SAP102 Switches the Mechanism of D_1R-Mediated ERK1/2 Activation from a PKA-Independent to PKA-Dependent Pathway

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Abstract

Hyperactivation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) by dopamine D_1 receptor (D_1R) in the striatum is a characteristic feature of several neuropsychiatric conditions, including drug addiction and L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia (LID) within individuals suffering from Parkinson's disease. However, the current mechanistic gap in understanding of D_1R-mediated regulation of ERK1/2, both in physiology and disease, hampers effective treatment of these conditions. One important factor that is underexplored in this regard is the role played by proteins that physically interact with the intracellular regions of D_1R. Using yeast two-hybrid screens and co-transfected human embryonic kidney 293 (HEK293) cells, our laboratory has recently characterized an interaction between the third intracellular loop of D_1R and synapse-associated protein 102 (SAP102), a member of the membrane-associated guanylate kinase family. Moreover, our lab identified endogenous D_1R-SAP102 complex within rat striatum and hippocampus. Interestingly, SAP102 regulates ERK signaling pathway within the hippocampus, and modulates adenosine A_{2A} receptor-mediated ERK1/2 activation within transfected HEK293 cells. Capitalizing on the above findings, I hypothesized a role for SAP102 in controlling D_1R-mediated ERK1/2 activation. Herein, I demonstrate using co-transfected HEK293 cells that SAP102 alters the temporal activation of ERK1/2 by D_1R. Intriguingly, experiments using the protein kinase A (PKA) inhibitors H89 and protein kinase inhibitor 14-22 amide myristoylated show that SAP102 also facilitates a switch in D_1R-mediated ERK1/2 activation from a PKA-independent to PKA-dependent pathway. Furthermore, SAP102 reduces basal ERK1/2 activation in HEK293 cells, reminiscent of a previously documented role of hippocampal SAP102. To the best of my

knowledge, my findings are the first to demonstrate scaffolding protein-mediated switching of a G protein-coupled receptor (GPCR) signaling pathway. Future studies aimed at uncovering the details of this process should provide valuable insight on the mechanisms contributing to D_1R-mediated ERK1/2 activation, and may also offer clues to the existence of similar phenomena for other GPCRs. These studies would also aid development of improved pharmacological treatment options for conditions with dysfunctional D_1R-dependent ERK1/2 activation.

