with no pretreatment ($p>0.05$; two-way ANOVA). **C**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells pretreated with cyclosporine A (CYC-A). Results are expressed as percent relative to control basal (no DA, no CYC-A). CYC-A had a significant effect on the extent of D3R phosphorylation under basal conditions and following DA exposure when compared with no pretreatment ($p<0.05$; two-way ANOVA).

KDa, kilodaltons; DA, dopamine.

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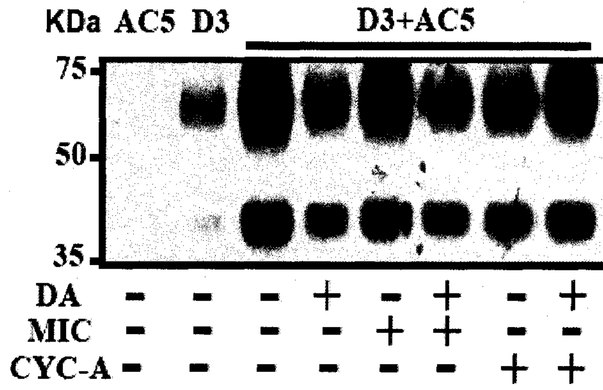

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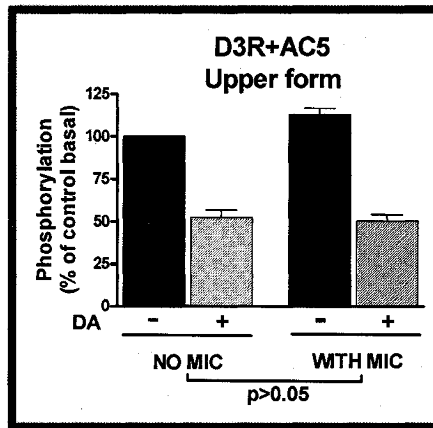

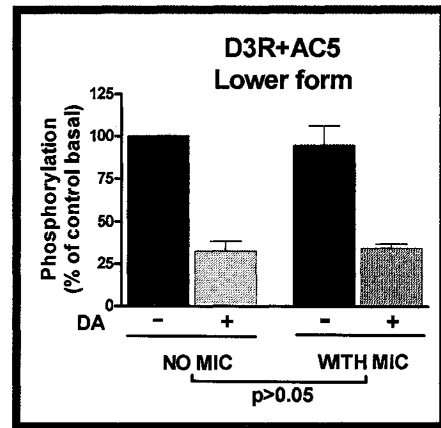

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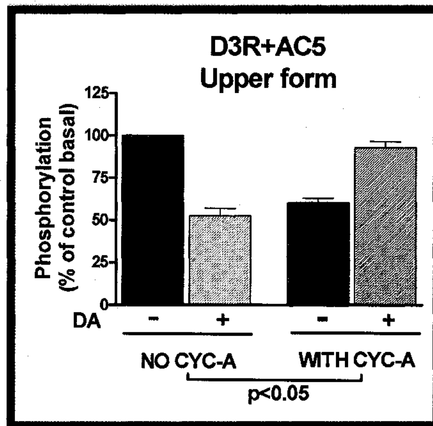

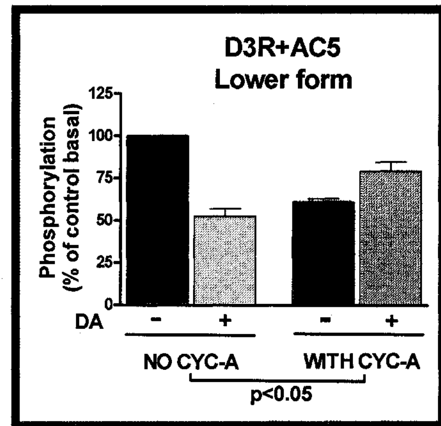

lines of evidence support our view. On the one hand, it has been shown that Gi-coupled melatonin receptor-mediated sensitization of AC activity in primary cells of ovine pars tuberalis is blocked by sodium orthovanadate, a tyrosine phosphatase inhibitor (122) suggesting that AC function is linked to a tyrosine phosphorylation/dephosphorylation pathway. On the other hand, a recent study (121) has demonstrated that the amyloid-β-dependent internalization of the NMDA (N-methyl-D-aspartate) receptor requires the dephosphorylation and activation of the tyrosine phosphatase STEP (striatal-enriched tyrosine phosphatase) in a PP2B-dependent manner, suggesting that serine/threonine and tyrosine phosphatase activities are intimately linked.

4.6 Blockade of tyrosine protein phosphatase activities inhibits AC5-dependent dopamine-induced D3R dephosphorylation.

To assess the role of protein tyrosine phosphatases (PTP) in the regulation of D3R phosphorylation status, HEK293 cells transfected with the receptor alone or with AC5 were pretreated with sodium orthovanadate (SOV 10 μM), a non-specific inhibitor of PTPs. In cells expressing D3R alone, our results show that inhibition of PTPs promotes a significant increase (~2-3 fold) in the extent of constitutive phosphorylation of both molecular forms of D3R (Figure 9). These results suggest that the constitutive phosphorylation of D3R is tonically inhibited by PTP activity. In striking contrast to PP2B inhibition studies (Figure 7), the DA-mediated D3R phosphorylation observed in untreated cells was converted into a DA-mediated D3R dephosphorylation following SOV

pretreatment (Figure 9). Similar results were obtained with both molecular forms of D3R. This effect was reminiscent of the DA-mediated D3R dephosphorylation observed in cells co-expressing the receptor and AC5. This raises an important question about the role of PTP activity in regulating the DA-mediated dephosphorylation of D3R in cells expressing AC5. The idea of DA receptors being linked to the regulation of PTP activity is not far-fetched. Indeed, DA-induced stimulation of PTP activity was demonstrated almost 15 years ago in a pitutitary cell line stably expressing the D2R subtype (123). We addressed this

Figure 9. Effect of sodium orthovanadate (SOV) on the regulation of receptor phosphorylation in HEK293 cells expressing D3R alone.

A, Representative example (n=2) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with the D3R alone pretreated with sodium orthovanadate (SOV, a PTP inhibitor). **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells pretreated with sodium orthovanadate (SOV). Results are expressed as percent relative to control basal (no DA, no SOV). SOV had a significant effect on the extent of D3R phosphorylation under basal conditions and following DA exposure when compared with no pretreatment ($p<0.05$; two-way ANOVA). KDa, kilodaltons; DA, dopamine.

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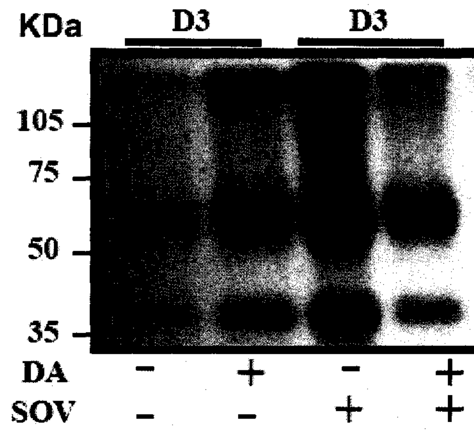

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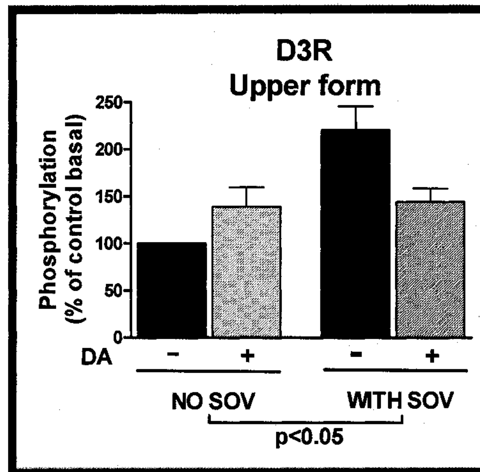

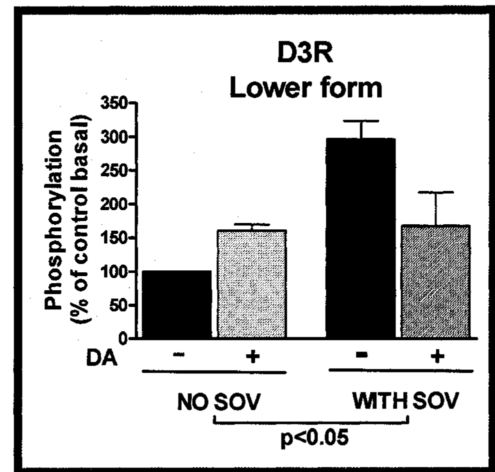

issue by investigating the D3R phosphorylation status in cells expressing AC5 following a pretreatment with SOV.

Our results show that SOV pretreatment blocks DA-mediated D3R dephosphorylation in cells expressing AC5 (Figure 10). In striking contrast to cells expressing the receptor alone, SOV pretreatment had no significant effect on the extent of basal phosphorylation of D3R (Figure 10). These results further support our view that the D3R signalosomes in cells expressing the receptor alone or with AC5 are regulated by distinct biochemical interplays between kinase(s) and phosphatase(s). While our observations made in cells expressing D3R alone treated with SOV are puzzling (Figure 9), we believe that they are not in contradiction with a role of PP2B (calcineurin) in the regulation of D3R phosphorylation status as suggested by our studies described in the previous section (Figure 7). In fact, while PP2B (calcineurin) activity depends on calcium and calmodulin, a large number of endogenous PP2B-binding proteins activating or inhibiting the phosphatase activity have been identified in recent years (124-127). These endogenous PP2B regulators are categorized into three major groups (125). Group I are made of PP2B inhibitors (cabin1/cain, FKBP38, CHP, A238L), which bind to and inhibit PP2B. Group II are made of dual regulators of PP2B (regulators of calcineurin or RCN1-3), which also bind to PP2B to mediate inhibitory or stimulatory effects on the phosphatase activity (124, 125, 127). Group III are composed of anchoring proteins of PP2B/calcineurin (A-kinase-

Figure 10. Effect of sodium orthovanadate (SOV) on the regulation of receptor dephosphorylation in HEK293 cells expressing D3R and adenylyl cyclase type 5 (AC5).

A, Representative example (n=2) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with the D3R and adenylyl cyclase type 5 (AC5) pretreated with sodium orthovanadate (SOV, a PTP inhibitor). **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells pretreated with sodium orthovanadate (SOV). Results are expressed as percent relative to control basal (no DA, no SOV). SOV had a significant effect on the extent of D3R dephosphorylation following DA exposure when compared with no pretreatment ($p<0.05$; two-way ANOVA). KDa, kilodaltons; DA, dopamine.

