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IDENTIFICATION OF NOVEL PROTEIN CONSTITUENTS OF THE DOPAMINE D2 RECEPTOR SIGNALPLEX: A STUDY OF TRPC1/D2R INTERACTION AND ITS ROLE IN SIGNALPLEX TRAFFICKING

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ABSTRACT

Dopamine exerts its effects through the activation of a family of dopamine receptors (DRs) and their subsequent coupling to downstream effector molecules. Dysfunction of dopaminergic neurotransmission is responsible for a myriad of diseases including Parkinson's disease, Tourette's syndrome, attention deficit hyperactivity disorder (ADHD), drug abuse, and schizophrenia. Schizophrenia is a devastating mental illness that affects approximately one percent of the population worldwide. It attacks its victims upon their emergence into adulthood and the workforce and leaves them severely disabled and dependent on life-long pharmacological treatment for. The majority of therapeutic antipsychotic drugs act as antagonists at D2R sites in the brain, but D2Rs are not genetically altered in schizophrenic individuals. We hypothesize that pathological dysregulation of dopaminergic signaling may stem from alterations in expression or function of proteins which interact with and regulate DR-mediated signaling, otherwise known as DRIPs (dopamine receptor interacting proteins) and DRAPs (dopamine receptor associated proteins).

Work presented in this thesis further investigates protein constituents of the D2R-signalplex and characterizes their effects on the regulation of dopaminergic signaling. A systematic survey of the literature has culminated in the collection of information concerning approximately fifty DRIPs and DRAPs. These proteins comprise the D2R-signalplex and affect various aspects of the life cycle of D2Rs, including regulation, trafficking, degradation, and recycling. Efforts were undertaken to investigate whether interacting partners of these known DRIPs could themselves bind the D2R. Isolation and

characterization of the cohort of proteins that comprise the D2R signalplex will provide insight into the mechanisms of receptor function in both healthy and diseased states.

TRPC1 was identified as a novel DRIP via yeast two-hybrid strategy. Here we validate the interaction of TRPC1/D2R in both heterologous and native protein expression systems. TRPC1, a nonselective, nonvoltage gated cation channel, colocalizes with D2R in cell culture systems and primate cortical neurons. TRPC proteins can function as scaffolding molecules as well as ion channels, and the TRPC1/D2R interaction affects trafficking of the D2R signaling complex to the plasma membrane. TRPC1 provides a direct link between D2R and intracellular calcium stores and may play a functional role in the pathogenesis of neuropsychiatric diseases.

Interactions between individual DRIPs NCS-1 and TRPC5 were further investigated. NCS-1 and TRPC5 were confirmed to interact through biochemical assays and native protein expression systems. NCS-1 regulates currents through TRPC5 channels, and both proteins were identified to function in a common pathway that operates to control the process of neurite extension. An interruption of this interaction would have significant neurodevelopmental consequences, and may contribute to neurological dysfunction in pathologic states.

zDHHC4, a newly discovered palmitoyltransferase, has been confirmed to interact with the D2R in a number of protein-interaction systems. Evidence is presented that suggests that zDHHC4/D2R interaction has different functional consequences for the two D2R isoforms, D2S and D2L. The zDHHC4/D2L interaction promotes the internalization of the receptor, whereas the zDHHC4/D2S interaction appears to have no effect on the trafficking of this isoform. This suggests that zDHHC4 differentially

palmitoylates the D2L on cysteine residues not present in the D2S. zDHHC4 may contribute to the differences in D2S/D2L trafficking observed in neurons.

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5HTR	serotonin receptor
A2AR	adenosine 2A receptor
AA	amino acid
AC	adenylyl cyclase
ADHD	attention deficit hyperactivity disorder
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
cAMP	cyclic adenosine monophosphate
CAPS	calcium-dependent activator of protein secretion
CB	cannabinoid
CNS	central nervous system
C-tail	carboxyl terminal
D2L	dopamine D2 receptor, long isoform
D2S	dopamine D2 receptor, short isoform
DA	dopamine
DAG	diacylglycerol
DAT	dopamine transporter
DHHC	asp-his-his-cys domain
DLPFC	dorsal lateral prefrontal cortex
DN	dominant negative
DR	dopamine receptor
DRAP	dopamine receptor associated protein
DRIP	dopamine receptor interacting protein
EC	extracellular region
ERK	extracellular signal-related kinase
FRET	Fluorescence resonance energy transfer
GASP	G-protein coupled receptor associated sorting protein
GFP	green fluorescent protein
GIPC	GAIP interacting protein C-terminus

GluR	glutamate receptor
GODZ	Golgi apparatus specific protein with a DHHC zince finger domain
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase
GST	glutathione S-transferase
GTP	guanine triphosphate
HEK	human embryonic kidney
H-FABP	heart fatty acid binding protein
IC	intracellular region
IP	immunoprecipitation
IP3R	inositol three-phosphate receptor
kD	kilodalton
KO	knockout
LDCV	large dense core vesicle
nACH	nicotinic receptor
NCS-1	neuronal calcium sensor -1
NGF	neuronal growth factor
NMDA	N-methyl-D-aspartate
NSF	N-ethylmaleimide sensitive factor
OD	optic density
ORF	open reading frame
Par-4	prostate apoptosis response-4
PAT	palmitoyltransferase
PDZ	PSD-95/Discs large/ZO-1 homology domain;
PKC	protein kinase C
PLC	phospholipase C
RGS	regulator of G-protein signaling
SSTR	somatostatin receptor
TM	transmembrane
TRP	transient receptor potential channel

TRPC	canonical transient receptor potential channel
VNTR	variable number tandem repeat
VTA	ventral tegmental area
Y2H	yeast two-hybrid

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Chapter 1

Literature Review

1.1 Dopamine Neurotransmission

Dopamine is a catecholamine neurotransmitter that regulates important cognitive and behavioral functions in the mammalian brain including locomotion, memory, attention, and reward [1-3]. Dopamine exerts its effects through the activation of dopamine receptors (DRs) and their subsequent coupling to downstream effector molecules [4]. The majority of DRs are found in four major tracts in the primate brain: 1) mesocortical, 2) mesolimbic, 3) nigrostriatal, and 4) tuberoinfundibular [4-6]. Each tract contains dopaminergic neurons that originate in the midbrain or striatum, project to different regions in the brain, and are responsible for control and modulation of distinct neurological functions. The mesocortical pathway contains dopaminergic neurons originating in the ventral tegmental area (VTA) that project to the cortex, primarily to the frontal lobes and dorsal lateral prefrontal cortex (DLPFC). Neurons in these projections control aspects of cognition, motivation and emotional processing. The mesolimbic pathway also originates in the VTA but its projections terminate in limbic areas of the brain, most notably the nucleus accumbens. Neurotransmission through this pathway modulates emotion, reward, and pleasure sensation. The nigrostriatal pathway originates in the substantia nigra and sends neuronal projections to the striatum. Dopaminergic neurotransmission in the nigrostriatal tract is primarily responsible for control and

coordination of movement. Finally, the tuberoinfundibular pathway contains neurons that reside in the arcuate nucleus of the hypothalamus and send projections to the pituitary gland. This pathway is a vital component of the neuroendocrine system and is responsible for controlling prolactin secretion under negative regulation by dopamine [4].

Alterations in dopaminergic neurotransmission have been implicated in a variety of neurological and neuropsychiatric conditions including schizophrenia [7-9], Parkinson's disease, Tourette's syndrome, and drug addiction [9]. In particular, common symptoms of schizophrenia have been attributed to aberrant dopaminergic signaling in mesocortical and mesolimbic pathways [10, 11]. Increased dopamine neurotransmission in mesolimbic projections appears responsible for the psychosis and positive symptoms of schizophrenia, whereas decreased dopaminergic signaling in the mesocortical pathway is linked to cognitive dysfunction and negative symptoms typical of the disease [10, 11]. In addition, the mesolimbic pathway is intimately intertwined with reinforcement of pleasurable emotion necessary for propagating addiction and drug abuse. In Parkinson's disease, the death of dopamine neurons in the nigrostriatal pathway causes decreased dopaminergic modulation of striatal activity and ultimately motor dysfunction resulting in tremors, rigidity and akathasia [12].

1.2 Dopamine Receptors

Dopamine receptors comprise one subgroup of a superfamily of G-protein coupled receptors (GPCRs) [7, 13, 14]. GPCRs are proteins that characteristically contain seven highly hydrophobic transmembrane regions, flanked by an extracellular

amino-terminal region and intracellular C-terminal tail. These proteins are responsible for transmission of extracellular signals across the plasma membrane to intracellular second messengers via coupling to heterotrimeric G-proteins [15]. The DR family, which consists of five subtypes grouped into D1-like (D1R and D5R) and D2-like (D2R, D3R, and D4R) subfamilies, has been shown to associate with diverse intracellular signaling pathways in neurons [4, 16]

1.2.1 Signal Transduction Pathways

Signaling through the five dopamine receptor subtypes has been shown to affect multiple downstream cascades in the cell (summarized in Table **1.1)**. In neurons, dopamine exerts its immediate effects on excitability by regulating various ligand- and voltage-gated ion channels and ion exchangers [1, 17]. Studies in both native and recombinant expression systems show that each DR subtype displays a unique profile in respect to its affinity for dopamine, potential for alternative splicing, and specificity of coupling to heterotrimeric GTP-binding G-proteins [4]. In addition, DRs appear to activate several non- G-protein associated pathways, including extracellular signal-regulated kinase (ERK) and receptor tyrosine kinase associated pathways [18].

Table **1.1**: **Overview of the Properties of Dopamine Receptor Subtypes [4, 19-21]**

AC, adenylyl cyclase; IC, intracellular region; C-tail, carboxyl terminus; nf, non-functional; AA, arachidonic acid.

	D1-Like Receptors		***D2-like Receptors***		
	D1R	**D5R**	**D2R**	**D3R**	**D4R**
Chromosomal Location	5q35.1	4p16.1	11q23	3q13.3	11p15.5
Introns	None	None	6 introns	5 introns	4 introns
Structure	Short IC3 Long C-tail	Short IC3 Long C-tail	Long IC3 Short C-tail	Long IC3 Short C-tail	Long IC3 Short C-tail
Major Splice Variants	One isoform: 1. D1R	One isoform: 1. D5R	2 isoforms: 1. D2R-short 2. D2R-long	2 isoforms: 1. D3R 2. D3Rnf	4 isoforms: 1. D4.2R 2. D4.4R 3. D4.7R 4. D4.11R
Amino Acids	446	477	414, 443	400	387 to 531
Brain Expression Profile	Caudate Putamen Nucleus Accumbens Olfactory Tubercule Cerebral Cortex Amgydala Subthalmic Nucleus	Hippocampus Lateral Mammillary Nucleus Thalamus	Caudate Putamen Nucleus Accumbens Olfactory Tubercule Substantia Nigra Ventral Tegmental Area Cerebral Cortex	Hypothalamus Cerebellum Thalamus Nucleus Accumbens	Cerebral Cortex Amgydala Olfactory Bulb Hippocampus Hypothalamus Mesencephalon
Neuronal Distribution	Post-synaptic	Post-synaptic	Pre- and Post-synaptic	Pre- and Post-synaptic	Post-synaptic
Signaling: **Adenylyl Cyclase**	Increase AC	Increase AC	Decrease AC	Decrease AC	Decrease AC
Ionic Currents	Increase L-type Ca^{++} currents Decrease N and P/Q type Ca^{++} currents Increase/ Decrease K^+ currents Decrease Type II Na^+ currents	Increase L-type Ca^{++} currents Decrease N and P/Q type Ca^{++} currents Increase/ Decrease K^+ currents Decrease Type II Na^+ currents	Inhibits Ca^{++} currents Decrease N and P/Q type Ca^{++} currents Increase/Decrease K^+ currents Increase/Decrease Na^+ Currents	Inhibits Ca^{++} currents Decrease N and P/Q type Ca^{++} currents Increase/Decrease K^+ currents Increase/Decrease Na^+ currents	Inhibits Ca^{++} currents Decrease N and P/Q type Ca^{++} currents Increase/Decrease K^+ currents Increase/Decrease Na^+ Currents
Arachidonic Acid	No effect	No effect	Stimulates AA production	Stimulates AA production	Stimulates AA production

Traditionally, it has been demonstrated that D1-like dopamine receptors (D1 and D5) couple to $G\alpha_s$ proteins and increase levels of cAMP in the cell, whereas D2-like receptors (D2, D3, and D4) couple to $G\alpha_{i/o}$ proteins and decrease levels of cAMP [4]. Interestingly, recent studies have found that most GPCRs are also capable of a significant amount of cross-talk mediated through heterodimerization with other subtypes of GPCRs, or promiscuous coupling to less preferred G-proteins and other downstream effectors [18]. Although D2Rs are primarily regulators of inhibitory $G\alpha_{i/o}$ pathways, they have been found to activate calcium associated signaling pathways, including the canonical $G\alpha_q$/PLC pathway, and can mediate the mobilization of intracellular calcium stores and activation of calcium-dependent phosphatases [22-24]. In particular, heterodimers of D1R/D2R have been known to activate $G\alpha_q$ –coupled pathways and increase levels of intracellular Ca^{2+} in a manner not typical of either receptor alone [25, 26].

1.2.2 Alternative Splicing

D1-like and D2-like receptors differ in respect to alternative splicing. D1-like receptor genes contain no introns and therefore only express a single isoform [4]. D2-like receptors all contain multiple introns and exons, resulting in expression of splice variants [4, 27]. Within the D2-like subfamily, the D4R displays the most diversity in isoform expression [28]. D4Rs contain a variable number tandem repeat region (VNTR) in their third intracellular loop (IC3) consisting of a 16 amino acid sequence [4, 28]. The VNTR in the D4R can be repeated up to 16 times, with the four most commonly seen D4R variants containing a direct repeat (D4.2R), four tandem repeats (D4.4R – the most

common variant), seven tandem repeats (D4.7R), or eleven tandem repeats (D4.11R) [28]. Various studies have linked increased VNTR number with such conditions as ADHD, novelty seeking, and extreme risk taking behavior [29]. The D4R variants are believed to have differential responsiveness to antipsychotics such as clozapine, and may contribute to variable responses to medication seen in certain populations of patients with schizophrenia [30]. In contrast, the D3R has been found to express only two splice variants – D3R and D3Rnf [31]. The D3Rnf (stands for non-functional) isoform contains a premature stop codon that truncates the protein after the fifth transmembrane domain [31]. This isoform can bind D3R and sequester the functional, full-length receptor in the cytoplasm [32].

The most intensely characterized D2-like receptor splice variants are those of the D2R. The D2R is alternatively spliced into two primary isoforms, the D2R-short (D2S) and D2R-long (D2L) [33]. These isoforms differ by the presence of a 29 amino acid insertion in the IC3 of the D2L [34]. Recent studies have shown that these isoforms have the ability to differentially bind to distinct subsets of G-proteins, resulting in divergent downstream effector functions [35]. The isoforms appear to be targeted to disparate compartments in neurons where they mediate distinct functions: the D2S is trafficked pre-synaptically and the D2L post-synaptically [36]. In the pre-synapse, the D2S functions as an autoreceptor and mediates regulatory aspects of dopamine neurotransmission, including neurotransmitter and peptide release [34].

1.2.3 Dopamine Receptor Knockouts

Due to the lack of specific pharmacological ligands which can discriminate between individual receptor subtypes, it had been impossible to differentiate functions of individual dopamine receptor subtypes *in vivo*. To understand the contributions of individual DR subtypes to complex neuronal processes, knockout mice have been generated for each receptor subtype (reviewed in [37]). Although phenotypic differences have been documented between strains of knockouts deficient in the same receptor subtype, it is possible to garner information and an understanding of the contributions of each individual receptor to dopamine neurotransmission from these animals. Most dopamine receptor knockouts have been created in a C57BL/6 strain genetic background.

Two D1R knockout (KO) strains have been generated to date, a genetic null [38] and a strain with the D1R disrupted in its fifth transmembrane domain [39]. These KO strains have an overall normal appearance with no obvious neurological defects. However, they do exhibit growth retardation and low survival after weaning [38, 39]. These characteristics may be due to deficits in fine motor control that impact the ability of these mice to feed properly. D1R KO mice also show subtle behavioral abnormalities, including alterations in basal and psychostimulant-induced locomotor activity, and are slow to habituate when challenged in an open field apparatus [38, 40-42]. Normal hyperactive and hypoactive motor responses to D1R-like (but not D2R-like) agonists and antagonists are abolished in these mice [38, 40-43]. In addition, D1R KO mice exhibit impairment in long-term potentiation in hippocampal and corticostriatal neurons, which may reflect a role for D1R in cognition and memory [44, 45].

Only one D5R KO strain has been reported [46]. An insertion of a premature stop codon in the IC2 of the D5R is the genetic deficit in this strain [46]. These mice are generally healthy and completely lack the growth retardation seen in D1R KO [46-48]. The only behavioral phenotype attributed to these mice is an overall increase in locomotor activity, suggesting a role for the D5R in the inhibition of locomotion [37, 48].

Three strains of D2R KO mice have been extensively studied; two that are complete null mutants [49, 50] and one that contains a sequence disruption resulting in the removal of part of the IC3 and transmembrane regions six and seven [51]. Upon examination, all D2R KO strains are approximately 15% smaller at birth than their wild-type littermates, have reduced fertility, and exhibit postnatal growth delay. The two genetic null strains show additional abnormalities, including a hunched posture, paw flattening, and sprawling of hindlegs [49, 50]. Behaviorally, all three KO strains show delayed initiation of movement and a decrease in overall locomotor activity when compared to wild-type controls [49-51]. Reward responses to drugs of addiction are also altered in these mice. Various studies have described a reduction of voluntary ethanol and morphine self-administration and consumption in D2R KO, as well as a failure to develop ethanol- or morphine-induced conditioned place preference [52-56]. These findings concur with emerging clinical data pointing to a critical, but complex, role for D2R in reward and addiction [57].

In addition to complete D2R KO, two laboratories have developed D2L-specific KO strains by deletion of exon six of the D2R [34, 58]. This alternatively spliced exon encodes the 29 amino acid insertion specific to the D2L isoform. Removal of the exon results in mice which are only capable of expressing D2S [33, 34, 58-61]. In addition,

D2R autoreceptor function, including the inhibition of dopamine synthesis and release, is intact in D2L KO mice, but absent in D2R KO [34]. These D2L KO mice exhibit behavioral abnormalities similar to D2R KO, which include diminished place preference for morphine, reduced aggression, and impaired avoidance learning [58, 59, 62]. Interestingly, haloperidol-induced cataplexy is significantly reduce in D2L KO, suggesting a specific role for the isoform in some side effects of antipsychotics [34, 60, 61]. Evidence taken from comparing the phenotypes of D2R versus D2L KO mice demonstrates that the D2S and D2L isoforms are responsible for discrete neuronal functions.

Three lines of D3R KO mutant mice have been generated. Two of the lines have disruption in the IC2 domain [50, 63], and the third line is a genetic null [64]. On gross examination, these mice are phenotypically normal with a slight propensity for increased locomotor investigation in a novel environment [50, 63, 64]. However, upon challenge with psychostimulants such as cocaine and amphetamines, D3R KO exhibit enhanced behavioral sensitivity compared to wild-type littermates as evidenced by statistically significant increases in locomotor activity, sniffing, licking, and biting [65].

Finally, one D4R KO strain has been produced [66] in which the deletion of exon two results in a truncated protein. As with the D3R KO, no gross morphological or neurological abnormalities are apparent in these mice. D4R KO mice possess superior motor coordination in comparison to wild-type littermates and exhibit exaggerated hyperactivity responses to both psychostimulants and ethanol [66, 67]. D4R KO mice have demonstrated a reduction in novelty-seeking behavior, which mimics the role this receptor appears to play in the human population as well [68].

1.3 Dopaminergic Neurotransmission and Schizophrenia

1.3.1 Pathology

Schizophrenia is a devastating mental illness that affects approximately 1% of the population worldwide [69, 70]. The age of onset of the disease ranges from the late teens to the early thirties, a time that typically corresponds with an individual's emergence into the workforce and adulthood. Women tend to have a later onset of disease than men, and overall, their course of illness is more benign in comparison [71, 72]. Schizophrenia ranks as one of the world's top ten causes of long-term disability [73]. The total cost of caring for schizophrenic patients is high. For instance, in the United States, the estimated direct- and indirect-costs of treatment were estimated to be ~$63 billion USD in 2002 [74].

Schizophrenia is a heterogeneous syndrome that is diagnosed based on a patient's history and presenting symptoms. There are no laboratory tests that definitively confirm the diagnosis of schizophrenia, and consequently there are many different disease phenotypes observed among schizophrenic patients. Schizophrenia is characterized by three main classes of symptoms: positive symptoms, negative symptoms, and cognitive dysfunctions [75-77]. Positive symptoms of schizophrenia include psychotic symptoms such as hallucinations (most commonly auditory), delusions, and bizarre behaviors, such as abnormal posturing and defects in personal hygeine. These symptoms occur in an episodic nature, and can wax and wane as the disease progresses. Negative symptoms comprise the 'deficit states' observed in schizophrenics, including flattening of affect, social dysfunction, avolition (loss of drive), anhedonia (loss of enjoyment), and alogia

(paucity of speech) [78]. These symptoms are more insidious than the positive symptoms of the disease, are more pervasive and tend to fluctuate less over time [79, 80]. Cognitive impairments in schizophrenia include deficits in working memory, attention, concentration, and other executive functions, such as initiation of and persistence in tasks, impulse control, and abstract reasoning [81].

1.3.2 Pharmacology

The dopaminergic system has long been implicated in the pathophysiology of schizophrenia for a variety of reasons. Drugs that increase dopaminergic signaling in the brain, such as amphetamines, are known to produce side effects that mimic the positive symptoms of schizophrenia [82]. Reciprocally, drugs that are used in the treatment of the disease (typical and atypical antipsychotics) selectively target and dampen dopaminergic signaling in the brain [83].

The treatment of schizophrenia has been largely pharmacological since the introduction of typical antipsychotics in the 1950s. These drugs (which include chlorpromazine, haloperidol, and perphenazine) act as competitive inhibitors of D2R and D3R and greatly reduce the psychotic symptoms of the disease [84]. These drugs exhibit serious extrapyramidal side effects which include Parkinsonism and tardive dyskinesia. Decades later, atypical antipsychotics emerged (such as clozapine and olanzapine), that had multiple antagonist targets including D2R, D3R, D4R and serotonin receptors (5HTR) [84]. These drugs were also effective at combating the positive symptoms of the disease. Atypical antipsychotics had fewer propensities to cause the extrapyramidal side

effects seen with typical antipsychotics. However, they also caused unwanted side effects, including weight gain, hyperlipidemia, and type II diabetes [84]. Neither drug class has proven effective at managing the cognitive or negative symptoms of the disease. The undesirable side effects and the uncertainty of therapeutic benefit contribute to the 50% non-compliance rate seen in patients prescribed these drugs; this rate is even higher in first-episode, first-time medicated schizophrenics [85].

1.3.3 Genetics of Schizophrenia

The risk of schizophrenia is higher among relatives of schizophrenic patients, which implies a genetic component of the disease. Typically, risk increases ten-fold when a first-degree family member is affected. Monozygotic twin studies have reported concordance rates for schizophrenia ranging from 48-80% [86]. No single gene mutations (including mutations in DR genes) have been linked to the disease to date. Schizophrenia is a complex disease that is not inherited in a Mendelian fashion. Rather, schizophrenia is most likely caused by many genes of small effect conferring susceptibility and acting in cooperation with epigenetic and environmental factors to promote the disease phenotype.

Numerous schizophrenia genomic association and linkage studies have isolated chromosomal regions that may contribute to the disease, but none are particularly compelling and most studies cannot be replicated in multiple populations. Table **1.2** lists a limited survey of the top candidate susceptibility genes to date. These genes show evidence for involvement in the pathophysiology of schizophrenia, including genetic

association with the disease and altered protein expression [87-89]. For an extensive list, refer to either the Genetic Association Database (GAD - http://geneticassociationdb.nih.gov) [90] or the Database for Schizophrenia candidate genes focusing on Variations (VSD - http://bioinfo.tsinghua.edu.cn:8080/vsd/index.php) [91].

Table **1.2**: **Catalog of Potential Schizophrenia Susceptibility Genes**

A survey of candidate genes with highest likelihood of contributing to schizophrenia pathophysiology.
Adapted from Straub and Weinberger, 2006, and Ross et al., 2006 [92, 93]

Gene	Protein	Locus
COMT	Catechl-O-methyltransferase	22q11
DTNBP1	Dysbindin	6p22
NRG1	Neuregulin 1	8p12-21
RGS4	Regulator of G-protein signaling4	1q21-22
GRM3	Glutamate Receptor type3	7q21-22
DISC1	Disrupted in Schizoprenia1	1q42
DAOA	D-amino acid oxidase activator	13q32-34
DAAO	D-amino acid oxidase	12q24
PPP3CC	Calcineurin gamma	8p21
CHRNA7	Alpha-7 nicotinic receptor	15q13-14
PRODH2	Proline dehydrogenase2	22q11
AKT1	V-akt murine thymoma viral oncogene homolog 1	14q22-32
GAD1	Glutamic acid decarboxylase1	2q31.1
ERBB4	Neuregulin1 receptor	2q34
FEZ1	Fasciculation and elongation of protein zeta1	11q24.2
MUTED	Subunit of BLOC-1	6p24.3
NPAS3	Neuronal PAS domain protein 3	9q34
GRIK4	Kainate-type glutamate receptor 4	11q23
zDHHC8	Putative Palmytolytransferase	22q11

1.4 Identification and Characterization of Receptor Signaling Complexes

1.4.1 Proteomics and Protein-Protein Interactions

Previously, the dogma surrounding GPCR-mediated signaling was one of a transient agonist-dependent interaction between a single GPCR and a specific heterotrimeric G-protein effector. It is now widely recognized that the signaling properties of GPCRs are dependent on their interactions with a myriad of proteins termed GPCR-interacting proteins (GIPs) [94-97]. The clustering of proteins around GPCRs is a mechanism that serves to physically stabilize the signalplex within specific cellular compartments or membrane domains, thus ensuring that the molecular basis of cellular operations can be sustained via highly regulated interactions among protein networks [98]. The physical assembly of signaling molecules into discreet macromolecular signaling complexes allows for biologically efficient means to integrate cellular signals. Using this method, there is no need to waste effort recruiting important proteins to cellular localizations and signaling should occur more rapidly compared to the case in which necessary components were free-floating in the cell [99].

It is well established that protein-protein interactions govern the structural and functional organization of GPCR-containing signalplexes [97, 100]. These structures contain protein components that interact with the primary GPCR either stably or ephemerally, allowing for a dynamic, fluid, and temporal response to environmental cues and signals. It has been suggested that the most effective design for a signaling complex appears to be a stable protein core associated with transiently interacting molecules [99]. This provides for multiple levels of control of GPCR signaling based on highly regulated

interactions between protein constituents of the signalplex. Depending on the tissue type and intracellular environment, a given GPCR- signalplex can respond to the expression or non-expression of various protein constituents, the differential trafficking of protein members, and the competition for binding domains between multiple protein partners. GIPs allow for precise control over many aspects of the life cycle of a GPCR, including localization, trafficking, desensitization, and recycling [94-96].

1.4.2 Methods for Identifying Interacting Proteins

Proteomics, which is defined as the research effort to establish the identities, interactions, and functions of all proteins in a particular cell, tissue, or organism, has culminated in the development of numerous molecular biological techniques aimed at the elucidation of protein networks. Historically, the yeast two-hybrid system [101] has been a common method for identifying and confirming individual protein-protein interactions. This approach has limitations. These include difficulty in detecting protein interactions involving highly hydrophobic proteins, as well as the fact that the protein-protein interaction is occurring in a yeast cell, which may differ from its native physiological environment. The use of truncation fragments of hydrophobic proteins can bypass some of these issues, but the question remains as to whether fragments of proteins can adequately reconstitute or mimic protein interactions between native full-length proteins. To address these limitations, a modified yeast two-hybrid method has been developed by Stajlar et al. [102-104], that utilizes proteomic cleavage of a reconstituted ubiquitin

molecule to survey for protein-protein interacts. This approach requires that one member of the protein interaction studied is an obligate membrane protein [102-104].

In addition to the yeast two-hybrid methods used to screen for direct interactors, current proteomic methods incorporate techniques that identify signaling complexes isolated from their native environment. Isolation of protein complexes through immunoprecipitation from native tissue are analyzed through a variety of techniques, including mass spectrometry, protein-chip analysis, and genome-based techniques [105, 106]. These methods have advantages over traditional and newer yeast two-hybrid genetic assays in that they isolate and identify a cohort of interactors in a given signaling complex. These approaches are useful in identifying vital signalplex components that are part of the complex, but do not directly interact with the primary receptor.

1.5 The Dopamine Receptor Signaling Complex

1.5.1 Overview

Similar to many GPCRs, DRs are known to function as part of a macromolecular complex [98]. A number of proteins have been identified that interact with DRs, referred to as dopamine receptor interacting proteins (DRIPs), and regulate the life cycle and signaling properties of individual DR subtypes. Recent studies suggest that alterations in DRIPs may be linked with CNS disease. For example, postmortem studies of schizophrenic patients indicate a significant increase in expression of the DRIP NCS-1 in the dorsolateral prefrontal cortex [107]. Transgenic mice expressing a mutated form of

the DRIP Par-4 (prostate apoptosis response-4) exhibit a depression-like syndrome [108]. The identification of DRIPs provides a useful platform for determining the diverse signaling mechanisms that contribute to dopamine-mediated neurotransmission.

1.5.2 DRIPs and DRAPs

Analysis of D2R/protein interactions has contributed to the finding that the D2R signalplex consists of proteins that directly or indirectly interact with the D2R itself. DRIPs directly interact with the receptor and modify its function through protein-protein interaction. Dopamine receptor associated proteins (DRAPs) do not directly interact with the D2R but still exert vital functions on the D2R signalplex. A visual schematic of the D2R showing the distinction between DRIPs and DRAPs is shown in Figure **1.1**. A complete summary (compiled by a thorough investigation of the literature and protein interaction databases) of confirmed DRIPs and DRAPs known to date is presented in **Table 1.3**

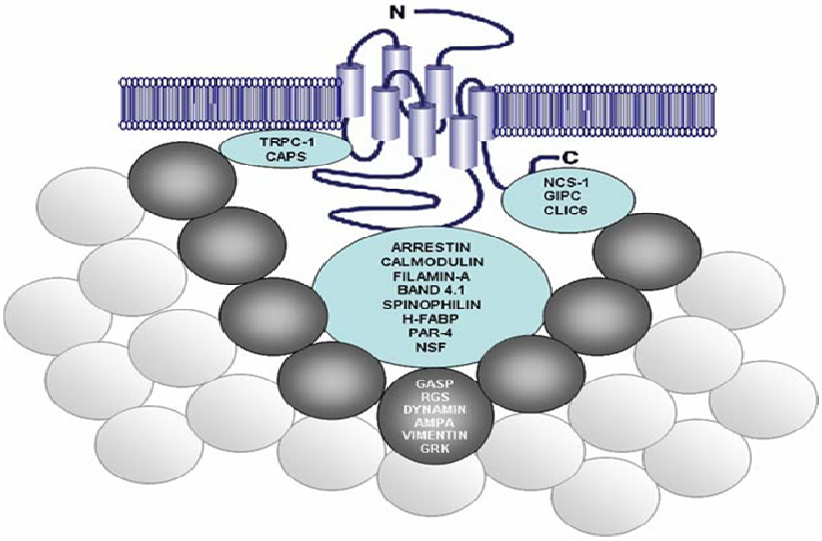

Figure **1.1**: **Schematic Illustration of the D2R Signalplex.**
Representative DRIPs and DRAPs are shown in blue and grey/white circles, respectively. DRAPs may interact either with DRIPs (grey circles) or with other DRAPs (white circles). From (Kabbani et al., 2005 [98]).