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List of Abbreviations

AA: Ascorbic acid

A_1R: Adenosine A_1 receptor

$A_{2A}R$: Adenosine A_{2A} receptor

AC: Adenylyl cyclase

AKAP5: A-kinase anchoring protein 5

AKAP-Lbc: A-kinase anchoring protein-lymphoid blast crisis

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

Arc: Activity-regulated cytoskeleton-associated protein

B_{max}: Maximal binding capacity

BSA: Bovine serum albumin

CA1: Cornu ammonis 1

cAMP: Cyclic adenosine monophosphate

Cav-1: Caveolin-1

CB_1R: Cannabinoid-1 receptor

CIHH: Chronic intermittent hypoxia-hypercapnia

CNK2: Connector enhancer of kinase suppressor of Ras 2

CNS: Central nervous system

CP-AMPAR: Ca^{2+}-permeable AMPAR

CPP: Conditioned place preference

CREB: cAMP-responsive element-binding protein

$CRFR_1$: Corticotropin-releasing factor receptor 1

CT: Cytoplasmic tail

D_1R: Dopamine D_1 receptor

D_1R-MSN: D_1R-expressing MSN

D_2R: Dopamine D_2 receptor

D_3R: Dopamine D_3 receptor

D_4R: Dopamine D_4 receptor

D_5R: Dopamine D_5 receptor

DA: Dopamine

DAR: Dopamine receptor

DARPP-32: DA- and cAMP-regulated phosphoprotein of 32 kDa

DLG: Discs large

DMSO: Dimethyl sulfoxide

EDTA: Ethylenediaminetetraacetic acid

EGR2: Early growth response protein 2

EMEM: Eagle's minimal essential medium

EPAC1: Exchange protein directly activated by cAMP 1

EPAC2: Exchange protein directly activated by cAMP 2

EphB2R: EphB2 receptor

ERK1/2: Extracellular signal-regulated kinase 1 and 2

FBS: Fetal bovine serum

FSK: Forskolin

$G\alpha_{i/o}$: G protein $\alpha_{i/o}$ subunit

$G\alpha_{s/olf}$: G protein $\alpha_{s/olf}$ subunit

Gβγ: G protein βγ subunit complex

$GABA_AR$: γ-aminobutyric acid type A receptor

GAP: GTPase-activating protein

GEF: Guanine nucleotide exchange factor

$G_{i/o}$: Inhibitory/o family heterotrimeric G proteins

GKAP: Guanylate kinase-associated protein

GluA1: AMPAR subunit 1

GluA2: AMPAR subunit 2

GluN1: NMDAR subunit 1

GluN2A: NMDAR subunit 2A

GluN2B: NMDAR subunit 2B

GPCR: G protein-coupled receptor

GPR30: G protein-coupled receptor 30

GRB2: Growth factor receptor-bound protein 2

$G_{s/olf}$: Stimulatory/olfactory family heterotrimeric G proteins

GUK: Guanylate kinase

HEK293: Human embryonic kidney 293

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP: Horseradish peroxidase

IL3: Third intracellular loop

IEG: Immediate early gene

IGFR: Insulin-like growth factor-1 receptor

JNK: c-Jun N-terminal kinase

Kal7: Kalirin-7

KD: Knockdown

KO: Knockout

KSR: Kinase suppressor of Ras

L-DOPA: L-3,4-dihydroxyphenylalanine

LID: L-DOPA-induced dyskinesia

LTD: Long-term depression

LTP: Long-term potentiation

M_4R: M_4 mAChR

mAChR: Muscarinic acetylcholine receptor

MAGI: Membrane-associated guanylate kinase inverted

MAGUK: Membrane-associated guanylate kinase

MAPK: Mitogen-activated protein kinase

MAP2K: Mitogen activated protein kinase kinase

MAP3K: Mitogen activated protein kinase kinase kinase

MDMA: 3,4-methylenedioxymethamphetamine

MEF: Mouse embryonic fibroblast

MEK: MAPK/ERK kinase

MEK1/2: MEK 1 and 2

mGluR: Metabotropic glutamate receptor

$mGluR_1$: Metabotropic glutamate receptor 1

$mGluR_2$: Metabotropic glutamate receptor 2

$mGluR_3$: Metabotropic glutamate receptor 3

$mGluR_5$: Metabotropic glutamate receptor 5

mPFC: Medial prefrontal cortex

mPins: Mammalian homolog of *Drosophila melanogaster* partner of inscuteable

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

MSN: Medium spiny neuron

Nbea: Neurobeachin

NMDAR: *N*-methyl-D-aspartate receptor

Nor-1: Neuron-derived orphan receptor 1

p38: p38 MAPK

PAK: p21-activated kinase

PBK: PDZ-binding kinase

PD: Parkinson's disease

PDZ: PSD-95/DLG/Zonula occludens-1

PFC: Prefrontal cortex

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PKD1: Protein kinase D1

PKI: Protein kinase inhibitor 14-22 amide myristoylated

PLC: Phospholipase C

PMSF: Phenylmethylsulfonyl fluoride

PP1: Protein phosphatase 1

PSD: Postsynaptic density

PSD-93: Postsynaptic density protein 93

PSD-95: Postsynaptic density protein 95

RapGEF1: Rap guanine nucleotide exchange factor 1

RapGEF2: Rap guanine nucleotide exchange factor 2

RapGEF3: Rap guanine nucleotide exchange factor 3

RapGEF4: Rap guanine nucleotide exchange factor 4

RasGRF1: Ras-specific guanine nucleotide-releasing factor 1

RasGRP2: Ras guanyl-releasing protein 2

Rap1GAP: Rap1 GTPase-activating protein

rpS6: Ribosomal protein S6

RTK: Receptor tyrosine kinase

SAP97: Synapse-associated protein 97

SAP102: Synapse-associated protein 102

SDS: Sodium dodecyl sulfate

SFK: Src family non-receptor tyrosine kinase

SH2: Src homology 2

SH3: Src homology 3

Shc: Src homology and collagen

SHANK: SH3 and multiple ankyrin repeat domains

Shp-2: SH2 domain-containing tyrosine phosphatase 2

SNc: Substantia nigra pars compacta

Sos: Son of sevenless

STDP: Spike timing-dependent plasticity

STEP: Striatal enriched phosphatase

SynGAP: Synaptic Ras GTPase-activating protein

TBS-T: Tris-buffered saline containing Tween 20

TH: Tyrosine hydroxylase

THC: Tetrahydrocannabinol

TrkBR: Tropomyosin-related kinase B receptor

$VPAC_2R$: Vasoactive intestinal polypeptide $VPAC_2$ receptor

VTA: Ventral tegmental area

WT: Wild-type

β_1AR: β_1-adrenergic receptor

β_2AR: β_2-adrenergic receptor

β-arr: β-arrestin

σ_1R: Sigma-1 receptor

5-HT: 5-hydroxytryptamine

$5\text{-}HT_{2B}R$: 5-HT 2B receptor

6-OHDA: 6-hydroxydopamine

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CHAPTER 1: OVERVIEW OF THE DOPAMINERGIC SYSTEM

1.1. The Dopaminergic System: Functions and Major Brain Pathways

The tyrosine-derived catecholamine 3,4-dihydroxyphenethylamine, commonly known as dopamine (DA), is a prominent neurotransmitter in the mammalian brain[1,2]. DA is crucial for regulating numerous central nervous system (CNS) functions including voluntary movement, positive reinforcement, cognition, affect, food intake and sleep[1–3]. DA also has effects outside the CNS where it is important for processes such as olfaction, vision, hormone secretion and regulation of cardiovascular, renal, gastrointestinal, immune, and sympathetic nervous system functions[1–3]. Given its widespread control over normal physiology, it is not surprising that dysregulation of the dopaminergic system is central to several pathologies, including Parkinson's disease (PD), L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia (LID), Huntington's disease and drug addiction[1–3]. Moreover, underlying alterations of dopaminergic function also contribute to conditions like schizophrenia, attention deficit hyperactivity disorder, bipolar disorder and major depression[1–3].

Three major groups of DA-synthesizing neurons exist in the brain. Two groups are located in the midbrain within the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). While the third group of dopaminergic neurons is located in the hypothalamus within the periventricular and arcuate nuclei[1,2,4]. Axonal projections from these neurons release DA throughout the entire forebrain along three main pathways. First, VTA DA neurons give rise to the mesocorticolimbic pathway that terminates within various cortical and limbic regions including the prefrontal cortex (PFC), nucleus accumbens and hippocampus. Second, SNc neurons give rise to the nigrostriatal pathway that terminates within the dorsal striatum. Third, the periventricular and arcuate nuclei DA neurons of the hypothalamus give rise to the

tuberoinfundibular pathway that releases DA into the median eminence through which it is able to act as a hormone on the anterior pituitary gland[1,2,4].

1.2. Family of Dopamine Receptors (DARs)

The actions of DA are mediated through the family of DARs (D_1R, D_2R, D_3R, D_4R and D_5R) which belong to the G protein-coupled receptor (GPCR) superfamily[1–3]. DARs are classified into two groups based on differences in their structure, pharmacology and canonical signaling pathway[1–3]. D_1R and D_5R constitute the D_1R-class, while D_2R, D_3R and D_4R comprise the D_2R-class[1–3]. D_1R-class genes do not contain introns, thus D_1R and D_5R each exist as a single isoform[1,2]. In contrast, introns are present within the coding sequence of D_2R-class genes, however only D_2R has more than one functional splice variant; namely, a short isoform and a long isoform that differ due to alternative splicing of a single 87-base pair exon[1,2]. Additionally, D_4R displays polymorphic variation via a 48 base pair sequence that is present as a different number of repeats, with variants containing either seven repeats, four repeats or a single repeat being the most common forms[1,2]. DARs are distributed throughout the entire forebrain, with D_1R and D_2R being the most highly expressed subtypes[1,2]. D_1R-class are present almost exclusively postsynaptically, while in contrast D_2R-class are present both postsynaptically and presynaptically where they function as inhibitory autoreceptors for regulating DA release from synaptic terminals[1,2].

Structurally, all DARs consist of seven α-helical membrane-spanning regions along with an extracellular N-terminal region, three extracellular loops, three intracellular loops and an intracellular C-terminal region also referred to as the cytoplasmic tail (CT)[1]. The major structural differences between D_1R-class and D_2R-class are present within the third intracellular loop (IL3)

and CT regions. D_1R-class have a relatively short IL3 and CT, while in contrast D_2R-class has a characteristically large IL3 but a CT that is approximately seven times shorter than that of D_1R-class[1]. Additionally, D_1R-class and D_2R-class differ in their post-translational modifications. The extracellular regions of all DARs are N-glycosylated, with two N-glycosylation sites present in D_1R-class, four present in D_2R, three present in D_3R and a single site present in D_4R[1]. Furthermore, both D_1R-class and D_2R-class contain a single cysteine residue within their intracellular CT region that is palmitoylated. This cysteine is located near the start of the CT in D_1R-class, but is present near the end of the CT in D_2R-class[1]. Finally, differences between D_1R-class and D_2R-class within their seven transmembrane regions results in much higher DA affinity for D_2R-class as a whole relative to D_1R-class, and has enabled the development of multiple selective agonists and antagonists that are capable of discriminating between D_1R-class and D_2R-class[1,2].

Functionally, D_1R-class is canonically linked to the activation of adenylyl cyclase (AC) through coupling to stimulatory/olfactory family heterotrimeric G proteins ($G_{s/olf}$), while in contrast D_2R-class is canonically linked to the inhibition of AC through coupling to inhibitory/o family heterotrimeric G proteins ($G_{i/o}$)[1–3,5]. Moreover, D_1R-class primarily acts via G protein $\alpha_{s/olf}$ subunit ($G\alpha_{s/olf}$)-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA)-DA- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32)-dependent pathways to reduce the activity of K^+ channels, N- and P/Q-type Ca^{2+} channels, Na^+ channels and γ-aminobutyric acid type A receptor ($GABA_AR$), and enhance the activity of L-type Ca^{2+} channel, *N*-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), which exerts an overall effect of increasing neuronal excitability[1,5]. On the

other hand, D_2R-class acts via both G protein $\alpha_{i/o}$ subunit ($G\alpha_{i/o}$)-mediated antagonism of the cAMP-PKA-DARPP-32 pathway and through G protein βγ subunit complex (Gβγ)-mediated signaling pathways to reduce the activity of N- and P/Q-type Ca^{2+} channels, Na^+ channels and $GABA_AR$, but enhance K^+ channel activity, and reduce L-type Ca^{2+} channel and NMDAR activity, which exerts an overall effect of reducing neuronal excitability[1,5]. D_1R-class and D_2R-class are both also capable of affecting gene expression through various pathways, including via the regulation of mitogen-activated protein kinases (MAPKs)[2,3,5]. Thus, overall, the major roles of D_1R-class and D_2R-class signaling pathways in neurons are to control cell excitability, with D_1R-class functioning to increase excitability and D_2R-class functioning in an opposite manner to reduce excitability, and to modulate gene expression through mechanisms that sometimes involve MAPK regulation.

1.3. D_1R-Class: Divergent Properties of D_1R and D_5R

D_1R-class as a whole are distributed throughout the entire forebrain[1,2], however differences in expression exist between D_1R and D_5R. D_1R is the most highly- and widely-expressed DAR in the brain. Dense D_1R expression is found in several brain regions including the dorsal striatum, nucleus accumbens, frontal cortex, amygdala, substantia nigra and olfactory bulb. Lower levels of D_1R are also observed in brain regions like the hippocampus, cerebellum, thalamus and hypothalamus. In contrast, D_5R expression is also reported in most of these regions, but in less quantity than D_1R overall[1,2,6,7]. Differences in subcellular expression pattern of D_1R and D_5R also exist. In particular, D_1R is enriched in dendritic shafts and spines, while D_5R is predominantly located in perikarya and specifically proximal dendrites[7]. Moreover, in cortical

pyramidal neurons, D_1R is reported to exhibit much greater expression within dendritic spines than shafts relative to D_5R[6,8].