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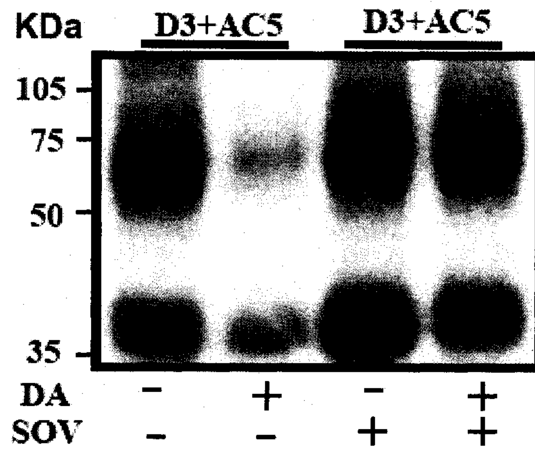

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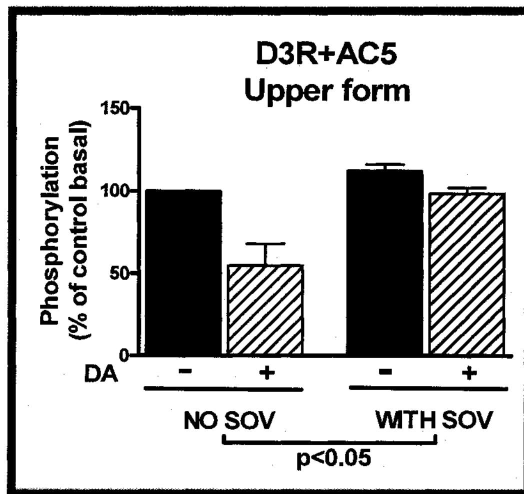
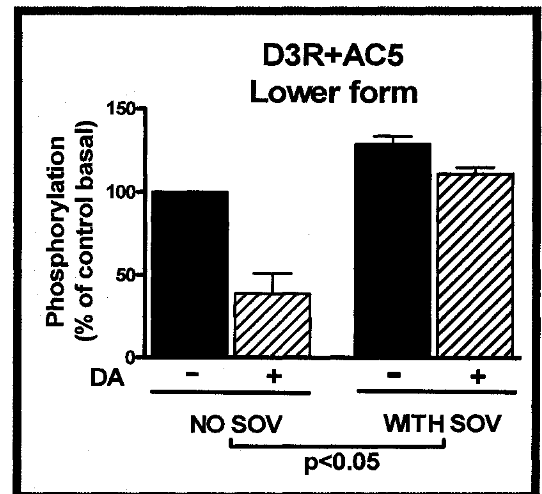




anchoring proteins or AKAPs; AKAP79), which serve as scaffolds to link PP2B physically to other signaling proteins or subcellular compartments (126).

Notably, the activity of RCN proteins (Group II) have been shown to be regulated by phosphorylation (124). In fact, unphosphorylated, hypophosphorylated and hyperphosphorylated forms of RCN (RCN, P-RCN and PP-RCN) have been proposed to mediate distinct inhibitory and stimulatory effects on PP2B/calcineurin activity (124). *In vitro* studies have shown that a conserved serine residue found in the SP-linker region of RCNs is phosphorylated by p42/44 MAP kinase to "prime" RCNs (124). Unphosphorylated RCNs and RCNs phosphorylated at the priming site (P-RCNs) bind to and inhibit PP2B/calcineurin activity. Moreover, P-RCN can be phosphorylated by members of the glycogen synthase kinase-3 (GSK3) family, which leads to an hyperphosphorylated form of RCN (PP-RCN). PP-RCN stimulates PP2B/calcineurin activity (124). Interestingly, PP2B can dephosphorylate PP-RCN and thus reverse the GSK3-mediated phosphorylation of P-RCN. However, the kinases and phosphatases regulating the priming and de-priming of RCN *in vivo* remains to be identified. Intringuinly, a recent study exploring the role of oxidative stress on the regulation of RCN1 (also known as (DSCR1/Adapt78/MCIP1/Calcipressin 1) have demonstrated that the peroxide-induced hyperphosphorylation of RCN1 was decreased by inhibitors of PTP suggesting that an upstream tyrosine dephosphorylation regulates the activity of downstream kinases involved in the phosphorylation of RCNs (128). This study

may help interpreting our data obtained with cells expressing D3R alone following SOV pretreatment.

Potentially, under basal conditions, PTP activity promotes the formation of PP-RCN and activation of PP2B/calcineurin regulating the dephosphorylation of D3R. Pretreatment with SOV would prohibit the formation of PP-RCN leading to higher intracellular levels of P-RCN and/or RCN, and thus inhibiting PP2B activity. This putative PP2B inhibition by RCN and P-RCN in HEK293 cells expressing D3R alone would then lead to an increase in the basal phosphorylation of the receptor as we observed (Figure 9). We believe that this model is also valid for the regulation of DA-mediated phosphorylation of D3R in cells pretreated with SOV. Indeed, D3R can stimulate MAP kinase activity (129). Therefore, DA-induced activation of the D3R signalosome may circumvent the PTP inhibition by virtue of a stimulation of MAP kinase activity culminating in the priming of RCN (P-RCN) and RCN hyperphosphorylation (PP-RCN) by GSK3 or other kinases. The PP-RCN potentially recruited to the DA-activated D3R-PP2B signalosome would promote PP2B stimulation and DA-mediated D3R dephosphorylation. It is worthwhile mentioning that *in vivo* administration of D2-like agonists has been shown to mediate inhibition of Akt and concomitant stimulation of GSK3 (130, 131). Additionally, we would like to propose that AC5 expression promotes a distinct regulation of the interplay between the PP2B/calcineurin pathway and D3R signalosome. In our working model discussed above, AC5 expression would impart a tonic inhibition to the PTP-dependent pathway regulating PP2B/calcineurin activity. In agreement with this model, SOV pretreatment would

have no effect on the basal phosphorylation status of D3R as we demonstrated (Figure 9). We propose that DA-mediated stimulation of the D3R-AC5 signalosome would relieve the tonic inhibition of the PTP-dependent activation of the PP2B/calcineurin pathway promoting SOV-sensitive D3R dephosphorylation.

Further studies are required to explore in more detail the molecular mechanisms underlying the interplay between AC5 expression and regulation of phosphorylation/dephosphorylation status of D3R. One important issue that remains to be investigated is whether the regulation of phosphorylation and dephosphorylation of D3R is a G protein-dependent process. Indeed, studies have now demonstrated that in addition to the classical G protein-dependent signaling pathways, GPCRs can mediate intracellular signaling in a G protein-independent manner (132). In the next section, we have addressed this issue.

4.7 The phosphorylation and dephosphorylation status of D3R is regulated by pertussis toxin-sensitive Gα proteins: role of $G\alpha_i$ activation.

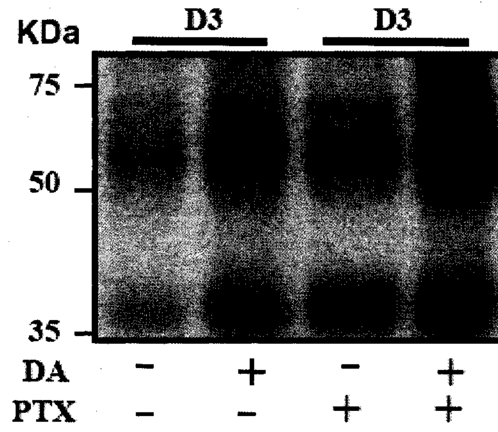
Previous studies have shown that D3R is a $G_{i/o}$-linked GPCR that selectively inhibits AC5 activity through $G\alpha_i$ subunits (66, 70). Whole cell phosphorylation assays were performed to assess whether D3R phosphorylation and AC5-dependent DA-mediated D3R dephosphorylation is controlled by a Gαi-dependent mechanisms. A day prior to the whole cell phosphorylation assays, HEK293 cells transiently transfected with HA-tagged human D3R alone or with AC5 were incubated in the absence or presence of pertussis toxin (PTX, a specific inhibitor of $G_{i/o}$ proteins) as described under "*Materials and Methods*". In

PTX-treated cells expressing D3R alone, our preliminary experiments show that the extent of basal and DA-induced phosphorylation of the two molecular forms of D3R is increased (Figure 11).

Figure 11. Effect of pertussis toxin (PTX) on the regulation of receptor phosphorylation in HEK293 cells expressing D3R alone.

A, Representative example (n=2) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with the D3R alone pretreated with pertussis toxin (PTX, $G_{i/o}$ protein inhibitor). **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells pretreated with pertussis toxin (PTX). Results are expressed as percent relative to control basal (no DA, no PTX). PTX had a significant effect on the extent of phosphorylation of the lower molecular form of D3R under basal conditions and following DA exposure when compared with no pretreatment ($p<0.05$; two-way ANOVA). KDa, kilodaltons; DA, dopamine.

A



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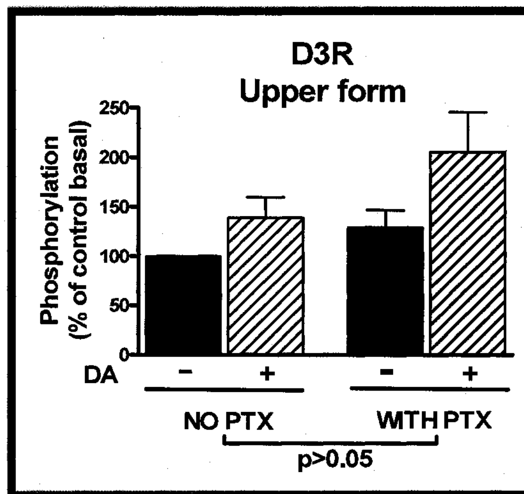

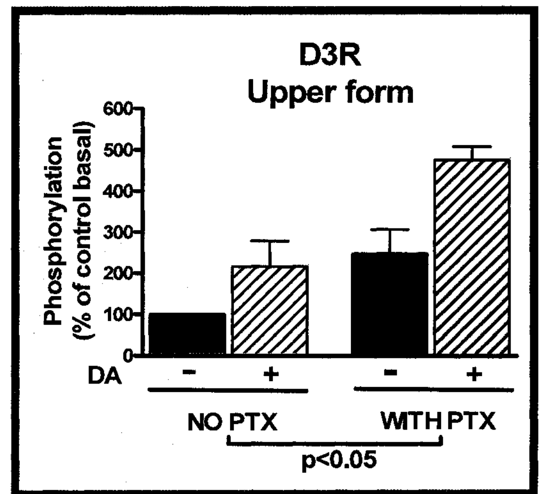

However, the statistical significance of PTX-mediated effects on receptor phosphorylation could only established for the lower molecular form of D3R. In cells transfected with D3R and AC5, PTX treatment had no significant effect on the basal phosphorylation of the receptor while completely abrogating DA-mediated D3R dephosphorylation (Figure 12). Interestingly, these results are reminiscent of those obtained with SOV-treated cells expressing D3R and AC5 (Figure 10).