Table **1.3**: **Summary of Known DRIPs and DRAPS.**

The list of DRIPs and DRAPs was compiled from PubMed (http://www.ncbi.nlm.nih.gov) and the database of interacting proteins (http://dip.doe-mbi.ucla.edu). The proteins are grouped into six classes based on biological function.

PROTEIN	INTERACTION	BINDING REGION	REFERENCE
Scaffolding, Anchoring, Trafficking			
4.1B	DRIP	N-term IC3	[109]
4.1N	DRIP	N-term IC3	[109]
4.1G	DRIP	N-term IC3	[109]
4.1R	DRIP	N-term IC3	[109]
Arrestin-2	DRIP	IC2, IC3	[110]
Arestin-3	DRIP	IC2, IC3	[110]
Clathrin	DRAP	---	[111]
Dynamin-2	DRAP	---	[112]
Filamin-A	DRIP	IC3	[113]
GASP	DRAP	---	[114]
GIPC	DRIP	C-term tail	[115]
H-FABP	DRIP	IC3(D2L)	[116]
Spinophilin	DRIP	IC3	[117]
Vimentin	DRAP	---	[118]
zDHHC4	DRIP	IC2	[119]
Signaling			
Calmodulin	DRIP	IC3	[120]
NCS-1	DRIP	C-term tail	[121]
Par-4	DRIP	IC3	[108]
Transient			
$G\alpha_{i(1-3)}$	DRIP	IC3	[35, 122, 123]
$G\text{-}\alpha_z$	DRIP	IC3	[122, 123]
$G\text{-}\alpha_o$	DRIP	IC3	[123, 124]
GRK2	DRAP	---	[121]
GRK3	DRAP	---	[121]
GRK5	DRAP	---	[121]
GRK6	DRAP	---	[121]
RGS9-2	DRAP	---	[125]
RGS19	DRAP	---	[126, 127]
Synaptic			
CAPS	DRIP	IC2	[128]
Dopamine Transporter	DRIP	IC3	[129]
NSF	DRIP	IC3	[130]
Receptors			
A2AR	DRIP	N/A	[131-133]
AMPAGluR1	DRAP	---	[130]
AMPAGlurR2	DRAP	---	[130]
CB1	DRIP	N/A	[134]
D1R	DRIP	N/A	[135, 136]
D2R	DRIP	N/A	[137-139]
D3R	DRIP	N/A	[140]
$nACH(\alpha_4)$	DRAP	---	[141]
$nACH(\beta_2)$	DRAP	---	[141]
NMDA(NR2B)	DRIP	IC3	[142]
SSTR5	DRIP	N/A	[138]
Ion Channels			
CLC6	DRIP	C-term tail	[143]
Kir3	DRAP	---	[144]
TRPC1	DRIP	IC2	[145]
TRPC4	DRIP	IC2	[145]
TRPC5	DRIP	IC2	[145]

1.5.2.1 Scaffold, Anchoring and Trafficking Interactors

The D2R maintains contact with the actin cytoskeleton of a cell through direct protein-protein interactions via motifs located in the third intracellular loop and the carboxyl terminus of the protein. Interaction between the D2R and cytoskeletal DRIPS provides both dynamic and stable regulation of the receptor and allows for proper trafficking and targeting of the receptor within the cell. Scaffolding and anchoring proteins act as templates to enhance nucleation of the signalplex within specific cellular microdomains or compartments. Through this spatial regulation, these components of the signalplex may aid in linking the receptor to differential signaling networks.

Included in this subset of DRIPs are members of the Band 4.1 family of cytoskeletal-associated proteins. 4.1N, as well as 4.1B, 4.1G, and 4.1R, were found to interact with the N-terminal segment of the third intracellular loop of D2R by directed yeast two-hybrid strategy [109]. These proteins are integral members of the spectrin-actin cytoskeleton that function as adaptor proteins and provide dynamic attachment between the cytoskeleton and the plasma membrane [146]. Co-expression of a dominant-negative mutant 4.1N protein was found to decrease cell surface expression of D2R in mouse neuroblastoma cells [109]. This implies a functional role for 4.1N in localizing and stabilizing D2 dopamine receptors at the plasma membrane.

Other members of the spectrin-actin cytoskeleton have been implicated in maintaining proper expression of the D2R at the cell membrane. Filamin A, or actin-binding protein 280, was identified as a DRIP in a yeast two-hybrid screen using the third intracellular loop of the D2R as bait [113]. Truncation mutants of filamin-A that lacked

the cytoskeletal binding domain reduced the number and half-life of exogenously expressed D2Rs present at the plasma membrane in HEK 293 cells. Point mutations of filamin-A that altered the D2R binding domain but not its ability to interact with the cytoskeleton prevented proper delivery of the D2Rs to the plasma membrane[147]. Interestingly, filamin-A and 4.1N can simultaneously bind D2Rs, implying non-overlapping or cooperative binding sites on the receptor [109].

Spinophilin (also known as neurabin II) contains a single PDZ domain and can serve as a direct link to the actin cytoskeleton as well as other PDZ binding proteins [148, 149]. Spinophilin was identified as a DRIP via yeast two-hybrid strategy using the third intracellular loop of the D2R as bait [117]. Although its exact role in D2R regulation remains unclear, the protein has been implicated in regulation of neuronal spine dynamics and has been found to contribute to antipsychotic-induced changes in spine morphology [150]. Spinophilin interacts with various GPCRs and can bind Regulators of G-protein signaling (RGS) via their amino terminal domains [151-153]. This implies that spinophilin acts as a molecular scaffolding protein and recruits various RGS proteins to the receptosome.

GIPC, or GAIP interacting protein C-terminus, is a PDZ domain-containing scaffolding protein that associates with the D2R carboxyl-terminus [115]. While associated with D2Rs, GIPC can recruit RGS19, a known G-protein regulator, to the signalplex resulting in the dampening of G-protein signaling [127]. Overexpression of GIPC can attenuate D2R signaling by mediating internalization of the receptor. Interaction of GIPC with the D2R allows for the protein complex to be sequestered in

intracellular vesicles and protected from degradation by lysosomes, in turn allowing for preferential recycling of the receptors [127].

In addition to stabilizing receptors at the plasma membrane, scaffolding proteins can target D2Rs to other intracellular compartments and play a vital role in the regulation of receptor recycling and degradation [154]. Arrestin-2 and arrestin-3 were found to interact with the D2R via GST-pulldown strategy [110]. The second and third intracellular loops and C-terminal tail of D2R were found to associate with arrestin-2 and arrestin-3 in vitro. Agonist stimulation of cells containing D2Rs showed a drastic decrease in receptor internalization when arrestin-2 was absent from the cell [110].

As mentioned previously, D2Rs exist as two functionally distinct isoforms (short and long) in the cell. Heart fatty acid binding protein (H-FABP) has been found to interact with D2L but not D2S via yeast-two hybrid strategy, suggesting that its binding site resides in the 29 amino acids present in the IC3 of the D2L [116]. Ectopically expressed D2S and D2L receptors have been shown to have different subcellular localization patterns in NG108-15 cells. H-FABP co-localizes with D2L but not D2S receptors in both NG108-15 cells and neurons, suggesting this protein plays a role in the differential regulation of the two D2DR isoforms [116].

GASP (GPCR-associated sorting protein) has been shown to associate with the D2R by coimmunoprecipitation from transfected HEK293 cells [114]. GASP is involved in the differential sorting of GPCRs to recycling endosomes or degradative lysosomes after their internalization [155]. An interaction between GASP and the carboxyl terminal of the D2R was suggested by GST-pulldown and antibody competition approaches [114]. However, a direct interaction could not be confirmed by these methods. GASP,

therefore, represents a DRAP. Expression of a dominant-negative GASP mutant in HEK293 cells implied a role for GASP in regulating postendocytotic sorting of D2Rs [114].

Dynamin II, a protein known to pinch off internalized clathrin-coated pits from the cellular membrane, has been identified as a DRAP [156, 157]. D2Rs have been shown to undergo dynamin II-dependent internalization in response to agonist treatment [112]. Immunoprecipitation of the D2R signalplex indicates that dynamin II associates with the D2R, and most likely regulates D2R signaling from within non-synaptic compartments [112].

1.5.2.2 Signaling and Transient Interactors

DRs activate downstream signaling cascades through differential coupling to various subtypes of heterotrimeric GTP-binding proteins known as G-proteins [4]. It is widely accepted that GPCRs can effectively couple to multiple classes of G-proteins [123]. D2Rs preferentially associate with $G\alpha_i$, $G\alpha_o$, and $G\alpha_z$ proteins, classes of G-proteins that are known to inhibit adenylyl cyclase activity and subsequently decrease intracellular levels of cAMP. G-proteins are thought to interact with D2Rs through motifs present on the third intracellular loop of the receptor. Evidence has been shown that point mutations in this region affect G-protein signaling through the receptor [35]. Receptor phosphorylation and other modifications in this region are thought to enhance or abolish G-protein signaling cascades through the receptor.

In addition to cAMP, intracellular calcium levels are known to function as second messengers in signaling cascades. Ca^{2+} has importance as a ubiquitous second messenger and is responsible for the transduction of a multitude of signals in a cell. Calmodulin, the prototypical calcium sensor, has an affinity for calcium in the range of 10^{-6}M and is known to interact with and regulate a wide variety of intracellular proteins (reviewed in [158]). A calmodulin-binding motif was identified in the D2R in the amino terminus of the third intracellular loop, and this region was found to bind calmodulin in a calcium-dependent manner [120]. Activation of purified $G\alpha_i$ by dopaminergic stimulation is inhibited by the presence of calmodulin in a calcium-dependent manner. As intracellular calcium levels rise, calmodulin is able to preferentially combine with the amino terminus of the third intracellular loop of the D2. This effectively blunts D2R signaling by blocking a site on the receptor necessary for Gα activation, without interfering with Gα binding [120].

Neuronal calcium sensor- 1 (NCS-1), another intracellular calcium sensing molecule, has been found to directly interact with the C-terminus of the D2R by a yeast two-hybrid strategy [121]. NCS-1 has an approximately 10-fold higher affinity for calcium when compared with calmodulin, providing a mechanism where NCS-1 can modulate D2R-mediated signaling at lower intracellular calcium concentrations than calmodulin. Overexpression of NCS-1 has been shown to decrease receptor internalization in the presence of dopamine stimulation, allowing for increased retention of D2 receptors at the plasma membrane [121]. NCS-1 appeared to reduce D2 receptor phosphorlyation in the presence of overexpressed G-protein receptor kinase 2 (GRK2), a

protein known to phosphorylate the receptor and target it for desensitization and internalization.

Par-4 (Prostate Apoptosis Response-4) is a leucine zipper-containing protein that was originally implicated in the regulation of cell survival during development [159]. Par-4 was identified as a DRIP in a yeast two-hybrid screen of a fetal embryonic cDNA brain library using the third intracellular loop of the D2L as bait [108]. It was found to directly interact with the D2R through the previously mentioned calmodulin binding domain, and was shown to function as an intracellular antagonist of calmodulin binding [108]. In this fashion, Par-4 may interact with the D2R and promote the receptor's coupling to downstream G-proteins.

GPCRs are tightly regulated by a select class of receptor kinases known as GPCR kinases (GRKs) [160]. D2R-kinase interactions, resulting in receptor phosphorylation, play an important role in regulating components of the life-cycle of the D2R [160]. GRKs have not been identified to directly interact with the D2R and have therefore been classified as DRAPs.

1.5.2.3 Synaptic Interactors

Calcium-dependent activator for protein secretion 1(CAPS1) is a novel DRIP found to interact with the second intracellular loop of the D2 dopamine receptor via a yeast two-hybrid screen of a human fetal cDNA brain library [128]. CAPS1, as well as the closely related protein CAPS2, are vital regulators of the large dense core vesicle (LDCV) trafficking pathway, making these proteins essential components for late stages

of exocytosis and neurotransmission [161, 162]. Interaction between the D2R and CAPS1 provides a direct link between receptor signaling and exocytotic pathways, implying that receptors themselves are able to modulate neurotransmitter release from vesicles in neurendocrine cells. Experiments involving an exogenously expressed peptide encoding the D2R binding domain of CAPS1 showed that K+-stimulated dopamine release in PC12 cells is inhibited by the presence of the peptide, implying that interaction between D2 dopamine receptors and CAPS1 is vital for proper neurotransmitter release [128].

A second protein closely associated with exocytosis in the cell is N-ethylmaleimide-sensitive factor (NSF), which was discovered to be a DRIP through a candidate search for adaptor proteins responsible for a functional AMPAGluR/ D2R interaction [130]. NSF plays an important role in intracellular membrane trafficking and vesicle fusion in the Golgi organelle and plasma membrane [163]. In addition to its role in exocytosis, NSF has been known to play a role in the regulation of the expression of GluR2s at the plasma membrane [130]. NSF directly interacts with the third intracellular loop of the D2R, and functions as a protein bridge that promotes cross-talk between the AMPA and D2R signaling pathways [130].

The dopamine transporter (DAT) is a membrane bound protein in the presynapse that facilitates the reuptake of extracellular dopamine and regulates dopaminergic neurotransmission. Using a GST-pulldown assay, the amino terminus of the DAT was found to directly interact with the third intracellular loop of the D2R [129]. This interaction facilitates the cell surface expression of the DAT and leads to an enhancement of dopamine reuptake. Interestingly, disruption of the DAT/D2R interaction in wild-type

mice by injection of an interfering peptide produced a hyperlocomotor phenotype, similar to that observed in a DAT KO mouse strain [129]. These findings imply a novel mechanism in which receptors can functionally modulate neurotransmitter transporter and thereby affect levels of neurotransmitter seen in the synapse.

1.5.2.4 Receptor Interactors

GPCRs function as either homo- or hetero-dimers. Dependent on the dimers that form, the resulting receptor complex will have unique functional and behavioral consequences for both the cell and organism. Dopaminergic signaling is considered to be neuromodulary in most instances; therefore, some of the modulation of neurotransmission may be due to direct protein-protein interactions between disparate receptor subtypes.

D2R proteins have been shown to heterodimerize with other membranes of the dopamine receptor subfamily, namely D3R [140]. D2R and D3R are closely related, sharing a high degree of amino acid homology with each other [4]. The D2R/D3R heterodimer has been identified to have unique functional properties as compared to the D2R or D3R homodimers. In particular, the D2R/D3R heterodimer seems to have an increased potency for D2-like receptor agonists, and in turn may couple more efficiently to adenylyl cyclase 6 [140].

D2Rs are also capable of heterodimerizing with D1Rs [135]. Anatomical studies suggest colocalization of D1R and D2R in the majority of striatal neurons [164]. Fluorescence resonance energy transfer (FRET) analysis revealed that D1R/D2R

heterodimers display unique cell surface localization, internalization, and novel phospholipase C-mediated signaling properties following co-activation in cell lines [135].

In addition to interacting with GPCRs in its own family, the D2R has been shown to interact with and affect the signaling properties of other, more distantly related GPCRs. Recently, the D2R has been shown to directly interact with the Adenosine A2 receptor (A2AR-[165, 166]). Functionally, the heterodimer has shown reciprocal antagonism at the level of adenylyl cyclase [167]. In particular, the stimulation of A2AR dramatically decreases the D2R affinity for dopaminergic agonists. Furthermore, the phosphorylation states of each receptor affect their heterodimeric interaction and subsequent downstream signaling, implying that the A2AR/D2R exists in a dynamic state of interaction that can constantly be modified [167].

D2Rs have been shown to functionally interact and modulate cannabinoid signaling [134, 168, 169]. Interactions between D2R and cannabinoid (CB1) receptors have been elucidated through co-immunoprecipitation and receptor activation strategies [134]. In the case of the CB1/D2R heterodimer, the functional consequence of hetermeric coupling was signaling through a non-preferred G-protein. The CB1/D2R heterodimer is shown to preferentially signal through $G\alpha_s$ instead of $G\alpha_{i/o}$ and causes an increase in activation of adenylyl cyclase levels [134]. In addition, active conformations of the CB1 or D2R singularly enhance their affinity to form the heterodimer. Upon simultaneous activation of both pathways, the CB1 and D2R are able to physically interact, participate in cross-talk, and engage in a third, unique cascade of intracellular signaling unavailable to the separate homodimer pairs alone [134].

D2R has been shown to interact with the somatostatin receptor 5 (SSTR5-[138]). SSTR5 preferentially signals through $G\alpha_i$ which causes an inhibition of adenylyl cyclase, similar to signaling through D2R [138]. The SSTR5/D2R heterodimer has a greater affinity for both dopamine and somatostatin agonists, enhancing the effect of agonist signaling through either pathway separately. The synergistic effect of the SSTR5/D2R heterodimer is demonstrated by a dramatic increase in downstream effector functioning unattainable by either dopaminergic or somatostatin signaling alone [138].

1.5.2.5 Ion Channel Interactors

The D2R modulates a wide variety of important ion channels, including voltage gated calcium [170, 171], potassium [172], and sodium [173] channels, as well as ligand gated channels such as NMDA, AMPA, GABA, and the nicotinic acetylcholine receptor [174-176]. Coimmunoprecipitation experiments have established that the D2R forms a stable complex with the Kir3 inwardly rectifying potassium channels [144]. CLIC6, a member of the intracellular chloride channel family, was identified as a DRIP in a two-hybrid screen of a rat brain cDNA library using the carboxyl-terminus of the D3R as bait [143]. CLIC6 was subsequently found to interact with the all members of the D2-like receptor subfamily [143]. The functional significance of this interaction remains unknown.

1.6 TRPC Ion Channel Family

1.6.1 Classification

Canonical transient receptor potential channels (TRPCs) comprise one family of a larger superfamily of transient receptor potential channels (TRPs) that function as calcium-permeable cation channels activated by various stimuli including temperature, mechanical stress, calcium store depletion, GPCR activation, as well as others [177, 178]. Most TRP channel subunits contain six transmembrane spanning domains, intracellular amino and carboxyl-terminal regions, and a pore-loop domain between the fifth and sixth transmembrane region believed to form the functional ion-conducting pore [179, 180]. TRP channels can function either as primary calcium entry portals in the plasma membrane, or they can secondarily mediate calcium entry from alternate pathways by modulating membrane polarization [180-182]. In addition, it is speculated that some TRPs may also play a role in calcium release from intracellular stores [183, 184].

TRPCs were first identified in mammals by their homology to the *Drosophila melanogaster* proteins TRP and TRPL [185, 186]. The seven identified TRPC channels share structural similarity; each member contains three or four amino terminal ankryin repeats and a TRP box, a six amino acid invariant sequence (EWKFAR) distal to the sixth transmembrane region [177, 178, 180]. Overall, TRPCs share approximately 30-45% sequence similarity over the amino terminal region of the protein, resulting in most of their diversity being confined to the carboxyl tail (Figure **1.2).** The TRPC subfamily can be further divided into three subgroups based on functionality and amino acid

sequence similarity: TRPC1/4/5, TRPC3/6/7, and TRPC2 (Figure **1.2)** [177, 180, 187]. In humans, TRPC2 is a pseudogene [178].

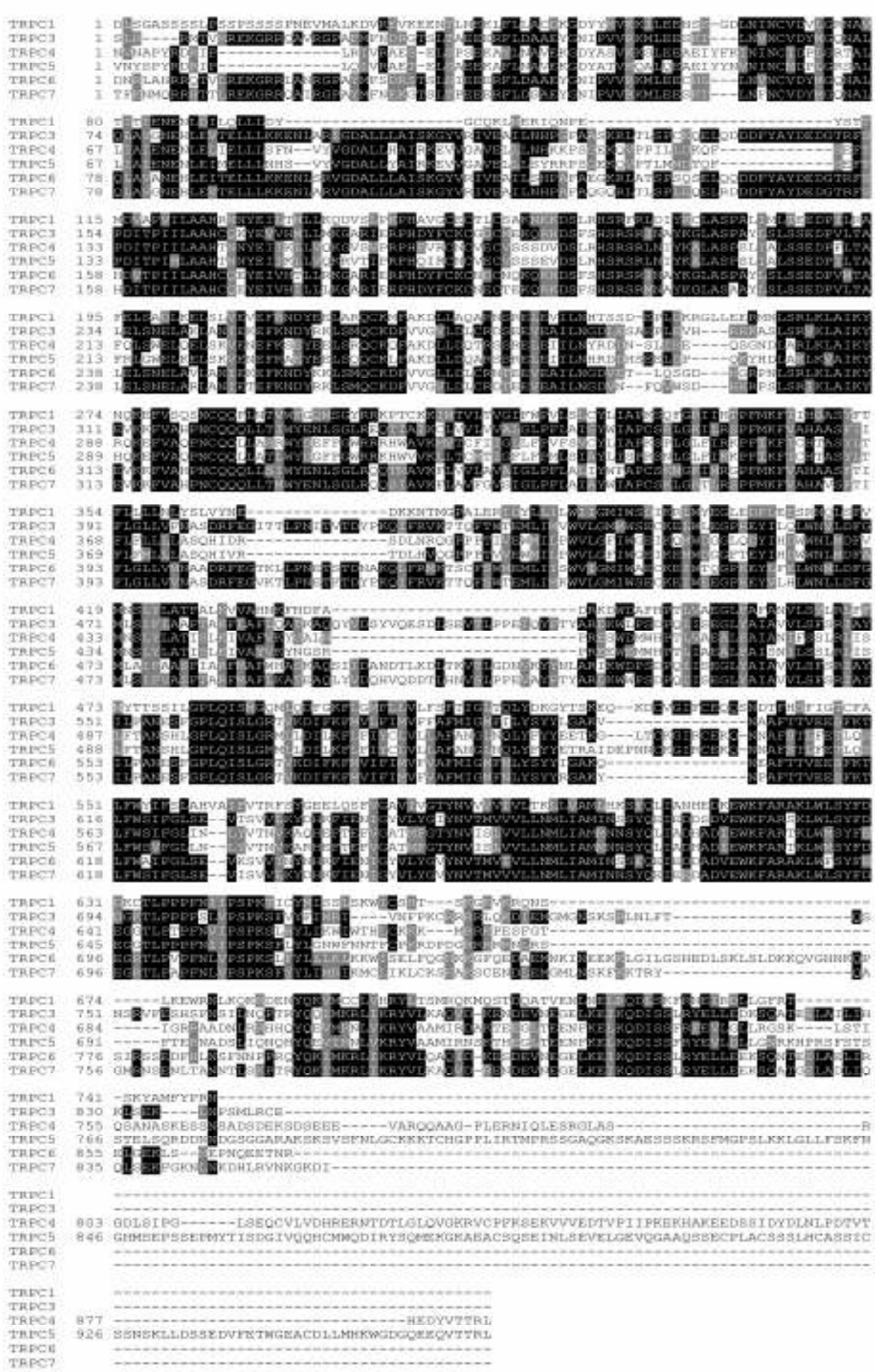

Figure **1.2**: **Amino Acid Alignment of Human TRPCs**

Amino acid sequence alignments of the six human TRPCs (1-7). TRPC2 is a pseudogene in humans and was omitted. Identical amino acids are in black boxes, conserved amino acids are in grey boxes.

1.6.2 Functionality of TRPCs

Within subfamilies, TRPCs either homo- or heterotetramerize to form functional ion channels with distinctive cation permeability ratios, current-voltage relationships, and modalities of activation [178, 180-182, 188]. TRPC channels are known to be activated by stimulation of receptors coupled to phospholipase C (PLC), calcium-store depletion, and various ligands [178]. TRPC3/6/7 channels are also responsive to diacylglycerol (DAG), independent of protein kinase C (PKC) activation [189, 190]. TRPC channels are typically activated by G_q protein coupled receptor activation, predominantly mediated by activation of PLC [178, 182]. For example, TRPC6 is known to exocytose to the plasma membrane upon muscarinic receptor stimulation in a PLC-dependent manner [191].

Of all the TRPC channels, TRPC1 remains the most enigmatic. Homomeric TRPC1 has been reported to function as either a store-operated, receptor-operated, or DAG-activated ion channel [178, 192]. On the other hand, TRPC1 has also been reported to be a non-functional channel subunit [184]. Most reports on TRPC1 have focused on its heterotetramirization with TRPC4, TRPC5, and occasionally TRPC3 [184, 193, 194]. These heteromeric TRPC channels have properties distinct from channels formed by TRPC3/4/5 subunits alone [193, 194]. TRPC1, when comprising a channel with either TRPC4 or 5, has been shown to be activated via G_q coupled receptors [181, 187, 194, 195]. The heteromeric TRPC1/4 and TRPC1/5 channels exhibit a simpler current-voltage relationship with outward rectification, which is markedly different than

the doubly rectifying current-voltage relationship observed when either TRPC4 or TRPC5 is expressed alone [193, 194].

1.6.3 Expression and Neurophysiology of TRPCs

All members of the TRPC family are highly expressed in mammalian brain, with TRPC4 and 5 being particularly abundant [194, 196]. TRPC1 is ubiquitously expressed, with protein levels in the brain being particularly robust. Specifically, TRPC1/4/5 homomers and heteromers are found in the cerebral cortex, hippocampus, cerebellum, and amygdala [193, 194, 197, 198]. TRPCs regulate calcium influx in both excitable and non-excitable cells [192].

Numerous TRPC proteins are implicated in various roles in neuronal processes. In *Xenopus* spinal neurons, TRPC1 plays a role in chemotaxis of growth cones toward netrin-1, brain derived neurotrophic factor, and myelin-associated glycoprotein [199]. TRPC1 functions as a mediator of Ca^{2+} influx in these cells, playing an important role in sensing extracellular neuronal-pathfinding cues and regulating guidance of axons during development [199]. TRPC5 is shown to form homomeric channels that are implicated in hippocampal growth cone morphology [200]. Homomeric TRPC5 channels, but not heteromeric TRPC1/5 channels, are expressed in cultured hippocampal growth cones [200]. These monomeric channels were found to control neurite length and growth *in vitro* via spatiotemporal regulation of Ca^{2+} influx in the cell [200]. TRPC4 has been shown to aid in depolarizing cells sufficiently to allow for exocytosis and release of neurotransmitter into the extracellular milieu in both mouse adrenal chromaffin cell and

PC12 cell lines [201]. In these cell lines, TRPC4 behaves as a GPCR-activated channel, and provides sufficient Ca^{2+} influx to elicit a secretory response [201].

TRPC1 is an integral component of the metabotropic GLUR1 signalplex in mammals [202, 203]. TRPC1 physically associates with mGLUR1, most likely mediated through the adaptor protein Homer, which is known to have independent scaffolding and gating functions for both proteins [203]. Disruptions of the TRPC1-mGLUR1 interaction inhibit mixed-cation excitatory postsynaptic conductance (EPSC) in Purkinjie cells, implying that TRPC1 mediated cation influx is responsible for these currents [204].

Recently, a new role for TRPC proteins has been discovered in *C. elegans*. *C. elegans* express three TRP channels, which most closely resemble mammalian TRPCs [205]. Wild-type worms challenged with the TRPC channel blocker 2-APB responded abnormally to acute nicotine exposure and nicotine withdrawal [206]. In addition, mutant worms lacking TRP-1 and TRP-2 proteins had a defective response to nicotine [206]. This response could be rescued by ectopic expression of human TRPC3, but not TRPC1 or TRPC4 [206]. This evidence shows that TRP channels modulate nicotine-induced behaviors of the worm, most likely by regulating calcium responses in interneurons that control locomotor behavior [206].

In *Drosophila,* TRP proteins function as a vital signaling and scaffolding component of the rhodopsin signalplex in retinal cells [207-209]. In addition to their involvement in Ca^{2+} homoestasis, TRP proteins in *Drosophila* play a role in retaining INAD, PLC, and PKC to their proper locations in rhadomeres [208, 209]. TRP mutants lacking the INAD binding domain show mislocalization of these core proteins of the rhodopsin-signalplex in rhabdomeres as the flies age [208]. Surprisingly, there are

reciprocal requirements for both INAD and TRP interaction in order for both to be correctly retained in rhabdomeres [208].

The interaction between individual TRPC family members and the D2R will be explored in detail elsewhere in this thesis.

1.7 DHHC Family of Proteins

Palmitate, a C16 saturated fatty acid and the most abundant fatty acid in the brain, is a common post-translational modification of membrane associated and integral membrane proteins [210]. Palmitoylation has been implicated in various cellular processes including aspects of protein trafficking, organelle inheritance, and vesicle fusion [211]. Until recently, the enzymes responsible for the reversible, thioester linkage of palmitate to cysteine residues (S-palmitoylation) of proteins remained unknown. Although palmitoylation of proteins was identified over 30 years ago, the membrane associated palmitoyltransferases (PATs) that catalyze this post-translational modification were first discovered in yeast less than five years ago [212, 213].

Recently, Fukata et al., reported the discovery of a 23 member family of DHHC-containing proteins in mammals [214]. These proteins are hoped to be the long sought-after palmitoyltransferases. These proteins are highly diverse, but all contain at least four transmembrane regions [215] and a conserved DHHC (Asp-His-His-Cys) domain thought to be the active site of the enzyme [216]. Cellular distribution studies of ectopically expressed yeast and human DHHC genes have shown localization to the endoplasmic reticulum or Golgi, with few proteins localized at the plasma membrane [217].

Interestingly, D2Rs are palmitoylated [218, 219]. Identification of the PAT responsible for the palmitoylation of the D2R will further aid in the understanding of receptor targeting and trafficking. Furthermore, the D2L isoform contains two additional cysteine residues not present in the D2S isoform [34]. Palmitoylation of cysteine residues present in the D2L but not the D2S may contribute to the differential cellular expression patterns exhibited by these two isoforms [220]. The interaction between zDHHC4, a newly identified potential PAT, and the D2R will be explored in detail elsewhere in this thesis.