D_1R and D_5R display similar structure and function overall, however several important differences between these two receptors have been characterized. D_1R-class are highly homologous within their transmembrane domains, exhibiting approximately 80% sequence identity within these regions, however they display significant divergence of sequence identity within their IL3 and C-terminal regions[1]. Differences in IL3 and CT sequence underlie differences in constitutive activation, DA binding affinity, DA potency for stimulating AC, and maximal binding capacity (B_{max}) measured with D_1R relative to D_5R[9–14]. Moreover, D_1R and D_5R interact with different $G_{s/olf}$ isoforms under certain circumstances[15], likely as a result of their different IL3 structure, given the known importance of IL3 for G protein coupling[16–21]. Furthermore, IL3 and CT are characteristically rich in phosphorylatable serine and threonine residues[22,23], thus divergence within these regions is likely to result in differential phosphorylation patterns between D_1R and D_5R that contributes to differences in receptor function. Indeed, protein kinase C (PKC) differentially affects D_1R-class receptor responsiveness to AC stimulation—facilitating D_1R sensitization but D_5R desensitization—in a manner which is fully dictated by IL3, and presumably involves phosphorylation of one or more of the known PKC sites present within the IL3 and CT of these receptors[24–26]. Finally, IL3 and CT differences of D_1R-class are implicated as crucial determinants underlying the unique complement of protein-protein interactions thus far characterized for D_1R and D_5R[27–35].

CHAPTER 2: OBJECTIVE AND HYPOTHESIS

2.1. Investigating the Role of Synapse-Associated Protein 102 (SAP102) in D_1R-Mediated ERK1/2 Activation

The Tiberi Lab used yeast two-hybrid assays to parse subtype-specific differences between IL3 and C-terminal region of D_1R and D_5R in the recruitment of unique binding partners. Screens with a bait derived from IL3 of human D_1R identified a potential new binding partner called SAP102, which belongs to the large family of membrane-associated guanylate kinases (MAGUKs)[36]. Interestingly, screens using IL3-derived bait from D_5R did not isolate SAP102, suggesting that this MAGUK family member selectively interacts with D_1R. Indeed, using co-transfected human embryonic kidney 293 (HEK293) cells our lab characterized the formation of a complex between the wild-type form of D_1R, but not that of D_5R or an IL3-deleted mutant form of D_1R, and SAP102. Moreover, we confirmed the presence of endogenous D_1R-SAP102 complex in rat striatum and hippocampus. SAP102 is a ubiquitously expressed neuronal protein present mainly within the postsynaptic density (PSD) that has been best characterized for its role in modulating glutamatergic receptor trafficking and function[37–40]. My M.Sc. thesis project is centered on assessing a potential role for SAP102 in modulating D_1R function.

Specifically, investigation into the role of SAP102 in modulating D_1R-mediated activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) of the MAPK family within co-transfected HEK293 cells was pursued for the following reasons. Firstly, SAP102 modulates ERK signaling pathway *in vivo* within the hippocampus[39,41], a brain region in which D_1R is known to be expressed[6,42]. Secondly, SAP102 alters the time course of ERK1/2 activation by adenosine 2A receptor (A_{2A}R)—another $G_{s/olf}$-coupled receptor—*in vitro* within a heterologous expression

system[43]. Lastly, D_1R-mediated activation of ERK1/2 has been identified as a crucial component to several physiological and pathological processes in the CNS [44–57]. However, the detailed mechanisms governing D_1R-mediated regulation of ERK1/2, including the observed variations within different brain regions and cell types, have not been clearly described. Thus, *in vitro* studies investigating basic processes of D_1R-mediated ERK1/2 activation, including potential involvement of SAP102, are warranted.

Taking these points into account, the overarching hypothesis for my M.Sc. thesis project is that SAP102 will exert an observable modulatory effect on D_1R-mediated ERK1/2 activation within co-transfected HEK293 cells.

Over the next two chapters of this thesis, I comprehensively review the literature surrounding both D_1R-mediated ERK1/2 activation and SAP102 functions in the CNS. Chapter three begins with an outline of basic MAPK properties and $G_{s/olf}$-linked GPCR-mediated ERK1/2 activation. It then delves specifically into what is currently known about the mechanisms underlying D_1R-mediated activation of ERK1/2, as well as the physiological and pathological processes associated with this D_1R-facilitated signaling pathway. Chapter four subsequently opens with an overview of MAGUKs, focusing on the discs large (DLG) subfamily to which SAP102 belongs. Details are then provided on the known roles of SAP102, with dedicated sections covering its established functions in glutamatergic transmission and ERK1/2 activation, as well as its strongly implied function in dopaminergic transmission through modulation of D_1R biology. The primary aim with these chapters is to highlight intersection points within the reviewed subject areas that support the likelihood and relevance of SAP102 involvement in D_1R-mediated ERK1/2 activation within the brain.

CHAPTER 3: D_1R-MEDIATED ERK1/2 ACTIVATION

3.1. Family of MAPKs

The MAPKs are a highly conserved family of proline-directed serine/threonine kinases that have important roles in diverse cellular processes including growth, proliferation, differentiation, motility, apoptosis and neuronal plasticity[58–60]. As such, dysregulated MAPK signaling is central to various pathological conditions like cancer, diabetes, inflammation and neurodegenerative disorders[59]. Mammals contain fourteen MAPKs that are grouped into seven subfamilies: ERK1/2 (also known as p44/p42 MAPK in accordance with their molecular weights), c-Jun N-terminal kinase (JNK)1/2/3, p38 MAPK (p38)α/β/γ/δ and extracellular signal-regulated kinase 5 constitute the conventional MAPKs, while extracellular signal-regulated kinase 3,4 and 7, and nemo-like kinase are considered atypical MAPKs because they are not activated through a canonical three tier sequential phosphorylation cascade like conventional MAPKs[60,61].

Numerous stimuli lead to activation of the conventional MAPKs. Specifically, growth factors, cytokines, cell adhesion molecules, ionotropic receptor ligands and GPCR ligands acting through their associated receptors are all capable of inducing MAPK activation[58,62–67]. Moreover, a diverse array of cellular stresses also potently activate MAPKs[62]. Nonetheless, all of these stimuli ultimately converge on the activation of Ras or Rho family small GTPases which initiate the canonical MAPK activation cascade[58,62,63,65,66]. This cascade consists of sequential activation of a MAPK kinase kinase (MAP3K) and a MAPK kinase (MAP2K) which brings about MAPK activation through dual phosphorylation of a threonine and tyrosine residue in the conserved TXY motif present within the MAPK activation loop[59,60]. Activated MAPKs subsequently phosphorylate many nuclear and cytoplasmic targets including MAPK-activated

protein kinases, transcription factors and membrane and cytoskeletal proteins, which ultimately leads to the overall MAPK-induced cellular effects[59,60].

The ERK1/2 module was the first identified MAPK pathway[68] and is one of the most highly-studied mammalian signaling pathways. Mitogens acting through their associated receptor tyrosine kinases (RTKs) are important contributors to cellular ERK1/2 activation[59,60]. The general mechanism underlying growth factor-induced activation of ERK1/2 through RTKs is well-understood and is considered a prototype of MAPK activation. Upon growth factor binding, RTKs undergo autophosphorylation of tyrosine residues within their intracellular domains that facilitates recruitment of the Src-homology 2 (SH2) domain-containing protein growth factor receptor-bound protein 2 (GRB2) along with its constitutively associated binding partner son of sevenless (Sos), a Ras guanine nucleotide exchange factor (GEF); subsequent to this, Sos promotes Ras-facilitated initiation of the canonical MAPK cascade specifically controlling ERK1/2 activation, which involves the MAP3K Raf and the MAP2K MAPK/ERK kinase (MEK)[60,65].

ERK1/2 activation can also be induced by GPCRs. All four families of heterotrimeric G proteins are capable of regulating ERK1/2 activation downstream of their associated GPCR, however $G_{s/olf}$, $G_{i/o}$, and G_q have much more prominent roles than G_{12}[66]. The following subsection summarizes the mechanisms underlying ERK1/2 regulation by GPCRs coupled to the $G_{s/olf}$ family.