Our results strongly suggest that D3R-mediated activation of PTX-sensitive $G_{i/o}$-like proteins regulates the phosphatase activity of HEK293 cells. Interestingly, in our transfection paradigm using D3R alone, phosphorylation data obtained in PTX-treated cells were similar to results observed with cyclosporine A-treated (Figures 7 & 11). In an opposite manner, phosphorylation experiments performed with D3R and AC5 transfection paradigm revealed that observations made with PTX-treated cells were reminiscent of the results obtained with SOV-treated cells (Figures 10 & 12).

Figure 12. Effect of pertussis toxin (PTX) on the regulation of receptor dephosphorylation in HEK293 cells expressing D3R and adenylyl cyclase type 5 (AC5).

A, Representative example (n=2) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with the D3R and adenylyl cyclase type 5 (AC5) pretreated with pertussis toxin (PTX, a $G_{i/o}$ protein inhibitor). **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells pretreated with pertussis toxin (PTX). Results are expressed as percent relative to control basal (no DA, no PTX). PTX had a significant effect on the extent of D3R dephosphorylation following DA exposure when compared with no pretreatment ($p<0.05$; two-way ANOVA).

KDa, kilodaltons; DA, dopamine.

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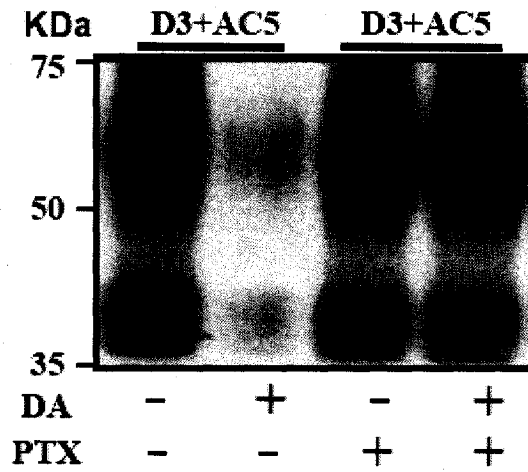

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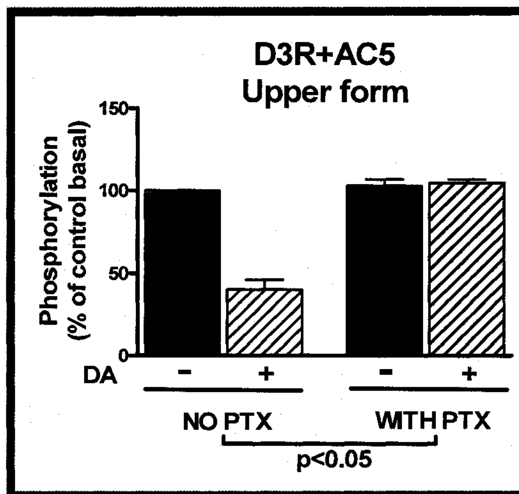

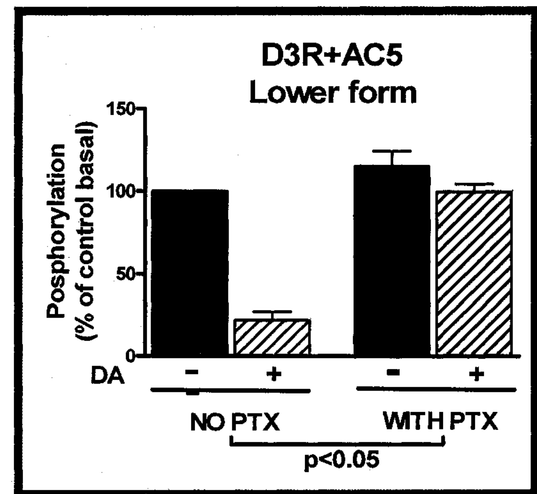

Overall, these studies support further the notion that the regulation of GPCR phosphorylation and dephosphorylation is differentially modulated by the cellular expression of AC repertoire. Meanwhile, the mechanisms involved in regulating PTP activity by PTX-sensitive G protein remains to be explored in more detail. Previously, GPCR-mediated PTX-sensitive stimulation of PTP activity has been associated with an increase in serine and tyrosine phosphorylation of PTPs (133, 134). Studies have shown that Gα subunits can bind to and stimulate activity of PTPs and serine/threonine phosphatases (135, 136). Alternatively, $G\alpha_{i2}$ has been shown to inhibit specific expression and activity of PTP1B but not other PTPs such as SHP-1 and SHP-2 (137). Gβγ subunits have also been shown to mediate GPCR-induced phosphorylation and activation of PTPs (138). However, it remains unclear if Gβγ subunits can directly bind to and activate PTPS. Gβγ subunits have been shown to interact directly with calmodulin, a modulator of calcineurin and alkaline phosphatase activity (139, 140, 141). Additionally, G protein-induced stimulation of phosphatase activity can be mediated through the activation of the non-receptor tyrosine kinases Src, Fyn and Pyk2 (142-145). Gα and Gβγ subunits can activate directly or indirectly non-receptor tyrosine kinase activity (145-148). Further studies using overexpression of dominant negative mutant forms of Gαi-like subunits (149-153) or Gβγ subunit scavengers (154-157) may help delineating the role of G protein subunits in the regulation of D3R dephosphorylation by phosphatases. In the next series of experiments, we tested the idea that non-receptor tyrosine kinases may be one of the downsteam G

protein-regulated effectors that participate in the regulation of D3R dephosphorylation.

4.8 The phosphorylation and dephosphorylation of the molecular forms of D3R are differentially regulated by the non-receptor tyrosine kinase Src.

Our D3R phosphorylation results obtained in cells expressing the receptor alone or with AC5 cells suggested that PTP-mediated regulation of D3R phosphorylation status is potentially controlled by non-receptor tyrosine kinases. We hypothesized that Src may be the non-receptor tyrosine kinase member involved as an upstream regulator of PTP activity in D3R-expressing cells. Importantly, a functional role for Src activity has been established in GPCR-mediated intracellular signaling (146). To test this hypothesis, we co-expressed D3R and a dominant negative mutant form of Src, Src-K298R (referred herein to as dn-Src), which has been shown previously to inhibit Src-mediated signaling effects (158).

HEK293 cells were transiently transfected with HA-tagged D3R alone or with AC5 in the absence or presence of the dn-Src expression construct DNA. Our preliminary studies performed under various transfection paradigms show that the phosphorylation and dephosphorylation of the two molecular forms of D3R (upper and lower bands) are differentially regulated by Src (Figure 13).

Figure 13. Effect of overexpression of dominant negative form of Src (dn-Src) on the regulation of receptor phosphorylation in HEK293 cells expressing D3R alone.

A, Representative example (n=2) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with D3R and a dominant negative form of Src (dn-Src). **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells co-expressing the receptor and a dominant negative form of Src (dn-Src). Results are expressed as percent relative to control basal (no DA, no dn-Src). dn-Src overexpression had a significant effect on the overall extent of D3R phosphorylation under basal conditions and following DA exposure when compared with no pretreatment ($p<0.05$; two-way ANOVA). KDa, kilodaltons; DA, dopamine.

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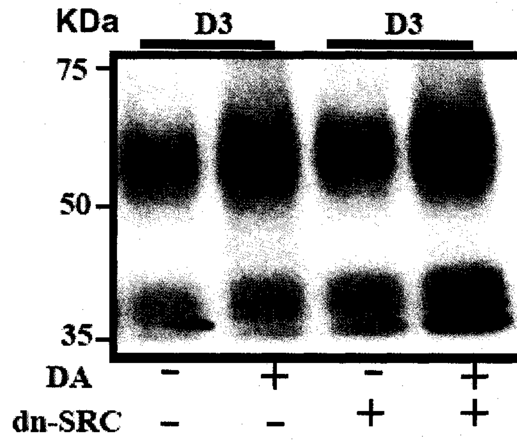

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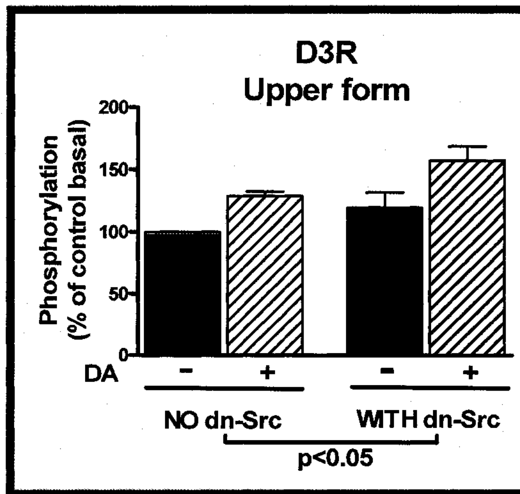

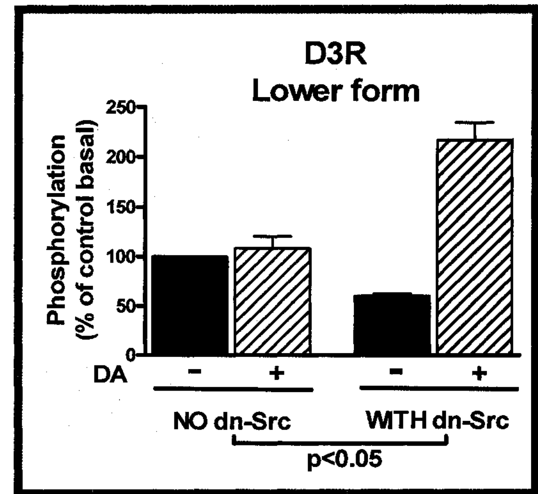

Regardless of the molecular form of the receptor examined, our statistical analysis (two-way anova followed by Bonferroni posttests) performed on data obtained in cells co-expressing D3R and dn-Src indicates that DA exposure leads to a statistically significant increase in the extent of D3R phosphorylation (Figure 13). However, Bonferroni posttests indicate that the extent of the basal and DA-induced phosphorylation of the high molecular form of the receptor was not significantly changed in comparison with corresponding values obtained in cells expressing D3R alone (no dn-Src). In contrast, Bonferroni posttests performed on data obtained from cells co-expressing the receptor and dn-Src, show a statistically significant increase in the extent of DA-induced phosphorylation of the low molecular form of D3R as compared to cells transfected with the receptor alone (Figure 13). The reduction in the basal phosphorylation of the low molecular form of D3R in cells expressing dn-Src was not statistically significant.