1.8 Rationale and Hypothesis

Pharmacologically, the dopaminergic system has been implicated in the disease pathogenesis of schizophrenia, but genetic association studies have yet to link any DRs robustly to the disease phenotype. Provided that DRs themselves are not genetically altered but dopaminergic signaling in general is disrupted in schizophrenia, we hypothesize that proteins involved in the regulation of the receptors contribute to aspects of the disease. Additionally, we hypothesize that these regulatory proteins will be directly associated with the D2R by membership in a common protein complex termed the signalplex. We have termed these proteins DRIPs and DRAPs, based on their direct or indirect binding, respectively, of the D2R.

Work presented in this dissertation explores the roles of two novel DRIPs uncovered by yeast two-hybrid strategy, namely TRPC1 and zDHHC4. Due to TRPC1's multiple functions as a scaffolding protein and an ion channel, we hypothesize that the

D2R/TRPC1 interaction promotes the cell surface expression of the D2R signalplex and provides a link for D2R-mediated activation of intracellular calcium levels. Given zDHHC4's potential as a palmitoyltransferase, we propose that the D2R/zDHHC4 interaction differentially modifies cell-surface trafficking of D2S and D2L by palmitoylation of cysteines present only in the IC3 of the D2L.

This thesis presents efforts to identify novel DRIPs and DRAPs in a rational manner by utilizing available resources in online databases. We hypothesize that we can identify proteins with high biological probability of interaction with the D2R by searching for proteins that bind to previously known DRIPs and DRAPs. These proteins have an enhanced likelihood of co-expression and trafficking with the D2R, and are likely to interact with the receptor itself.

Through identifying and functionally characterizing novel DRIPs and DRAPs, a more comprehensive understanding of dopamine signaling in both normal and pathological states will be attained. In disease states such as schizophrenia, DRIPs may offer effective drug targets that could reduce and/or modify the undesirable side effects of current pharmacologic therapies. In addition, DRIPs may offer biomarkers for schizophrenia that could aid in disease diagnosis and help tailor treatment to individual patients.

Chapter 2

Interaction Between D2R and TRPC1 Mediates Delivery of TRPC1 to the Cell Surface

2.1 Introduction

The DR family, consisting of the D1-like and D2-like receptors, has been shown to associate with diverse intracellular signaling pathways in neurons. D2Rs are primary regulators of the inhibitory $G_{i/o}$ pathways but have been shown to also activate calcium-associated signaling pathways, including the canonical G_q/PLC pathway, and can mediate the mobilization of intracellular calcium stores and activation of calcium-dependent phosphatases [22-24]. Interaction of D2Rs with calcium binding proteins such as NCS-1 [121] and CAPS [128] plays an important role in D2R desensitization and dopamine secretion, respectively. The identification of DRIPs therefore provides a novel platform for elucidating the diverse signaling mechanisms that contribute to dopamine-mediated neurotransmission.

In the present study, we used the second intracellular loop of the D2R (D2IC2) as bait in a yeast two-hybrid screen and identified TRPC1 (transient receptor potential channel1) as a novel D2R interactor. In mammals, seven TRPC subtypes (TRPC1-7) have been identified based on sequence similarities to *Drosophila* TRP (reviewed in [178] and have been shown to function in a variety of physiological processes including Ca^{2+} and Na^{+} entry, receptor/phospholipase C (PLC) signaling, lipid raft integrity, cell volume regulation, and cell proliferation (reviewed in [183]). TRPCs form tetrameric

non-selective, non-voltage gated ion channels in excitable and non-excitable cells. Among mammalian TRPCs, TRPC1/4/5 and TRPC3/6/7 comprise two interaction subgroups whose members can hetero- or homotetramize to form functional ion channels with distinct properties [178]. In humans, TRPC2 is a pseudogene. Our data indicate that TRPC1 interacts with the D2R in transfected cells as well as native brain tissue, and that this interaction enhances the delivery of TRPC1 to the cell surface. Ultrastructural analysis of native TRPC1 and D2R proteins using electron microscopy shows that these proteins colocalize in postsynaptic compartments of cortical neurons in the primate cortex. These findings reveal a novel link between D2Rs and TRPC channels in neurons, and suggest a role for TRPC channels in neuropsychiatric disease.

2.2 Experimental Procedures

2.2.1 DNA Constructs and Protein Interaction Assays

All constructs were generated by subcloning PCR amplified or restriction enzyme fragments into appropriate expression vectors, and each construct was verified by automated DNA sequencing. For the yeast two-hybrid screen, the second intracellular loop of the human D2R (D2IC2, amino acids 131-151) was subcloned into the yeast GAL4 DNA-binding domain vector pAS2-1 (BD Biosciences Clonetech) and used as bait to screen a human fetal brain cDNA library expressed in the GAL4 activation domain vector pACT2 (BD Biosciences Clonetech). Bait and prey constructs were simultaneously cotransformed into the yeast strain MaV103, and 1 X 10^6 independent clones were screened by selective growth on Leu-/Trp-/His-/Ade- plates as

described previously [128]. Protein interaction was assayed for by β-galactosidase activity via the nitrocellulose filter lift method [113].

Sites within human DRs and TRPCs that contribute to the TRPC/DR interaction were mapped using a directed two-hybrid assay. The IC2 domain of the D2R (in pAS2-1) was assayed for interaction against truncation fragments of TRPC1, TRPC4, or TRPC5 (in pACT2). IC2 constructs of D1R (amino acids 127-149), D3R (amino acids 127-149). D4R (amino acids132-151) and D5R (amino acids 137-158) were subcloned into the pAS2.1 vector and used in a directed yeast two-hybrid screen with the C-termini of TRPC1, TRPC4, and TRPC5. TRPC cDNAs were generously provided by Drs. Michael Schaefer (University of Berlin), Craig Montell (Johns Hopkins University), and David Beech (University of Leeds). Bait and prey plasmids were sequentially transformed into the yeast strain MaV103 and interactions were assayed as described above.

2.2.2 Cell Culture and Colocalization

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. HEK293 cells stably expressing FLAG-tagged D2L (long-splice isoform) dopamine receptors (HEK293-D2L) were provided by Dr. Mark von Zastrow (University of California San Francisco). HEK293-D2L cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 300 μg/ml Geneticin (Invitrogen, Grand Island, NY). Cells were transiently transfected with either enhanced green fluorescent protein (EGFP)-tagged full length human TRPC1, TRPC4, or mouse TRPC5 constructs 24 hours prior to fixation. Cells were transfected

using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) under conditions described by the manufacturer. FLAG-tagged D2L was detected by immunostaining cells with an anti-FLAG M2 monoclonal antibody (1:1000 dilution; Sigma-Aldrich, St. Louis, MO). Immunostaining was visualized with a Texas red conjugated goat anti-mouse secondary antibody (1:200 dilution; Jackson ImmunoResearch), while GFP epifluorescence was visualized directly. For D2IC2-GFP coimmunoprecipitation experiments, the second intracellular loop of the D2R was fused to the C-terminus of GFP in a pEGFP-C1 vector (Clontech) and transfected into HEK293 and HEK293-D2L cells using Effectine (Qiagen, Valencia, CA) according to manufacturer's protocol.

2.2.3 Immunogold Electronmicroscopy

In collaboration with Dr. Constantinos Paspalas, University of Crete, two adult rhesus monkeys (*Macaca mulatta*) were perfused, and brain tissue was prepared as described previously [221]. Affinity-purified polyclonal rabbit antibodies directed against human D2R (amino acids 284-311) and human TRPC1 (amino acids 557-571) were purchased from Chemicon (Temecula, CA). Sections of the dorsolateral prefrontal cortex (Walker's area 46) were processed for electron microscopy as previously described [222].

For single gold-based immunocytochemistry, sections were incubated with either anti-D2R (diluted 1:500) or anti-TRPC1 (diluted 1:200) antibodies, and transferred to nanogold–anti-rabbit Fab′ (Nanoprobes, Yaphank, NY) or biotinylated anti-rabbit $F(ab')_2$ (Jackson ImmunoResearch) and nanogold–anti-biotin Fab (Nanoprobes), as previously

described [221, 222]. Nanogold was enhanced with silver autometallographic developer (HQ Silver; Nanoprobes).

For dual gold-based immunocytochemistry, sections were incubated with anti-D2R (1:300 dilution) antibody, followed by nanogold–anti-rabbit Fab′ and gold enhancement (GoldEnhance; Nanoprobes). An excess of unconjugated goat anti-rabbit Fab (1:10-50; Jackson ImmunoResearch) and mild glutaraldehyde fixation was used to neutralize any remaining rabbit proteins. Sections were incubated with anti-TRPC1 (1:100 dilution) antibody and a second series of nanogold conjugates, followed by gold autometallography to enhance the TRPC-1-bound nanogold as previously described [223]. TRPC1 was labeled in the second series using biotinylated anti-rabbit $F(ab')_2$ followed by unconjugated anti-rabbit Fab and fixation. TRPC1 was visualized with peroxidase and diaminobenzidine chromogen, while D2Rs were visualized with nanogold as detailed above for single immunocytochemistry [222], and sampled for thin-sectioning and analysis under a JEM 1010 (Jeol, Tokyo, Japan) transmission electron microscope operated at 80kV.

2.2.4 Glutathione-S-Transferase Pulldown Assays

Glutathione S-transferase - D2IC2 fusion protein (D2IC2-GST) and D2LIC3-GST fusion protein (amino acids 211-373) were constructed in the bacterial expression vector pGEX-4T-1 vector (Amersham Pharmacia, Piscataway, NJ), while carboxyl terminal truncations of TRPC1 (amino acids 638-759), TRPC4 (amino acids 621-893), and TRPC5 (amino acids 619-973) were constructed in the pET30C vector (Amersham Pharmacia) to generate S-tagged protein

fragments. All fusion proteins were induced in *E. coli* strain BL-21 (DE3). D2IC2-GST and D2LIC3-GST fusion proteins were purified using glutathione-sepharose (Amersham) according to the manufacturer's instructions. GST pull-down assays were performed as described previously [113]. Eluted proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) filter and probed with an anti-S-tag polyclonal antibody conjugated to horseradish peroxidase (1:5000 dilution; Novagen, San Diego, CA). Immunoreactivity was detected by enhanced chemiluminescence with an ECL Plus kit (Amersham).

2.2.5 Coimmunoprecipitation

For coimmunoprecipitation experiments, striatal and cortical tissue were isolated from 10-day-old Sprague-Dawley rats. Crude membranes were prepared and membrane proteins solubilized as previously described [112]. Immunoprecipitation of D2R complexes was performed using a goat polyclonal anti-D2R antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were separated by SDS-PAGE, transferred to a PVDF filter, and the filter sequentially probed with a monoclonal anti-TRPC1 antibody (1:1000 dilution; generous gift from Dr. Leonidas Tsiokas, University of Oklahoma Health Sciences Center), a rabbit polyclonal anti-TRPC4 antibody (1:700 dilution; Chemicon, Temecula, CA), or a chicken polyclonal anti-NCS-1 antibody (1:700 dilution; Rockland Immunochemicals, Gilbertsville, PA). Peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Immunoreactivity was detected using an ECL Plus kit (Amersham).

Specificity of the monoclonal anti-TRPC1 antibody has been demonstrated previously [224]. In Western blot analysis of HEK293 cell membrane lysate and rat brain lysate, three commercially available polyclonal anti-TRPC1 antibodies (Alomone, Sigma, and Chemicon) identified a protein of the same molecular weight as the protein detected by the monoclonal anti-TRPC1 antibody. Using the same technique, the polyclonal anti-TRPC4 antibody (Chemicon) detected a protein at the predicted molecular weight of TRPC4, whereas the polyclonal anti-TRPC5 antibody (Chemicon) cross-reacted with an unknown protein of a smaller molecular weight than predicted for TRPC5 (data not shown).

2.2.6 Reverse Transcriptase PCR

HEK293T cells were grown in a 10 mm cell culture dish until confluent. RNA was extracted using standard phenol:chloroform protocols. Briefly, cells were lysed in 1 mL of TRIZOL reagent (Invitrogen, Carlsbad, CA) and transferred to a microcentrifuge tube. 200 μL of chloroform was added per 1 mL of TRIZOL reagent. Cells were shaken vigoruously and centrifuged at 12000 rpm at for 15 minutes to separate aqueous and organic phases. The aqueous (upper phase) was transferred to a new tube, and RNA precipated by addition of isopropanol. RNA was pelleted by centrifugation at 12000 rpm for 10 minutes, and the pellet washed with 75% ethanol and resuspended in RNAse-free water. 5 μg of RNA was used to create single-stranded cDNA from random hexamer primers using a Superscript II first strand synthesis for RT-PCR kit (Invitrogen). PCR for endogenous TRPC1 was performed with REDTaq DNA polymerase (Sigma; St. Louis, MO) using primers that amplified the the final 365 nucleotides of TRPC1 cDNA;

mah07 (5'-GGGGAATTCGAGATGACAAATGTACGTTACCTCC-3') and mah04 (5'-GGGGAATTCGAGAAAACTATCAAAAAGTGATG-3'). PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

2.2.7 Biotinylation Assays

Cell surface labeling assays were performed using a modification of the cleavable biotin method described previously by Vickery and von Zastrow [225]. Briefly, cells were labeled with 1 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford IL) for 30 min at 4^{o}C. Cells were washed three times with ice cold phosphate buffered saline (PBS) to remove unbound biotin, and crude membrane fractions prepared as described previously [32]. Biotin-labeled TRPC1 proteins were immunoprecipitated using polyclonal anti-TRPC1 antibodies (Alomone Labs, Jerusalem, Israel), while D2R proteins were immunoprecipitated with polyclonal anti- D2R antibodies (Santa Cruz). Complexes were washed repeatedly with ice cold PBS, resolved by SDS-PAGE, and transferred to a PVDF membrane. The biotinylated proteins were complexed with avidin conjugated horseradish peroxidase (Vectastain Elite ABC detection system; Vector Laboratories), then detected by enhanced chemiluminescence with an ECL Plus kit. Immunoblots were quantitated using a laser densitometer (Molecular Dynamics, Sunnyvale, CA) and analyzed using the Quantity One software package (PDI, Inc., Huntington Station, NY)

2.3 Results

2.3.1 Interactions of TRPCs with DRs

A yeast two-hybrid screen was carried out using the 22 amino acid-long second intracellular domain of the D2R (D2IC2) as bait to screen a human brain cDNA library. The D2IC2 domain has previously been shown to interact with CAPS1 (calcium activated protein for secretion), a presynaptic calcium binding protein that links D2Rs with components of the exocytotic machinery [128]. In control experiments, neither the D2IC2 construct nor any of the other bait constructs used in these studies was found to autoactivate β-galactosidase expression (data not shown). Using the D2IC2 construct as bait, a positive interacting cDNA clone was identified (Figure **2.1**A) that contained an ~200 base pair cDNA insert. Analysis of the predicted amino acid sequence revealed 100% identity with the carboxyl-terminal 65 amino acids of TRPC1 (transient receptor potential channel1). The interaction between TRPC1 and the D2IC2 appeared to be specific, as the TRPC1 cDNA failed to interact with the D2LIC3 domain, and the D2IC2 domain failed to interact with protein 4.1N (Figure **2.1**A), which has previously been shown to interact with a region within the D2LIC3 domain [109]. TRPC1 is one of seven (TRPC1-TRPC7) mammalian TRPC channels involved in agonist-stimulated Ca^{2+} influx (for reviews see [181, 183, 226]) and store-operated Ca^{2+} release (reviewed in [183]).

The TRPC1/D2R interaction was initially validated using pulldown techniques. A lysate prepared from bacteria expressing an S-tagged TRPC1 carboxyl-terminal cDNA fragment (amino acids 638-759) was tested for the ability to associate with a GST fusion

protein containing the D2IC2 domain (D2IC2-GST). As shown in Figure **2.1**B (top panel) a Western blot containing lysate from bacteria expressing the S-tagged TRPC1 construct produced an immunoreactive band of ~18 kD when probed with an anti-S-tag antibody (lane 1). This band corresponds to the expected size of the *C*-terminal TRPC1 fragment encoded by the cDNA construct. The same band was detected by pulldown after the bacterial lysate was incubated with the D2IC2-GST fusion protein (lane 2), but not when the lysate was absorbed onto beads alone (lane 3). These results support the validity of the TRPC1/D2R interaction.

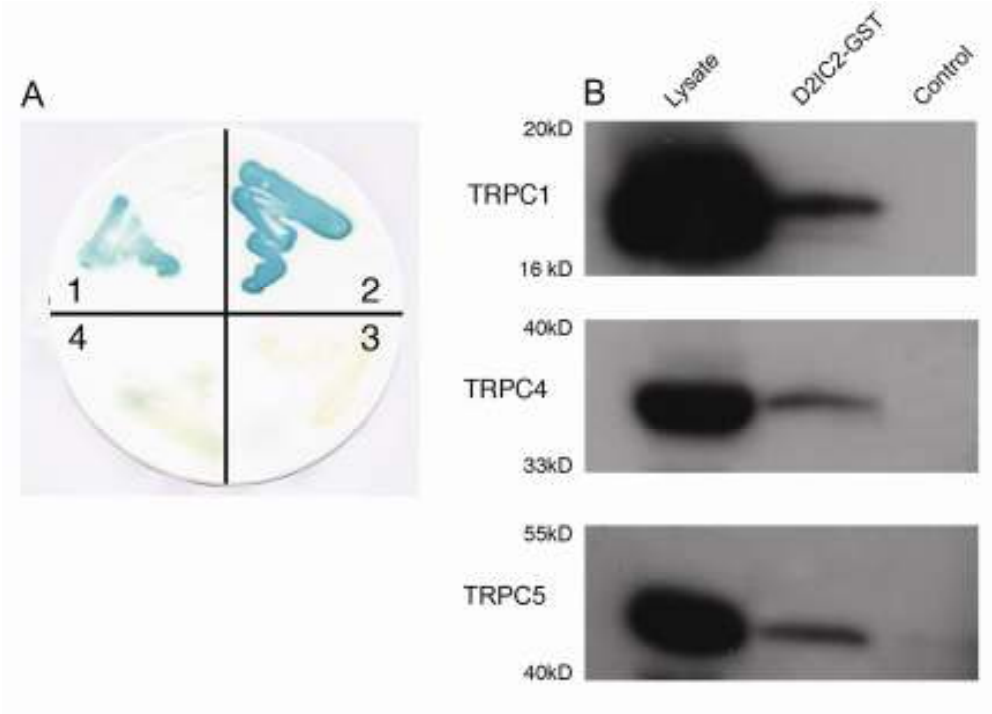

Figure **2.1**: **Interaction Between the D2R and TRPC proteins**.

(**A**) Results from a yeast two-hybrid screen showing interaction between TRPC1 and the D2IC2 domain (1) but not with an unrelated bait (D2LIC3; 3) or protein 4.1N (4). Interaction between CAPS and the D2IC2 domain (2) was included as a positive control. **(B)** The D2R associates with TRPC1, TRPC4, and TRPC5 proteins. D2IC2-GST fusion protein pulled down S-tagged TRPC1 (amino acids 638-759; top panel), TRPC4 (amino acids 621-893; middle panel), or TRPC5 (amino acids 619-973; bottom panel) from bacterial lysates. No immunoreactive bands were detected when lysates were absorbed onto beads alone (control lane).

TRPC1 is most closely related in amino acid sequence to TRPC4 and TRPC5 and can form functional heteromeric channels with either of these TRPC subtypes [178]. Based on these considerations, we used the GST-pulldown assay to investigate whether the D2R might also interact with TRPC4 and/or TRPC5. As shown in Figure **2.1**B, Western blots containing lysates prepared from bacteria expressing S-tagged TRPC4 (amino acids 621-893) or TRPC5 (amino acids 619-973) *C*-terminal fragments were probed with anti-S-tag antibodies. Immunoreactive bands of the expected sizes were visualized in the bacterial lysates (lane1 of middle and lower panels) and after pulldown with the D2IC2-GST fusion protein (lane 2 of middle and lower panels). No immunoreactive bands were visible when the lysates were incubated either with beads alone (lane 3 of each panel) or with a D2LIC3-GST fusion protein (data not shown). These results indicate that the IC2 of the D2R interacts directly with TRPC4, TRPC5, as well as TRPC1.

2.3.2 Mapping Binding Site of D2R on TRPCs

Deletion mapping was performed to localize sites within TRPC1 that contribute to D2R interaction. A series of truncation fragments of the *C*-terminal domain of TRPC1 (Clone 44, amino acids 638-759) were tested for interaction with the D2IC2 domain in a directed yeast two-hybrid screen. As shown in Figure **2.2**B, TRPC1 truncation constructs A (amino acids 638-693) and B (amino acids 638-726) failed to interact with D2IC2 in the two-hybrid system, indicating that the D2R binding site resides within the C-terminal

portion of Clone 44. In contrast, TRPC truncation constructs D (amino acids 638-759) and C (amino acids 638-745) tested positive with D2IC2 in the β-galactosidase assay. These mapping studies suggest that the region in TRPC1 spanned by amino acids 726-745 is required for D2R binding. The ability of construct E (amino acids 726-745) to directly interact with the D2R (Figure **2.2**B, Figure **2.3**) provides very strong evidence that this region spans a portion of the D2R binding site.

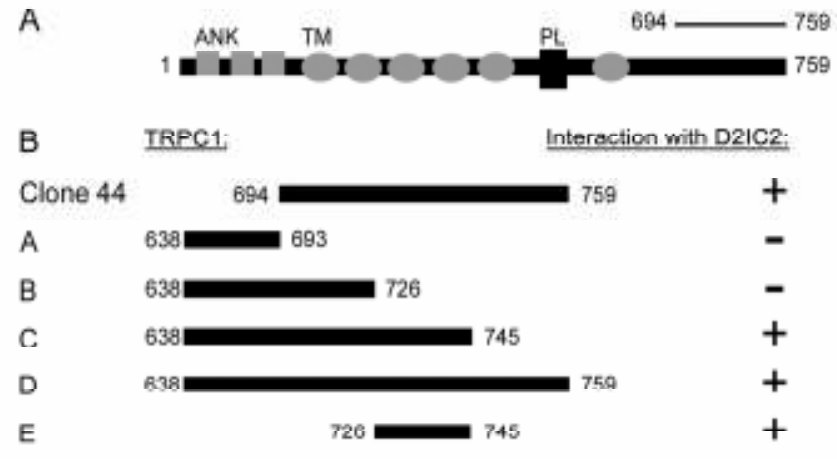

Figure **2.2**: **Localization of the D2R Binding Site on TRPC1**.
Schematic representation of constructs encoding truncations of TRPC1. (**A**) Full-length TRPC1 protein. Shaded boxes depict ankyrin repeats (ANK), shaded ovals represent transmembrane (TM) segments, and black box depicts the pore loop domain (PL). Amino acids 694-759 encompass the *C*-terminal fragment (Clone 44) identified in the original yeast two-hybrid screen. (**B**) TRPC1 *C*-terminal truncation fragments A-E were tested for interaction with the D2IC2 domain in the two-hybrid assay. Interaction is indicated by the presence (+) or absence (-) of β-galactosidase activity.

Amino acid sequence alignments were used to predict potential D2R binding sites on TRPC4 and TRPC5 (Figure **2.3**A). Within the *C*-terminal domain of TRPC1, the region encompassing the D2R binding domain (amino acids 726-745) showed 70% amino acid similarity to the comparable region in TRPC4 (amino acids 735-754) and

75% amino acid similarity to the comparable region in TRPC5 (amino acids 742-761). We used a directed two-hybrid approach and tested for interaction between the D2IC2 and truncation fragments of TRPC4 and TRPC5 analogous to the D2R binding domain present in TRPC1. As shown in Figure **2.3**B, the TRPC4 and TRPC5 truncation fragments each displayed a positive interaction with the D2IC2 domain, and produced detectable, albeit lower levels of β-galactosidase activity compared to the TRPC1/D2IC2 interaction. Taken together, these results support the view that TRPC1, TRPC4, and TRPC5 are all capable of interaction with the D2R, and that this interaction is mediated via a conserved binding domain located in the *C*-terminal tail of each of the three TRPC family members.

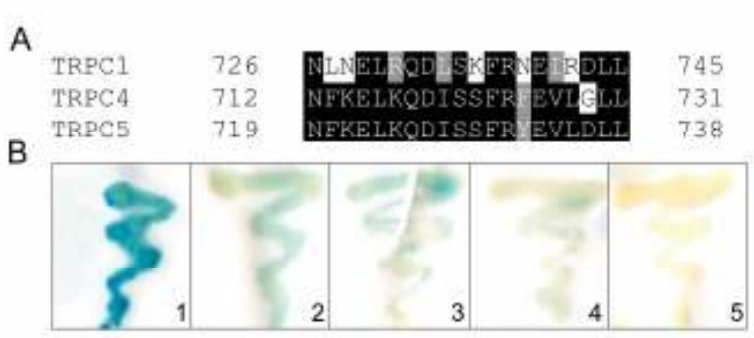

Figure **2.3**: **D2R interacts with TRPC1, TRPC4, and TRPC5 Truncation Fragments**. (A) Amino acid sequence alignment of TRPC1, TRPC4, and TRPC5 in the *C*-terminal region found to contain the D2IC2 binding site. Amino acids are numbered to the left and right of each line. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in grey. (B) Representative β-galactosidase assays comparing the interaction of D2IC2 with Clone 44 (amino acids 694-759 of TRPC1; 1), TRPC1 (amino acids 726-745; 2), TRPC4 (amino acids 712-731; 3), and TRPC5 (amino acids 719-738; 4). Interaction between TRPC1 (amino acids 726-745) and the D2LIC3 is shown as a negative control (5).

Figure **2.4**: **Second Intracellular Loops of DRs.**
Amino acid sequence alignments of the five subtypes of dopamine receptors. Identical amino acids are in black boxes. Conserved amino acids are in grey boxes

The specificity of interaction between TRPC1, 4, and 5 and the five subtypes of dopamine receptors (DR) was examined. DR subtypes exhibit a high amount of sequence conservation in their second intracellular loops, especially among amino acids comprising their N-terminal sequences (Figure **2.4)**. The second intracellular loops of D1, D3, D4, and D5 were constructed in the pAS2.1 bait vector and tested for interaction with truncation fragments of TRPC1, TRPC4, and TRPC5, which spanned from the end of transmembrane region six to the C-terminus of each protein. The results, depicted in Table **2.1**, show that TRPC1 binds all DR subtypes. In contrast, TRPC4 and TRPC5 displayed a more restricted, subtype-specific binding. TRPC4 interacted only with D1IC2 and D2IC2, whereas TRPC5 interacts with D1IC2, D2IC2, and D3IC2.

Table **2.1**: **TRPCs Interact with Multiple Subtypes of DRs**.

The C-terminal tails of TRPC1, TRPC4, and TRPC5 were tested for interaction with the second intracellular loops (IC2) of D1R, D2R, D3R, D4R, and D5R. + indicates interaction, - indicates no interaction observed.

DRs	**TRPC1**	**TRPC4**	**TRPC5**
D1IC2	+	+	+
D2IC2	+	+	+
D3IC2	+	-	+
D4IC2	+	-	-
D5IC2	+	-	-

2.3.3 Expression profile of TRPC1 mRNA and Protein

HEK293T cDNA was isolated, and RT-PCR was performed using primers to detect endogenous TRPC1. As shown in figure **2.5**, TRPC1 mRNA is present in HEK293T cells.

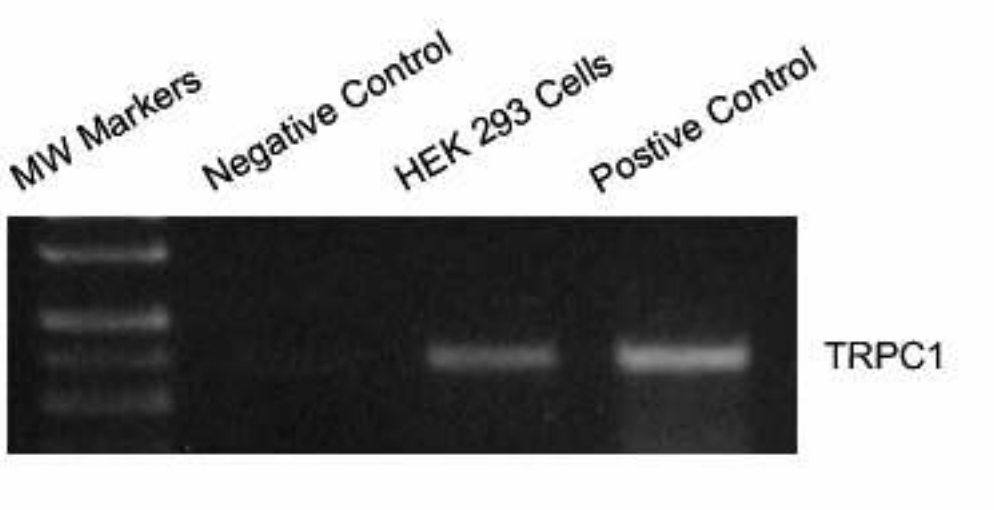

Figure **2.5**: **HEK293T Cells Endogenously Express TRPC1 mRNA**

Total cDNA was prepared from HEK293T cells. To detect TRPC1, HEK293T cDNA was amplified using primers mah07 and mah04. A plasmid containing TRPC1 cDNA was used as a positive control.

To confirm this finding, we tested numerous anit-TRPC1 antibodies to determine if we could detect native TRPC1 protein in cell lysates and rat cortex and striatum membrane preparations. As shown in Figure **2.6,** all antibodies tested identified a protein that ran at the expected molecular weight of TRPC1 (between 75 kD and 100 kD). This protein band was identified in HEK293T cells, HEK293-D2L cells, and rat cortex and striatum, indicating that TRPC1 protein is endogenously expressed in these cell lines and native tissues.

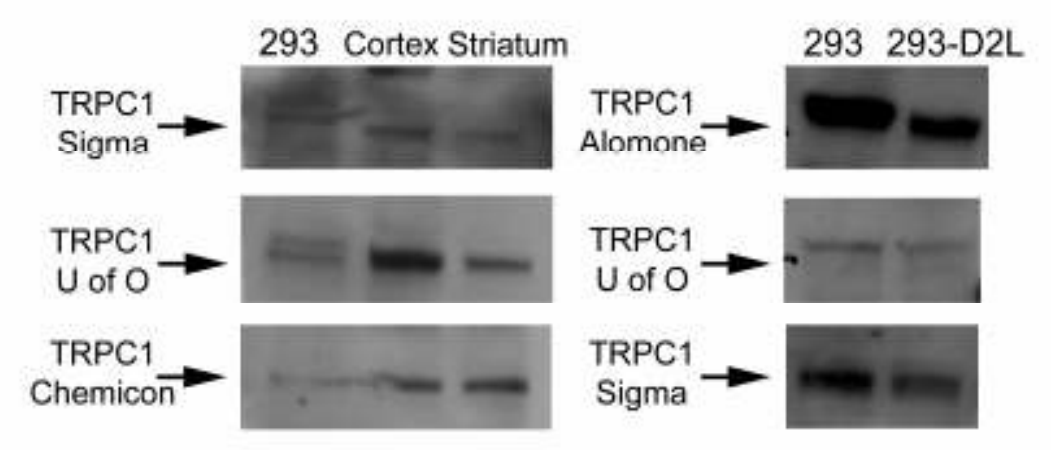

Figure **2.6**: **HEK293 Cells and Rat Brain Endogenously Express TRPC1 Protein**
Membrane lysates were prepared from HEK293T, HEK293-D2L, and rat cortex and striatum. Proteins were separated by SDS-PAGE, and transferred to a PVDF membrane. Blots were then probed with available TRPC1 antibodies to test for present of TRPC1 in lysates as well as antibody specificity. All antibodies tested (Alomone, Chemicon, Sigma, and U of O) detected a band that ran between MW markers 75 kD and 100 kD in all lysates tested.