3.2. $G_{s/olf}$-Linked GPCR-Mediated Regulation of ERK1/2

3.2.1. $G\alpha_{s/olf}$-Induced ERK1/2 Activation

$G_{s/olf}$-linked GPCRs are capable of regulating the activity of all four conventional MAPKs[66], however their role in ERK1/2 regulation has been studied the most extensively. Primarily, they act to promote ERK1/2 signaling via several distinct $G\alpha_{s/olf}$-mediated pathways involving multiple GEFs and at least one GTPase activating protein (GAP) that all converge on the activation of Ras or Rap[46,69–85] **(Fig. 1)**. However, under certain circumstances $G_{s/olf}$-linked GPCRs instead act to inhibit ERK1/2 activation[86–92].

The specific pathways utilized, and their resulting downstream effects ultimately promoting or inhibiting ERK1/2 activation, differ in a cell type-specific manner. This presumably depends in large part on differential expression of the various components contributing to cAMP-mediated ERK1/2 signaling pathways, and is likely also influenced by distinct properties of the particular $G_{s/olf}$-linked GPCR involved. Differential expression of the three Raf isoforms (A-Raf, B-Raf and C-Raf) is one important factor imparting cell type-specific differences in cAMP-mediated ERK1/2 signaling. Specifically, the relative levels of A-Raf and C-Raf to B-Raf largely dictate the relative levels of Ras- and Rap-dependent signaling pathways present within a given cell type. Because Ras is capable of activating all three Raf isoforms but Rap can only activate B-Raf[93], low levels of B-Raf serve to bias $G_{s/olf}$-linked GPCRs toward Ras-dependent pathways of ERK1/2 activation, while in contrast both Ras- and Rap-dependent pathways are likely to be utilized when B-Raf is abundant. Varying expression levels of the different Ras isoforms (H-Ras, K-Ras and N-Ras) and Rap isoforms (Rap1A, Rap1B, Rap2A, Rap2B and Rap2C), as well as the different GEFs and GAPs controlling Ras- and Rap-activation, are likely also major factors

contributing to differential properties of $G_{s/olf}$-linked GPCR-mediated ERK1/2 regulation between cell types. Finally, a key factor underlying receptor-specific variations in ERK1/2 signaling pathway regulation by $G_{s/olf}$-linked GPCRs may be interactions with different protein binding partners via their intracellular regions.

Several GEFs that are activated downstream of $G\alpha_{s/olf}$-mediated increases in cAMP promote either specific activation of Ras or Rap, or non-specific activation of both Ras and Rap, leading to ERK1/2 signaling pathway activation **(Fig. 1)**. Namely, the Ras-specific GEF Ras-specific guanine nucleotide-releasing factor 1 (RasGRF1)[70,83,85], the Rap-specific GEFs RapGEF3 (exchange protein directly activated by cAMP 1 [EPAC1])[75,80,82] and RapGEF4 (exchange protein directly activated by cAMP 2 [EPAC2])[71], and the dually functional Ras/Rap GEFs RapGEF1[76,78,79], RapGEF2[69,73,74,76,77,81,84] and Ras guanyl-releasing protein 2 (RasGRP2)[46] are all implicated in regulating ERK1/2 activity downstream of $G_{s/olf}$-linked GPCRs. In contrast, $G\alpha_{s/olf}$-mediated inactivation of the Rap-specific GAP Rap1GAP also facilitates ERK1/2 activation[72] **(Fig.1)**.

$G\alpha_{s/olf}$-mediated pathways leading to activation of ERK1/2 can be classified into two groups based on whether or not they require PKA for GEF activation/GAP inactivation **(Fig. 1)**. Pathways utilizing RasGRF1[70,83,85], RasGRP2[46], RapGEF1[76,78,79] or Rap1GAP[72] all appear to be PKA-dependent, requiring GEF/GAP phosphorylation either directly by PKA or through indirect PKA-mediated mechanisms involving downstream Src family non-receptor tyrosine kinases (SFKs). In contrast, pathways utilizing RapGEF2[69,73,74,76,77,81,84], EPAC1[75,80,82] or EPAC2[71] occur independently of PKA, instead proceeding via GEF activation through direct cAMP binding. However, to complicate matters slightly, it is possible that $G\alpha_{s/olf}$-mediated pathways facilitating ERK1/2 activation via PKA-independent GEFs may still require PKA under certain circumstances.

In particular, PKA has a role in SFK activation downstream of $G_{s/olf}$-linked GPCRs[79,94], thus suggesting potential involvement of PKA in SFK-mediated phosphorylation of C-Raf and A-Raf, an event which is necessary for completely activating these two Raf isoforms to initiate the ERK1/2 cascade[95].

Both Ras- and Rap-dependent ERK1/2 activation downstream of $G\alpha_{s/olf}$ can occur through PKA-dependent and PKA-independent pathways depending on the cellular context. A PKA-dependent RasGRF1-Ras pathway leading to ERK1/2 activation has been characterized within HEK293 cells[83]. In contrast, PKA-independent Ras-mediated ERK1/2 activation in HEK293 cells, HEK293T cells and mouse B16 melanoma cells has been shown to occur via RapGEF2[69,76,81,84]. Within HEK293 cells, Rap1-mediated ERK1/2 activation appears to require sequential activation of PKA, Src and RapGEF1 in some cases[76,78,79]. Two additional PKA-dependent pathways leading to Rap1-mediated ERK1/2 activation involving either RasGRP2[46] or Rap1GAP[72] have been demonstrated in mouse striatal medium spiny neurons (MSNs). A role for RapGEF2 in PKA-independent Rap1-mediated ERK1/2 activation was identified within Neuroscreen-1 cells[73,74]. Moreover, EPAC1 is implicated in PKA-independent ERK1/2 activation through Rap1 within HEK293 cells and rat kidney cortical collecting duct cells[75,80], while EPAC2 is involved in PKA-independent Rap1-facilitated activation of ERK1/2 within cortical pyramidal neurons[71].

The demonstrated existence of several distinct $G\alpha_{s/olf}$-mediated pathways controlling ERK1/2 activation within a single cell type, as described above for HEK293 cells[75,76,78,79,83], suggests that under certain circumstances more than one pathway downstream of $G\alpha_{s/olf}$ could work in concurrence to regulate ERK1/2 within some cell types. Indeed, specifically within

HEK293 cells, parallel $G\alpha_{s/olf}$-mediated pathways through Ras and Rap were demonstrated to coordinately facilitate activation of ERK1/2[76]. In particular, two distinct temporal components of ERK1/2 activation were isolated and shown to be the direct result of discrete K-Ras- and Rap1-mediated pathways. A transient component of large magnitude was attributed to a PKA-independent RapGEF2-K-Ras pathway, while a prolonged component of small magnitude was attributed to a PKA-dependent SFK-RapGEF1-Rap1 pathway.

Further inquiry into the observed temporal differences between K-Ras- and Rap1-mediated ERK1/2 activation downstream of $G\alpha_{s/olf}$ in HEK293 cells uncovered a novel underlying mechanism involving 14-3-3 protein and kinase suppressor of Ras (KSR)[96]. Initial $G\alpha_{s/olf}$-mediated ERK1/2 activation was found to occur through direct Raf interactions with both K-Ras and Rap1; however, these interactions were rapidly abrogated through PKA-mediated Raf phosphorylation, thereby accounting for the transiently activated pool of ERK1/2. Moreover, while termination of direct Raf interactions with K-Ras served to completely eliminate further K-Ras-dependent ERK1/2 signaling, termination of said interactions with Rap1 failed to prevent continued ERK1/2 activation through Rap1. This persistent Rap1-mediated ERK1/2 activation was found to occur as a result of PKA-mediated phosphorylation of Rap1A-Ser180 and Rap1B-Ser179. More specifically, phosphorylation of these residues by PKA facilitated indirect linkage of Rap1 and B-Raf through a unique Rap1 binding interaction with 14-3-3 protein bound to KSR and present overall within a constitutive tripartite complex also containing B-Raf; in turn, this indirect Rap1-B-Raf linkage enabled extended Rap1-mediated ERK1/2 activation in the absence of direct Rap1-B-Raf interactions.