Similar statistical analysis was done on data obtained from cells transfected with D3R, AC5 and dn-Src. The analyzed data suggest that expression of dn-Src promotes a reduction in the extent of basal receptor phosphorylation, which was more important for the low molecular form of D3R (Figure 14). Additionally, our data show that the extent of DA-induced dephosphorylation (measured as the inhibition of the basal receptor phosphorylation) of the molecular forms was differentially regulated by dn-Src expression. In fact, dn-Src mediates a 61% and 38% inhibition of the DA-induced dephosphorylation of the high and low molecular form of D3R, respectively.

Figure 14. Effect of overexpression of dominant negative form of Src (dn-Src) on the regulation of receptor dephosphorylation in HEK293 cells expressing D3R and adenylyl type 5 (AC5).

A, Representative example (n=2) of an autoradiogram obtained from whole cell phosphorylation studies using HEK293 cells transfected with D3R, adenylyl cyclase type 5 (AC5) and a dominant negative form of Src (dn-Src). **B**, Quantification of the phosphorylation of the upper (right panel) and lower (left panel) molecular forms of D3R in cells co-expressing the receptor, adenylyl cyclase type 5 (AC5) and a dominant negative form of Src (dn-Src). Results are expressed as percent relative to control basal (no DA, no dn-Src). dn-Src overexpression had a significant effect on the extent of D3R phosphorylation under basal conditions and D3R dephosphorylation following DA exposure when compared with no pretreatment ($p<0.05$; two-way ANOVA).

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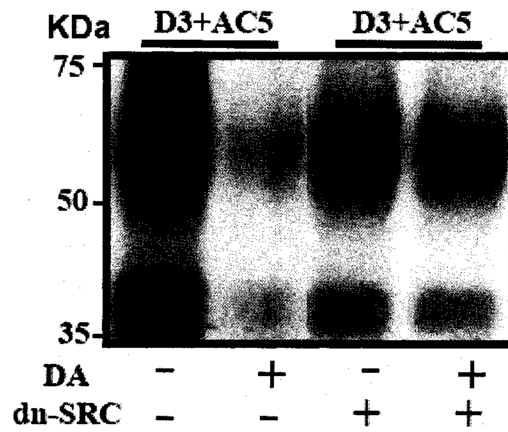

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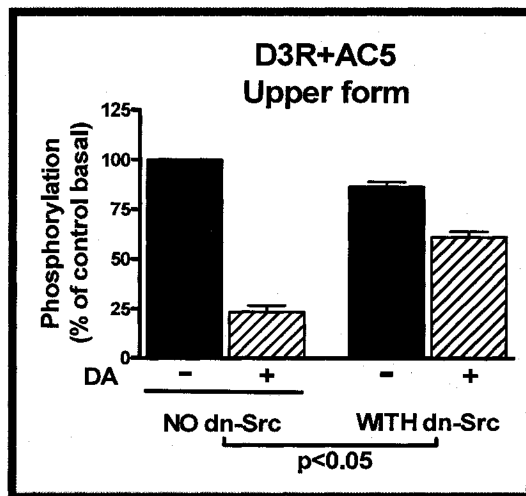

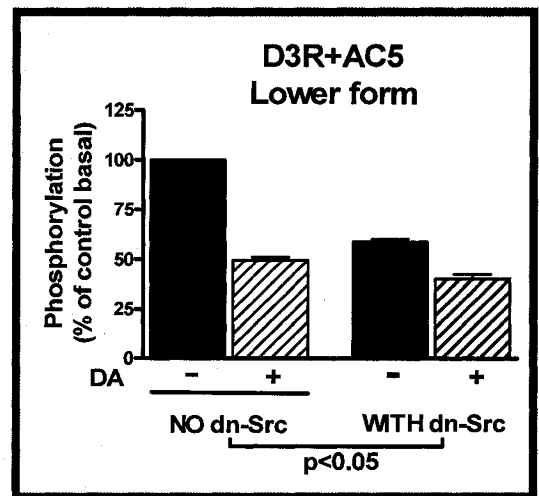

The differential regulation of the phosphorylation and dephosphorylation of the low and high molecular forms of D3R by dn-Src suggests that these two forms of D3R may represent distinct functional receptor entities. However, it remains to be firmly established whether the molecular forms of D3R exhibit additional distinct signaling and regulation properties in HEK293 cells as well as *in vivo*. Importantly, different molecular forms of D3R have been shown to be expressed in brain preparations but their physiological relevance has yet to be determined (159, 160). Moreover, it remains unclear whether results showing different molecular forms of D3R in mammalian cells and tissues are indicative of differences in D3R homo-oligomerization, hetero-oligomerization and/or glycosylation (159-162). Studies combining co-immunoprecipitation and proteomic approaches may be help elucidating this issue.

Notwithstanding the differential regulation of the two molecular forms of D3R by dn-Src, more general conclusions can be drawn with respect to the role of Src in the modulation of phosphatase activity. Indeed, our results obtained in cells co-expressing D3R and dn-Src are reminiscent of those obtained in HEK293 cells expressing D3R alone pretreated with the PP2B inhibitor cyclosporine A (Figures 7 & 13). Indeed, like in cells expressing dn-Src, an increase in the extent of DA-induced D3R phosphorylation was observed with cyclosporine A treatment suggesting that Src inhibits PP2B/calcineurin activity. Furthermore, the inhibition of DA-induced dephosphorylation of D3R by dn-Src in cells expressing AC5 is similar to that observed in cells transfected with D3R and AC5 following a pretreatment with SOV or PTX (Figures 10, 12 & 14). These

results imply that Src activity is required for PTP-mediated D3R dephosphorylation. The partial inhibition observed in our dn-Src studies suggest that an additional signaling partner (e.g another tyrosine kinase) is potentially required for the full expression of PTP activity. Importantly, we do not believe that results obtained with dn-Src are suggestive of a direct Src-mediated phosphorylation of D3R. Preliminary studies using a phosphotyrosine immunoblotting approach indicate that D3R does not undergo tyrosine phosphorylation in HEK293 cells expressing the receptor alone or with AC5 (data not shown).

4.9 Regulation of FSK-induced stimulation of endogenous ACs by D3R activation: evidence for D3R coupling to multiple G protein classes.

Our phosphorylation studies raise an important issue regarding the role of receptor phosphorylation status in the D3R-mediated regulation of AC activity. Prior to investigating the role of receptor phosphorylation and dephosphorylation in the control of D3R-induced regulation of AC activity, we first assessed whether coupling of D3R to AC is mediated through PTX-sensitive $G_{i/o}$ proteins under our HEK293 cell culture conditions. This is an important issue as previous studies have shown that G protein-mediated signaling through D3R can be regulated in PTX-dependent manner (70, 163, 164). Results obtained using the PTX paradigm in cells expressing D3R alone are shown in Figure 15.

Figure 15. Effect of pertussis toxin (PTX) on the regulation of D3R-mediated regulation of forskolin-induced activation of endogenous adenylyl cyclases in HEK293 cells.

A, Representative example (n=3) of a whole cell cAMP assay in HEK293 cells transfected with D3R alone using raw data obtained before and after a treatment with pertussis toxin (PTX). **B**, Averaged values using data normalized relative to control forskolin (FSK) stimulation (no DA, no PTX). DA exposure leads to a significant increase of the FSK-induced stimulation of adenylyl cyclase (AC) activity in comparison to FSK alone ($p<0.05$; two-way ANOVA). PTX had a significant effect on the D3R-mediated increase of FSK-induced AC activation when compared with no pretreatment ($p<0.05$; two-way ANOVA). **C**, Quantification of FSK stimulation in cells before and after PTX treatment. PTX had a significant effect on the amount of FSK-induced stimulation of AC activity when compared with no pretreatment ($p<0.05$; unpaired t-test).

KDa, kilodaltons; DA, dopamine.

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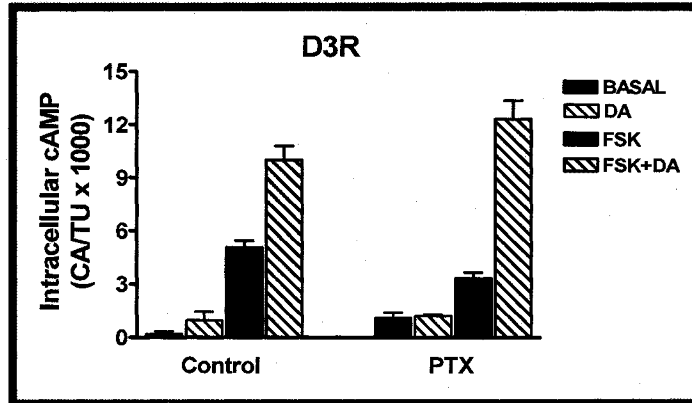

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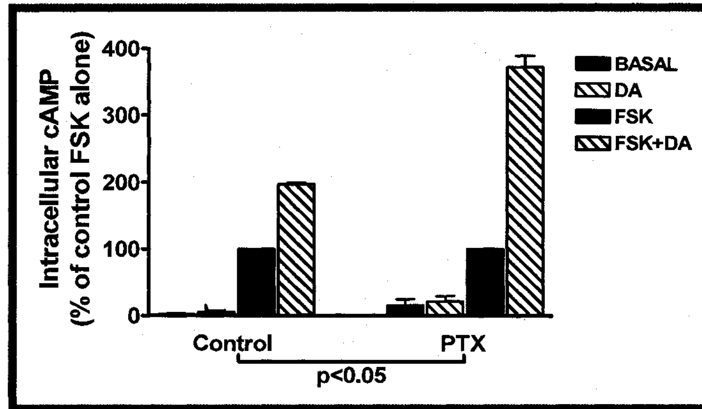

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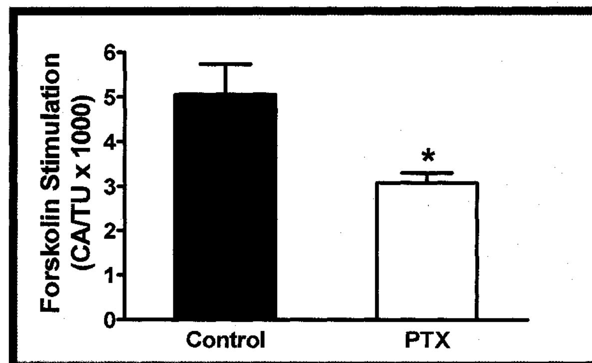