2.3.4 TRPC1 and TRPC4 Colocalize with D2R in HEK293 Cells

The intracellular distribution of transiently transfected EGFP-TRPC1 or EGFP-TRPC4 and stably expressed FLAG-tagged D2L was examined in HEK293-D2L cells by confocal laser microscopy. Images of representative cells are shown in Figure **2.7**. FLAG-D2L exhibited colocalization with either EGFP-TRPC1 (panels A-C) or EGFP-TRPC4 (panels D-F) when coexpressed in HEK cells. Expression of EGFP-TRPC1 and EGFP-TRPC4 was predominantly punctate and cytosolic in cultured HEK cells in the absence of D2R expression (data not shown). In HEK293-D2L cells however, EGFP-TRPC1 and EGFP-TRPC4 appeared to cluster at polarized regions of the cell also exhibiting intense D2R expression (panels A-F). TRPC1 was also found to colocalize with the D2R at the plasma membrane in these cells (panel C). Although similar results were obtained for EGFP-TRPC5 (data not shown), EGFP-TRPC5 expression was generally weak in HEK293-D2L cells.

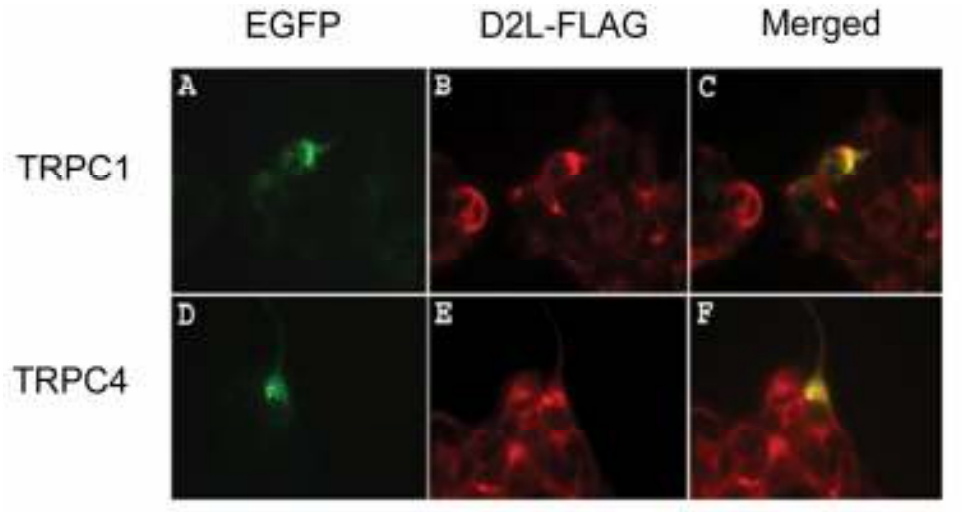

Figure **2.7**: **D2R and TRPC1 Colocalize in HEK293 Cells**.
HEK293-D2L cells were transiently transfected with either EGFP-TRPC1 (A-C) or EGFP-TRPC4 (D-F). Cellular localization of D2L-FLAG (A,D), EGFP-TRPC1 (B), and EGFP-TRPC4 (E) was determined by confocal laser microscopy. Merged images are shown in C and F

2.3.5 TRPC1 is Present in a Complex with D2R in Native Tissue

We examined the interaction between the D2R and TRPCs in rat brain by coimmunoprecipitation experiments. Crude membrane fractions from 10-day-old striatal and cortical rat tissue were immunoprecipitated using an anti-D2R antibody and immunocomplexes sequentially probed with anti-TRPC1 antibodies. As shown in Figure **2.8**, two independent TRPC1 antibodies (top and middle panels, University of Oklahoma; bottom panel, Sigma) were able to detect TRPC1 in an immunocomplex with D2R. In each instance, the D2R was immunoprecipitated with one of three independent commercially available anti-D2R antibodies (Abcam, Santa Cruz, or Calbiochem). The

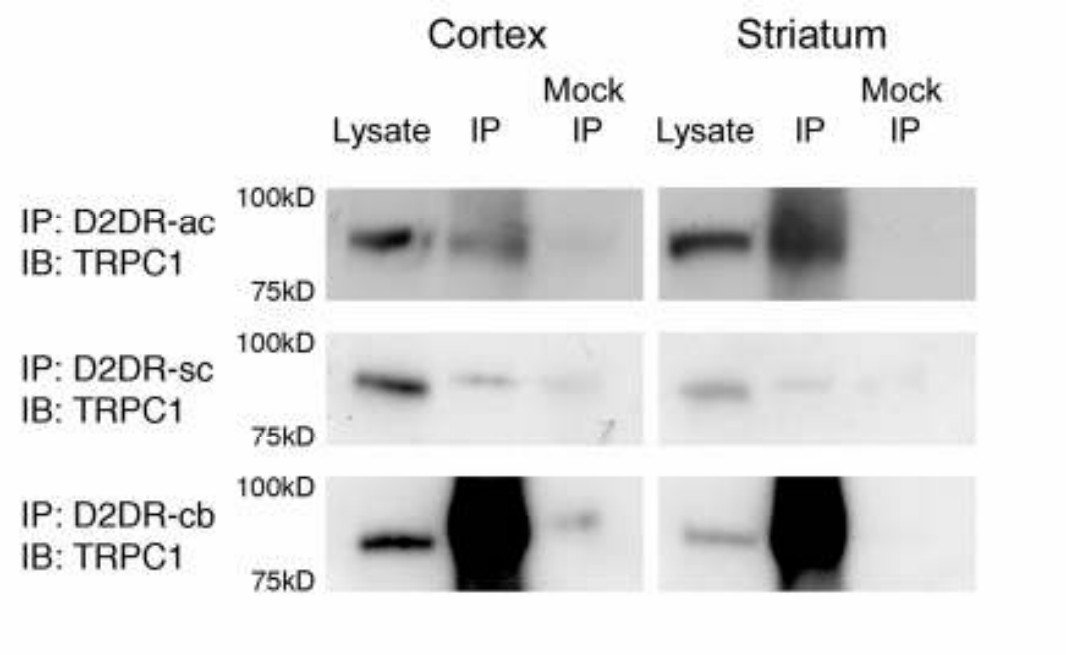

Figure **2.8**: **TRPC1 Interacts with D2R in Rat Cortex and Striatal Tissue**

Anti-D2R antibodies were used to immunopreciptate D2Rs from cortex and striatum of rat (ac-Abcam; sc-Santa Cruz; cb-Calbiochem). Blots containing immunocomplexes (IP) were sequentially probed with anti-TRPC1 antibodies (top panels – University of Oklahoma, bottom panel – Sigma). The position of TRPC1 endogenously expressed in cortical and striatal tissue is shown (lysate lane). No signal was seen in lysate adsorbed onto beads alone (Mock IP)

blots were stripped and re-probed to detect other DRIPs. Immunoreactive bands corresponding in size to D2R (Figure **2.9,** top panel), TRPC1 and TRPC4 were detected in these complexes (Figure **2.9,** middle panels). The D2R runs as a dimer on Western blots from native tissue, with bands at 50kD and 75kD representing the receptor monomer and higher order aggregates, respectively. As shown in Figure **2.9** (bottom panel), D2R-containing immunocomplexes obtained from cortex and striatum also contained the D2R interacting protein NCS-1 [121]. Although our two-hybrid and pulldown results indicate that TRPC5 and D2Rs also interact, we were unable to convincingly detect TRPC5 in the immunoprecipitate.

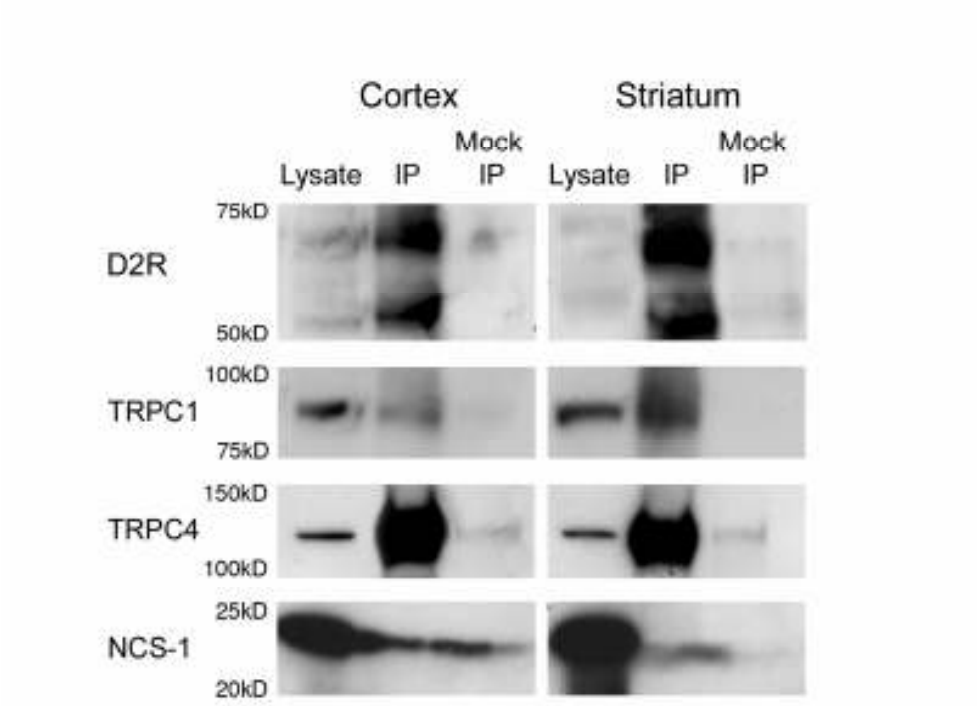

Figure **2.9**: **TRPCs Interact with D2R and Other Components of the D2R Signalplex**. An anti-D2R antibody was used to immunopreciptate D2Rs from cortex and striatum of rat. Blots containing immunocomplexes were sequentially probed with a separate anti-D2R (upper panel) anti-TRPC1, anti-TRPC4 (middle panels), and anti-NCS-1 (lower panels) antibodies. The positions of D2R, TRPC1, TRPC4, and NCS-1 endogenously expressed in cortical and striatal tissue is shown (lysate lanes). No signal was seen in lysate adsorbed onto beads alone (Mock IP)

Due to the controversy surrounding whether TRPC1 can form functional homomeric ion channels, we wanted to confirm whether another TRPC subtype could coimmunopreciptiate with TRPC1 in native tissue. We immunoprecipitated TRPC1 from rat cortical lysate using an anti-TRPC1 antibody, and separated immunocomplexes as previously described. Blots containing TRPC1 immunocomplexes were probed for TRPC4, and an immunoreactive band at the appropriate predicted size for TRPC4 was identified in the immunoprecipitate lane, but not in the negative control (Figure **2.10).** This demonstrates that TRPC4 and TRPC1 are present in the same signalplex in rat cortical tissue, suggesting the possibility that these two TRPC proteins could interact to form functional channels. TRPC1/TRPC4 heterotetramers have been previously identified to form ion channels with unique properties [193]. Thus, these results suggest that D2Rs, TRPC1, TRPC4, and NCS-1 form a signaling complex within cortical and striatal tissue.

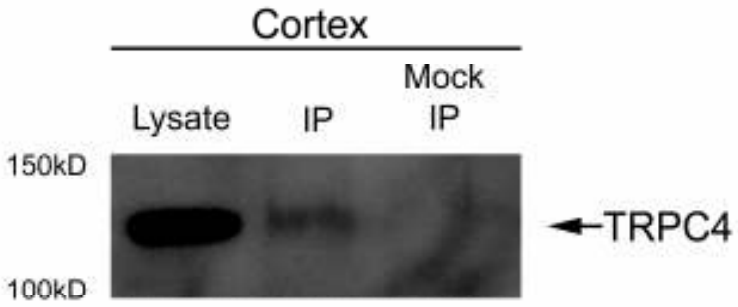

Figure **2.10 TRPC4 coimmunoprecipitates with TRPC1 in Rat Cortex**

An anti-TRPC1 antibody was used to immunoprecipitate TRPC1 from rat cortex. Blots containing immunocomplexes were probed with an anti-TPC4 antibody. Rat cortex lysate (lysate) was used as a positive control to identify TRPC4 at the expected molecular weight. TRPC4 was immunoprecipitated with TRPC1 (IP), but not with beads alone (Mock IP)

2.3.6 TRPC1 Colocalizes with D2R in Individual Neurons in Monkey Cortex

Immunohistochemical analysis of monkey prefrontal cortex (PFC) was used to gain insight into the subcellular distribution of TRPC1 and D2Rs. As shown in Figure **2.11**A, TRPC1 immunoreactivity was detected in both the cell body and the dendritic shafts of PFC pyramidal neurons, where immunoparticles were found in association with the *trans*-Golgi network and with the endoplasmic reticulum. A fraction of TRPC1 immunoreactivity was also localized at the plasma membrane, and often occurred at sites opposite smooth reticular endomembranes. Similarly, D2R immunolabeling was also found in the cell body (where it often decorated cisternae of the rough endoplasmic reticulum) and extrasynaptically in higher order dendrites (Figure **2.11**B). Consistent with earlier studies [221], D2Rs were found associated with the plasma membrane as well as intracellular compartments (Figure **2.11**B). In general, D2R expression within the PFC was considerably less than TRPC1 expression.

Double immunoelectron microscopic analysis of monkey PFC was used to examine the subcellular colocalization of TRPC1 and D2Rs. Peroxidase labeling of TRPC1 produced a diffuse precipitate that contrasted with the D2R immunogold particles (Figure **2.12**, A-C). TRPC1 labeling was found to colocalize with D2R expression within dendritic branches expressing both cytoplasmic and membrane bound D2Rs (Figure **2.12**B, C). In addition, TRPC1 labeling colocalized with D2R immunolabeling at the plasma membrane (Figure **2.12**A). Reversal of the immunocytochemical sequence (i.e., peroxidase-labeled D2R, gold-labeled TRPC1) corroborated these findings and

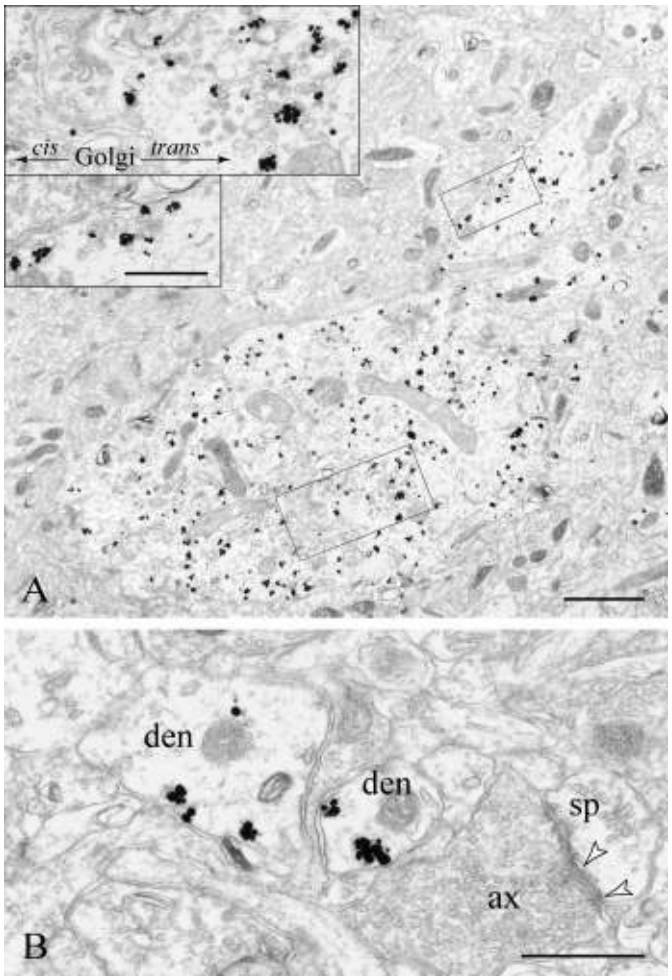

Figure **2.11**: **Expression profile of TRPC1 and D2Rs in Neurons of the PFC**.

(**A**) Immunolabeling of TRPC1 subunits in the perikarya and dendrites of cortical neurons. TRPC1 immunolabeling was found in association with the (granular and agranular) endoplasmic reticulum and along the *trans*-Golgi axis (large inset). A limited plasmalemmal expression was also found at or near sites of juxtaposition of smooth endomembranes (small insets). (**B**) D2R expression was predominantly localized to (extrasynaptic) membrane-bound compartments of high-order dendritic branches (den), and virtually absent in nearby axo-spinous (ax-sp) synapses. Scale bars: (**A**) 1 μm; (**A**-insets) 200 nm; (**B**) 400 nm.

demonstrated that the two immunoprobes clearly overlap on endoplasmic membranes (Figure **2.12**D).

We next exploited the resolution power of postfixed nanogold to label TRPC1 and D2Rs on single sections of monkey PFC (Figure **2.12**E, F). TRPC1 and D2Rs exhibited comparable distribution patterns within individual subcellular compartments and organelles (Figure **2.12**E, F). Spatial co-expression of TRPC1 and D2R immunosignals (i.e. those cases where the two immunoprobes could be found separated by less than 20 nm as determined by the maximum linear dimension of both immunocomplexes) was often observed at extrasynaptic sites of the plasma membrane (Figure **2.12**F). The close spatial proximity of TRPC1 and D2R immunoreactivity demonstrates that the two proteins colocalize within neurons, and provides compelling evidence for a physical interaction between these binding partners in the primate cortex.

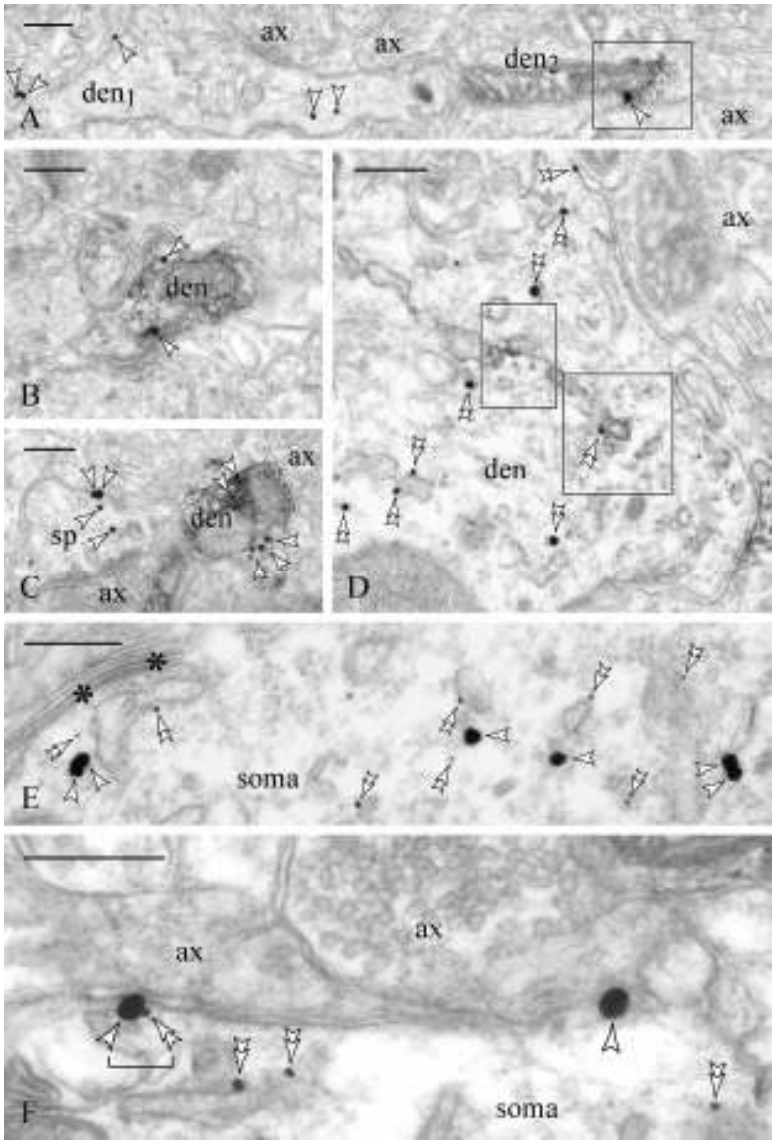

Figure **2.12**: **Colocalization of D2R and TRPC1 in the Cortex**.

(**A-C**) TRPC1 labeled with immunoperoxidase is shown in dendritic branches expressing both cytoplasmic and plasmalemmal D2R-immunoparticles (arrowheads). Note that TRPC1 labeling in **A** is restricted to a portion of the dendrite and overlaps with the expression of D2Rs at the plasma membrane (frame). Other profiles reactive for D2R alone are seen in **A** and **C**. To test for reagent and method selectivity, we reversed the immunocytochemical sequence. In **D**, D2R labeling with immunoperoxidase overlaps with TRPC1 immunoparticles (double arrowheads) within membranes of intracellular compartments of a primary dendrite (lower inset). In **E** and **C**, dual nanogold labeling for D2Rs (large particles; arrowheads) and TRPC1 (small particles; double arrowheads) shows that immunoprobes are often separated by fewer than 50 nm. (**F**) Spatial colocalization of D2R and TRPC1 immunoparticles strongly suggests that the two proteins are physically associated at the plasma membrane of dendrites. Asterisks mark a subsurface cistern; ax, axon; den, dendrite; sp, spine. Scale bars: 200 nm

2.3.7 Presence of D2R Mediates TRPC1 Cell Surface Expression in HEK293 Cells

To determine the functional significance of the TRPC1/D2R interaction, we used a cell surface biotinylation assay to analyze the effect of D2R expression on the levels of TRPC1 at the plasma membrane. We took advantage of the fact that wild-type HEK293 and HEK293-D2L cells endogenously express similar levels of TRPC1 (Figure **2.13**B), whereas only HEK293-D2L cells express D2Rs. HEK293 and HEK293-D2L cell surface proteins were biotinylated by incubation with NHS-SS-biotin. Biotinylated membrane proteins were immunoprecipitated using an anti-TRPC1 polyclonal antibody (Alomone) and TRPC1 proteins quantified by densitometric analysis. As shown in Figure **2.13** (A and C), HEK293-D2L cells displayed a two-fold increase in the numbers of cell surface TRPC1 channels compared with wild-type HEK293 cells. Similar results were obtained with an independent (Sigma) anti-TRPC1 antibody (data not shown). These results suggest that TRPC1/D2R interaction is required for the proper trafficking and/or expression of TRPC1 channels at the plasma membrane.

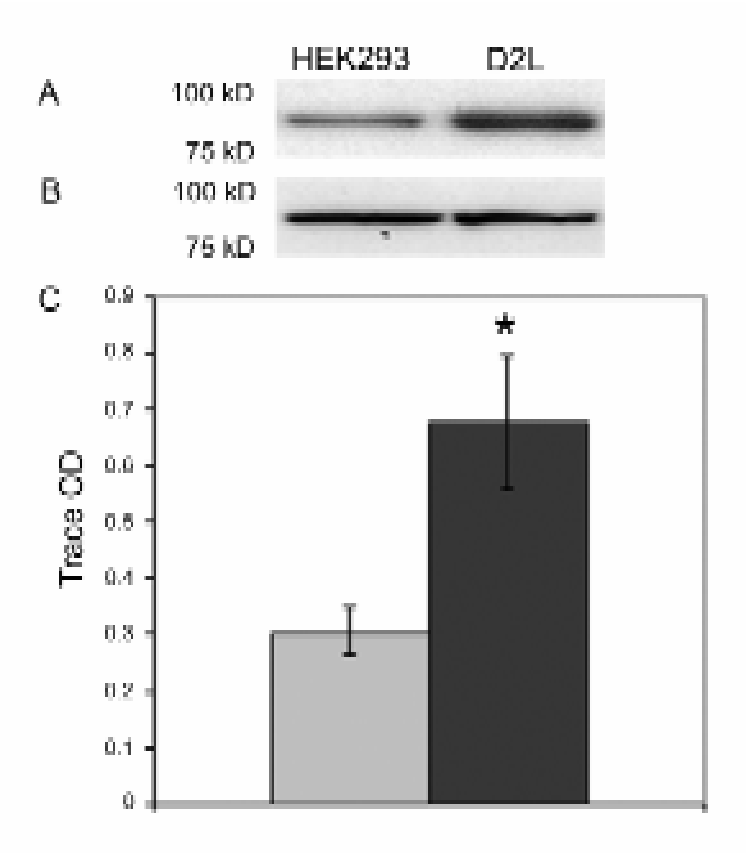

Figure **2.13**: **TRPC1/D2R Interaction Mediates Cell Surface Expression of TRPC1**. A cell surface biotinylation assay was used to examine the effect of the D2R on plasma membrane expression of TRPC1. HEK293 and HEK293-D2L cell surface proteins were biotinylated by incubation with NHS-SS- biotin. Biotinylated membrane proteins were immunoprecipitated using an anti-TRPC1 antibody and TRPC1 proteins quantitated by densitometric analysis. (**A**) Anti-TRPC1 antibody was used to immunoprecipitate TRPC1 proteins from HEK293 and HEK293-D2L cells. Immunoblots were probed for the presence of biotin to identify cell surfaced- expressed TRPC1. (**B**) Total expression of TRPC1 in crude membrane fractions prepared from HEK293 and HEK293-D2L cells. (**C**) Biotinylated TRPC1 proteins in A were quantitated by laser densitometry. HEK293-D2L cells stably expressing D2Rs show an approximately 50% increase in the level of cell surface TRPC1 proteins (Students two-tailed *t* test, n=6, *p< 0.05) compared to HEK293 cells which do not express the D2R.

We next analyzed whether overexpression of TRPC1 could lead to an increase in D2R cell surface expression. Wild-type HEK293 cells and HEK293-D2L cells were transiently transfected with TRPC1. Cells were grown for 48 hrs, biotin-labeled, and

membrane fractions immunoprecipitated with anti-D2R antibodies. D2R-containing complexes were then probed with horseradish peroxidase-conjugated avidin. As shown in Figure **2.14**, overexpression of TRPC1 resulted in an approximate two-fold increase in the number of cell-surface D2L receptors compared with untransfected HEK293-D2L cells. Due to its highly hydrophobic nature, the D2R, as typical of GPCRs, runs as a dimer on a Western blot. As mentioned previously, the 50kD band represents the monomeric form of the receptor. A second band present at 75kD corresponds to higher order aggregates of the receptor (Figure **2.14)**. These results indicate that while D2Rs mediate trafficking of TRPC1, overexpression of TRPC1 can also promote cell surface expression of D2Rs.

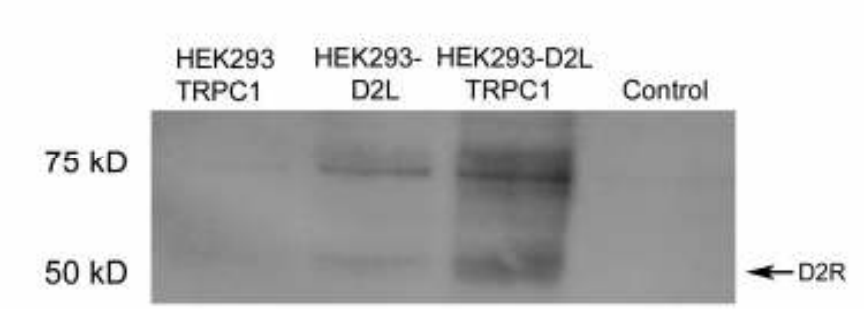

Figure **2.14**: **TRPC1 Overexpression Increases Cell Surface Expression of D2Rs**. HEK293 and HEK293-D2L cells were transiently transfected with TRPC1. Cell surface proteins were biotinylated and immunoprecipitated with a polyclonal anti-D2R antibody, and immunoblots were probed for the presence of biotin. Biotinylated receptors were quantitated by laser densitometry. Arrow shows position of D2R monomer. Overexpression of TRPC1 produced a 50% increase in the number of cell-surface D2Rs compared with untransfected HEK293-D2L cells

We employed a dominant-negative approach to determine the functional consequences of interrupting the interaction between TRPC1 and the D2R. An

ectopically expressed construct containing a D2IC2-GFP fusion protein (D2IC2-GFP) could act as a dominant-negative peptide by competing against the D2R for binding of endogenous TRPC1, disrupting the native interaction. Overexpression of the D2IC2-GFP would result in more TRPC1 bound to the competing peptide rather than D2R, which may have functional consequences on the trafficking of TRPC1. To determine whether the D2IC2-GFP could bind endogenous TRPC1, an anti-TRPC1 antibody was used to immunoprecipitate TRPC1 from cell lysates from wildtype HEK293-D2L and HEK293-D2L transfected with the D2IC2-GFP fusion protein. Immunocomplexes were then separated via SDS-PAGE, and blots were probed with an anti-GFP antibody to detect D2IC2-GFP. As seen in Figure **2.15**, D2IC2-GFP was detected in lysate lanes alone (top panel), or nonspecifically in lysate, IP, and mock IP lanes (bottom panel), indicating that the D2IC2-GFP did not bind endogenous TRPC1 in this context. Immunoprecipitation with an anti-GFP antibody and subsequent immunoblotting with an anti-TRPC1 antibody produced similar results (data not shown). Multiple attempts failed to detect binding between endogenous TRPC1 and the D2IC2-GFP. It is possible that the large size of the GFP moiety interfered with the TRPC1/peptide interaction. The native D2R/TRPC1 interaction could be sufficiently strong that the D2IC2-GFP peptide was unable to compete with the native receptor for binding. In addition, wild-type HEK293-D2L cells and HEK293-D2L cells transfected with D2IC2-GFP showed similar cell surface expression of TRPC1 as assessed by biotinylation assay (data not shown). These results indicate that D2IC2-GFP cannot function as a dominant-negative in this experimental system.