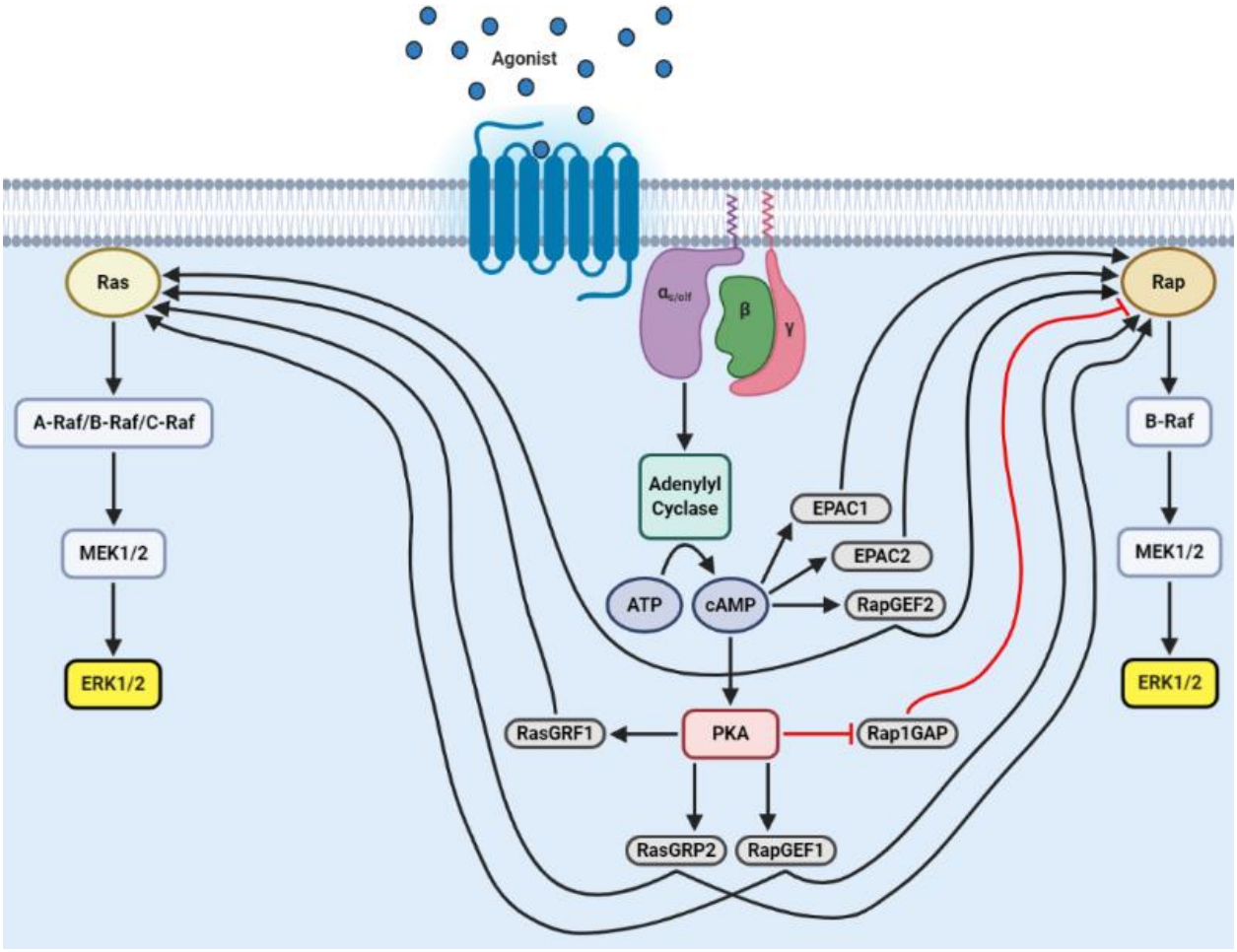


Figure 1. Canonical Mechanisms of $G_{s/olf}$-Linked GPCR-Mediated ERK1/2 Activation
Black lines denote activation; red lines denote inhibition. Created with BioRender.com.

3.2.2. $G\alpha_{s/olf}$-Induced ERK1/2 Inhibition

In some cases, $G\alpha_{s/olf}$-mediated signaling leads to inhibition of ERK1/2[86–92]; two major mechanisms underlying this inhibition have been characterized **(Fig. 2)**. PKA-dependent phosphorylation of B-Raf-Ser365 and C-Raf-Ser259 has been established to disrupt direct B/C-Raf binding interactions with Ras and Rap1[92,95,97]. Within certain cell types such as H1299 lung cancer cells this PKA-mediated effect predominates over other $G\alpha_{s/olf}$-mediated pathways positively regulating ERK1/2 signaling, thus resulting in overall inhibition of ERK1/2 activation[92]. The second mechanism factoring in to $G\alpha_{s/olf}$-mediated ERK1/2 inhibition involves Rap1-mediated antagonism of Ras-C-Raf signaling. This inhibitory effect of Rap1 depends on the level of B-Raf expression within a cell. Namely, in some cases, low cellular B-Raf expression levels promote promiscuous Rap1-C-Raf interactions that act to sequester C-Raf away from Ras[91,98,99].

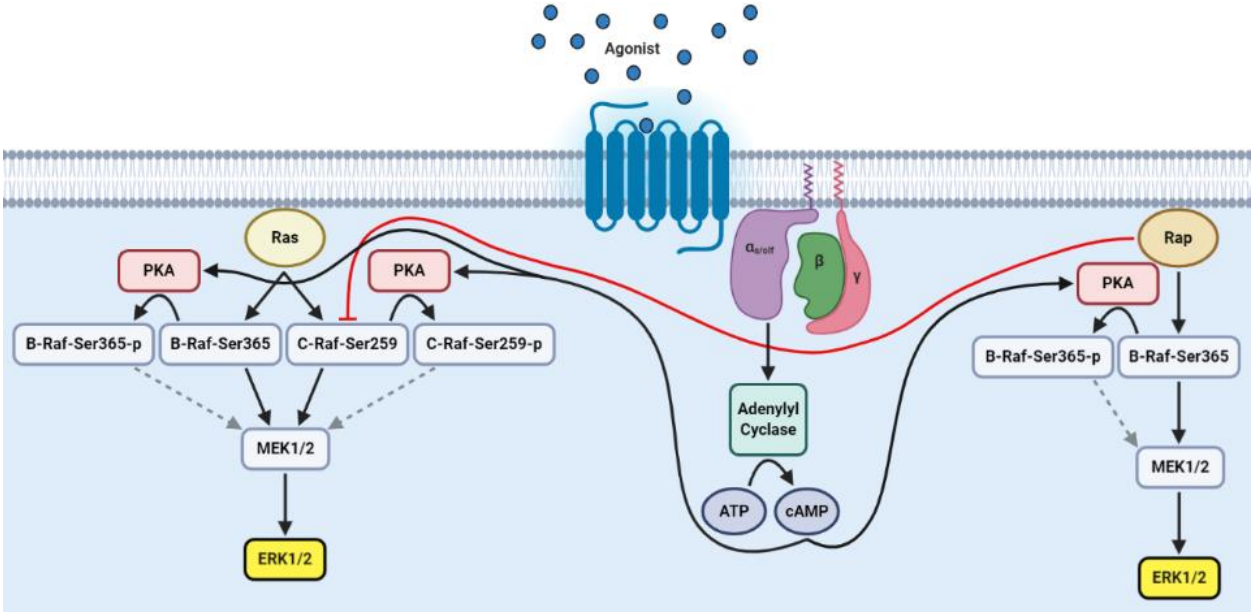


Figure 2. Mechanisms of $G_{s/olf}$-Linked GPCR-Mediated ERK1/2 Inhibition
Black lines denote normal activation; dashed, faded lines denote reduced activation; red line denotes inhibition. Created with BioRender.com.

3.2.3. Additional Mechanisms of $G_{s/olf}$-Linked GPCR-Induced ERK1/2 Activation

3.2.3.1. Gβγ

Potential $G\alpha_{s/olf}$-independent modes of ERK1/2 activation by $G_{s/olf}$-linked GPCRs have also been demonstrated **(Fig. 3)**. A mode involving Gβγ is implicated in at least two cases. Within HEK293 cells, Gβγ-dependent activation of ERK1/2 occurs through a Src-induced Src homology and collagen (Shc)-Sos-Ras-Raf-MEK pathway[100] **(Fig. 3)**. Similarly, Gβγ initiates a SFK-dependent pathway leading to ERK1/2 activation within striatal neurons[101].