Figure 13

Figure 15A depicts a representative example using raw data. Stastitical analysis (two-way anova) were performed on data normalized relative to FSK stimulation alone under control and PTX conditions, respectively (Figure 15B). In cells transfected with D3R alone (no PTX treatment), DA did not significantly altered the intracellular cAMP levels in comparison with basal conditions (Figure 15B). Intracellular cAMP levels were significantly increased following a 15-min exposure to FSK (10 µM) when compared with basal and DA conditions ($p<0.05$). Surprisingly, DA-induced D3R activation elicits a fairly robust potentiation of FSK-induced AC stimulation ($p<0.05$). Interestingly, the potentiation of FSK-induced stimulation of endogenous ACs was significantly increased ($p<0.05$) in cells pretreated with PTX (Figure 13B). Notably, the higher potentiation of FSK stimulation by D3R observed in PTX treated cells cannot be explained by an increase in FSK stimulation. In fact, PTX-treated cells exhibit a significant reduction ($p< 0.05$) in the level of stimulation of endogenous ACs by FSK when compared with untreated cells (Figure 15C). These results indicate that PTX-sensitive $G_{i/o}$ proteins regulate the extent of FSK stimulation of endogenous ACs in cells expressing D3R. Moreover, these data strongly suggest that PTX-sensitive $G_{i/o}$ proteins are to some extent activated in untreated cells (no PTX) expressing D3R alone under basal conditions (no DA). This view is consistent with studies reporting that D3R has some degree of constitutive activity (165-167). Further studies are required to establish unequivocally that D3R is constitutively activated in our cellular paradigm. Nevertheless, it is unlikely that activation of endogenous $G_{i/o}$ proteins by D3R under basal conditions would also

be involved in the DA-mediated potentiation of FSK-induced AC stimulation. Indeed, DA-induced potentiation of FSK stimulation is increased rather than blocked in PTX-treated HEK293 cells. To explain the potential mechanism(s) involved in the multifaceted regulation of ACs by D3R and FSK, it is important at this point to highlight the AC repertoire of HEK293 cells as well as the previously reported G protein coupling properties of D3R.

As I mentioned in "Introduction", there are 9 membrane-bound AC isoforms (AC1-AC9), which can be classified into 5 groups. Studies have shown that HEK293 cells endogenously expressed AC1, AC2, AC3, AC6 and AC7, which are differentially regulated by G protein subunits (168-170). While all these AC isoforms are stimulated by $G\alpha_s$ subunit and FSK, *in vitro* studies suggest that members of Group 2 ACs (such AC2 and AC7) are not inhibited by $G\alpha_{i/o}$-like subunits (171-170). However, βγ subunits released from activated heterotrimeric $G\alpha_{i/o}$-like complexes interact directly with and stimulate these AC isoforms (173-170). Importantly, the βγ-mediated stimulation of Group 2 ACs is conditional upon the co-activation of $G\alpha_s$-linked receptors i.e. the $G\alpha_s$-mediated stimulation of AC2 and AC7 is synergistically increased by βγ subunits released from activated heterotrimeric $G\alpha_{i/o}$ proteins (173-170). In contrast, *in vitro* studies have demonstrated that βγ subunits bind to and inhibit the calmodulin/calcium-activated AC1 and potentially AC3 and AC8, which belong to Group 1 ACs. Interestingly, a previous study using purified ACs and G proteins has reported that FSK-induced activation of Group 2 ACs is insensitive to βγ dimers (172). However, co-expression studies in HEK293 cells have demonstrated that βγ

subunits can also potentiate FSK-mediated stimulation of Group 2 ACs (70). Therefore, βγ-mediated potentiation of FSK stimulation previously observed in HEK293 cells (70) suggest that as previously seen with Group 2 ACs activated by $G\alpha_s$, βγ subunits may also be capable of stimulating FSK activation through a direct interaction with Group 2 ACs (AC2 and AC7) in intact cells. On the other hand, we cannot rule out that α and/or βγ subunits released from $G_{i/o}$ proteins regulate indirectly FSK-mediated stimulation of Group 2 ACs through downstream signaling partners.

With respect to the regulation properties of AC by $G_{i/o}$ protein subunits and the AC expression pattern in HEK293 cells (AC1, AC2, AC3, AC6 and AC7), we believe that the basal activation (DA-independent) of PTX-sensitive $G_{i/o}$ proteins by D3R exerts a positive effect mainly on the FSK-induced stimulation of AC2 and AC7. Consistent with this idea is the reduction of the FSK-induced stimulation of AC activity under basal conditions (no DA) in cells treated with PTX treatment, which precludes the basal activation of $G_{i/o}$-like proteins by D3R. Importantly, because studies have shown that $\alpha_{i/o}$-like subunits can inhibit α_s- and FSK-activated AC1, AC6 and possibly AC3 (170-171), we believe that these specific AC isoforms are not regulated in HEK293 cells by the D3R-evoked activation of $G_{i/o}$-like proteins under basal conditions. Indeed, if these AC isoforms expressed in HEK293 cells were tonically inhibited by D3R under basal conditions, PTX inactivation of $G_{i/o}$-like proteins would then lead to an increase (or no change) rather than a decrease of the FSK-induced stimulation.

Interestingly, the potentiation of FSK stimulation observed in untreated and PTX-treated cells expressing D3R alone following DA exposure (Figure 15B) suggests that the agonist-bound D3R can stimulate the FSK-induced AC activity through PTX-insensitive G proteins. Therefore, our results support the notion that unbound and DA-bound D3R exhibit distinct conformational states, which potentially couple to and activate different subsets of G proteins. Importantly, multiplicity of G protein coupling has been demonstrated for several GPCRs (176, 177). We think that DA binding imparts an active conformation to D3R allowing the receptor coupling to switch from a PTX-sensitive G protein to PTX-insensitive G protein-dependent pathways.

Three independent research groups have provided evidence that D3R can couple to multiple G proteins (164, 178, 179). Studies have demonstrated that the mouse D3R can couple to $G\alpha_z$ (a member of the $G\alpha_{i/o}$ family insensitive to PTX treatment which is not expressed in HEK293 cells) and $G\alpha_s$ in COS-7 and CHO cells using different experimental paradigms (178). Additionally, Newman-Tancredi *et al* (164) have shown that the human D3R could also couple to G_q/G_{11} proteins (PTX-insensitive) using $[^{35}S]GTP\gamma S$ binding assays on membrane preparations derived from CHO cells. The D3R coupling to G_q/G_{11} proteins remains, however, controversial, as previous studies have failed to measure a direct activation of phosphatidylinositol phospholipase C (PI-PLC) in numerous cell lines including CHO and HEK293 cells (163, 179, 180). Overall, our studies using PTX on D3R-expressing cells support the notion that D3R can mediate intracellular signaling independently of $G_{i/o}$ protein coupling. Therefore, unbound

D3R (constitutively active) may preferentially couple to $G_{i/o}$-like proteins whereas DA-bound D3R may preferentially couple to G_s in HEK293 cells. This assertion is in agreement with the enhancement of FSK-induced stimulation of AC observed in our studies. In fact, previous studies have demonstrated that activated $G\alpha_s$ and $G\alpha_s$-linked GPCRs can enhance either in an additive or synergistic manner FSK stimulation of AC (170, 181, 182). In our study, DA-bound D3R increases the FSK-induced AC activation in a synergistic manner as DA alone does not elicit any significant changes in the intracellular cAMP levels (Figure 15). Another interesting observation made in our studies is the greater synergistic effect measured in PTX-treated cells (Figure 15B). This is potentially explained by the activation state of ACs prior to FSK and D3R stimulation. For instance, depending on the activation state of ACs (e.g. α_s-activated ACs, calcium/calmodulin-activated ACs, FSK-activated ACs), a high degree of specificity among members of $G_{i/o}$ proteins has been observed for the inhibition of AC activity (172, 183). In a similar fashion, the extent of FSK stimulation observed in cells is not only linked to type and amount of AC isoforms expressed but also to their activation states (170, 182, 184). Consistent with this idea, PTX-treated cells display a lower FSK stimulation than that measured in untreated cells while exhibiting a higher FSK stimulation upon D3R activation.

It has been reported that GPCR-induced activation of phospholipase D (PLD) can be mediated by $\beta\gamma$ subunits released from PTX-sensitive $G_{i/o}$ proteins (185-187). In striking contrast and of potential significance for our studies is the recent demonstration that PLD activation by the human D3R in HEK293 cells is

mediated by PTX-insensitive G proteins (179). Interestingly, a previous study has shown that GPCR-mediated AC stimulation is associated with PLD activation (188). Therefore, the synergistic activation of FSK by DA-bound D3R may also be explained by a D3R-mediated PLD activation. An alternative explanation for our results may be suggested by studies showing that GPCRs can elicit intracellular signaling in a G protein-independent manner (189). Recent studies have shown that D3R interacts as well with intracellular molecules unrelated to G proteins such as filamin A (ABP-280), an actin-binding protein (106, 190-195). The interaction of D3R with filamin A is potentially important for regulation of PLD activation as studies have shown that actin polymerization mediates stimulation of PLD (196). Meanwhile, further studies are required to explore in more detail the precise mechanisms underlying the D3R-mediated increase of FSK-induced stimulation of AC.

4.10 Activation of D3R mediates bidirectional effects on FSK-induced stimulation of AC5: further evidence for PTX-sensitive and PTX-insensitive dependent pathways.

Previously, Robinson and Caron (70) have shown that D3R selectively inhibits AC5 activity through the activation of $G_{i/o}$ proteins. In agreement with Robinson and Caron (70), we have shown that in HEK293 cells co-transfected with D3R and AC5, DA elicits a 50% inhibition of FSK-stimulated AC5 activity (Figure 14, $p<0.05$). These results are in contrast to those obtained with cells expressing the D3R alone (Figure 15) in which DA exposure leads to a

synergistic activation of FSK-induced stimulation of ACs. Interestingly, PTX treatment of cells co-transfected with D3R and AC5 not only abrogates DA-mediated inhibition of FSK stimulation but promotes a synergistic activation of

Figure 16. Effect of pertussis toxin (PTX) on the regulation of D3R-mediated regulation of forskolin-induced activation of adenylyl cyclase type 5 (AC5) in HEK293 cells.

A, Representative example (n=4) of a whole cell cAMP assay in HEK293 cells transfected with D3R aand adenylyl cyclase type 5 (AC5) using raw data obtained before and after a treatment with pertussis toxin (PTX). **B**, Averaged values using data normalized relative to control forskolin (FSK) stimulation (no DA, no PTX). DA exposure leads to a significant decrease of the FSK-induced stimulation of adenylyl cyclase (AC) activity in comparison to FSK alone before PTX treatment ($p<0.05$; two-way ANOVA). PTX had a significant effect on the D3R-mediated decrease of FSK-induced AC activation when compared with no pretreatment ($p<0.05$; two-way ANOVA). **C**, Quantification of FSK stimulation in cells before and after PTX treatment. PTX had no significant effect on the amount of FSK-induced stimulation of AC activity when compared with no pretreatment ($p>0.05$; unpaired t-test).

KDa, kilodaltons; DA, dopamine.