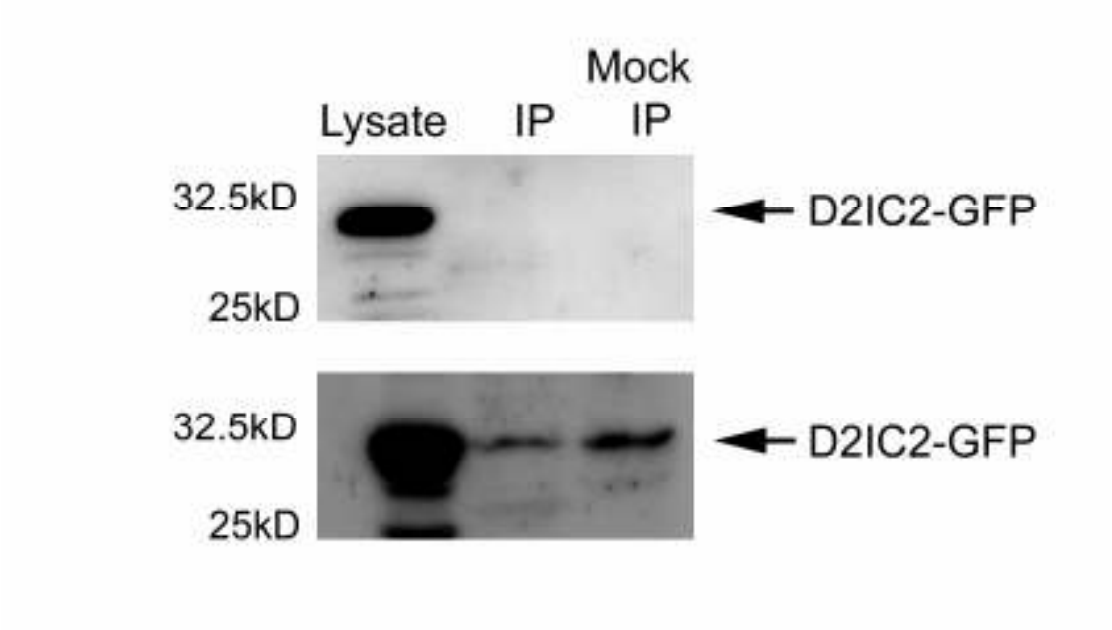

Figure **2.15**: **TRPC1 and D2IC2-GFP do not Interact in HEK293-D2L Cells.** D2IC2-GFP was transiently transfected into HEK293-D2L cells. Endogenous TRPC1 was immunoprecipitated using an anit-TRPC1 antibody. Blots containing immunocomplexes were then probed for D2IC2-GFP using an anti-GFP antibody (Sigma). A band was detected between 25 and 32.5kD in lysate alone lanes, corresponding to the expected size of D2IC2-GFP. Using a high stringency wash (top panel), no D2IC2-GFP was detected in the immunoprecipitation lane (IP) or beads alone (Mock IP). Using a lower stringency wash (bottom panel), D2IC2-GFP was detected nonspecifically in both the immunoprecipitation lane (IP) and beads alone lane (Mock IP). N=5.

2.3.8 Cell Surface Biotinylation and D2R Activation

To further perturb the TRPC1/D2R interaction, we investigated whether the activation state of the receptor had an effect on the cell surface expression of endogenous TRPC1 in both HEK293 and HEK293-D2L cells. Briefly, HEK293 and HEK293-D2L cells were stimulated with 100 μM of dopamine hydrochloride (DA) for a period of time ranging from 2.5 minutes to 30 minutes. The zero time point represents unstimulated

cells. At the end of the incubation period, cell surface proteins were biotinylated as previously described, and endogenous TRPC1 was immunoprecipitated with an anti-TRPC1 antibody. Immunocomplexes were separated via SDS-PAGE and probed with avidin to identify TRPC1 proteins expressed on the cell surface. Signal intensity was determined by densitometric analysis. To control for inter-experimental variation, all signals were normalized to the unstimulated data point (t=0 min) for each series, and relative ratios, rather than raw data points, were used in statistical analysis.

Stimulation of HEK293T cells shows no significant effect on the cell surface expression of TRPC1 protein (Figure 2.16). Stimulation of HEK293-D2L cells shows a slightly different trend. The cell surface expression of TRPC1 in this cell line increases at 5 minutes and 10 minutes, and sequentially decreases at 15 minutes and thereafter. However, after analysis using paired student t-tests, none of these time points show statistical significance between the HEK293T and HEK293-D2L sample groups, implying that agonism of D2Rs does not affect the cell surface expression of TRPC1. Similar results were obtained with stimulation of HEK293T and HEK293-D2L by quinpirole, a potent and specific D2-agonist. Based on these findings, cell surface expression of TRPC1 does not appear to be affected by the activation state of the D2R in this experimental system. Interestingly, at time points t=10 min and t=15 min, althought the p value is not significant, the error bars do not over-lap between the HEK293T control group and the HEK293-D2L experimental group (Figure 2.16). Statistical analysis of this experiment was conducted on N=5 replications. It would be of value to increase the replication size, especially at these two data points, to determine if the presence of the D2R can potentially affect the trafficking of TRPC1. These time points

approximately coincide with the time frame needed for internalization and desentization of the D2R. The D2R may potentially internalize TRPC1 as it goes through its normal life cycle of activation, internalization, and subsequent internalization and recycling or degradation.

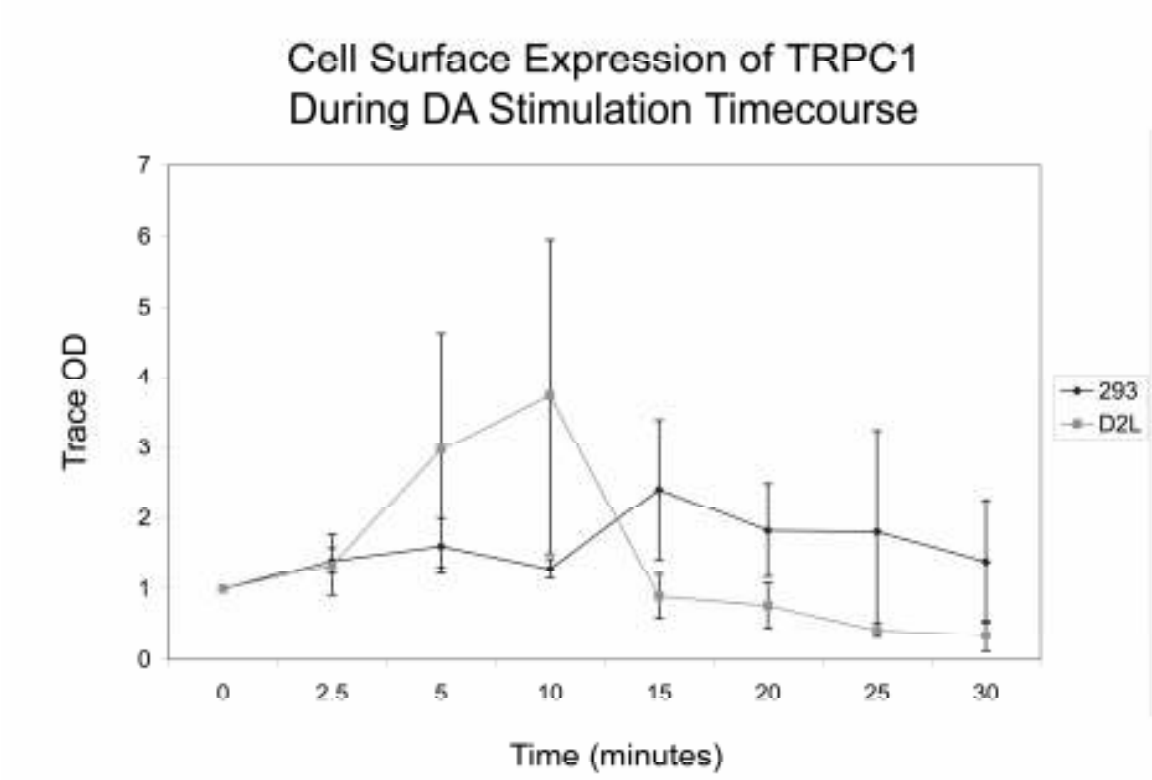

Figure **2.16**: **Stimulation with DA did not Significantly Change Cell Surface Membrane Localization of TRPC1.**

HEK293T and HEK293-D2L cells were stimulated with 100 μM DA for timepoints of 0-30 minutes. Cell surface proteins were then biotinylated, cell membranes harvested, and TRPC1 immunoprecipitated using an anti-TRPC1 antibody. Immunocomplexes were separated by SDS-PAGE and transferred to PVDF. Blots were subsequently probed for biotin to detect cell surface TRPC1, and quantitated via laser densitometry. Relative intensity of signals were standardized to unstimulated cells (time = 0). Averages from n=5 trials were plotted. Analyzed by paired student's t test, double tailed.

2.4 Discussion

In this study, we present evidence that TRPC1, TRPC4, and possibly TRPC5, are bona-fide DRIPs. Among the seven mammalian TRPC family members, TRPC1, TRPC4 and TRPC5 comprise a sequence-related subgroup in which TRPC1 can form heteromeric channels with either TRPC4 or TRPC5 (reviewed in [178, 226]). In GST-pulldown experiments, we detected interaction of D2R with the C-terminus TRPC1, TRPC4 or TRPC5. This region of TRPCs is known to bind multiple proteins such as the IP3R and CaM, and plays an important role in the cellular regulation of TRPC channel activity [181, 226-229]. The D2R/TRPC1 and D2R/TRPC4 interactions were further substantiated by cellular colocalization and brain-derived coimmunoprecipitation experiments. However, the evidence supporting a direct interaction between the D2R and TRPC5 was not as convincing. First, we were unable to detect D2R/TRPC5 interaction in either coimmunoprecipitation or colocalization experiments within HEK cells. However, the inability to validate the D2R/TRPC5 interaction using these immunohistochemical based detection methods may reflect the fact that TRPC5 is not robustly expressed in HEK cells, either endogenously or after transfection. Second, the commercially available TRPC5 antibodies we employed reacted very weakly, and in some cases non-specifically, with TRPC5 thus making it difficult to draw firm conclusions about the D2R/TRPC5 interaction from these validation studies.

Truncation mapping identified a region within TRPC1 responsible for interaction with the D2R. Sequence comparisons identified a potential D2R interaction site within comparable segments of TRPC4 and TRPC5. Interaction between the D2R and

fragments of each of the three TRPC subtypes containing the putative interaction domain was confirmed by using a directed two-hybrid assay. The fragment containing the minimal TRPC1 binding domain produced β-galactosidase activity, although the strength of the interaction between this fragment and the D2R appeared less robust than that of Clone 44, the *C*-terminal segment of TRPC1 identified in the original two-hybrid screen. This result suggests that within TRPC1, the region spanning amino acids 726-745 contributes to the D2R/TRPC1 interaction. Multiple regions in the c-terminus of TRPC1 likely contribute to increase the total strength of interaction with the D2IC2, indicating the possible need for secondary structure in order to preserve maximal binding between the two proteins. In the directed two-hybrid assay, the comparable fragment within TRPC4 produced somewhat weaker but readily detectable β-galactosidase activity. In contrast, the minimal TRPC5 truncation fragment produced relatively low levels of β-galactosidase activity. It is possible that sequence variations between the binding domains identified in TRPC1, TRPC4, and TRPC5 could account for differences in the levels of β-galactosidase activity produced in the directed two-hybrid assay. Alternatively, the segments we tested within the TRPC channels may constitute only a portion of the D2R binding site, and additional sequences may be necessary to produce stronger β-galactosidase activity. Finally, our truncation mapping results are consistent with the idea that TRPC1 interacts directly with the D2R and may form a bridge between the D2R and TRPC4 and/or TRPC5. In this scenario, TRPC4 and TRPC5 would also be in direct physical contact with the D2R, but the D2R/TRPC4 and D2R/TRPC5 interactions would encompass a smaller surface interface and be substantially weaker

(energetically) than the D2R/TRPC1 interaction. Molecular modeling algorithms may help to elucidate the structural basis underlying the interaction between the D2R and each of the TRPC proteins.

It is well established that TRPC channels interact with scaffolding and calcium signaling proteins and are assembled into multimeric protein complexes within Ca^{2+} signaling microdomains (reviewed in [181, 226, 230]). Differences in the localization and regulation of these signaling complexes result from their interaction with regulatory and scaffolding molecules. Interestingly, TRPC channels themselves may play a role in anchoring Ca^{2+} signaling complexes to the plasma membrane. For example, in *Drosophila* photoreceptor cells, TRP has several non-channel functions including an anchoring role in retention of the phototransduction signalplex to the rhabdomere [208, 231, 232]. TRP channel mutants that cannot bind to INAD (a signalplex scaffolding protein), mislocalize the phototransduction complex [208]. These findings implicate TRP as a core scaffolding component of the phototransduction signalplex [233].

The results reported in the present study demonstrate that TRPC proteins interact directly with components of the D2R signalplex. Initial brain-derived coimmunoprecipitation experiments we performed revealed that TRPC1 and TRPC4 form a complex with the calcium signaling DRIP NCS-1, while pulldown experiments demonstrated a direct interaction between TRPC1 and NCS-1 (Chapter 3). NCS-1 has previously been shown to physically associate with TRPC5, an interaction that contributes to the inhibition of neurite outgrowth [234]. These results suggest that the D2R/NCS-1/TRPC interaction may serve to couple dopamine and calcium signaling pathways within neurons.

It will clearly be of interest to determine whether the D2R/TRPC interaction can modulate the electrophysiological properties of TRPC channels. In the present study, overexpression of TRPC proteins appears to have detrimental effects on HEK293 cells and membrane integrity of cells transiently transfected with the proteins is compromised. Transient overexpression of TRPC1 in the presence of stably overexpressed D2R seems to have additive deleterious effects on cells making whole cell patch clamping in this instance technically challenging. In addition, there is continuing debate surrounding the functionality of homomeric TRPC1 ion channels. Various reports have documented disparate findings. Claims have been made that TRPC1 is a nonfunctional channel subunit, TRPC1 monomeric channels are functionally active/inactive, or TRPC1 subunits form functional channels only in the presence of TRPC3/4/5 binding partners. HEK293 cells may lack the proper protein chaperones or binding partners necessary to ensure proper physiological functioning of TRPC1, adding to the difficulty encountered with attempts at whole-cell patch analysis of TRPC1 in this system. TRPC1 may have to be co-expressed with either TRPC4/5 to generate functional ion channels in these cells. Further attempts at characterizing the effects of TRPC1/D2R interaction on the electrophysiological properties of TRPC1 will have to address these issues.

Interestingly, a recent study by Aman et al. suggests that TRP-like currents can be evoked via D2-like dopaminergic stimulation of rat dorsal raphe serotonin neurons [235]. Stimulation of these cells with D2R-specific agonists elicits an inward current that exhibits a linear I-V relationship and reverses polarity at around 15 mV. This profile suggests an involvement of mixed cationic conductance and strikingly resembles currents known to be produced by TRPC channel involvement [194, 235]. Further *in vitro*

electrophysiological studies of rat brain slices containing the dorsal raphe nucleus neurons demonstrate a reversible slow membrane depolarization in response to stimulation with dopamine (3 to 100 μM) or quinpirole (30 μM). This current is not evoked by the D1-like agonist SKF38393, and is abolished in the presence of the D2-like antagonist sulipride and profoundly attenuated by the TRP channel inhibitors 2-APB and SKF-96365. In addition, the current appears dependent on G-protein signaling pathways and activation of phospholipase C (PLC), but is not affected by inhibition of adenylyl cyclase. Dorsal raphe neurons have been shown to express D2R, D3R, as well as TRPC1, TRPC3, TRPC5, and TRPC6 [236-238]. Taken together, these results suggest that TRPC channels are responsible for the dopamine stimulated slow membrane depolarization current in dorsal raphe nucleus serotonin neurons, and provide evidence for an electrophysiological relationship between the D2R and one or more TRPC channels [235].

Our biotinylation studies indicate an increase in cell surface expression of TRPC1 in HEK293 cells stably expressing the D2R. Our findings suggest that overexpression of TRPC1 can promote an increase in D2R expression at the plasma membrane. It is not clear whether such an upregulation in the membrane expression of either the D2R or TRPC1 is the result of a net increase in protein trafficking and/or stabilization at the plasma membrane. In this context it is worth noting that treatment of cells with the D2R agonists dopamine or quinpirole did not promote a statistically significant increase in cell surface expression TRPC1 proteins. It appears that in the HEK293 cellular model the conformational state of the D2R has no effect on localization of TRPC1. Upon interaction, the D2R/TRPC1 complex moves to the cell surface where the interaction

appears stable and ultimately unaffected by the activation state of the receptor. It will be interesting to determine if activation or inhibition of the D2R promotes ion flux through the TRPC1 channel or changes its ion permeability properties without affecting its trafficking. Furthermore, trafficking of the D2R/TRPC1 complex in HEK293 cells may not mimic trafficking of the complex in neurons. TRPC4 and TRPC5 have been shown to translocate from the plasma membrane to the cytosolic compartment upon stimulation of DHPG, a group I metabotropic glumate receptor agonist [239]. This translocation was observed initially in mouse hippocampal slices and was determined to be PLCβ1-dependent [239]. Perhaps investigation of dopaminergic stimulation of native tissue, such as striatum, would show differences in trafficking of TRPC1 not evident in HEK293 cells.

Competition experiments with a potential dominant negative peptide were unable to disrupt the native D2R/TRPC1 interaction, and showed no effect on cell surface expression of TRPC1. Perhaps a peptide corresponding to the D2R binding domain of TRPC1 could compete for interaction with the receptor. More likely, the interaction between the D2R and TRPC1 is formed early in the endoplasmic reticulum and is relatively stable; a peptide consisting of only a portion of either protein may not contain all secondary structure and/or post translational modifications necessary for proper binding in the cellular environment. Together, these results suggest that physical assembly of the TRPC1/D2R complex is sufficient for promoting TRPC1 trafficking to the plasma membrane, and may be a key determinant in the receptor–mediated activation of these channels.

Previous studies have shown that expression of NCS-1, a D2R interacting protein, is altered in patients with schizophrenia [107, 240] as well as in mice treated with antipsychotic drugs [241]. It is of particular interest that TRPC1 and TRPC5 also interact with NCS-1. In view of the critical roles assumed by TRPCs in regulating key neural functions, including neurite extension and growth cone guidance [200, 242, 243], it will be important to determine if alterations in expression or function of TRPC occur in neuropathologies associated with dysregulation of dopamine neurotransmission. Alterations in TRPC protein expression or function could have a profound effect on the proper trafficking and/or signaling properties of the D2R-signalplex. In this context, it is interesting to consider that alterations in TRPC7 expression have recently been reported in patients with bipolar affective disorder [244].

The present results provide compelling evidence that TRPC channels are integral components of the D2R signalplex. Within the signalplex, TRPC channels interact directly with the D2R as well as the calcium signaling molecule NCS-1, suggesting a link between dopamine and calcium signaling pathways within neurons. It is not yet clear whether the D2R/TRPC interaction serves to modulate the electrophysiological or calcium permeability properties of TRPC channels. Our data indicate that the D2R/TRPC interaction mediates trafficking of signalplex components to the cell surface. These results raise the possibility that TRPC channels play multifunctional roles within mammalian cells.

Chapter 3

Interaction between NCS-1 and TRPC Proteins has Functional Consequences for Neurite Growth Extension

3.1 Introduction

In addition to interacting with and regulating the D2R, there is evidence that members of the D2R signalplex also interact with and influence each other [109, 112]. This is not surprising, considering the close proximity in which these proteins exist in the signalplex. It is of interest to determine how these secondary protein-protein interactions affect the functioning of the D2R signalplex and D2R-mediated neurotransmission. We investigated whether a subset of calcium signaling DRIPs, namely NCS-1, TRPC1, and the related but unconfirmed interactor TRPC5, could interact with and influence the functioning of each other. TRPC1 and TRPC5 are known to interact and form functional heteromeric ion channels with properties distinctive from homomeric ion channels formed by TRPC1 or TRPC5 subunits alone [193, 194]. These interactions imply a dynamic interplay between protein partners that sense and respond to calcium levels within the cell.

TRPCs are widely expressed in neurons and have been implicated in a variety of neuronal processes [200, 203]. In particular, a role for TRPCs in neurodevelopment and the extension of neuronal processes has been the topic of a number of investigations [199, 200, 242, 243, 245-247]. TRPC5 has been discovered to have an inhibitory effect on neurite extension [200, 248]. However, the mechanism that regulates activation of

TRPC5 in this process remains poorly understood [194, 249, 250]. NCS-1, a calcium sensing molecule that functions in the same calcium concentration range as TRPC5, emerged as a potential candidate for regulating TRPC5 channels [251, 252]. In addition, NCS-1 has an overlapping expression pattern with TRPC5 and is a known modulator of other types of ion channels [252-254].

The C-terminal regions of TRPC1 and TRPC5 were tested for interaction with NCS-1 in a directed yeast two hybrid assay. The interaction between the TRPCs was confirmed via GST-pulldown and coimmunoprecipation experiments in HEK293 cells as well as in native rat brain. In collaboration with the laboratories of David Beech, Jamie Weiss, and Damian McHugh at the University of Leeds and the University of Sheffield, we have shown that NCS-1 and TRPC5 contribute to the regulation of neurite outgrowth pathways [234]. Mutants of both NCS-1 and TRPC5 have non-additive detrimental effects on patterning and signaling between neurons, suggesting direct interaction and involvement in a common mechanism affecting neuronal growth cone morphology [234].

3.2 Experimental Procedures

3.2.1 DNA Constructs and Directed Yeast Two-Hybrid Assays

All fragments inserted into vectors were generated by PCR amplification and verified by automated DNA sequencing. cDNA encoding the complete neuronal calcium signaling molecule -1 (NCS-1; accession #NM_014286) open reading frame (ORF) was subcloned into the Gal4 DNA-binding domain vector pAS2-1 (BD Biosciences Clontech)

and used as bait. cDNA encoding the carboxyl terminal truncations of human TRPC1 (TRPC1; accession #X89066) and human TRPC5 (TRPC5; accession #AF054568) were constructed in the Gal4 DNA-activation domain vector pACT2 (BD Biosciences Clontech) and used as prey. Bait and prey constructs were sequentially transformed into yeast strain MaV103 by standard lithium acetate protocol [113]. Transformants were identified via growth on -Leu/-Trp selection plates. Protein-protein interactions were detected using α – galactosidase assays as per the manufacturer's instructions (BD Biosciences) or β-galactosidase filter-lift assays as previously described [113].

3.2.2 GST-Pulldowns

A cDNA encoding the complete NCS-1 ORF was constructed in pGEX-4T-1 (Amersham Pharmacia) to generate GST-tagged NCS-1 (NCS-1-GST). The IC3 (amino acids 211-373) of the human D2R, long isoform (D2L) was constructed in pGEX-4T-1 to generate a GST-tagged D2L receptor (D2LIC3-GST) and was used as a negative control. Carboxyl-terminal truncations of TRPC1 and TRPC5 were subcloned in the vector pET30C (Novagen) to generate a set of S-tagged constructs. All fusion proteins were induced in *E. coli* strain BL-21 (DE3) and purified using glutathione-sepharose beads (Amersham) according to the manufacturer's instructions. Eluted proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was probed for presence of S-tagged carboxyl-terminal TPRC fusion proteins using an anti-S-tag polyclonal antibody (1:5000 dilution)

conjugated to horseradish peroxidase (Novagen). Immunoreactivity was detected by ECL (Amersham).

3.2.3 Cell Culture and Transfection

Tetracycline-inducible expression of human TRPC5 in HEK293 (TRex-TRPC5) cells has been previously described [250]. Cells were maintained at 37^{o}C and 5% CO_2 in Dulbecco's modified Eagle's medium-F12 (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Cells were transfected with wt-NCS-1 or the dominant negative (DN) mutant (E120Q) NCS-1 in a pcDNA3 vector using the Fugene 6 transfection reagent (Roche). Red fluorescent protein (pDsRed2-N1) was co-transfected to monitor transfection efficiency.

PC12 were obtained from the European tissue collection (ECACC no. 88022401) and cultured according to the instructions supplied. Cells were plated onto type IV-collagen coated coverslips and transfected with either wild-type or dominant negative NCS-1 or TRPC5 constructs using the Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions. The dominant-negative TRPC5 (DN-TRPC5) contains a triple alanine mutation of a conserved sequence in the pore-loop region and has been described previously [193]. Neuronal growth factor (NGF – Invitrogen) was added after two days to induce neurite formation. Cells were fixed, and immunocytochemistry was performed using a polyclonal rabbit anti-NCS-1 antibody (Biomo/Affiniti, Exeter, UK), a polyclonal affinity-purified chicken anti-frequenin

antibody (Rockland Immunochemicals), or monoclonal TUJ1 neuronal class III β-tubulin (Covance) at dilutions of 1:500.

3.2.4 Coimmunoprecipitation

TRPC5/NCS-1 coimmunoprecipitations were conducted in collaboration with Damian McHugh, Hui Hui, and David Beech at the University of Leeds, Leeds, UK [234]. Briefly, 36 hours after tetracycline induction (1 μg/mL) of TRPC5 expression in TRex-TRPC5 cells, the cells were washed three times in chilled phosphate-buffered saline (PBS), harvested by scraping, and centrifuged for five minutes at 500g. Cells were homogenized at 4^{o}C in ice cold lysis buffer (containing 20 mM Tris/acetate, 1 mM EDTA, 1 mM EGTA, 10 mM sodium β-glycerophospate, 1 mM sodium orthovanadate, 1% Triton X-100, 270 mM sucrose and protease inhibitors). Rats (> postnatal day 12) were killed by cervical dislocation according to Schedule 1 procedures outlined in the Code of Practice, UK Animals Scientific Procedures Act 1986. Rat brain membranes were isolated with an ultracentrifugation step. Protein concentrations of lysates were determined by the Bradford method using BSA as the standard. For immunoprecipitation experiments, 600 μg of cell/brain lysate were precleared with protein G-sepharose beads (Amersham Biosciences, UK) by incubating for one hour at 4^{o}C. The beads were pelleted by centrifugation, and 4 μg of anti-NCS-1 antibody were added to the precleared lysate and incubated with nutation for two hours at 4^{o}C. Fresh protein G-sepharose beads were added to the lysate with nutation overnight at 4^{o}C. Proteins were eluted with 50 μL of sample buffer (Sigma, UK) and resolved by SDS-PAGE. Custom-made and affinity-

purified anti-TRPC5 antibodies were generated in chicken to peptide VFETWGEACDLLMHKWGDGQ. Blots were incubated with appropriate secondary antibody coupled to horseradish peroxidase and visualized via ECL detection.

3.2.5 Image Analysis and Neurite Outgrowth Quantification

Representative PC12 cell images were chosen at random from three separate transfections for each experimental group. Forty cells were analyzed for each experimental group. Total neurite extensions were tallied for each cell, and neurite length was measured directly from the cell body to the end of each process.

3.2.6 Biotinylation Experiments

PC12 cells cotransfected with TRPC5 and either wild-type or dominant-negative NCS-1 were cell surface labeled with Sulfo-NHS-SS-Biotin (Pierce). Cells were lysed according to standard procedure and a Bradford assay was performed to determine total protein concentration. Equal amounts of protein were incubated with NeutrAvidin sepharose beads (Pierce), washed numerous times to remove unbound protein, and separated via 10% SDS-PAGE. Anti-TRPC5 antibodies were used to identify cell surface expressed TRPC5 proteins by Western blot analysis.

3.2.7 Calcium Imaging and Patch Clamp Recording

Experiments were performed as previously described [250]. For calcium imaging experiments, cells were preincubated with 1 μM of the fluorescent Ca^{2+} indicator fura PE3-AM (Calbiochem) at 37°C for one hour in standard bath solution. Cells were washed for 30 min at room temperature. Fluorescence was observed with an inverted microscope (Zeiss, Martinsried, Germany). Recordings were taken alternatively from test and control cells. Images were sampled every 10 sec in pairs for the two excitation wavelengths (340 and 380 nm) and analyzed off-line using Openlab 2 software (Image Processing and Vision Company Ltd., UK). $[Ca^{2+}]_i$ is expressed as the ratio of the emission intensities for 340 and 380 nm ($R_{340/380}$) [250].

For electrophysiological experiments, voltage clamp was performed at room temperature using the whole-cell patch configuration. Signals were amplified with an Axopatch 200 A patch clamp amplifier and controlled with pClamp software 6.0 (Axon). Patch pipettes were made from borosilicate glass capillary tubing with an outside diameter of 1 mm and resistance of 3-5 MΩ (Clark Electromedical Instruments, Reading, UK) [250].

Standard bath solution contained (in mM): NaCl 130, KCl 5, D-glucose 8, HEPES 10, $MgCL_2$ 1.2, and $CaCl_2$ 1.5. The pH was titrated to 7.4 with NaOH. Internal pipette solution contained (in mM): CsCl 100, HEPES 10, Na_2ATP 5, EGTA 10, and $MgCl_2$ 2. $CaCl_2$ was titrated at 0, 4.3, 6.9, 8.17, and 8.84 mM to obtain unbound Ca^{2+} concentrations of 0, 100, 300, 600, and 1000 nM, respectively. Osmolarity was adjusted

to 290 mOsm with mannitol, and pH was maintained at 7.2. 2-Aminoethoxydiphenyl borate (2-APB, Sigma) was prepared as a 75 mM stock in dimethylsulfoxide (DMSO).

3.3 Results

3.3.1 TRPC1 and TRPC5 Interact Directly with NCS-1

We used a directed yeast two-hybrid assay to test for interaction between NCS-1 and a C-terminal fragment of TRPC1 (amino acids 694-759) and a C-terminal fragment of TRPC5 (amino acids 619-973). Protein interaction between NCS-1/TRPC1 and NCS-1/TRPC5, as measured by growth on selection plates and expression of either α- or β-galactosidase activity, was detected in eight total independent experiments (data not shown). Individual constructs did not exhibit autologous activation of the galactosidase system. For negative controls, we failed to detect interaction between NCS-1 and protein 4.1N, as well as between the C-termini of TRPC1 and TRPC5 and the third intracellular loop of the D2L dopamine receptor (DR). To further refine the NCS-1 binding site on TRPC5, truncation fragments of the C-terminus of TRPC5 (Figure **3.1**) were tested for interaction with NCS-1 in a directed Y2H screen. The complete C-terminal fragment of TRPC5 (construct A) interacted robustly with NCS-1, as did two truncation fragments (constructs B and C) that together deleted 170 amino acids from the TRPC5 C-terminus. Truncations of the N-terminal portion of the TRPC5 fragment significantly reduced interaction with NCS-1 (constructs D and E). A fragment of TRPC5 with N- and C-terminal truncations (construct F) failed to interact. These results suggest that regions in

the amino-terminus (amino acids 619-711) as well as the carboxyl half of the TRPC5 fragment (amino acids 803-973) contribute to the NCS-1/TRPC5 interaction. TRPC5 truncations with proximal C-terminal sequences (Trunc A, B, and C) displayed greater strength of interaction with NCS-1, indicating a need for these sequences for maximal protein-protein binding. This suggests possible coopertivity of binding between the TRPC5 C-terminal truncations and NCS-1, redundancy of binding sites, and/or indicates a requirement for secondary structure provided by these sequences to replicate maximal strength of binding between TRPC5 and NCS-1.