3.2.3.2. β-Arrestin (β-arr)

β-arr contributes to $G_{s/olf}$-linked GPCR-mediated ERK1/2 activation[54,102–106], although some $G\alpha_{s/olf}$ function is likely required for these β-arr-dependent effects under most circumstances. In particular, β-arr is capable of scaffolding all three kinases in the ERK1/2 module (Raf, MEK and ERK1/2) and is thought to facilitate signal propagation among these kinases[107] **(Fig. 3)**; however, β-arr appears unable to independently promote Raf signal initiation which indeed has been found to require G protein-dependent pathways such as those downstream of $G\alpha_{s/olf}$[107,108].

3.2.3.3. RTK Transactivation

Transactivation of RTKs underlies $G_{s/olf}$-linked GPCR-mediated activation of ERK1/2 in several instances. In one report, ERK1/2 activation downstream of a $G_{s/olf}$-linked GPCR within HEK293 cells necessitates insulin-like growth factor-1 receptor (IGFR) transactivation through an unclear process involving phosphoinositide 3-kinase (PI3K)[109] **(Fig. 3)**. Another study demonstrates $G_{s/olf}$-linked GPCR-induced ERK1/2 activation requiring IGFR transactivation within both HEK293 cells and rat medullary collecting duct cells, which occurs in a manner

entirely independent of $G_{s/olf}$ but instead contingent on direct Src function[110] **(Fig. 3)**. Additionally, ERK1/2 activation requiring $G_{s/olf}$-linked GPCR-induced transactivation of c-Kit exists within HEK293 cells and melanoma cell lines that also depends completely on Src in a fashion not involving cAMP-mediated processes[111]. Finally, via mechanisms involving Src and increased intracellular Ca^{2+}, $G_{s/olf}$-linked GPCRs induce transactivation of tropomyosin-related kinase A receptor in PC12 cells[112,113], and tropomyosin-related kinase B receptor (TrkBR) in striatal[114] and hippocampal neurons[112,113], that leads to ERK1/2 activation.

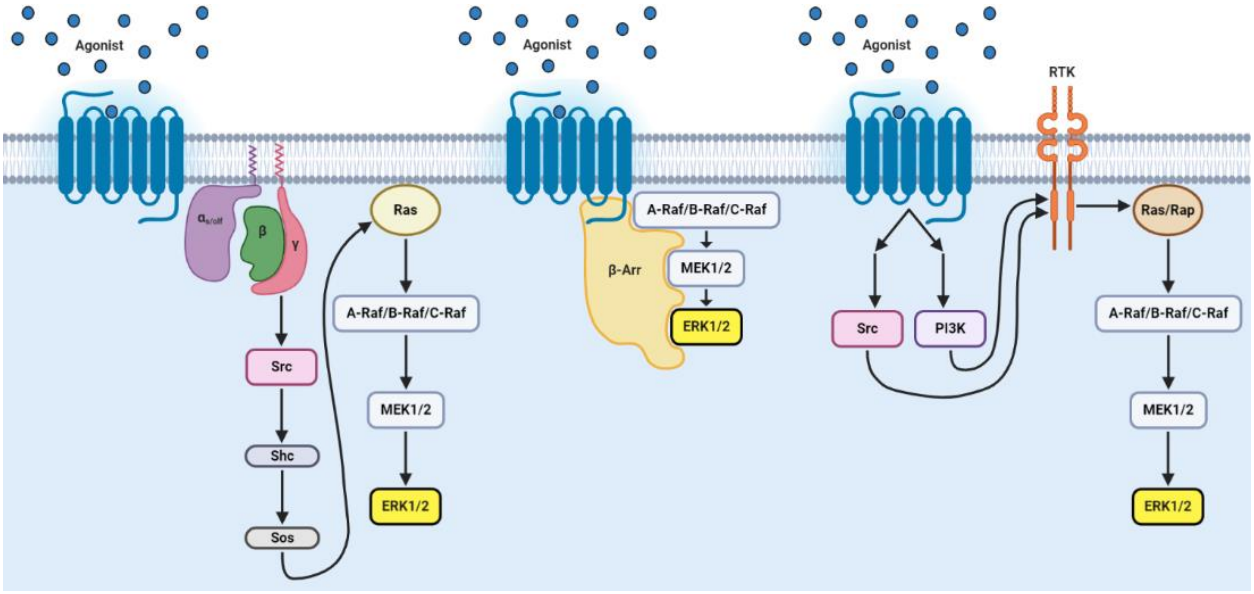


Figure 3. Non-Canonical Mechanisms of $G_{s/olf}$-Linked GPCR-Mediated ERK1/2 Activation
Black lines denote activation. Created with BioRender.com.

3.3. Mechanisms of D_1R-Mediated ERK1/2 Activation

Involvement of D_1R in the regulation of ERK1/2 signaling pathway has been investigated in various cell types both *in vitro* and *in vivo*, with identified roles for D_1R differing in a cell-type specific manner. An early study in SK-N-MC neuroblastoma cells found that D_1R-mediated pathways elicit no change in the activation status of ERK1/2 but instead actually facilitate JNK and p38 activation[115]. Another study using vascular smooth muscle cells reported that D_1R reduces the level of activated ERK1/2[116]. In contrast to these studies, initial investigations of D_1R-mediated modulation of ERK1/2 signaling in HEK293 cells[35] and non-cancerous brain cells—specifically, striatal[47,117–119], hippocampal[45,55,120] and prefrontal cortical neurons[45,121,122]—demonstrate a clear role for D_1R in promoting ERK1/2 activation. Many additional studies, both *in vitro* and *in vivo*, using these same cell types[44,51,53,54,71,77,114,123–129] and others, including neurons from the amygdala[53,77] and various cortical regions[53,122], have confirmed that D_1R predominantly acts to facilitate ERK1/2 activation, with the specific mechanisms utilized undoubtedly depending on the neuronal context **(Fig. 4** and **5)**.

3.3.1. Role of PKA and DARPP-32

Like other $G_{s/olf}$-linked GPCRs, D_1R is capable of activating ERK1/2 through $G\alpha_{s/olf}$-cAMP-mediated signaling cascades **(Fig. 4)**. Several *in vitro* studies examining D_1R-mediated ERK1/2 activation using cultured striatal neurons[35,54,118,127,130] and hippocampal cornu ammonis 1 (CA1) neurons from brain slices[120] demonstrate that this process occurs in a PKA-dependent fashion **(Fig. 4)**. Similarly, *in vivo* studies in the dorsal striatum and nucleus accumbens[46,131–136] also find a PKA requirement for D_1R-mediated ERK1/2 activation.

Indeed, PKA-mediated phosphorylation of DARRP-32-Thr34 is found necessary for activation of ERK1/2 by D_1R in both dorsal striatum and nucleus accumbens in response to psychostimulants[131,133] **(Fig. 4)**. On the other hand, the role of DARPP-32-Thr34 phosphorylation for dorsal striatal D_1R-mediated ERK1/2 activation in the context of PD and LID remains controversial. Three studies using 6-hydroxydopamine (6-OHDA)-lesioned rodent models of PD demonstrate a requirement for PKA-p-DARRP-32-Thr34 signaling in dorsal striatal D_1R-mediated ERK1/2 activation underlying LID[132,134,135]. In contrast, two other reports offer completely contradictory results, suggesting that the supersensitive D_1R-mediated ERK1/2 activation within the DA denervated dorsal striatum of rodents that leads to LID occurs entirely independent of a PKA-p-DARRP-32-Thr34 pathway[133,137].

Phosphorylated DARPP-32-Thr34 promotes ERK1/2 activation through combined actions both upstream and downstream of MEK[131]. While the specific details of p-DARPP-32-Thr34 actions upstream of MEK are incompletely understood, it is well known to act downstream of MEK by inhibiting protein phosphatase 1 (PP1), thereby reducing ERK1/2 dephosphorylation through the resultant reduction in PP1-mediated activation of the ERK1/2 phosphatase striatal enriched phosphatase (STEP) **(Fig. 4)**.