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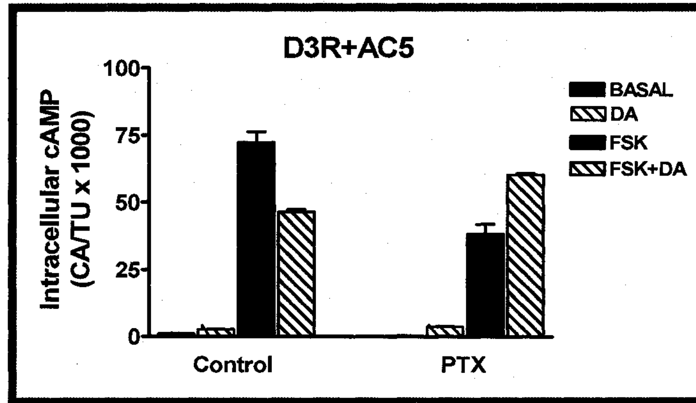

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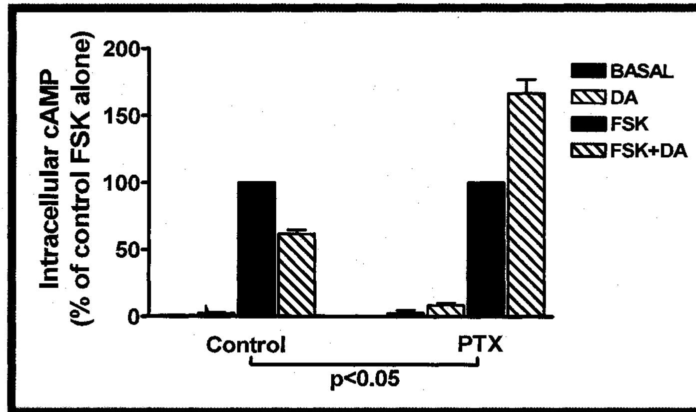

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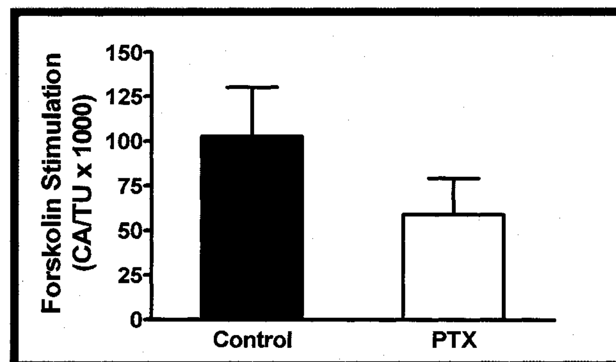

FSK stimulation of AC following DA exposure (Figure 16). In the context of a D3R/AC5 signalosome, these results suggest that the PTX-independent signaling pathway controlled by D3R is still effective. In a similar fashion to cells expressing D3R alone, we observed a reduction of FSK stimulation in HEK293 cells co-transfected with the receptor and AC5 following PTX treatment (Figure 16C), although it did not reach statistical significance. These results, as those described in the previous section, suggest the intriguing possibility that PTX-sensitive $G_{i/o}$ proteins also tonically regulate the activation state of AC5, which potentially affect the extent of FSK stimulation. Importantly, the DA-mediated enhancement of FSK-induced stimulation of AC is largely mediated through AC5. Indeed, we believe that the contribution of endogenous AC activity as measured with FSK is too low to explain the synergism observed in co-transfected cells (compared Figures 15C and 16C). How can we then explain in the context of a D3R/AC5 signalosome the tonic regulation of AC activity by $G_{i/o}$ proteins and the switch from a D3R-mediated inhibition of FSK-activated AC5 (PTX-dependent pathway) to a D3R-mediated stimulation of FSK-activated AC5 (PTX-independent pathway)? Collectively, our cAMP data combined with the phosphorylation results obtained in HEK293 cells expressing AC5 suggest that the distinguishing functional features of the D3R/AC signalosome may be mediated through the formation of a stable D3R complex with AC5, $G_{i/o}$ and G_s. In the cellular context of a stable D3R complex with AC5, $G_{i/o}$ and G_s, it is possible that D3R preferentially couples to PTX-sensitive $G_{i/o}$-like proteins. This precoupling of D3R to PTX-sensitive $G_{i/o}$-like proteins may lead to a D3R

conformation prohibiting the coupling to and activation of G_s proteins following DA binding. We would like to propose that this preferential precoupling of $G_{i/o}$-like proteins to D3R is regulated in a PTX-dependent manner. In PTX-treated cells the precoupling of $G_{i/o}$-like proteins to D3R would be blocked and $G_{i/o}$-free D3R would then be able to adopt a conformation facilitating the potentiation of FSK-stimulated AC5 presumably *via* G_s coupling (PTX insensitive). Alternatively, in HEK293 cells, two distinct D3R/AC5 signalosomes may work in parallel through stable receptor/AC5 complexes containing $G_{i/o}$ and G_s proteins, respectively. Importantly, recent studies have lend support to the idea that GPCRs forms stable complexes with heterotrimeric G proteins and effectors under basal conditions and following receptor activation (197-200). Interestingly, a study has shown that a stable complex between the β2-adrenergic receptor and L-type calcium channel (Ca(v)1.2) contain AC and G proteins as well as PKA and the counterbalancing phosphatase, PP2A (198).

Overall, our phosphorylation studies have unequivocally demonstrated that the D3R is controlled dynamically by phosphorylation and dephosphorylation in a AC5-specific manner. However, what is the role of the receptor phosphorylation and dephosphorylation on D3R ability to couple to G proteins and regulate AC activity in HEK293 cells? This question has been addressed in the next series of experiments in intact cells using pharmacological blockers of phosphatases.

4.11 Blockade of PP2B or PTP activity completely prohibits D3R-mediated inhibition of FSK-induced AC5 stimulation.

To assess the impact of phosphatase activity on D3R-mediated regulation of FSK-induced stimulation of AC, whole cell cAMP assays were performed in HEK293 cells transiently transfected with HA-tagged human D3R alone or with AC5. Cells were pretreated with either microcystin-LR (a potent inhibitor of PP1/PP2A; MIC), cyclosporine A (a potent inhibitor of PP2B; CYC-A) or sodium orthovanadate (an inhibitor of PTP activity; SOV) for 15 min prior to DA stimulation (15 min). Our results indicate that inhibition of PP1/PP2A activity by microcystin-LR has no significant effect on D3R-mediated regulation of AC in cells expressing the receptor alone (Figure 17) or with AC5 (Figure 18).

Figure 17. Effect of microcystin-LR (MIC) on the regulation of D3R-mediated regulation of forskolin-induced activation of endogenous adenylyl cyclases in HEK293 cells.

A, Averaged values (n=3) from whole cell cAMP assays performed in HEK293 cells transfected with D3R alone incubated in the absence or presence of microcystin (MIC). Data are normalized relative to control forskolin (FSK) stimulation (no DA, no MIC). DA exposure leads to a significant increase of the FSK-induced stimulation of adenylyl cyclase (AC) activity in comparison to FSK alone ($p<0.05$; two-way ANOVA). MIC had no significant effect on the D3R-mediated increase of FSK-induced AC activation when compared with no pretreatment ($p>0.05$; two-way ANOVA). **B**, Quantification of FSK stimulation in cells incubated in the absence or presence of MIC. MIC had a significant effect on the amount of FSK-induced stimulation of AC activity when compared with no pretreatment ($p>0.05$; unpaired t-test). KDa, kilodaltons; DA, dopamine.

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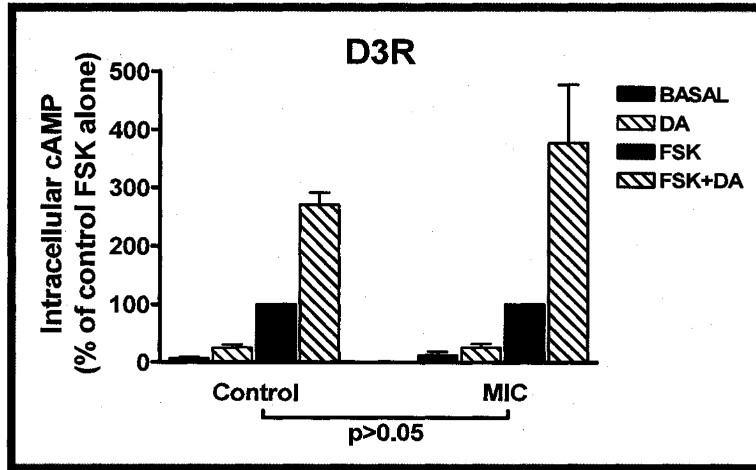

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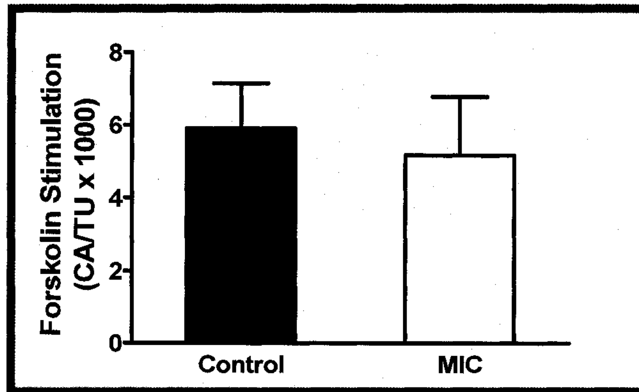

Figure 18. Effect of microcystin-LR (MIC) on the regulation of D3R-mediated regulation of forskolin-induced activation of adenylyl cyclase type 5 (AC5) in HEK293 cells.

A, Averaged values (n=3) from whole cell cAMP assays performed in HEK293 cells transfected with D3R and adenylyl cyclase type 5 (AC5) incubated in the absence or presence of microcystin (MIC). Data are normalized relative to control forskolin (FSK) stimulation (no DA, no MIC). DA exposure leads to a significant decrease of the FSK-induced stimulation of AC5 activity in comparison to FSK alone ($p<0.05$; two-way ANOVA). MIC had no significant effect on the D3R-mediated decreased of FSK-induced AC activation when compared with no pretreatment ($p>0.05$; two-way ANOVA). **B**, Quantification of FSK stimulation in cells incubated in the absence or presence of MIC. MIC had a significant effect on the amount of FSK-induced stimulation of AC activity when compared with no pretreatment ($p>0.05$; unpaired t-test). KDa, kilodaltons; DA, dopamine.