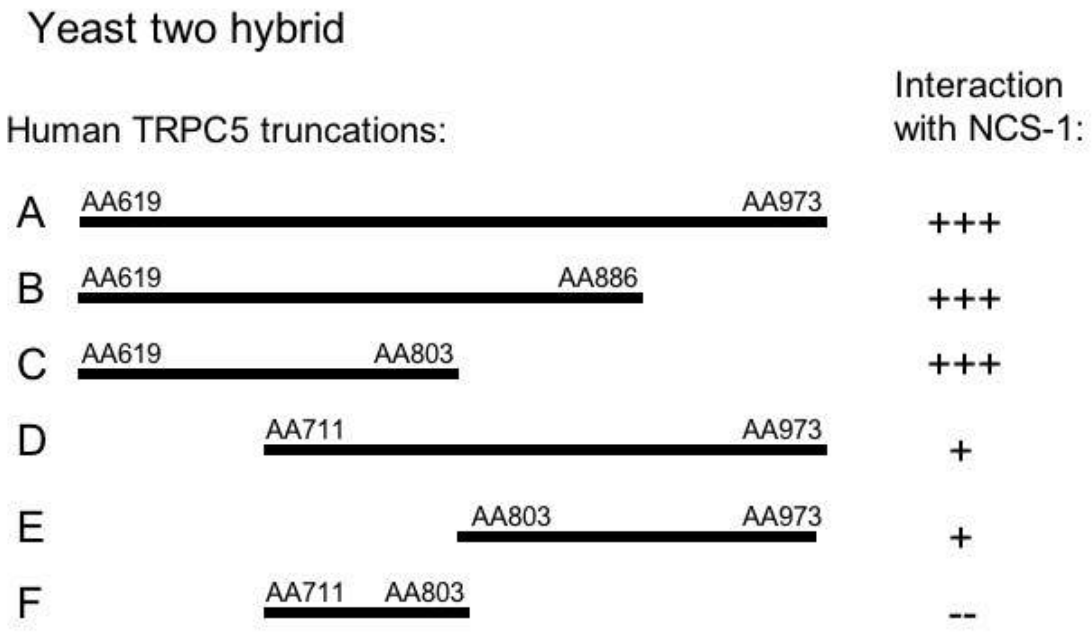

Figure **3.1**: **Directed Yeast Two-Hybrid between TRPC5 and NCS-1**

Schematic representation of constructs encoding for C-terminal truncations of TRPC5 (AA, amino acid). C-terminal truncations A-F of TRPC5 were tested in a directed yeast two-hybrid screen for interaction with NCS-1. Interaction is indicated by the strength of α-galactosidase activity (+++, strong activity; +, weak activity; --, no activity).

To further validate the NCS-1/TRPC5 interaction, we performed pulldowns using NCS-1-GST as bait to pull down truncation fragments encompassing amino acids 619-973, 619-803, and 803-973 of the TRPC5 C-terminus (Figure **3.2**). A fragment of the D2L DR (D2LIC3- GST) was used as a negative control. As shown in Figure **3.2**, the entire TRPC5 C-terminal fragment (amino acids 619-973) exhibited considerable non-specific binding with the D2LIC3-GST negative control (Figure **3.2**). However, an N-terminal truncation fragment of TRPC5 (trunc B; amino acids 619-803) showed strong interaction with NCS-1 in four separate pulldown experiments (Figure **3.2**). A C-terminal truncation (trunc A; amino acids 803-973) of TRPC5 also interacted with NCS-1 (Figure **3.2**). Neither of these TRPC5 truncation fragments interacted with the D2LIC3-GST negative control. These results suggest the presence of potential binding sites for NCS-1 within the proximal and distal segments of the TRPC5 C-terminal domain.

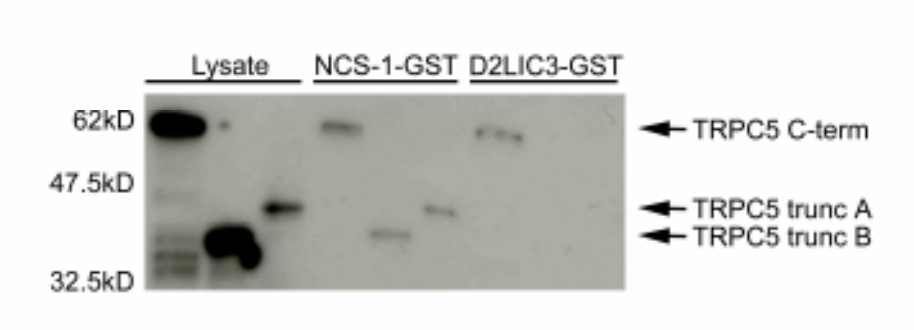

Figure **3.2**: **NCS-1 Binds to Multiple Sites on the C-terminus of TRPC5**

NCS-1 fusion proteins were able to pulldown S-tagged TRPC5 C-term (amino acids 619-973), TRPC5 trunc A (803-973), and TRPC5 trunc B (619-803) from bacterial lysates. TRPC5 trunc A and trunk B did not react with a D2L-GST protein, whereas TRPC5 C-term reacted nonspecifically with D2LIC3-GST, a negative control. Lysate lanes show S-tagged proteins run at predicted molecular weight.

GST-pulldown techniques were used to confirm that TRPC1 and NCS-1 also directly interact. A lysate prepared from bacteria expressing an S-tagged TRPC1 C-terminal fragment (amino acids 638-759) was tested for the ability to associate with an NCS-1-GST fusion protein. As shown in Figure **3.3**, the lysate produced an immunoreactive band of ~18 kDa, the expected size of the TRPC1 fragment, when probed with an anti-S-tag antibody. The same band was detected after the bacterial lysate was incubated with the NCS-1-GST fusion protein, but not when the lysate was absorbed onto either GST or beads alone. These results support the finding that TRPC1 interacts directly with NCS-1.

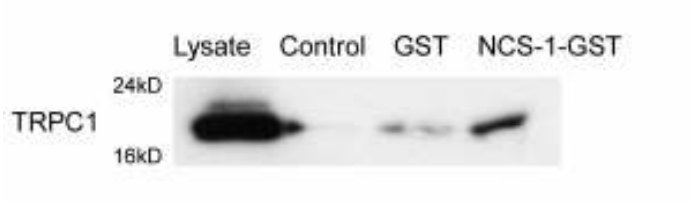

Figure **3.3**: **TRPC1 C-terminus Interacts with NCS-1-GST Fusion Protein**.
NCS-1-GST fusion protein was used to pull down S-tagged TRPC1 (residues 638-759) from a bacterial lysate. S-tagged TRPC1 was pulled down in the presence of NCS-1-GST, but not with GST or beads alone. The position of the TRPC truncation fragment is shown in the lysate lane

3.3.2 TRPC1 and TRPC5 Coimmunoprecipitate with NCS-1 in Cultured Cells and Primary Tissue

TRPC1 has been shown to exist in a complex with the D2R and NCS-1 in rat cortical and striatal tissue (Chapter 2 - Figure **2.9**). We examined the interaction between TRPC5 and NCS-1 in cell culture by coimmunoprecipitation experiments. Crude membrane preps from HEK293 cells stably expressing TRPC5 were immunoprecipitated

using an anti-NCS-1 antibody and immunocomplexes probed with an anti-TRPC5 antibody. As shown in Figure **3.4,** a band corresponding to the predicted molecular weight of TRPC5 was present in the NCS-1 immunoprecipitation lane but not in the negative control lane (post-IP supernatant). Similarly, anti-NCS-1 antibody coimmunoprecipitated TRPC5 in rat brain lysates (data not shown [234]). Collectively, these data demonstrate that TRPC5 and NCS-1 exist in a protein complex in native tissue.

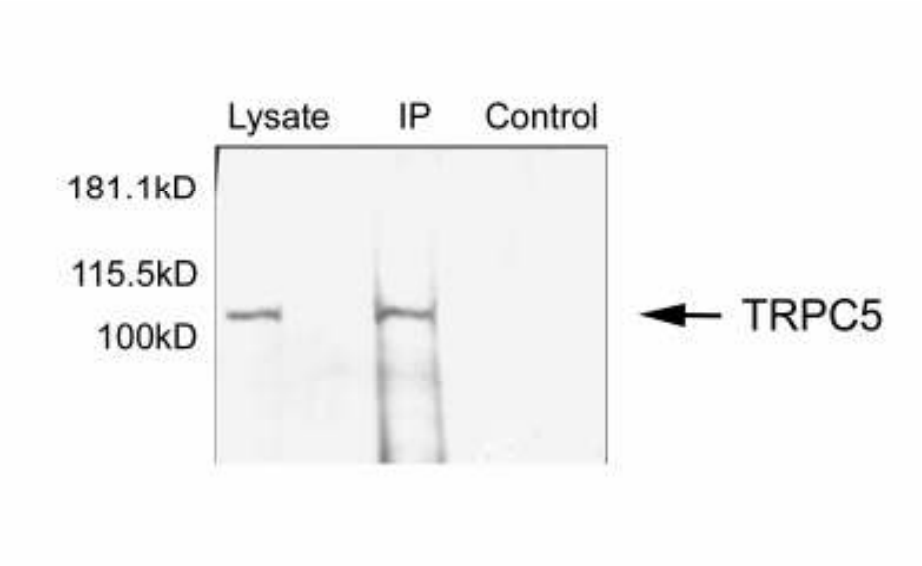

Figure **3.4**: **TRPC5 Interacts with NCS-1 in HEK293 Cells**

Anti-NCS-1 antibodies were used to immunoprecipitate NCS-1 from HEK293 cells stably expressing TRPC5. Immunocomplexes were separated via SDS-PAGE, and blots were probed with an anti-TRPC5 antibody generated in the McHugh laboratory. The position of TRPC5 is shown (Lysate). TRPC5 interacts specifically with NCS-1 (IP), and is not seen in the negative control.

3.3.3 Effects of NCS-1 and TRPC5 on Neurite Outgrowth

PC12 cells were tested for endogenous expression of NCS-1 by immunohistochemistry with an anti-NCS-1 antibody (Figure **3.5A**, **B**). NCS-1 expression in PC12 cells exhibited characteristic perinuclear staining [255] that extended into neuronal projections (Figure **3.5A**, **B**). To explore the role of NCS-1 in PC12 cell morphology, cells were transfected with a dominant negative (DN) form of NCS-1 containing the E120Q mutation, which is known to inactivate the third EF-hand and render the protein incapable of proper calcium binding [256]. DN-NCS-1 enhances neurite extension (Figure **3.5 C-F, K**), whereas overexpression of wild-type NCS-1 had little effect (Figure **3.5G,H,K**). These results suggest that NCS-1 is normally saturating and serves to inhibit neurite outgrowth. The effect of DN-TRPC5 was also explored in PC12 cells (Figure **3.5J,I,K**), which produced similar results. Overall, no significant effect on total neurite number was observed [234]. The effects of DN-NCS-1 and DN-TRPC5 are non-additive (Figure **3.5K**), which supports their involvement in a common pathway. Interestingly, DN-NCS-1 slightly increases the amount of TRPC5 at the plasma membrane (Figure **3.6A**), which suggests that endogenous NCS-1 has either no effect on or inhibits the rapid trafficking of TRPC5 to the cell surface. This finding does not fully explain the inhibitory effect of NCS-1 on neurite extension (Figure **3.5K**). Therefore, we needed to explore whether NCS-1 had a modulating effect on TRPC5 ion channel function in this context.

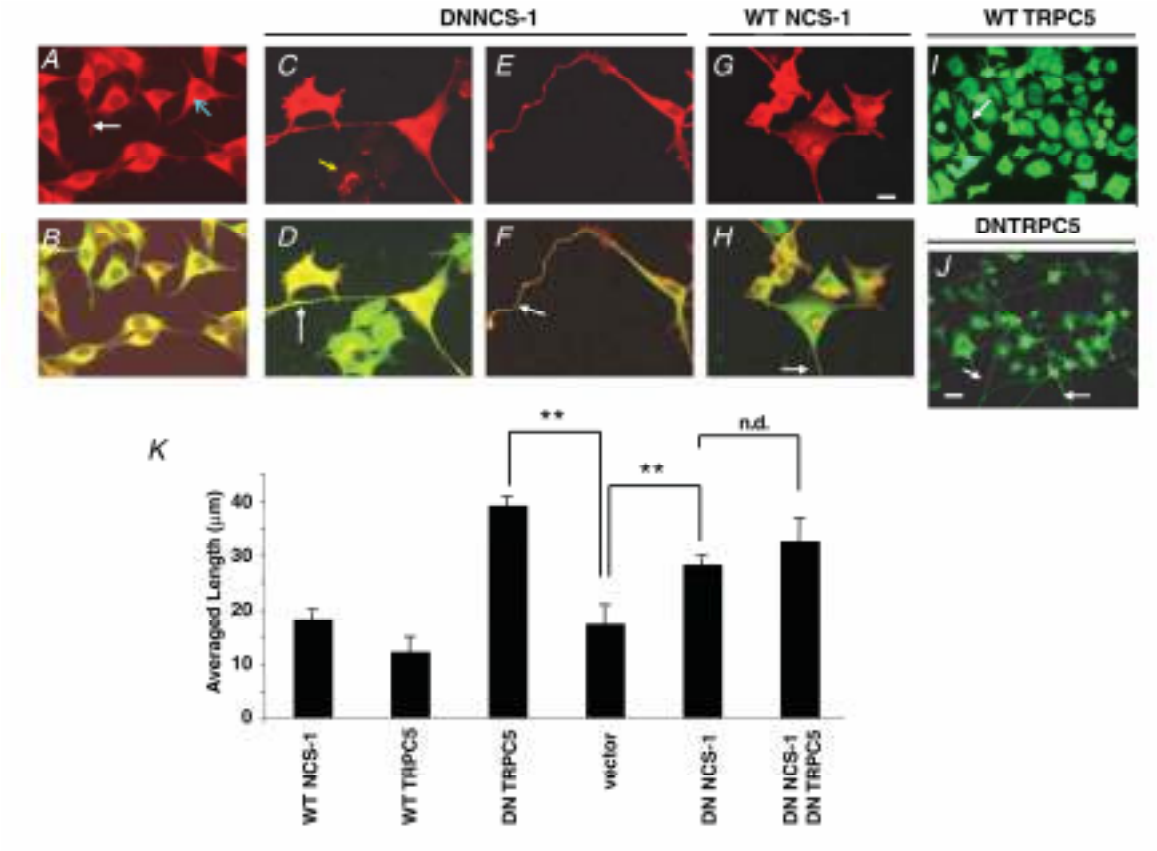

Figure **3.5**: **Overlapping Effects of TRPC5 and NCS-1 on Neurite Outgrowth (From Hui et. al., 2006 [234])**

(A-E) Images of PC12 cells exhibiting fluorescence after anti-NCS-1 antibody labeling (A-H), co-labeling for β-tubulin (B,D,F,H), or labeling for β-tubulin alone (I-J). (A.B) PC12 cells showing endogenous NCS-1 staining. (C-H) PC12 cells transfected with DN-NCS-1 (C-F), WT-NCS-1 (G,H), WT-TRPC5 (I) or DN-TRPC5 (J). Scale bar in G is 5 µm and applies to A-H; the bar in J is 10 µm and applies to I-J. White arrows point to neurite extensions. Blue arrow points to perinuclear NCS-1 staining. Yellow arrow points to untransfected cell. (K) Mean+/- s.e. mean (n=40, paired student's t-test) neurite length (in µm) for PC12 cells transfected with indicated cDNA constructs. Control is pcDNA3 vector without insert. ** $p \leq 0.001$, n.d., not different

To explore this possibility, a tetracycline-inducible HEK293 cell expression system (TRex-TRPC5) was employed. This cell system demonstrates functional TRPC5 signals that are clearly distinguishable from background [250] and endogenously expresses NCS-1 as demonstrated by Western blot [234]. TRPC5 activity was previously characterized using this system and was shown to be evoked by a multiplicity of signals, including GPCR agonists, store-depletion and external lanthanides including gadolinium [250]. Calcium imaging experiments demonstrated that DN-NCS-1 inhibits the activation of TRPC5 by any of these signals (Figure **3.6B,C**). This implies a role for NCS-1 in the regulation of TRPC5 function.

TRPC5 shows modest activation in response to 200 nM intracellular calcium [194, 250], which corresponds the calcium-binding range of NCS-1 [251]. To explore the possibility that the function of TRPC5 depends on the calcium-sensing ability of NCS-1, whole cell-patch clamp recordings were performed. Intracellular calcium levels could be controlled via the patch pipette. DN-NCS-1 inhibits TRPC5 current when the calcium concentration in the patch pipette is in the range of 300 – 600 nM, but has weak effects outside this range (Figure **3.6D**). These findings are consistent with TRPC5 dependence on NCS-1 as a calcium-sensor.

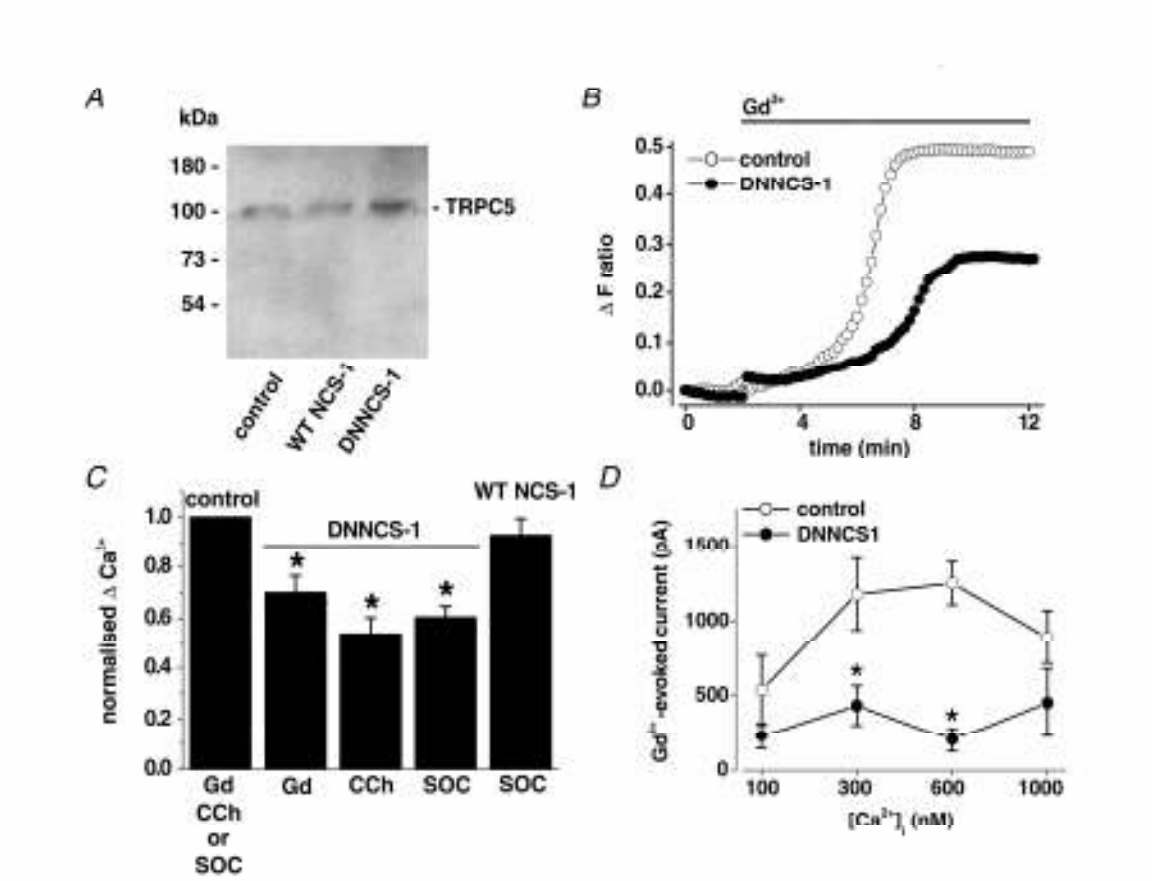

Figure **3.6**: **NCS-1 Affects TRPC5 Function at the Membrane (From Hui et. al., 2006 [234])**

(A) Representative Western blot from cell surface biotinylation experiments. PC12 cells were cotransfected with TRPC5 and either WT-NCS-1, DN-NCS-1, or nothing (control). Cell lysates were immunoprecipitated with NeutrAvidin, probed with anti-TRPC5 antibody. (B-D) Data from analysis of TRex-TRPC5 cells. (B) Illustrative examples of simultaneous measurements of intracellular calcium in two cells, one transfected with DN-NCS-1, the other untransfected (control). Calcium concentration is shown as the change in fura-PE3 fluorescence (F) ratio above baseline. TRPC5 activity was evoked by 0.1 mM gadolinium (Gd^{3+}). (C) As for (B) but normalized mean data ($n \geq 123$ cells from 6-11 independent experiments). Each experiment was paired, comparing the response to a TRPC5 activator in untransfected cells (control) or in cells transfected with either DN-NCS-1 or WT-NCS-1. Activators employed were: 0.1mM Gd^{3+} (Gd); 0.1 mM carbachol (CCh); or Ca^{2+}-influx after treatment with 1μM thapsigargin (SOC; store-operated channel). (D) Mean whole-cell patch-clamp data showing amplitudes of currents evoked at +80mV by 10 μM Gd^{3+} 10-min after starting whole-cell recording. The Ca^{2+} concentration in patch pipette is indicated on x-axis. For each concentration, currents were compared on the same day in cells transfected with DsRed2 (control) or DsRed2 plus DN-NCS-1 (n= 3-9 cells per point, *$p<0.05$, student's t-test).

3.4 Discussion

We provide evidence that NCS-1 acts as a direct protein binding partner of TRPC1 and TPRC5. TRPC1 and TRPC5 are known to form functional ion channels with one another [193, 194]. In yeast two-hybrid experiments both TRPC1 and TRPC5 were shown to interact with NCS-1. The interaction between TRPC5 and NCS-1 was further explored, and directed yeast two-hybrid assays showed the possibility of two binding sites of NCS-1 on TRPC5. These results were corroborated by GST-pulldown experiments in which the C-terminus of TRPC1 and two truncation fragments of the C-terminus of TRPC5 were able to bind NCS-1 but did not bind an unrelated GST-fusion protein (D2LIC3-GST) or GST alone. TRPC5 was also found to be present in a protein complex with NCS-1 in a HEK293 cell culture system, as well as in native rat brain tissue. TRPC1 is known to interact with NCS-1 in the context of the D2R-signalplex (chapter 2). NCS-1 and TRPC1 are known to directly interact with the D2R, and TRPC5 has a high probability of interaction as well [98, 121].

The NCS-1/TRPC5 interaction was further explored in collaboration with the laboratories of David Beech, Jamie Weiss, and Damian McHugh at the University of Leeds and the University of Sheffield [234]. TRPC5, functioning as a homotetrameric channel, has been shown to affect growth cone morphology of hippocampal neurons [200]. Using a dominant-negative NCS-1 mutant that cannot bind calcium (E120Q), activation of TRPC5 and subsequent neuronal extension in a PC12 cell line were explored. In the presence of mutant NCS-1, neurite extension is enhanced in PC12 cells, implying that wild-type NCS-1 is normally saturating and serves to inhibit neurite

outgrowth. In addition, dominant-negative NCS-1 was found to inhibit current through TRPC5 when extracellular levels of calcium ranged between 300 and 600 nM. These results suggest that NCS-1 forms a direct and functional protein partnership with TRPC5, with NCS-1 regulating the effectiveness of TPRC5 to respond to activating signals [234]. In regard to neurite extension, NCS-1 provides a negative feedback mechanism to retard neuronal outgrowth in response to calcium transients mediated by TRPC5 [234].

Further investigation is needed to determine what impact the functional relationship between NCS-1/TRPC5, as well as NCS-1/TRPC1 has on the regulation and signaling properties of the D2R signalplex. Individual protein constituents of the D2R signalplex most likely exist in a dynamic equilibrium with one another, where DRIPs compete for limited binding space on the D2R. Depending on the intracellular environment immediately surrounding the signalplex, some proteins may bind the D2R differentially, allowing other DRIPs to usurp their interaction sites on the D2R. In addition, certain DRIPs may bind to the D2R very strongly, and may influence the D2R in a more static and stable fashion than other proteins. Protein modifications, such as the binding of calcium or palmitoylation, may affect the confirmation, trafficking, and binding properties of DRIPs [98, 241]. It may be found that in specific cellular milieus, DRIPs such as NCS-1 and the TRPCs, bind preferentially to one another, rather than the D2R. This competition for binding partners between the three proteins would allow for differential signaling consequences downstream of both the receptor and ion channel. NCS-1, when bound to the D2R, is known to inhibit D2R desensitization and enhance cAMP signaling downstream of the receptor [121]. In contrast, when NCS-1 is bound to TRPC5, the interaction inhibits currents through the channel that are responsible for

calcium transients important in growth cone guidance [234]. NCS-1 binding to the D2R and TRPCs is known to be calcium dependent [121, 234]. Determining the K_D and calcium dependency of each interacting pair will further enhance our knowledge of how proteins behave in a complex system such as the D2R signalplex.

NCS-1 protein has been found to be upregulated in the dorsolateral prefrontal cortex of patients with schizophrenia and bipolar disorder [107, 240]. This increased amount of NCS-1 protein is thought to affect signaling through the D2R; enhancing dopaminergic transmission by inhibiting desensitization of the receptor and contributing to the diseased state. Knowing that NCS-1 also has regulatory and functional effects on TRPC5 may provide clues to other signaling pathways that contribute to dysfunction in disease states. An increase of NCS-1 protein may affect neurodevelopment and the formation of synapses between neurons by impeding calcium transients through TRPC5 that are responsible for proper neurite outgrowth. By perturbing interactions between NCS-1, TRPC5, and the D2R through alterations of protein levels, general calcium homeostasis may be changed in the cell, resulting in dysfunction and potentially contributing to a diseased state. Studying networks of proteins, rather than the individual proteins themselves, may provide greater insight into the pathological process of complex diseases. Additional targets for therapeutic intervention can be identified and unintended consequences of intervention, such as unwanted side effects, could be better understood and prevented.

Chapter 4

Investigation into the Potential Role of zDHHC4 in the Differential Trafficking of D2S and D2L Isoforms

4.1 Introduction

D2Rs are known to be alternatively spliced and expressed as two major isoforms, D2S and D2L [4]. The two isoforms differ by the presence of an alternatively spliced exon six in the D2L that encodes a 29 amino acid sequence present in the third intracellular loop of the receptor [4, 34]. These two isoforms appear to be identical in regard to their ligand binding profiles [4, 18]. Evidence has shown that although the D2S and D2L are closely related, they exhibit differential trafficking and sorting in neurons and are responsible for diverse roles in dopaminergic signaling [4, 33, 36]. D2S and D2L isoforms show distinct expression patterns in the primate brain [220]. The D2L is most prominently expressed in neurons of the striatum and nucleus accumbens, whereas the D2S is predominantly expressed in the cell bodies and axons of neurons that project from the mesencephalon and hypothalamus [220]. These findings imply that D2L are mainly distributed post-synaptically, in contrast to the apparent pre-synaptic distribution of D2S [220]. Evidence from D2R knockout (KO) mice corroborates this finding. D2L KO strains, which exhibit D2S expression in the absence of D2L expression, retain dopamine autoreceptor functions such as inhibition of dopamine synthesis and release [34, 58]. These responses are absent from complete D2R KO strains [49-51]. In addition to these

observations, D2S and D2L receptors have been shown to be differentially trafficked in various cell systems [36, 116, 257].

Although there is abundant evidence for differential trafficking of the D2R splice isoforms, the mechanisms by which this occurs remain largely unknown. D2S and D2L proteins interact with the same cohort of DRIPs and DRAPs, with few exceptions [98]. Studies have shown the D2S and D2L can bind distinct sets of G-proteins, which affects signaling cascades downstream of the receptor [258]. Binding to different sets of G-proteins is not likely to be responsible for differences in trafficking seen between the two receptor subtypes, due to the fact that D2S and D2L signal through overlapping sets of G-proteins [123, 259-261]. Heart fatty acid binding protein (H-FABP) has been discovered to interact with the D2L, but not D2S, via a yeast two-hybrid screen [116]. In NG108-15 cells, overexpressed and endogenous H-FABP was found to colocalize with D2L immunostaining in the perinuclear region associated with the Golgi apparatus [116]. In these same cells, the D2S did not colocalize with H-FABP and was found to be expressed primarily at the plasma membrane [29]. D2L was found to colocalize with H-FABP in rat striatal neurons [29]. These findings imply a role for H-FABP in the separate trafficking patterns of the D2S and D2L. It is likely that additional factors are necessary for the proper localization of D2R isoforms in their native environments.

Recently, a split-ubiquitin screen using the entire D2R as bait uncovered a novel interaction between the D2R and zDHHC4 [262]. Directed yeast two-hybrid assays localized the binding domain of zDHHC4 to the second intracellular loop of the D2R [262]. zDHHC4 is one member of a newly identified family of potential mammalian palmitoyltransferases (PATs) [214]. zDHHC4 has not yet been confirmed as a functional

PAT. It is highly likely that this protein is a PAT due to the presence of a highly conserved DHHC domain (stands for Asp-His-His-Cys) [214]. The DHHC domain is highly conserved in known PATs and is assumed to contain the catalytic site of the enzyme [214]. PATs are a group of enzymes discovered in yeast. They are thought to be responsible for the reversible thioester linkage of palmitate to specific cysteine residues in a protein (S-palmitoylation) [212, 213]. Palmitoylation has been implicated in aspects of protein trafficking, organelle inheritance, and vesicle fusion [211].

D2Rs have been known to be palmitoylated, although the cysteine residues that undergo palmitoylation in these proteins remain unknown [218, 219]. The D2L contains two cysteines in its third intracellular loop that are absent from the D2S. Palmitoylation prediction software which compares known palmitoylated protein sequences to a query sequence indicates that at least one of these two cysteines in the D2L is highly likely to undergo palmitoylation [263]. Specific palmitoylation concensus sequences have not been precisely established [263]. We hypothesize that palmitoylation of this D2L-specific cysteine will contribute to the differential trafficking of the D2S and D2L isoforms. We employed a cell surface biotinylation strategy to investigate the effects of ectopically expressed zDHHC4 on the cell surface expression of D2S and D2L in HEK293 cells.