3.3.2. Role of Ras and Rap

The importance of Ras in D_1R-mediated ERK1/2 activation within the dorsal striatum and nucleus accumbens has been demonstrated by several studies that identify a requirement for the Ras-specific GEF RasGRF1 in this process **(Fig. 4)**. One study finds that *in vivo* cocaine-induced ERK1/2 activation downstream of D_1R within both dorsal striatum and nucleus accumbens is greatly reduced in RasGRF1 knockout (KO) mice[70]. Two other studies show that

RasGRF1 KO mice and wild-type (WT) mice lacking striatal RasGRF1 secondary to short hairpin RNA-mediated RasGRF1 knockdown (KD) whom had together been lesioned with 6-OHDA have reduced dorsal striatal D_1R-mediated ERK1/2 activation in response to chronic L-DOPA treatment[49,138]. Furthermore, KD of RasGRF1 in the dorsal striatum of a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned monkey model of PD with existing LID is able to reverse the already elevated dorsal striatal D_1R-mediated ERK1/2 activation exhibited by these animals[138]. Finally, it is observed that direct inhibition of Ras function with lovastatin reduces the ability of repeated L-DOPA treatment to promote D_1R-facilitated ERK1/2 activation in the dorsal striatum of 6-OHDA-lesioned rats[139].

Rap1-dependent processes underlying D_1R-mediated activation of ERK1/2 have also been implicated in several brain regions. Both *in vitro* and *in vivo* experiments within the nucleus accumbens identify a mode of ERK1/2 activation through D_1R involving a PKA-RasGRP2-Rap1-dependent pathway[46] **(Fig. 4)**. Results from two additional reports imply the existence of another striatal D_1R pathway contributing to PKA-dependent ERK1/2 activation through Rap1. In particular, D_1R promotes PKA-mediated phosphorylation of the Rap1-specific GAP Rap1GAP, which facilitates Rap1GAP inactivation and thereby enhances Rap1 activity, presumably leading to downstream activation of ERK1/2[72,140] **(Fig. 4)**.

In contrast, a different *in vivo* study within the nucleus accumbens finds D_1R-mediated ERK1/2 activation to occur via a PKA-independent mechanism involving RapGEF2 and presumed downstream activation of Rap1[77] **(Fig. 4)**. This same mechanism is also determined to account for *in vivo* D_1R-mediated activation of ERK1/2 within the hippocampus and amygdala[77].

Additionally, a PKA-independent mechanism of D_1R-mediated ERK1/2 activation through Rap1 involving EPAC2 appears to occur within cortical neurons[71] **(Fig. 4)**.

3.3.3. Role of SFKs, SH2 Domain-Containing Tyrosine Phosphatase 2 (Shp-2) and TrkBR

SFKs and Shp-2 have been implicated as important signaling components in D_1R-mediated ERK1/2 activation. One study finds a requirement of SFKs for ERK1/2 activation downstream of D_1R *in vitro* within striatal slices[141]. Moreover, demonstration in this study of much higher striatal Fyn expression than that of Src, another major SFK within the striatum, along with the finding of increased striatal Fyn phosphorylation following D_1R activation but no concurrent D_1R-induced change in phosphorylation of Src, suggest selective involvement of Fyn in D_1R-mediated activation of ERK1/2 with the striatum. Results from another study[101] also link Fyn to striatal D_1R-mediated ERK1/2 activation; intriguingly, involvement of Fyn in this instance is found to require a cAMP-independent Gβγ-mediated upstream pathway **(Fig. 4)**.

In contrast, D_1R-mediated ERK1/2 activation within HEK293 cells is specifically shown to require PKA-dependent activation of Src for the purpose of phosphorylating the tyrosine phosphatase Shp-2[35] **(Fig. 4)**. Indeed, a Shp-2-D_1R complex has been identified in HEK293 cells stably expressing D_1R[35]; specifically, this complex results from direct Shp-2 interaction with its canonical binding motif, the immunoreceptor tyrosine-based inhibitory motif (S/I/V/LXYXXI/V/L), present within the extreme N-terminal region of D_1R-IL3 **(Fig. 4)**. Moreover, the same group demonstrates that a PKA-Src-Shp-2-dependent pathway proceeding through Ras and C-Raf underlies D_1R-mediated ERK1/2 activation within cultured striatal neurons[35] **(Fig. 4)**. Offering further support for a role of Shp-2 in ERK1/2 activation by D_1R, another study finds

that KD of Shp-2 in 6-OHDA-lesioned mice prior to an L-DOPA treatment course reduces L-DOPA-induced ERK1/2 activation downstream of D_1R within the dorsal striatum[142].

Interestingly, in one instance D_1R-mediated ERK1/2 activation within striatal neurons has also been shown to require transactivation of TrkBR[114]. The mechanism underlying TrkBR transactivation by D_1R is demonstrated to depend on increased intracellular Ca^{2+} through a yet uncharacterized pathway **(Fig. 5)**. Given the known involvement of Fyn, Src and Shp-2 in TrkBR signaling[143–146], D_1R-facilitated transactivation of TrkBR could also conceivably involve these three proteins, however this remains to be investigated.

3.3.4. Role of Scaffolding Proteins and D_1R Trafficking

Few studies have examined the relationship between D_1R trafficking and D_1R-mediated ERK1/2 activation; in particular, the contributing roles of β-arr and other scaffolding proteins in this interplay remain scarcely investigated. One study using SK-N-MC neuroblastoma cells[102] demonstrates that the majority of phosphorylated ERK1/2 downstream of D_1R is retained in the cytoplasm rather than translocated to the nucleus, which coincides with D_1R activation-induced formation of a D_1R-β-arr2-p-ERK1/2 tripartite complex **(Fig. 4)**. Indeed, β-arr2 is also implicated in D_1R-mediated ERK1/2 activation within primary striatal neurons in another study that similarly detects the presence of cytoplasmic D_1R-β-arr2-p-ERK1/2 complexes following activation of D_1R[54]. Additionally, another report characterizes β-arr2 involvement in morphine induced D_1R-mediated ERK1/2 activation within the striatum[106]. Finally, β-arr2-dependency is implicated in striatal D_1R-mediated ERK1/2 activation elicited by D_1R-histamine H_3 receptor (H_3R) heterodimers[147] **(Fig. 5)**.

Two other scaffolding proteins, postsynaptic density protein 95 (PSD-95) and caveolin-1 (Cav-1), also known to modulate ERK1/2 pathway in certain contexts[148,149] and physically interact with D_1R[32,33], are implicated in D_1R-mediated ERK1/2 activation within the dorsal striatum **(Fig. 5)**. PSD-95 appears to positively regulate ERK1/2 activation downstream of D_1R based on the finding that PSD-95 KD in a rat model of LID greatly attenuates the elevated D_1R-mediated ERK1/2 activation within these animals[150]. In contrast, Cav-1 may act to antagonize D_1R-mediated activation of ERK1/2, in an indirect fashion, by limiting D_1R surface expression. This notion is supported by the previously established role of Cav-1 in D_1R trafficking[32], combined with the demonstration that extended access methamphetamine self-administration leads to a major reduction in the level of Cav-1 in concomitance with a large increase in both D_1R expression and D_1R-mediated ERK1/2 activation[151].

Finally, another mechanism regulating D_1R surface expression appears to indirectly impact ERK1/2 activation downstream of D_1R within HEK293A cells. Specifically, protein kinase D1 (PKD1)-mediated phosphorylation of Ser421 within the C-terminus of D_1R promotes concurrent enhancements in D_1R surface expression and D_1R-facilitated activation of ERK1/2[152] **(Fig. 5)**.

3.3.5. Role of D_1R Interactions with D_2R-class: D_2R and D_3R

D_1R is co-expressed with D_2R-class in various neuronal populations throughout the forebrain[153,154] and has been demonstrated to form heterodimeric signaling complexes with both D_2R[154,155] and D_3R[153,156]. Moreover, clear evidence has been provided for a role of D_1R interactions with D_2R[157,158] and D_3R[56,159–163] in modulating D_1R-mediated ERK1/2 activation **(Fig. 5)**. It is found that D_3R normally acts to antagonize D_1R-mediated activation of ERK1/2 within

the dorsal striatum, nucleus accumbens and PFC[56,159,160]. Using D_3R KO mice, it is shown that cocaine treatment leads to significantly increased D_1R-mediated ERK1/2 activation in the dorsal striatum when D_3R is not present, implying that D_3R functions to reduce the magnitude of dorsal striatal ERK1/2 activation by D_1R. Moreover, cocaine-induced ERK1/2 activation downstream of D_1R in the nucleus accumbens and PFC of D_3R KO mice exhibits a sustained time course, suggesting that D_3R acts to decrease the duration of D_1R-mediated ERK1/2 activation in these brain regions.