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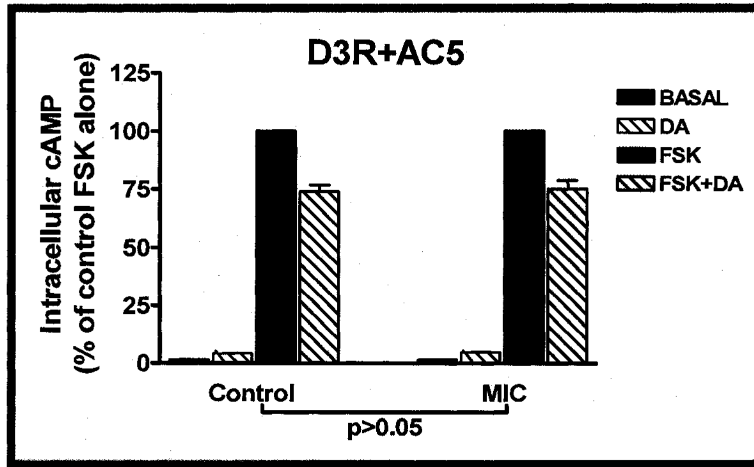

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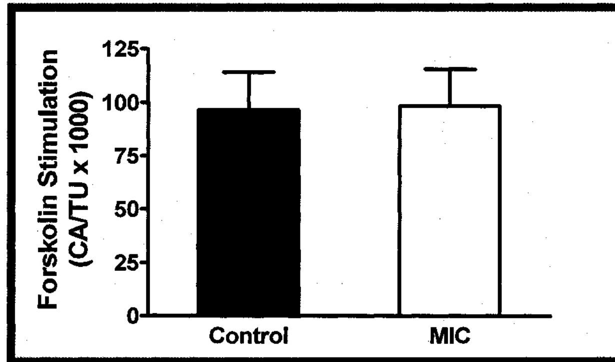

Additionnally, no effect on DA-mediated regulation of FSK-induced AC stimulation was observed in cells expressing the receptor alone pretreated with cyclosporine A (Figure 19).

Figure 19. Effect of cyclosporine A (CYC-A) on the regulation of D3R-mediated regulation of forskolin-induced activation of endogenous adenylyl cyclases in HEK293 cells.

A, Averaged values (n=4) from whole cell cAMP assays performed in HEK293 cells transfected with D3R alone incubated in the absence or presence of cyclosporine A (CYC-A). Data are normalized relative to control forskolin (FSK) stimulation (no DA, no CYC-A). DA exposure leads to a significant increase of the FSK-induced stimulation of adenylyl cyclase (AC) activity in comparison to FSK alone ($p<0.05$; two-way ANOVA). CYC-A had no significant effect on the D3R-mediated increase of FSK-induced AC activation when compared with no pretreatment ($p>0.05$; two-way ANOVA). **B**, Quantification of FSK stimulation in cells incubated in the absence or presence of CYC-A. CYC-A had no significant effect on the amount of FSK-induced stimulation of AC activity when compared with no pretreatment ($p<0.05$; unpaired t-test). KDa, kilodaltons; DA, dopamine.

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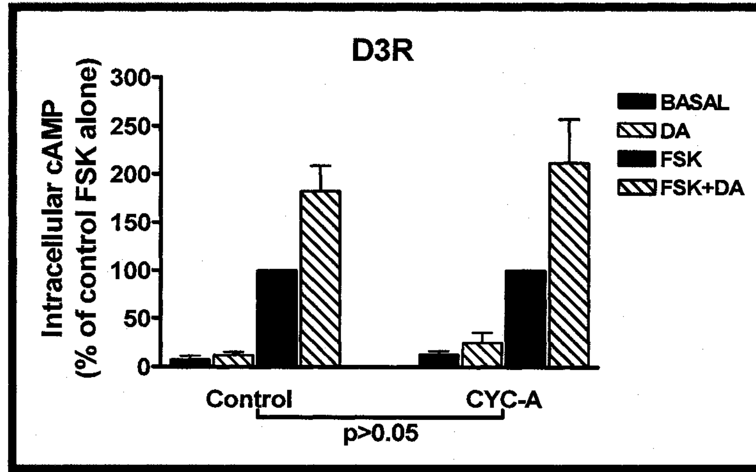

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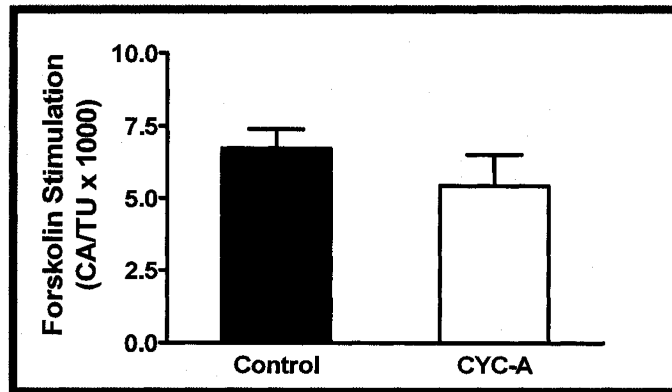

These results indicate that the PP2B-induced tonic inhibition of D3R phosphorylation in cells not expressing AC5 (as suggested by our previous results reported in Figure 7) does not play a role in the enhancement of FSK stimulation by D3R. In striking contrast, pretreatment of cells expressing D3R and AC5 with cyclosporine A completely abrogates DA-mediated inhibition of FSK-induced stimulation of AC5 (Figure 20). These studies strongly suggest that PP2B-mediated D3R dephosphorylation plays an important role in the regulation of DA-induced inhibition of FSK-activated AC5.

Figure 20. Effect of cyclosporine A (CYC-A) on the regulation of D3R-mediated regulation of forskolin-induced activation of adenylyl cyclase type 5 (AC5) in HEK293 cells.

A, Averaged values (n=3) from whole cell cAMP assays performed in HEK293 cells transfected with D3R and adenylyl cyclase type 5 (AC5) incubated in the absence or presence of cyclosporine A (CYC-A). Data are normalized relative to control forskolin (FSK) stimulation (no DA, no CYC-A). DA exposure leads to a significant decrease of the FSK-induced stimulation of AC5 activity in comparison to FSK alone ($p<0.05$; two-way ANOVA). CYC-A had significant effect on the D3R-mediated decreased of FSK-induced AC activation when compared with no pretreatment ($p>0.05$; two-way ANOVA). **B**, Quantification of FSK stimulation in cells incubated in the absence or presence of CYC-A. CYC-A had no significant effect on the amount of FSK-induced stimulation of AC activity when compared with no pretreatment ($p>0.05$; unpaired t-test). KDa, kilodaltons; DA, dopamine.

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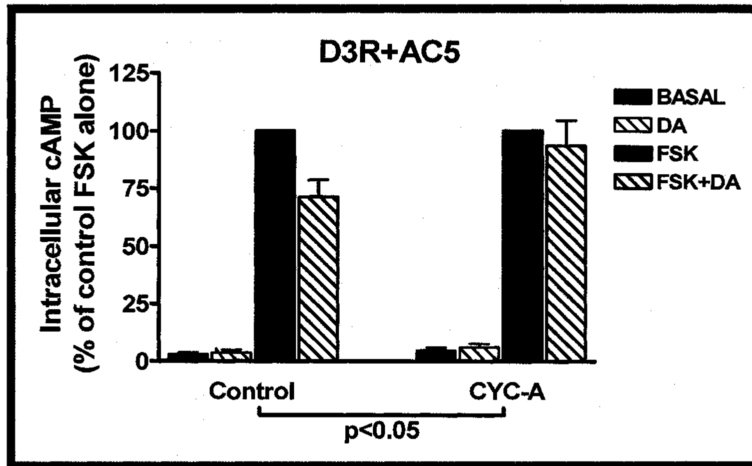

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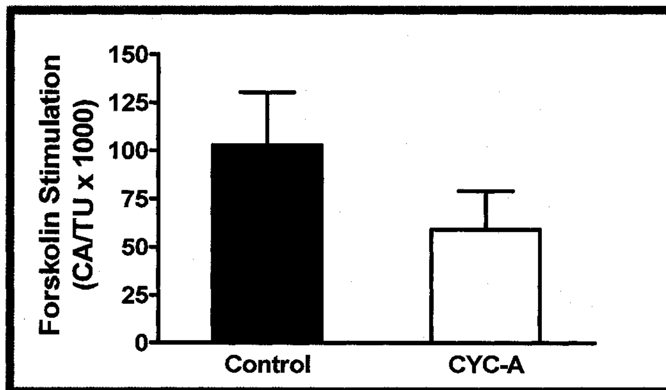

Interestingly, the DA-mediated potentiation of FSK-induced AC activation in cells expressing D3R alone was significantly reduced following SOV pretreatment (Figure 21) suggesting that PTP activity regulate positively the D3R coupling to AC.

Figure 21. Effect of sodium orthovanadate (SOV) on the regulation of D3R-mediated regulation of forskolin-induced activation of endogenous adenylyl cyclases in HEK293 cells.

A, Averaged values (n=6) from whole cell cAMP assays performed in HEK293 cells transfected with D3R alone incubated in the absence or presence of sodium orthovanadate (SOV). Data are normalized relative to control forskolin (FSK) stimulation (no DA, no SOV). DA exposure leads to a significant increase of the FSK-induced stimulation of adenylyl cyclase (AC) activity in comparison to FSK alone ($p<0.05$; two-way ANOVA). SOV had a significant effect on the D3R-mediated increase of FSK-induced AC activation when compared with no pretreatment ($p>0.05$; two-way ANOVA). **B**, Quantification of FSK stimulation in cells incubated in the absence or presence of SOV. SOV had no significant effect on the amount of FSK-induced stimulation of AC activity when compared with no pretreatment ($p<0.05$; unpaired t-test). KDa, kilodaltons; DA, dopamine.