4.2 Experimental Procedures

4.2.1 Identification of Potential Palmitoylation Domains in D2R

Recently, Zhou et al. developed software to predict which cysteines in a protein could be palmitoylated [263]. The software is available for public use in a web-based format at http://bioinformatics.lcd-ustc.org/css_palm/. We utilized this software to determine which cysteines residues of the D2L and D2S proteins were most likely to be palmitoylated. Amino acid sequences of the D2L (accession #AAB26274) and D2S (accession #NP_057658) were used in the determination of potential palmitoylation sites.

4.2.2 Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. HEK293 cells stably expressing FLAG-tagged D2L (long-splice isoform) dopamine receptors (HEK293-D2L) were provided by Dr. Mark von Zastrow (University of California San Francisco). HEK293 cells stably expressing FLAG-tagged D2S (short-splice isoform) dopamine receptors (HEK293-D2S) were provided by Dr. Jonathon Javitch (Columbia University). HEK293-D2L and HEK293-D2S cells were maintained in DMEM supplemented with 10% fetal bovine serum and 300 µg/ml Geneticin (Invitrogen, Grand Island, NY). Cells were transiently transfected with either enhanced green fluorescent protein (EGFP)-tagged full length mouse zDHHC4, myc-tagged full length mouse zDHHC4, or a GFP control plasmid. Cells were transfected using the Effectine transfection reagent (Qiagen, Valencia, CA) under conditions described by the manufacturer.

4.2.3 Cell Surface Biotinylation Assays

Cell surface labeling assays were performed using a modification of the cleavable biotin method described previously by Vickery and von Zastrow [225]. Briefly, cells were labeled with 1 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford IL) for 30 min at 4^{o}C. Cells were washed three times with ice cold phosphate buffered saline (PBS) to quench and remove unbound biotin, and crude membrane fractions prepared as described previously [32]. Biotin-labeled D2R proteins were immunoprecipitated using either polyclonal anti-FLAG antibodies (Sigma) or polyclonal anti-D2R antibodies (Santa Cruz). Complexes were washed repeatedly with ice cold PBS, resolved by SDS-PAGE, and transferred to a PVDF membrane. The biotinylated proteins were complexed with streptavidin conjugated horseradish peroxidase (Rockland) and visualized by enhanced chemiluminescence with an ECL Plus kit.

4.3 Results

4.3.1 Identification of Palmitoylation Sites on the D2R

The D2R was found to interact with zDHHC4 in a split-ubiquitin yeast two-hybrid screen [262]. The interaction was confirmed by coimmunoprecipitation of zDHHC4 with the D2S and D2L in a HEK293 cell culture system [119]. Further experiments revealed that the binding site of zDHHC4 localized to the second intracellular loop of the D2R [262]. This portion of the D2R was found to interact with the second intracellular loop of the zDHHC4, which contains the DHHC domain [119]. The second intracellular loop is identical between the D2S and D2L forms of the

receptor. However, due to additional cysteine residues present in the D2L that are not found in the D2S, the potential for differential palmitoylation of the two isoforms exists.

To test this hypothesis, we utilized a web-based palmitoylation prediction software program (http://bioinformatics.lcd-ustc.org/css_palm/) to predict which cysteine residues in the D2R proteins were likely to be palmitoylated. The D2L contains twelve total cysteine residues, two of which are absent in the D2S. Three cysteines in the D2L were predicted to undergo palmitoylation: C#56, C#244, and C#443 (Table **4.1**). Of the potentially palmitoylated cysteines, only C#244 was not present in the D2S. This *in silico* assessment of potential palmitoylation sites provides evidence that the D2S and D2L isoforms can be differentially palmitoylated. Further investigation using molecular and biochemical methods will need to be employed to confirm that these identified cysteine residues undergo S-palmitoylation.

Table **4.1**: **Cysteine Residues in D2R Available for Palmitoylation**

Prediction program http://bioinformatics.lcd-ustc.org/css_palm/ [263]
C – Cysteine Residue; TM – Transmembrane Region, EC- Extracellular loop, IC- Intracellular loop, C-term – Carboxyl terminus.

Position of C-residues in D2L	Location	Also present in D2S isoform?	Predicted to be palmitoylated?
C # 56	TM1	Yes	**YES**
C# 107	EC2	Yes	No
C# 118	TM3	Yes	No
C# 126	TM3	Yes	No
C# 168	TM4	Yes	No
C# 182	EC3	Yes	No
C# 244	IC3	**NO**	**YES**
C# 253	IC3	**NO**	No
C# 385	TM6	Yes	No
C# 399	EC4	Yes	No
C# 401	EC4	Yes	No
C# 443	C-term	Yes	**YES**

4.3.2 Cell Surface Biotinylation Assay

To determine whether zDHHC4 expression causes differences in membrane trafficking of D2S and D2L, we employed a cell surface biotinylation assay to assess the effect of co-expression of zDHHC4 in HEK293 cell lines stably expressing D2S- or D2L-FLAG tagged receptors. Wild-type, D2S-, and D2L-FLAG expressing HEK293 cell lines were transiently transfected with myc-tagged mouse zDHHC4. Untransfected cell lines were used as controls. Cell surface proteins were biotinylated by incubation with NHS-SS-biotin. Biotinylated membrane proteins were immunoprecipitated using an anti-FLAG polyclonal antibody (Sigma). Immunocomplexes were separated via SDS-PAGE

and immunoblotted with streptavidin conjugated to horseradish peroxidase to visualize cell surface expressed D2Rs. As shown in Figure **4.1**, expression of zDHHC4-myc did not affect cell surface expression of D2S-FLAG. Since D2S was not detected at the plasma membrane whether it was expressed with zDHHC4-myc or not, it cannot be determined if zDHHC4 would promote internalization of the D2S in this context. However, co-expression of zDHHC4-myc clearly decreased the amount of D2L-FLAG at the plasma membrane in comparison to untransfected controls (Figure **4.1**). This effect was not seen when cell lines were transfected with a control plasmid expressing GFP alone (data not shown). This provides preliminary evidence that the interaction between zDHHC4 and D2L functionally differs from the interaction between zDHHC4 and D2S. The zDHHC4/D2R interaction may play an interesting role in the differential trafficking of D2R isoforms.

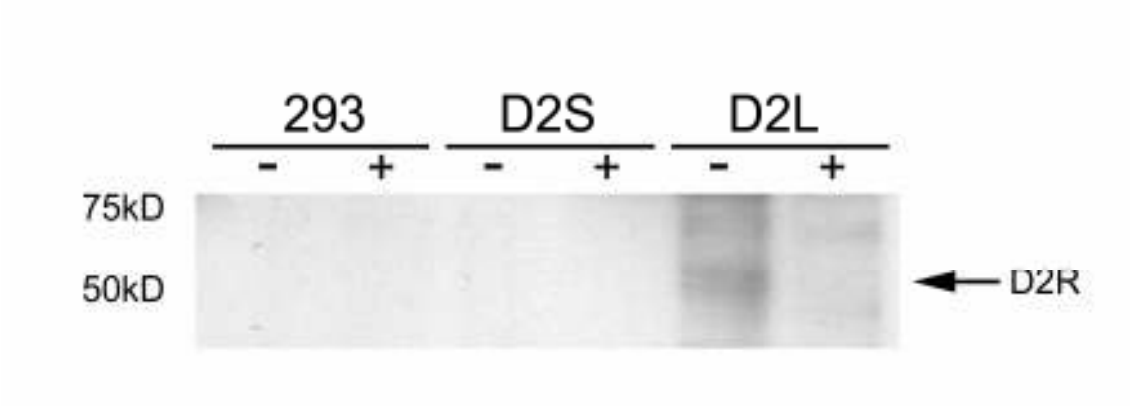

Figure **4.1**: **zDHHC4/D2R Interaction Promotes Internalization of D2L**

A cell surface biotinylation assay was utilized to examine the effect of zDHHC4 expression on plasma membrane localization of D2S and D2L. Wild-type HEK293, HEK293-D2S and HEK293-D2L cell lines were transfected with zDHHC4-myc (+). Untransfected cell lines were used as control (-). Wild-type HEK293 cells were included to demonstrate antibody specificity. Cell surface proteins were biotinylated by incubation with NHS-SS- biotin. Biotinylated membrane proteins were immunoprecipitated using an anti-FLAG antibody. Biotinylated D2R proteins were visualized by immunoblotting with strepavidin conjugated to horseradish peroxidase. -, untransfected cells, +, cells transfected with zDHHC4-myc. Arrow indicates position of D2R monomer.

4.4 Discussion

These results provide encouraging evidence supporting the hypothesis that the interaction between zDHHC4/D2R has different functional consequences for D2S and D2L, the two splice variants of the D2R. A palmitoylation prediction software identified three cysteine residues that are highly likely to be S-palmitoylated in the D2R – C #56, C#244, and C#443. Of the three residues, only C# 244 is present in the D2L isoform but absent from the D2S. This provides initial evidence that the D2R splice variants have the potential to be differentially palmitoylated. Although the D2Rs are known to be palmitoylated, the residues that undergo palmitoylation remain unknown [218, 219].

Further experiments with D2R cysteine mutants are necessary to determine which amino acids are actually palmitoylated. It will be interesting to determine which residues of the D2R are palmitoylated once the PAT responsible for the post-translational modification of the D2R is positively identified.

Cell surface biotinylation experiments demonstrated a difference in plasma membrane expression of D2L when zDHHC4 was co-expression with this variant. In HEK293-D2S cells, D2S proteins are not detectably expressed at the plasma membrane. Co-expression of zDHHC4 in these cells did not increase D2S localization to the cell surface. In contrast, HEK293-D2L cells demonstrated considerable D2L expression at the cell surface. Co-expression of zDHHC4 in this instance caused a dramatic decrease in D2L localization at the plasma membrane, implying that the presence of zDHHC4 in this system is sufficient to produce a shift in D2L localization from the cell surface to intercellular sites. There may be multiple explanations for these effects. First, a functional effect of the zDHHC4/D2S may not be detectable in HEK293-D2S cells because another protein is preventing the D2S from trafficking to the cell membrane. In that case, the functional consequence of the zDHHC4/D2S interaction remains unknown due to the interfering protein. Second, the co-expression of zDHHC4 in HEK293-D2L cells may affect another aspect of the D2R, such as globally suppressing D2R protein expression in the cell line. Finally, zDHHC4 may palmitoylate the D2L on a cysteine residue not present in the D2S that promotes the internalization of the D2L. Further experimentation is needed to determine if the effects demonstrated in this preliminary experiment are due to differential palmitoylation of the D2S/D2L isoforms or to other

causes. First and foremost, the zDHHC4 will have to be confirmed as an active PAT, and the D2R will have to be demonstrated to be one of its substrates.

Other GPCRs undergo palmitoylation. These include serotonin, nicotinic, GABA, and AMPA glutamate receptors [210, 264-266]. The AMPA receptor subunits (GluR1, GluR2, GluR3, and GluR4) interact with the confirmed PAT, Golgi apparatus specific protein with a DHHC zinc finger domain (GODZ) [266]. GODZ, also known as zDHHC3, was determined to upregulate palmitloylation of the AMPA GluR2 at a cysteine in the second transmembrane domain of the protein [210]. Coexpression of GODZ and GluR2 in HEK293T cells promotes excess palmitoylation of this cysteine. This leads to the accumulation of the AMPA GluR2 in the Golgi, and a subsequent reduction of the receptor at the plasma membrane [210]. Co-expression of a GODZ mutant with a nonfunctional DHHC domain had no effect on the cell surface trafficking of GluR2 in HEK293T cells, indicating this internalization of the receptor was specific to the properties of the functional GODZ protein. zDHHC3 is a member of the same family as zDHHC4, so the question remains as to whether zDHHC4 functionally trafficks the D2R in a manner similar to the trafficking of AMPA receptors by GODZ.

It is of interest to determine if defects in D2R palmitoylation contribute to aberrant dopaminergic signaling states. Genetic association between zDHHC4 and various diseases caused by pathological D2-mediated neurotransmission can be explored. Intriguingly, human zDHHC8, a related potential PAT, is known to localize to a schizophrenia susceptibility region on chromosome 22 [267-269] zDHHC8 has been identified to interact with the D2R in a yeast two-hybrid screen, but its interaction with the receptor has not been explored further [119]. Also of interest, a common D2R

polymorphism that causes an amino acid change from serine to cysteine at residue 311 has been found to correlate with a predisposition to schizophrenia in a number of studies [270, 271]. It would be exciting to determine if this novel cysteine variant could undergo anomalous palmitoylation, and if dysregulation of D2R palmitoylation could contribute to the pathophysiology of schizophrenia.

Chapter 5

Proteomic Analysis of the D2R Signalplex

5.1 Introduction

Although dopaminergic augmentation has been the mainstay of treatment in schizophrenia, no mutations in dopamine receptor genes have been discovered in any patients to date [272]. Dopamine receptors are known to function in a large macromolecular complex known as the signalplex [98, 273]. These signalplexes are comprised of a collection of proteins that either interact directly with the dopamine receptor (dopamine receptor interacting proteins, DRIPs), or are associated with the receptor through secondary protein-protein interactions (dopamine receptor associated proteins, DRAPs – [98]). DRIPs and DRAPs interact stably, transiently, or dynamically with the receptor and regulate its localization, signaling properties, and life cycle [98]. This subset of proteins, which are intimately involved with the D2R, can potentially serve as novel drug targets and genetic markers for schizophrenia. Identification and characterization of DRIPs and DRAPs will therefore be important to the understanding of the pathophysiology of schizophrenia and related diseases.

Here we employ a novel method to identify new DRIPs and DRAPs. Using available bioinformatics resources, we mined the literature and protein-protein interaction databases for novel proteins that have high biological probability of interacting with the D2R. We have identified a subset of proteins that, due to their interaction with

previously identified DRIPs, have a high likelihood of interacting or associating with the D2R itself. We have tested these candidates for direct interaction with the D2R via directed yeast two-hybrid and GST-pulldown assays, and for native association with the D2R via coimmunoprecipitation experiments. Using directed yeast two-hybrid and GST-pulldown approaches, we can test candidate proteins for a direct interaction with the intracellular portions of the D2R and begin to gather support that the proteins are DRIPs. These DRIPs can be confirmed via coimmunoprecipitation with the D2R signalplex from cellular and native tissue lysates, giving further evidence that the candidate proteins interact with the D2R in a cellular environment. In addition, coimmunoprecipitation techniques can be used to identify candidate proteins that are associated with the D2R but do not directly bind the receptor. Employing these methods, we found that candidate DRIPs have the highest probability of interacting with the D2R if they belong to the same subfamily as a previously identified DRIP.

5.2 Experimental Procedures

5.2.1 Literature Search

PubMed (http://www.ncbi.nlm.nih.gov) and the Database of Interacting Proteins (http://dip.doe-mbi.ucla.edu) were queried to identify known dopamine D2 receptor interacting proteins (DRIPs; refer to Table **1.3** for extensive list). Also included in this search were unpublished DRIPs confirmed in our laboratory. From this list of published and well-characterized DRIPs, we queried each individual DRIP to determine its unique

assemblance of protein interactors. From this subset of proteins, we identified fifteen candidate DRIPs that have high biological plausibility of interacting with the D2R (Table **5.1**). Biological plausibility was estimated by the candidate DRIP's known function, overlapping expression profile with the D2R, and interaction with a previously characterized DRIP.

Table **5.1**: **Candidate DRIPs Identified by Database Query.**

The list was compiled by searching PubMed and the Database of Interacting Proteins for previously published DRIPs. These DRIPs were then individually queried and each cohort of their interacting proteins was identified. Fifteen candidate proteins with high biological plausibility were identified for further characterization as potential DRIPs or DRAPs.

Candidate DRIP	Known DRIP Interactor	Biological Plausibility	Reference
DynaminIIab	D2R	• Coimmunoprecipitates with D2R signalplex • Not identified as DRIP	[112]
FLJ12242	D2R	• Out-of-frame FLJ12242 originally identified in Y2H screen • Potential Potassium Channel	[145]
GRK2/3/5/6	Dynamin NCS-1 Calmodulin	• Phosphorylates D2R • Coimmunoprecipitates with D2R signalplex • Not identifed as DRIP	[121]
Homer1/2	TRPC1	• Established role in trafficking of GPCRs	[227]
IP3R	NCS-1 4.1N	• Potential link between D2R and intracellular calcium stores	[136, 274]
PI4Kinaseβ	NCS-1	• Potential link between D2R and lipid signaling pathways	[275]
TRPC4/5	TRPC1	• TRPC1/4/5 interact to form functional ion channels with distinct properties	[193]
TRPC3/6/7	-----	• TRPC3/6/7 are remaining members of the TRPC subfamily • May interact with TRPC1 in embryonic brain	[178]

5.2.2 Directed Yeast-Two Hybrid Screen

All constructs were generated by PCR amplification and verified by automated DNA sequencing. Five bait constructs encoding the first (D2IC1, amino acids 61-71), second (D2IC2, amino acids 131-151), and third intracellular loops (both short and long isoforms - D2IC3-S, amino acids 211-344; D2IC3-L, amino acids 211-373) and carboxyl terminal tail of the D2R (D2-tail, amino acids 430-443) were subcloned into the DNA-binding domain vector pAS2-1 (BD Biosciences Clontech) and used as bait. Full-length DynaminIIab, FLJ12242, GRK2, GRK3, GRK5, GRK6, Homer1, Homer2, and PI4kinaseβ, and the carboxyl termini of TRPC3, TPRC4, TRPC5, TRPC6 and TPRC7, were constructed in the DNA-activation domain vector pACT2 (BD Biosciences Clontech) and used as prey. Please refer to Table **5.2** for detailed prey construct information. Bait and prey constructs were sequentially transformed into yeast strain *MaV103* by standard lithium acetate protocol [113]. Transformants were identified via growth on -leu/-trp selection plates. Protein-protein interactions were detected using β-galactosidase filter lift assays as previously described [113]. Positive interactions were identified by monitoring β-galactosidase activity over a period of 8-12 hours.

Table **5.2**: **Directed Yeast-Two Hybrid Prey Construct Information (pACT2)**

Protein Name	GenBank Accesion#	Species	Amino Acids	Restriction Enzyme Sites (5'/3')
DynaminIIab	L25605	Rat	1-870	XmaI/EcoRI
FLJ12242	BC025403	Human	1-290	EcoRI/KpnI (klenow)
GRK2	M34019	Bovine	1-689	BamHI/EcoRI
GRK3	BC036797	Human	1-387	EcoRI/XhoI
GRK5	L15388	Human	1-590	BamHI/EcoRI
GRK6	L16862	Human	1-576	EcoRI/XmaI
Homer1	BC015502	Human	1-354	NcoI/EcoRI
Homer2	BC012109	Human	1-343	EcoRI/XhoI
PI4Kinaseβ	BC040300	Human	1-801	XmaI/XhoI
TRPC3	NM_003305	Human	660-848	EcoRI/XhoI
TRPC4	AF421359	Human	621-893	EcoRI/SacI
TRPC5	AF054568	Human	619-973	EcoRI/SacI
TRPC6	NM_004621	Human	710-931	EcoRI/XhoI
TRPC7	NM_020389	Human	670-862	EcoRI/XhoI

5.2.3 Glutathione S-Transferase Pulldown Assay

Glutathione S-transferase (GST)-D2IC2 fusion protein (D2IC2-GST) and D2LIC3-GST fusion protein were constructed in the bacterial expression vector pGEX-4T-1 vector (Amersham Pharmacia). Full-length GRK2, GRK3, GRK6, Homer1 and Homer2 were constructed in the pET30C vector (Amersham Pharmacia) to generate S-tagged proteins. All fusion proteins were induced in *E. coli* strain BL-21 (DE3). D2IC2-GST and D2LIC3-GST fusion proteins were purified using glutathione-sepharose (Amersham) according to the manufacturer's instructions. GST pull-down assays were performed as described previously [113]. Eluted proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) filter and probed with an anti-S-tag

polyclonal antibody conjugated to horseradish peroxidase (1:5000 dilution; Novagen). Immunoreactivity was detected by enhanced chemiluminescence with an ECL Plus kit (Amersham).

5.2.4 Cell Culture

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS; Gibco). HEK293 cells stably expressing FLAG-tagged D2L (long-splice isoform) dopamine receptors (HEK293-D2L) were provided by Dr. Mark von Zastrow (University of California San Francisco). HEK293 cells stably expressing FLAG-tagged D2S (short-splice isoform) dopamine receptors (HEK293-D2S) were provided by Dr. Jonathon Javitch (Columbia University). HEK293-D2L and HEK293-D2S cells were maintained in DMEM supplemented with 10% fetal bovine serum and 300ug/ml Geneticin (Invitrogen, Grand Island, NY).

5.2.5 Crude Membrane Preparations and Coimmunoprecipitation assay

Confluent HEK293, HEK293-D2L, and HEK293-D2S cells were washed twice with 5mL phosphate buffered saline (PBS) and incubated for five minutes in 2.5mL PBS supplemented with a mammalian protease inhibitor cocktail (PBS+PIs; Sigma-Aldrich). Cells were subsequently scraped, homogenized and centrifuged at 4000rpm for 30 minutes at 4^{o}C. The pellet was discarded, and the supernatant transferred to a fresh tube

for ultracentrifugation at 32,5000rpm for one hour at 4^{o}C. The supernatant was discarded, and the pellet resuspended in PBS+PIs and sonicated three times in fifteen to twenty second bursts. Sonicated membranes were solubilized overnight with nutation at 4^{o}C in an equal volume of lysis buffer containing 1% Triton-X-100 (Sigma-Aldrich). Insoluble membrane constituents were pelleted at 13,000rpm for 10 minutes at 4^{o}C, and the supernatant was collected. Protein concentration was determined using a Bradford protein assay (BioRad). Immunoprecipitations of D2R protein complexes were performed using either a monoclonal (M2 or M5) or polyclonal rabbit anti-FLAG antibody (Sigma-Aldrich).

Striatal and cortical tissues were isolated from 10-day-old Sprague-Dawley rats. Briefly, rat pups were decapitated and striatal and cortical tissues were dissected and resuspened in Hanks buffered saline solution (HBSS). Crude membranes were prepared and membrane proteins solubilized as previously described [112]. Immunoprecipitation of D2R complexes was performed using a goat polyclonal anti-D2R antibody (Santa Cruz Biotechnology).

Immunocomplexes were separated by SDS-PAGE, transferred to a PVDF filter, and the filter sequentially probed with antibodies against various candidate DRIPS: polyclonal rabbit anti-PI4Kinaseβ antibody (Upstate), monoclonal anti-IP3R antibody (generous gift from Dr. Sol Synder, Johns Hopkins University), polyclonal rat anti-Homer1 antibody (Chemicon) and polyclonal rabbit anti-Homer1 antibody (Santa Cruz). Peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Immunoreactivity was detected using an ECL Plus kit.

5.3 Results

5.3.1 Literature Search

Through database query, fifteen candidate DRIPs were identified for testing for direct binding of the D2R (Table **5.1**). Each protein was chosen for its biological plausibility of interacting with the D2R, which was determined by the candidate DRIP's known function, overlapping expression profile with the D2R, and interaction with a previously characterized DRIP.

DynaminIIab was chosen for further investigation due to its previous identification as a DRAP [112]. DyanminIIab has been shown to coimmunoprecipitate with the D2R and regulate internalization of D2R, but has not been shown to directly interact with the receptor itself [112].

FLJ12242 was identified as a candidate DRIP in our laboratory in a yeast-two hybrid screen of a cDNA brain library using the D2IC2 as bait [276]. The sequence of the FLJ12242 clone identified in the screen was not intact, so a full-length EST was procured from the I.M.A.G.E. consortium in order to confirm interaction with the D2R. FLJ12242 was identified as a hypothetical protein with homology to putative potassium channel tetramerization domains which could potentially function as an ion channel.

G-protein Receptor kinases (GRK) are known to phosphorylate GPCRs and regulate their signaling properties [160, 277, 278]. In addition, GRKs interact with numerous neuronal calcium sensors, including the DRIPs NCS-1 and calmodulin [121, 279]. GRK2 was found to coimmunoprecipitate with the D2R from HEK293-D2L cells,

indicating its status as a DRAP [121]. GRK2/3/5/6 were chosen for further investigation to determine if these proteins exerted their functions as direct interactors of the D2R.

Homer proteins are known to be components of the metabotropic glutamate receptor 1 (mGLUR1) signalplex, another GPCR which is important in neurologic signaling pathways [280-283]. Homer1 proteins exist as both a short and a long isoform [284, 285], and protein translation can be induced by exposure to antipsychotics, which are known to have effects at the D2R [286, 287]. Homer1 also interacts with the DRIP, TRPC1 [227]. In addition, Homer1 and Homer2 proteins have been shown to have important functions in pathways known to be influenced or modulated by dopaminergic signaling [288-290]. Homer1 knockout mice show evidence of endophenotypes seen in schizophrenia [291], and Homer2 knockout mice have deficits in alcohol processing and reward [292]. Taken together, these proteins have strong biological plausibility for interaction with or regulation of the D2R

The inositol (1,4,5) trisphosphate receptor (IP3R) plays an important role in the regulation of intracellular calcium stores [293]. D2R receptors are known to be sensitive to intracellular calcium levels [273, 294], and interact directly with a number of calcium sensing proteins (CAPS and NCS-1[121, 128]). In addition, IP3Rs are known to interact with the DRIPs 4.1N, calmodulin, and NCS-1 [136, 274, 295].

PI4Kinaseβ catalyzes the initial synthesis process of lipid regulators with important cellular functions [296]. PI4Kinaseβ interacts directly with the DRIP NCS-1 [275, 297, 298]. Association of PI4Kinaseβ with the D2R would link dopaminergic signaling to intracellular lipid signaling pathways.

TRPCs are non-selective, non-voltage gated ion channels which are responsible for a myriad of signaling functions in the cell [178]. TRPC1 was identified as a DRIP in a yeast two-hybrid screen of a human brain cDNA library using the D2IC2 as bait, and was later confirmed to be a bona-fide DRIP (Chapter 2). Discrepency remains as to whether TRPC1 can form functional homomeric ion channels, and it is thought that TRPC1 heterotetramerizes with TRPC4 or TPRC5 in order to form functional channels with distinctive properties [193, 194]. TRPC1 has also been found to interact with TRPC3 in the embryonic mammalian brain [193]. Due to the finding that TRPC1 is a legitimate DRIP, and its promiscuous nature of interacting with members of its immediate subfamily, all mammalian TRPC proteins were tested for interaction with the D2IC2 in the directed yeast two-hybrid screen.

5.3.2 Directed Yeast-Two Hybrid Screen

Fifteen candidate DRIPs were identified through query of PubMed and the Database of Interaction Proteins (Table **5.1**). Fourteen candidate proteins were subcloned into the pACT2 vector (Table **5.2**) and used as prey in a directed yeast-two hybrid screen against the intracellular portions of the D2R. Full-length cytosolic proteins were subcloned into the pAS2.1 bait vector. Highly hydrophobic proteins, such as the TRPCs, were truncated, and the fragment most likely to interact was subcloned into the pAS2.1 vector and utilized in the screen (Table **5.2**). The IP3R was excluded from this preliminary screen because of its extremely hydrophobic nature. Candidate DRIPs did not exhibit autologous β-galactosidase activity. Only the D2IC1 construct showed slight

autoactivation of β-galactosidase, identified by a light blue hue in individually transformed yeast colonies. Each construct pairing tested was replicated by at least five independent yeast cotransformations. A positive result was identified by comparison to the intensity of β-galactosidase acitivity of a previously identified DRIP (Figure **5.1**). Interaction of a candidate DRIP with D2IC1 was scored positive if the β-galactosidase activity was greater than that observed in D2IC1 single transformants. The results of the directed yeast two-hybrid screen are depicted in Table **5.3**. FLJ12242 was screened against the D2IC2 because this was the segment of the receptor that pulled out FLJ12242 in the original yeast two-hybrid screen. TRPC1 and Homer1 were among the first constructs screened for interaction with the D2R. Members of the TRPC1 and Homer1 families were preferentially tested against segments of the D2R which interacted with TRPC1 and Homer1 to determine if these interactions where subtype specific. Previous experience in our lab found that closely related members of the same family are more

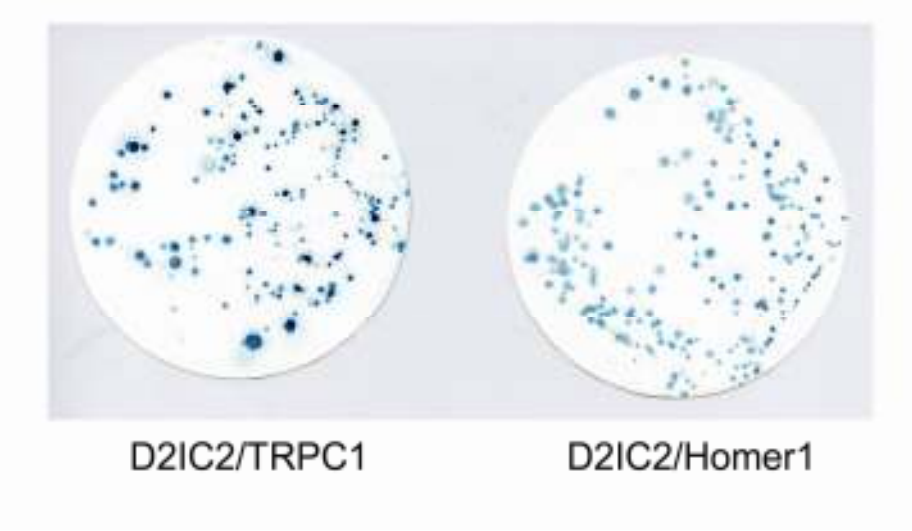

Figure **5.1**: **Representative β-galactosidase Assays of Directed Yeast-two Hybrid Screen.**

Representative β-galactosidase assay depicting positive protein-protein interaction between the D2IC2 and TRPC1, and the D2IC2 and Homer1, respectively.

likely to interact with the same rather than disparate regions of the receptor due to sequence conservation and similar protein conformation. Proteins that showed a positive interaction with one or more intracellular loops of the D2R (DynaminIIab, GRK3, GRK5, GRK6, Homer1, PI4Kinaseβ, TRPC4, TRPC5, and TRPC6) were scored as potential DRIPs and were subjected to further confirmatory experiments.

Table **5.3**: **Directed Yeast-Two Hybrid Screen of Candidate DRIPs with D2R**

D2R truncations were tested for interaction with fourteen candidate DRIPs identified by bioinformatics approaches. + indicates interaction, - indicates no interacation, N/A indicates not tested. For each construct pairing tested, n = 5-10.