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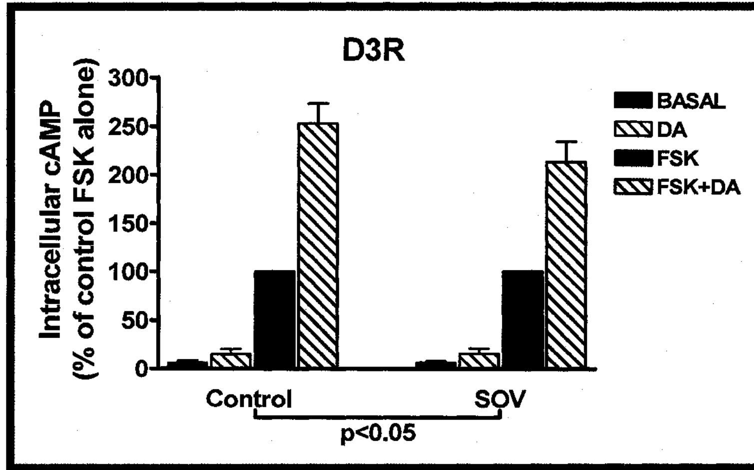

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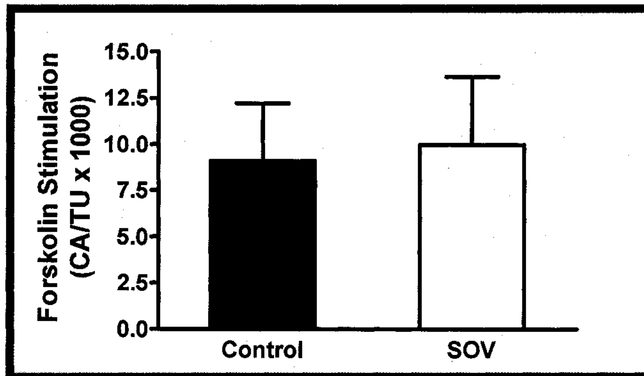

The role of PTP activity in D3R coupling to AC was further highlighted with studies using SOV pretreatment in cells expressing D3R and AC5. Indeed, SOV pretreatment leads to a complete blockade of DA-mediated inhibition of FSK-induced AC stimulation (Figure 22). Importantly, these results strengthen the importance of the D3R phosphorylation/dephosphorylation status in the absence and presence of DA in controlling the receptor ability to potentiate or inhibit AC activation by FSK. Overall, our results further strenghten a potential interplay between PP2B and PTP in regulating the coupling of D3R to G proteins in cells expressing AC5. As no tyrosine phosphorylation could be detected on D3R in cells expressing the receptor alone or with AC5 (data not shown), we believe that the phosphorylation detected on D3R is mainly explained by a phosphorylation of Ser and/or Thr residues. Therefore, we would like to propose that PP2B mediates a direct dephosphorylation of Ser and/or Thr residues on D3R whereas PTP regulates indirectly the D3R phosphorylation status via a downstream D3R signaling partner, potentially PP2B. Overall, our phosphorylation and cAMP studies in cells expressing AC5 strongly suggest that D3R is constitutively desensitized under basal conditions. This constitutive desensitization is alleviated upon DA binding and D3R activation by virtue of an AC5-dependent stimulation of PTP and PP2B leading to dephosphorylation and functional $G_{i/o}$ protein coupling of D3R.

Figure 22. Effect of sodium orthovanadate (SOV) on the regulation of D3R-mediated regulation of forskolin-induced activation of adenylyl cyclase type 5 (AC5) in HEK293 cells.

A, Averaged values (n=6) from whole cell cAMP assays performed in HEK293 cells transfected with D3R and adenylyl cyclase type 5 (AC5) incubated in the absence or presence of sodium orthovanadate (SOV). Data are normalized relative to control forskolin (FSK) stimulation (no DA, no SOV). DA exposure leads to a significant decrease of the FSK-induced stimulation of AC5 activity in comparison to FSK alone ($p<0.05$; two-way ANOVA). SOV had a significant effect on the D3R-mediated decrease of FSK-induced AC5 activation when compared with no pretreatment ($p>0.05$; two-way ANOVA). **B**, Quantification of FSK stimulation in cells incubated in the absence or presence of SOV. SOV had no significant effect on the amount of FSK-induced stimulation of AC activity when compared with no pretreatment ($p<0.05$; unpaired t-test). KDa, kilodaltons; DA, dopamine.

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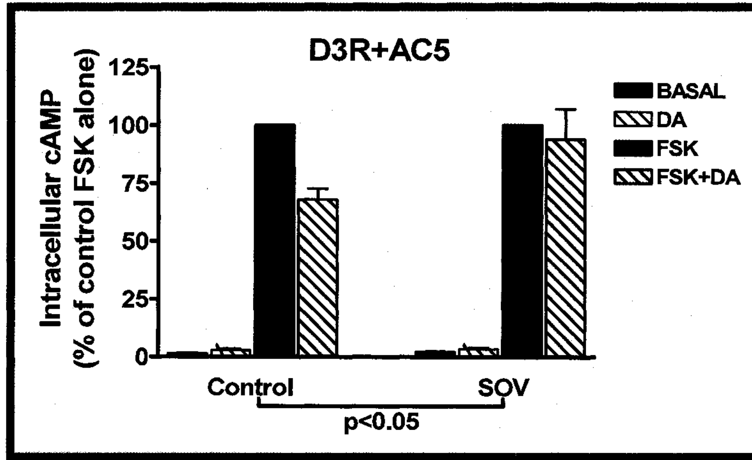

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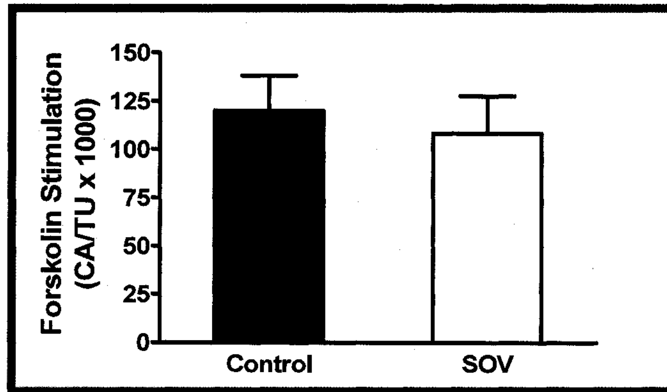


General Conclusions

5.1 Physiological relevance and hypothetical models of D3R/AC5 signalosome.

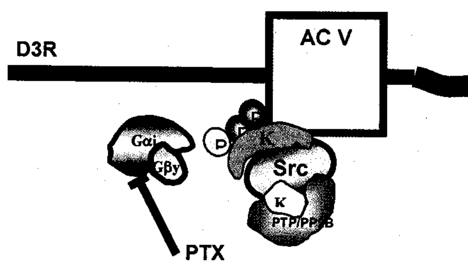
Parkinson's disease is a neurodegenerative disorder with an insidious onset and a prolonged course over many years. The primary cause of the symptoms of this illness is the death of DA-producing neurons of the SN and the resultant depletion of DA in the striatum (70). It is believed that activation of postsynaptic dopaminergic receptor (D2-like) by dopamine derivatives provides an efficient therapeutic approach. Although D2R stimulation is necessary for antiparkinsonian activity, but in many cases dopamine agonists have high (or higher than D2R) affinity for D3R. Thus, it is possible that the mesolimbic D3R could play a role in antiparkinsonian relief. While the motor striatum is involved in sensorimotor function and integration of motor movements, the limbic striatum is also involved in aspects of movement, such as goal-directed behaviors and locomotor activity. The D3-preferring agonist pramipexole (PPX) can reverse muscle rigidity produced by the combination of reserpine and α-methyl-p-tyrosine (leading to DA depletion) or haloperidol (blocking DA receptors) (201). The D3-preferring agonist 7-OH-DPAT also reverses catalepsy evoked by reserpine or DA receptor blockers in rats (202). Thus, the mesolimbic DA system might also be involved in "motor" aspects of Parkinson's disease. Many investigators have proposed that the limbic regions of the striatum play a particularly important role in neuronal processing in schizophrenia. The original hypothesis is that the levels of DA are elevated in schizophrenic patients since this, in turn, is modulated by

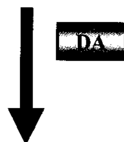
the mesolimbic DA system, several groups have proposed that the mesolimbic D3R mediates this effect (203, 204). In fact, postmortem studies have shown that D3R mRNA and binding sites are expressed by neurons within the ventral striatum and at several sites in the limbic ventral striatalpallidal-thalamo circuit (205, 206). Furthermore, there is overproduction of D3R in the ventral striatum of unmedicated schizophrenic patients and those removed from antipsychotics, but D3R down-regulation in those remaining on medication (207).

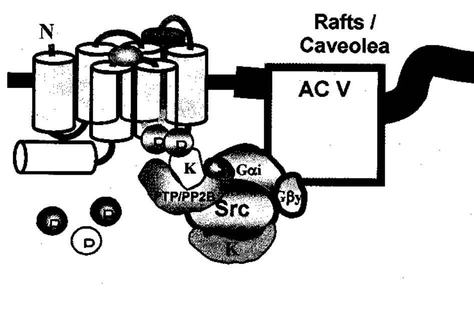
Although involvement of D3R has been reported in other severe diseases but its regulation still needs to be clearly understood. Moreover, it is currently unknown whether regulation of D3R responsiveness and signaling works through classical mechanisms or not. In fact, the distribution of D3R and AC5 in brain is overlapping; they both coexpressed in striatum. Furthermore, D3R can selectively inhibit AC5 in HEK293 cells. So, we hypothesized that AC5 might be involved in D3R regulation. The agonist-induced D3R dephosphorylation observed in cells expressing AC5 is the strongest evidence supporting our hypothesis. This is a novel mechanism in GPCR regulation. In addition, because AC5 is located in caveolae regions of the plasma membrane, agonist-induced D3R dephosphorylation mediated by AC5 suggests that D3R may be sorted to the lipid rafts/caveolae region where G proteins, filamin-A (D3R can be connected to the cytoskeleton), AKAP, Src-tyrosine kinase, Trk B and several RTKs are found. Previous studies could not show D3R internalization via clathrin-coated vesicle. It is possible that D3R may be desensitized and internalized by calveolin-dependent pathway. One of the aspect of our D3R dephosphorylation

studies is the clear involvement of different types of phosphatases. Both PP2B (Calcineurin) and tyrosine phosphatases are involved in dephosphorylation (see our hypothetical model for HEK293 cells and nucleus accumbens, Figure 23). Importantly, we have shown that the phosphatase effects are linked to AC5 expression. In other words, AC5 may serve as a scaffolding complex containing phosphatases. The role of phosphatases in the regulation of D3R dephosphorylation is an important issue that need to be further explored. Because most of the D3R agonists and antagonists have some affinity to other D2-like receptors, this novel mechanism will open a new field of drug designing to regulate D3R in the brain through the targeting of phosphatases.

Figure 23. Hypothetical model of the D3R signalosome in HEK293 cells expressing AC5.


D3R
AC V
Src
PTX


DA


N
Rafts /
Caveolea
AC V
Src
Threonine
Serine
Tyrosine

5.2 Future studies

It is still not known whether AC5-mediated effects on receptor phosphorylation is specific to the D3R or can be generalized to other DA receptors and GPCRs. Furthermore, it is very important to establish whether other AC isoforms can elicited similar effects on D3R phosphorylation status. Moreover, establishing unequivocally that D3R can localized to lipid rafts/calveolae region of the plasma membrane will provide precious information about the mechanisms underlying the regulation of D3R responsiveness by desensitization and internalization processes. Finally, genetic studies may prove useful in linking AC5 to pathophysiologies for which D3R has implicated such as schizophrenia and Parkinson's disease, and thus providing researchers and clinicians with a new therapeutic target.

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