Candidate DRIPs	***Truncation Fragments of D2R***				
	D2-IC1	**D2-IC2**	**D2S-IC3**	**D2L-IC3**	**D2-Tail**
DynaminIIab	+	-	-	-	-
FLJ12242	N/A	-	N/A	N/A	N/A
GRK2	-	-	-	-	-
GRK3	-	+	-	-	-
GRK5	+	-	-	-	-
GRK6	-	+	-	-	-
Homer1	-	+	-	-	-
Homer2	N/A	-	N/A	-	N/A
PI4Kinaseβ	+	-	-	-	-
TRPC3	N/A	-	N/A	-	N/A
TRPC4	N/A	+	N/A	-	N/A
TRPC5	N/A	+	N/A	-	N/A
TRPC6	N/A	+	N/A	-	N/A
TRPC7	N/A	-	N/A	-	N/A

Confirmation of TRPC4 and TRPC5 as potential DRIPs is described previously (Chapter 2). A potential binding site of D2R had been identified on TRPC1, TRPC4 and TRPC5, although other sites in the C-termini of these proteins may also contribute to D2R binding (Figure **2.3**). Sequence alignments of all six members of the TRPC

subfamily show high conservation of this D2R binding domain (Figure **5.2**). Due to the fact that larger segments of TRPC3, TRPC6, and TRPC7 were used in the initial directed yeast-two hybrid screen, additional sequences in these proteins may have had positive or negative effects on binding with the D2R.

Figure **5.2**: **Putative D2R Binding Domains on TRPC Subfamily C-terminus**
Amino acid sequence alignments of the putative D2R binding domain for the seven members of the TRPC family. Identical amino acids are in black boxes. Conserved amino acids are in grey boxes.

5.3.3 GST-Pulldown

To further confirm the directed yeast-two hybrid results, five proteins (GRK3, GRK6, Homer1, TRPC4 and TRPC5) were chosen to test for interaction with the D2R in a GST-pulldown assay. TRPC4 and TRPC5 GST-pulldown assays have been described (Chapter 2). GRK3, GRK6, and Homer1 were chosen to confirm interaction due to the finding that they all interacted with the D2IC2 specifically in the directed yeast-two hybrid screen (Table **5.3**). GRK3, GRK6, and Homer1 S-tagged fusion proteins were constructed and induced in bacteria. GRK2 and Homer2 S-tagged fusion proteins were

created to use as negative controls due to lack of binding with any D2R intracellular loop in the directed yeast two-hybrid screen. As shown in Figure **5.3** Western blots containing lysates prepared from bacteria expressing full-length S-tagged GRK2, GRK3, and GRK6 were probed with anti-S-tag antibodies. Bacterial lysates (lane 1, all panels) displayed immunoreactive bands at the expected molecular weights for all fusion proteins tested. In the case of GRK2, no immunoreactivity was detected after pulldown with D2IC2-GST (lane 2, top panel) or with D2LIC3-GST (lane 3, top panel) as expected. GRK2 also did not interact with glutathione-sepharose beads alone (data not shown). Immunoreactive bands corresponding to the expected molecular weights of S-tagged GRK3 and GRK6 were identified in both the D2IC2-GST lane and the negative control lane (lanes 2 and 3, middle and bottom panels). GRK3 and GRK6 fusion proteins interacted with beads as well (data not shown). These results indicate that GRK2 does not interact with the D2R, and GRK3 and GRK6 nonspecifically interact with the D2R. GRK2, GRK3, and GRK6 are not bona-fide DRIPs, although they most likely are an integral part of the D2R signalplex and function as DRAPs.

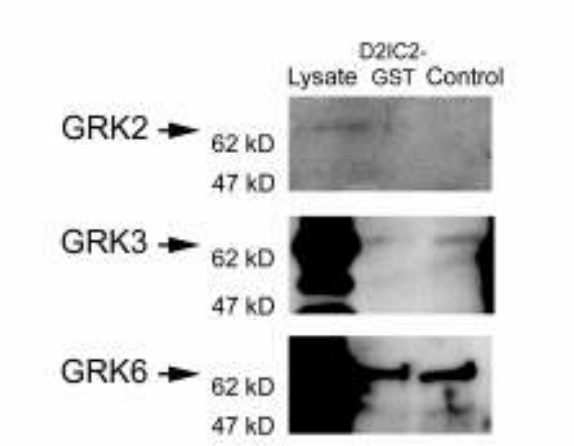

Figure **5.3**: **Representative Blot of GRK2, GRK3, and GRK6 GST-Pulldown Results.** D2IC2-GST and D2LIC3-GST fusion proteins were used to pull down S-tagged GRK2, GRK3, and GRK6. Blots were probed with an anti-S-tag antibody (Novagen). Lysate lane identifies GRK S-tagged proteins running at expected molecular weight. GRK2 was used as a negative control for interaction with the D2IC2 and D2LIC3 truncations based on directed yeast two-hybrid data. GRK3 and GRK6 both interacted positively with the D2IC2 but not the D2LIC3 in directed yeast two-hybrid experiments. GRK3 and GRK6 interacted non-specifically with the D2IC2, D2LIC3 and beads alone in n=4 experimental trials.

Homer1 and Homer2 S-tagged fusion proteins were tested for interaction with D2IC2-GST in a GST-pulldown. As shown in Figure **5.4A**, an anti-S-tag antibody detected an immunoreactive band in bacterial lysates that corresponded to the 51kD expected size of Homer1 and Homer2 (lane 1, both panels). A 51kD band is also seen when Homer1 and Homer2 were incubated with D2IC2-GST, or with beads alone (lanes 2 and 3, both panels). More stringent washes with higher salt concentration lysis buffer (150uM or 350uM NaCL) did not prevent Homer1 from binding nonspecifically to beads alone (data not shown). In n=5 trials, Homer1 and Homer2 proteins nonspecifically reacted with glutathione sepharose beads, indicating that interaction with the D2IC2 is non-specific in this experimental context.

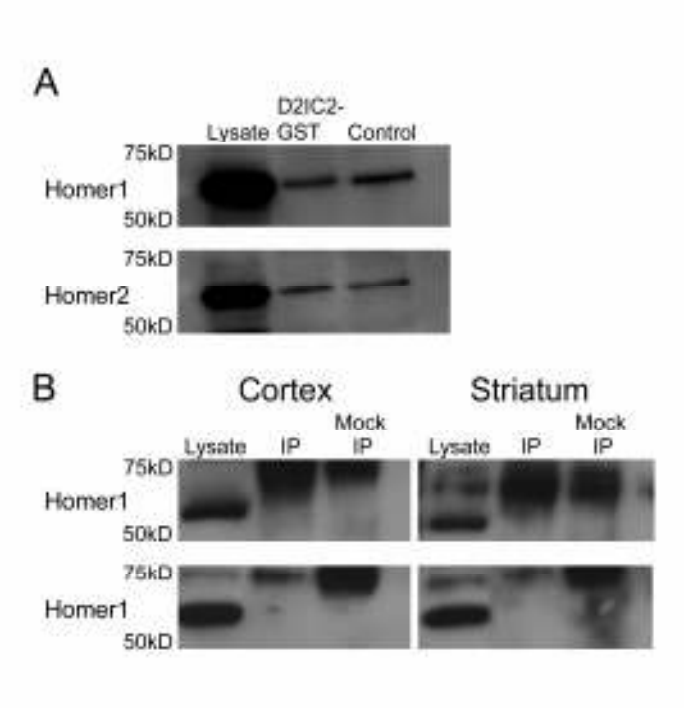

Figure **5.4**: **Representative Blots of Homer Proteins and D2R-GST-pulldown and Coimmunoprecipitation Experiments.**

(A) Full-length S-tagged Homer1 and Homer2 proteins were incubated with D2IC2-GST proteins or beads alone (control). Blots were probed with anti-S-tag antibody (Novagen). Homer proteins reacted non-specifically with beads alone. (n=5) **(B)** Coimmunoprecipitation attempt of Homer1 with D2R. A rabbit anti-D2R polyclonal antibody was used to immunoprecipate D2R from crude membrane preparations from 10 day old rat pup cortex and striatum. Immunocomplexes were separated via SDS-PAGE and blots were probed with a rabbit anti-Homer1 antibody (Santa Cruz, top panel) or a rat anti-Homer1 antiboday (Chemicon, bottom panel). Homer1 did not coimmunoprecipitate with D2R in rat cortical or striatal tissue (n=8 trials).

5.3.4 Coimmunoprecipitation

We next examined candidate DRIPs to determine if they exist in a multiprotein complex with the D2R in either cell culture or native tissue. Coimmunopreciptiation of candidate DRIPs with the D2R would confirm direct interactions discovered by either

GST-pulldown or yeast two-hybrid assay. A positive coimmunoprecipitation experiment in the context of negative results in a directed yeast two-hybrid or pulldown assay would indicate that the candidate protein was a DRAP; direct protein-protein interaction cannot be detected by coimmunoprecipitation alone. We decided to investigate five proteins for interaction with the D2R by this method (Homer1, IP3R, PI4Kinaseβ, TRPC4 and TRPC5). Results for TRPC4 and TRPC5 are discussed previously (Chapter 2).

Homer1 proteins were tested for interaction with the D2R in the rat cortical and striatal tissue. Crude membrane fractions were harvested from the cortex and striatum of 10-day-old rat pups. D2R immunocomplexes were isolated using an anti-D2R antibody and separated via SDS-PAGE. Blots were probed for the presence of Homer1 by two independent, commericially available antibodies (Figure **5.4B**). As shown in Figure **5.4B**, Homer1 protein was present at the expected molecular weight in rat cortical and striatal membrane lysate (lysate lanes, all panels). However, Homer1 was unable to be detected in either the immunoprecipitate lanes in n=8 experimental trials, indicating that it was not present in a signalplex with the D2R in native tissue (Figure **5.4B**, IP and Mock IP lanes, all panels).

Two other proteins were tested for the ability to coimmunoprecipitate with the D2R. As mentioned earlier, the IP3R has numerous transmembrane domains, is highly hydrophobic, and was therefore excluded from the initial directed yeast two-hybrid screen. PI4Kinaseβ was shown to interact with the D2IC1 in the directed yeast two-hybrid screen (Table **5.3**). Crude membrane preparations were harvested from HEK293, HEK293-D2L, and HEK293-D2S cells. An anti-FLAG M2 or M5 monoclonal antibody was used to immunoprecipitate FLAG-tagged D2R, and immunocomplexes were

separated via SDS-Page. HEK293 cells were used as negative controls in order to confirm specificity of the anti-FLAG antibodies. Blots were probed with either an anti-IP3R antibody, or an anti-PI4Kinaseβ antibody. Cell membrane lysates alone indicated that all three HEK293 cell lines endogenously expressed IP3R (Figure **5.5,** top panels, lysate lanes) and PI4Kinaseβ (Figure **5.5**, bottom panels, lysate lanes). No band corresponding to the IP3R or PI4Kinaseβ was detected specifically in the lanes containing immunoprecipitated FLAG-tagged D2Rs in n=4 independent trials (293-D2S and 293-D2L, IP lanes). Similar results were seen when the D2Rs were immunoprecipiated from cell membrane lysates with an anti-D2R antibody (data not shown). These results indicate that the IP3R and PI4Kinaseβ are not part of a D2R-signaling complex in HEK293 cell lines.

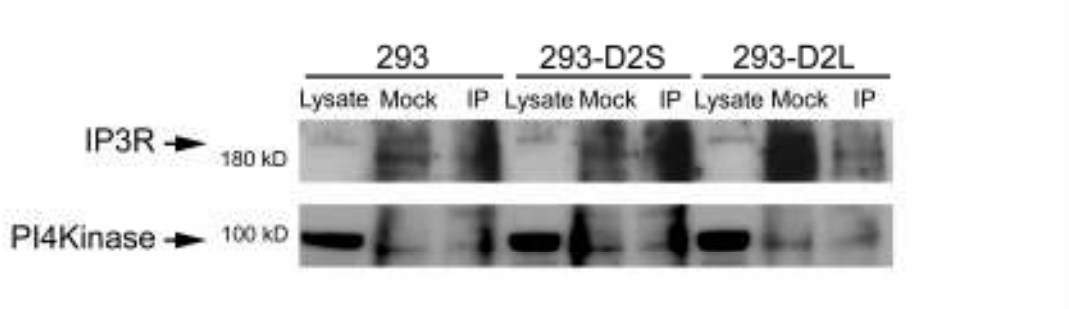

Figure **5.5**: **Representative Blot of Attempted Coimmunoprecipitation of the D2R with Candidate DRIPs in HEK293 Cell Lines.**

D2R were immunoprecipitated from HEK293-D2S and HEK293-D2L cell lines with either a monoclonal M5 anti-FLAG (top panel - Sigma) or a monoclonal M2 anti-FLAG (bottom panel - Sigma) antibody. HEK293 wild-type cells were immunoprecipitated with the same antibodies and used as a negative control. Blots were then probed for endogenously expressed candidate DRIP proteins. Top panel – polyclonal rabbit anti-IP3R antibody (Dr. Synder, Johns Hopkins University) Bottom panel – polyclonal rabbit anti-PI4Kinaseβ (Upstate). Lysate lanes were run for proper identification of IP3R and PI4Kinaseβ. Blots show no identifying bands that are not also present in either mock immunoprecipitation lanes or in the negative control lanes, indicating that IP3R and PI4Kinaseβ could not be coimmunoprecipitated with the D2R in this cell culture system. N = 4 trials.

5.4 Discussion

The protein constituents of GPCR signalplexes have important consequences for the regulation, trafficking, and life cycle of individual GPCRs, including the D2R [96, 98, 273]. Uncovering novel receptor-protein interactors will aid in the understanding of D2 receptor regulation and lend insight into complex cellular signaling processes. Utilizing the vast amount of electronic information available concerning proteins and their interacting partners, candidate DRIPs can be identified *in silico* and confirmed for interaction by *in vitro* and *in vivo* techniques. Through systematic query of available databases, proteins with a high biological probability of modulating various properties of the D2R can be identified and tested for direct interaction with the receptor itself. DRIP-status could be identified by detection of protein-protein interaction in directed yeast two-hyrbid and GST-pulldown assays. Coimmunoprecipitation with the D2R in a tissue or cell culture system would confirm that the interaction occurred in a biologically relevant system.

We have identified fifteen proteins through electronic database screening with high biological probability of interacting with the D2R. High biological probability was defined as containing one or more of the following characteristics: 1.) interaction with a known DRIP, 2.) membership in the same subfamily of proteins as a known DRIP, 3.) association with the D2R but unconfirmed DRIP-status, or 4.) involvement in a signaling pathway downstream of the receptor. Candidate proteins were tested for interaction with the D2R by directed yeast-two hybrid screen, GST-pulldown assay, or coimmunoprecipitation experiments.

The directed yeast two-hybrid screen offered an effective mechanism for detecting strong interactions between the D2R and a candidate DRIP. However, due to the hydrophobic nature of the D2R and the limitations of the traditional yeast-two hybrid system, the D2R could not be used in its native confirmation to screen for interacting proteins. Instead, intracellular truncations of the D2R lacking the hydrophobic transmembrane domains had to be used in the initial validation of the candidate DRIP/D2R interaction. This requirement removed D2R-secondary structure and would result in a false negative if the native confirmation of the D2R was necessary for candidate DRIP/D2R interactions. In addition, smaller truncations of the D2R, especially the D2IC1, appeared to auto-activate the yeast two-hybrid system in the absence of an interacting partner, resulting in false positives.

GST-pulldown assays were employed to confirm interactions identified in the directed yeast two-hybrid screen. Drawbacks to the GST-pulldown system are similar to those for a traditional yeast two-hybrid approach. Highly hydrophobic proteins are difficult to induce full-length in a bacterial cell due to their tendency to form insoluble aggregates, therefore intracellular truncations of the D2R were utilized. High and low stringency washing protocols were employed in order to detect either strong- or weak protein-protein interactions, allowing for more flexibility in detecting protein interactions as compared to the cellular environment of the yeast two-hybrid system.

Coimmunoprecipitation experiments use tissues or cell culture systems to identify proteins that coexist in a native protein complex with the D2R. However, this technique cannot distinguish between direct protein-receptor interactions and indirect associations via an intermediary protein.

Employing these methods, only a small subset of *in silico* identified candidate proteins (TRPC4 and TRPC5 – Chapter 2) could be confirmed as direct interactors of the D2R. These proteins were members of the same subfamily as the previously identified DRIP, TPRC1. A conserved D2R putative binding domain was identified upon amino acid sequence comparison of all mammalian TRPC proteins, lending credibility to the finding that multiple TRPCs interact with the D2R (Figure **5.6**). No other candidate proteins were found to directly interact with the D2R in our screening system. Negative results in these protein interaction screens does not preclude interaction with the D2R in a native system; rather, our experimental protocols were most likely not sensitive enough to identify protein-protein interactions of a weak or transient nature. GRK proteins, which are known to phosphorylate D2Rs, may only transiently interact with the receptor and therefore were not identified in our screening process which selected for stronger protein-protein interactions. Homer1 proteins interacted strongly in the directed yeast two-hybrid screen and non-specifically in the GST-pulldown assay; however, these proteins could not be isolated in a protein complex with the D2R in native tissue, implying that these interactions were experimental artifacts rather than a true interaction with the D2R. Only proteins that showed a high homology with previously known DRIPs, ie membership in the same subfamily, were able to be confirmed as DRIPs. This implies that screening multiple members of a subfamily where one protein member is a known DRIP will yield a high probability of identifying novel DRIPs.

Chapter 6

Closing Discussion

6.1 Characterization of Novel DRIPs

This thesis details efforts to advance understanding of the regulation of D2Rs and identification of the protein components of the D2R signalplex. Work presented here represents the first complete compilation of the D2R signalplex. This macromolecular complex is comprised of up to fifty proteins which interact with the D2R and regulate aspects of its life cycle. To expand our existing knowledge of the receptor complex, proteins with a high biological likelihood of interacting with the D2R were identified through database and literature query. These candidate proteins were screened for interaction with the receptor through various protein-interaction assays to determine their status as a potential DRIP or DRAP. The positive hits discovered from this biologically rational approach to identifying novel interactors consisted of proteins which had a family member previously proven to interact with the D2R. This indicates that proteins closely related to known DRIPs should be screened for interaction with the D2R themselves. It is reassuring to discover that not all proteins in a signaling pathway interact with the D2R. This demonstrates that although signaling proteins have been discovered to function in large macromolecular complexes, not every protein expressed in a cell clusters together and distinct, individual signaling pathways can still be discerned.

Two novel interacting proteins, TRPC1 and zDHHC4, were confirmed as DRIPs. The TRPC1/D2R interaction was characterized, and TRPC1 was found to promote trafficking of the D2R signalplex to the plasma membrane in HEK293 cells. The possibility remains that D2Rs may modulate the functionality of TRPC channels. D2R stimulation could directly activate TRPC channels, or alter the ion conducting and electrophysiological properties of the channel itself. Further experiments are needed to address these intriguing possibilities.

This is the first instance in which a direct interaction has been observed between a TRPC channel and a GPCR. TRPC channels, which are known to be activated by G_q-protein coupled receptor stimulation, have never before been shown to directly associate with a receptor. The established dogma had assumed a second messenger activated by the GPCR, such as a lipid intermediate, was responsible for the stimulation of current through TRPC channels. This novel finding suggests that activation of the receptor itself may directly and instantly modify the functionality of the ion channel. This evidence suggests that D2Rs are intimately associated with calcium signaling pathways and could play a role in the maintenance of calcium homeostasis. The D2Rs, thought to signal primarily through cAMP stimulation, are more diverse in their coupling to multiple downstream signaling cascades than previously realized.

TRPC1/D2R interaction promotes the upregulation of both proteins at the plasma membrane, making both accessible for activation by extracellular signals. Due to the fact that increases in dopaminergic signaling in schizophrenia contribute to symptoms of the disease, it will be interesting to determine if the TRPC1/D2R interaction is correlated with the diseased state. TRPC channel polymorphisms need to be investigated in humans

to determine if any TRPC single nucleotide polymorphsisms (SNPs) or haplotypes show genetic association with the schizophrenic disease state.

It will be intriguing to establish if aberrant or overactive TRPC currents in dopaminergic neurons associate with schizophrenia. Interruption of the TRPC/D2R interaction may have therapeutic benefits if disruption initiates internalization of the receptor and the ion channel. Alternatively, the development of specific TRPC channel inhibitors may have therapeutic benefits in schizophrenia. TRPC1 has been found to interact with all DR subtypes; therefore a specific TRPC1 channel inhibitor may have unwanted side effects due to the lack of unique interaction with the D2R. TRPC4 and TRPC5 have more restricted interaction profiles with DR subtypes. TRPC4 interacted with D1R and D2R, whereas TRPC5 interacted with D1R, D2R, and D3R. Specific interruptions of the TRPC4 or TRPC5/D2R interactions or specific inhibition of TRPC4, TRPC5, or heteromeric TRPC1/4/5 channels may prove to be important targets for rational drug design. Further experiments will be needed to determine which TRPC subunits interact with the D2R under which circumstances. It is likely that ion channels formed by D2R interacting with TRPC1, TRPC4, TRPC5, or a combination of the three will have distinct and discernible properties from one another.

It will be of interest to determine the stoichiometry of TRPC/D2R molecules in the D2R signalplex. Current protein models predict that most GPCRs function as dimers, whereas TRPC channels are thought to function as heterotetramers. Due to the small length of the D2IC2, it is likely that only a single TPRC protein can bind a single D2R. Competition for binding sites and availability of binding partners are factors which will govern the makeup of the components of the D2R signalplex. The D2R may compete

with other TRPC proteins to bind and sequester TRPC subunits and prevent functional ion channels from forming. D2Rs could also act as a nucleation point for TRPC channels. A D2R dimer could bind and stabilize two TRPC channel subunits in the signalplex and allow for recruitment of additional TRPC subunits to create a functional channel. Different TRPC proteins may compete with each other for binding to the D2R. TRPC1 appears to interact most strongly with the D2R, followed by TRPC4 and TRPC5. The presence of TRPC1 in a given cell type may prevent other TRPC subunits from binding the D2R, or TRPC1 may be necessary to recruit other TRPC subunits to the D2R signalplex.

Additionally, the TRPC/D2R interaction is likely to prevent other constituents of the signalplex, such as CAPS, from binding the D2R. Binding of TRPCs to D2Rs can prevent binding of other DRIPs to the D2R directly by occupying a binding site, or indirectly by sterically blocking neighboring binding sites or causing a conformational change in the D2R protein itself. TRPCs and CAPS share and compete for a common binding site present on the D2IC2. CAPS primarily localizes to the presynapse, where TRPCs exhibit a distributed plasmallemal expression pattern in neurons. Factors such as the intercellular environment directly surrounding the signalplex may contribute to which proteins comprise the D2R signalplex at various locations in the cell. Additional proteins which are present at these sites may aid in one DRIP preferentially binding the D2R over another. DRIPs that are able to compete for binding to the D2R under changing cellular environments allow for specialization and flexibility of function without the need to completely form and re-form the signalplex to perform each new role.

zDHHC4 was found to differentially interact with D2S/D2L isoforms of the D2R. The zDHHC4/D2L interaction caused the internalization of the receptor, an effect that was not demonstrated by the zDHHC4/D2S interaction. This finding will need to be confirmed in native expression systems, but it offers interesting options for potential therapy of schizophrenia. Palmitoylation is a highly reversible post-translational modification which has consequences for the trafficking of proteins to various intracellular compartments. If a drug was discovered that could stabilize the palmitate group on cysteines in the D2L, this could offer therapeutic benefits for schizophrenia. A drug which stabilized global palmitoylation would likely have unwanted side effects, so this drug would need to be specific for palmitoylation of D2L, or for stabilizing the interaction between zDHHC4/D2L. Palmitoylation of D2L would cause the receptor to be downregulated at the plasma membrane, resulting in an overall decrease in dopaminergic signaling. Due to the finding that the zDHHC4/D2R interaction has functional specificity for the D2L, the effects of stabilization this interaction and promoting palmitoylation of the D2L would only effect post-synaptic dopaminergic signaling. A small molecule or drug that would stabilize or irreversibly palmitoylate the D2L would not affect D2S-mediated signaling, including autoregulatory functions of D2Rs.

In addition to zDHHC4, one of the other 23 newly identified potential mammalian palmitoytransferases could interact with the D2R. The expression profiles of these proteins are unknown. It is plausible that multiple DHHC proteins could be responsible for palmitoylating different cysteine residues on D2Rs in different cell types, and therefore could have cell-specific effects on trafficking of D2Rs. Alternatively, it would

be interesting to determine if zDHHC4 is the only mammalian PAT that can interact with and post-translationally modify the D2R or if zDHHC4 can interact with and palmitoylate additional members of the DR family. Overall, the study of TRPCs and zDHHC proteins are rapidly exploding fields, and the more that is known about how these individual proteins function, the better their interactions with the D2R can be understood.

We have investigated the interaction of two DRIPs, NCS-1 and TRPC5, and have discovered that they interact and functionally influence one another. Dominant negative mutants of NCS-1 and TRPC5 were discovered to have effects on the extension of neurite processes in PC12 cells. These finding suggest implications of these DRIPs in neuronal patterning and development. It will be interesting to further investigate the NCS-1/TRPC5 interaction in the context of the D2R to determine how interactions between these proteins would affect neuronal processes. PC12 cells are known to express dopamine receptors, so it would be fascinating to determine the effects of dopaminergic antagonism or agonism on the extension of neurites in this system. Findings such as this will add to the growing evidence of DRIPs and DRAPs working in concert to affect cellular processes.

6.2 DRIPs and Schizophrenia: Potential for New Therapies and Diagnostic Techniques

The identification of novel DRIPs has numerous implications for the diagnosis and treatment of schizophrenia and other diseases associated with dysfunctional dopaminergic signaling. Although D2Rs have not been found to be mutated in or

genetically associated with schizophrenia, the possibility remains that certain DRIPs could be disrupted in or correlated with the disease state. DRIPs can be screened for alterations in mRNA and protein expression levels in the disease state. If such a correlation between DRIPs and disease could be uncovered, such DRIPs would offer clues to the heritability of schizophrenia and can be used as biomarkers to more definitively diagnose patients. The more that can be uncovered about the genes which contribute to the disease state, the more precisely the contributions of genetics, epigenetics, and environment to schizophrenia can be delineated. Schizophrenia is a genetic disorder of complex inheritance, so the contribution of multiple genes is likely to cause the disease phenotype. It will be exciting to determine if two or more proteins in a common pathway, such as DRIPs responsible for dopamine signaling, could be identified as genetically influencing each other and contributing to the disease state.

By better understanding the proteins which contribute to the regulation of dopaminergic signaling, these proteins could be monitored to determine how they respond to pharmacological intervention. Differences in protein expression levels and activation states of DRIPs in the presence and absence of antipsychotic exposure will aid in the understanding of how these drugs function. Additionally, individuals could be genotyped for haplotypes or polymorphisms in a set of DRIPs which could aid in the identification of the pharmaceutical treatment that would offer the greatest therapeutic benefit to the patient. The development of a microarray gene chip containing various polymorphisms of the protein components of the D2R signalplex, termed a DRIP-chip, could be developed and utilized in diagnosis of schizophrenia. Research correlating an individual's DRIP profile with the antipsychotic treatment that ultimately provided the

most benefits for that individual, could help to identify patterns between specific DRIP-expression profiles and corresponding effective therapeutic treatment.

Due to the heterologous nature of signaling through D2Rs, it will be interesting to determine exactly how drugs which modulate D2R-mediated neurotransmission, such as antipsychotics, produce their therapeutic effects. By blocking signaling through the D2R, antipsychotics could modulate numerous downstream cascades. Inhibition of certain signaling cascades is likely to produce therapeutic benefits, while others may cause untoward side effects. It will also be useful to know if antipsychotics can affect the functioning of DRIPs as well. If the benefits of antipsychotics were contributed primarily through modulation of one aspect of D2R signaling, such as decreasing cAMP stimulation or modulating calcium flux downstream of receptor activation, there arises the possibility that novel drugs can be discovered which preferentially inhibit that cascade downstream of the D2R. A drug such as this may prove to more effective in treating schizophrenia with fewer side effects. This is corroborated by differences between D2R activation profiles of typical and atypical antipsychotics already in use. For example, haloperidol, a typical antipsychotic, is thought to produce its effects through strict D2R antagonism. Haloperidol is known to produce severe extrapyramidal side effects. One of the more recently developed atypical antipsychotics, aripiprazole, exerts its benefits through partial antagonism/agonism of the D2R. This drug, although newer on the market than haloperidol, claims to produce fewer side effects than competing traditional therapies. It will be interesting to determine which signaling cascades downstream of the D2R are affect by treatment with aripiprazole compared to haloperidol. Perhaps by modulating D2R coupling to functional DRIPs such as TRPC1,

dopamine mediated neurotransmission could be tweaked to signal preferentially through pathways which would produce therapeutic benefits.

6.3 Future Research Directions

Ultimately, the study of protein networks and the relationships between multiple proteins could prove to be more interesting than the individual proteins themselves. The study of protein network interactions will provide new directions for the study of neurotransmission in general. The days where a single receptor was thought to signal through a single downstream effector molecule are long gone. Novel techniques will need to be developed to conquer research into signaling networks as a whole. Individual protein contributions to signaling cascades are vital to the understanding of cellular signaling, but these studies will need to be extended into a bigger context. Thinking about proteins in terms of networks, instead of focusing on one or two individual proteins, will be essential for understanding complex diseases such as schizophrenia. By identifying the complex of proteins associated with the D2R, we have taken the first step towards establishing a dopamine signaling interaction network. The crucial part will be in the identification of the subtle differences and nuances in interactions between proteins in the network which contribute to the disease state and differentiate it from the healthy state.

Due to the fact that schizophrenia is a disorder of complex inheritance, it is highly unlikely that mutations or alterations in a single gene will be found to cause the disease. Instead, schizophrenia may be caused by subtle disruptions in groups of multiple genes of

small effect. These genes, when present in an individual in a precipitating environment, could combine to produce the disease phenotype. Intriguingly, these combinations of genes of subtle effect may also produce deficits in an individual that correlate with, but do not expressly cause, the disease state. This thinking has led to the study of endophenotypes in schizophrenia. Endophenotypes are defined as deficits seen in schizophrenics and their first degree relatives that correlate with schizophrenia but are not necessarily pathogenic in themselves. Examples of endophenotypes include deficits in smooth eye pursuit and alteration of the startle reflex, termed prepulse inhibition. Endophenotypes are intriguing because they allow for the systematic laboratory investigation of single aspects of a complex disease state. Animal models of human endophenotypes, such as prepulse inhibition, are more easily identifiable in a laboratory setting than animal models of psychosis. Additionally, these defects can be measured and quantified, allowing for a more systematic understanding of the processes which potentially contribute to schizophrenia. It would be interesting to determine if alterations in one or more DRIPs contribute to established endophenotypes, or if alterations in DRIPs could cause novel endophenotype themselves.

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