Intriguingly, recent studies using 6-OHDA-lesioned mice as a PD model[161,162] have identified that D_1R-mediated ERK1/2 activation within the DA-depleted dorsal striatum is modulated by D_3R in an opposite manner relative to that observed within the intact dorsal striatum. Namely, D_3R acts to facilitate rather than inhibit ERK1/2 activation downstream of D_1R within the DA-depleted dorsal striatum. A specific role for D_1R-D_3R heteromers in D_1R-mediated activation of ERK1/2 is demonstrated in another study using co-transfected Chinese hamster ovary cells[163]. In particular, synergistically enhanced ERK1/2 activation is observed when both receptors within D_1R-D_3R heteromers are activated simultaneously with subtype-selective agonists. Interestingly, this synergistic enhancement of ERK1/2 activation by D_1R appears to arise secondarily to a D_3R-facilitated switch in the D_1R signaling modality controlling ERK1/2 activation, specifically eliciting a change from G protein-dependent to G protein-independent D_1R-mediated ERK1/2 activation. Furthermore, *ex vivo* experiments within the striatum demonstrate that D_1R-D_3R synergism regulating ERK1/2 activation occurs selectively in the most ventromedial portion of the nucleus accumbens shell[163].

D_1R-mediated ERK1/2 activation within the nucleus accumbens is also affected by D_1R interactions with D_2R. The majority of striatal MSNs selectively express D_1R or D_2R; these neurons respectively send their projections along either the direct- or indirect-basal ganglia output pathway[52,157,164]. However, the nucleus accumbens contains a relatively large population of MSNs that exhibit co-expression of D_1R and D_2R, along with associated axonal projections that terminate in more diverse basal ganglia output nuclei[157,164]. Indeed, Hasbi and colleagues[157] show that specific activation of D_1R-D_2R heteromers by SKF83959 prior to cocaine treatment greatly reduces the resultant accumbal D_1R-mediated ERK1/2 activation, thus implying an inhibitory role for these heteromers in ERK1/2 activation by D_1R within the nucleus accumbens. Compellingly, another study[158] finds that mice lacking D_2R specifically within striatal indirect-pathway MSNs exhibit a switch in D_1R-mediated signaling preference selectively within the nucleus accumbens direct-pathway MSNs. In particular, accumbal D_1R signaling in WT mice is primarily involved in pathways not related to ERK1/2 regulation, whereas mice without indirect-pathway MSNs expressing D_2R show a striking bias toward D_1R-mediated ERK1/2 activation. The authors believe that this phenomenon could arise from heightened indirect-pathway MSN-mediated lateral inhibition of direct-pathway MSNs in D_2R mutant mice. They hypothesize that the heightened lateral inhibition triggers a yet to be characterized mechanism promoting enhanced ERK1/2 activation by D_1R in order to facilitate activity of direct-pathway MSNs and motor output in an otherwise depressed state.

3.3.6. Role of D_1R Interactions with Glutamatergic Transmission: NMDAR and Metabotropic Glutamate Receptors (mGluRs)

Signaling pathways downstream of D_1R can be intricately linked with glutamatergic transmission to control ERK1/2 activation within the brain[45,51,101,119,121,122,127,136,165–170]. Of paramount importance in this interplay is the relationship between D_1R and NMDAR **(Fig. 5)**, known to encompass bidirectional D_1R-NMDAR functional modulation through second messenger-dependent protein kinases as well as direct D_1R-CT physical interactions with both NMDAR subunit 1 (GluN1) and NMDAR subunit 2A (GluN2A)[171–180]. NMDAR-dependency for D_1R-mediated ERK1/2 activation is observed within the striatum[45,101,119,165,166,169,170], hippocampus[51] and PFC[122]. In the dorsal striatum and nucleus accumbens, D_1R-mediated activation of ERK1/2 induced by either cocaine or D_1R agonist is found to require D_1R-NMDAR co-activation[45,101,119,165,169,170]. Moreover, accumbal ERK1/2 activation downstream of D_1R elicited by an appetitive conditioned stimulus also necessitates the function of NMDAR[166]. Interestingly, in stark contrast to the dorsal striatum of naïve rats, D_1R-mediated ERK1/2 activation within the dorsal striatum of 6-OHDA-lesioned rats occurs independently of NMDAR[47,167]. Thus, dopaminergic denervation of the striatum appears to facilitate a switch from NMDAR-dependent to NMDAR-independent D_1R-mediated ERK1/2 activation in the dorsal striatal region.

A few studies have highlighted the significance of NMDAR subunit composition in NMDAR-facilitated modulation of ERK1/2 activation by D_1R, identifying NMDAR subunit 2B (GluN2B) as a crucial component for this process. Both striatal and hippocampal D_1R-mediated ERK1/2 activation are reported to specifically require the presence of GluN2B-containing

NMDAR. A GluN2B-dependency for D_1R-mediated activation of ERK1/2 is observed within D_1R-class agonist-treated striatal neurons *in vitro*, and in the dorsal striatum and nucleus accumbens of cocaine-treated mice[101]. Interestingly, ERK1/2 activation by D_1R within these experimental settings does not require a cAMP-dependent pathway. Instead, the particular mechanism imparting necessitation of GluN2B for this process involves Gβγ-induced Fyn activation and consequent Fyn-mediated phosphorylation of GluN2B, which acts to enhance Ca^{2+} influx through GluN2B-containing NMDAR. On the other hand, GluN2B-dependent D_1R-mediated ERK1/2 activation within the hippocampus, specifically demonstrated within dentate gyrus granule cells, appears to occur in a PKC-dependent manner[51], although additional details of the underlying mechanism remain uncharacterized.

Additionally, striatal ERK1/2 activation by D_1R can depend on D_1R physical interaction with GluN1. Specifically, experiments using a D_1R-GluN1 interfering peptide identify a requirement for D_1R-GluN1 complexes in D_1R-mediated ERK1/2 activation both in cultured striatal neurons and in mouse nucleus accumbens[165].

Several studies also demonstrate an important modulatory role for mGluRs in ERK1/2 activation downstream of D_1R within the striatum[127,136,167,168], hippocampus[51] and PFC[121]. Group I mGluRs ($mGluR_1$ and $mGluR_5$) in particular have been identified as key facilitators of striatal D_1R-mediated ERK1/2 activation. Within primary striatal neurons, co-application of D_1R agonist and non-selective group I mGluR agonist leads to greatly enhanced D_1R-mediated activation of ERK1/2 relative to that elicited by D_1R agonist alone[127]. Moreover, $mGluR_5$, rather than $mGluR_1$, is predominantly responsible for potentiating ERK1/2 activation by D_1R in primary striatal neurons[127]. Indeed, complex formation between D_1R and $mGluR_5$ has recently been identified in

rat striatum, and is critically involved in pathological D_1R-mediated ERK1/2 activation underlying LID in 6-OHDA-lesioned rats[168]. Interestingly, D_1R-mGluR$_5$ heteromers couple to G_q and activate ERK1/2 through a phospholipase C (PLC)-dependent process within these animals **(Fig. 5)**. The existence of striatal D_1R-mGluR$_5$ complex sheds light on previous reports implicating selective mGluR$_5$ involvement in hyperactivated D_1R-mediated ERK1/2 activation within the striatum of 6-OHDA-lesioned rodents. Specifically, pharmacological inhibition of either mGluR$_1$ or mGluR$_5$ reduces striatal ERK1/2 activation downstream of D_1R in 6-OHDA lesioned rats, however, this reduction is much more robust with blockade of mGluR$_5$[167]. In line with this finding, 6-OHDA-lesioned mice lacking mGluR$_5$ in D_1R-expressing MSNs (D_1R-MSNs) experience severely reduced striatal D_1R-induced ERK1/2 activation[136].

Group I mGluR-facilitated potentiation of D_1R-mediated ERK1/2 activation is also observed in the hippocampus. In particular, selective antagonism of either mGluR$_1$ or mGluR$_5$ significantly reduces ERK1/2 activation by D_1R in granule cells of the dentate gyrus[51]. In contrast, both mGluR group I and group II (mGluR$_2$ and mGluR$_3$) stimulation augments D_1R-mediated activation of ERK1/2 within the PFC[121] **(Fig. 5)**.

3.3.7. Role of D_1R Interactions with Neuromodulatory Systems

3.3.7.1. Cholinergic Transmission

Functional interactions between D_1R and several neuromodulatory systems also regulate D_1R-mediated activation of ERK1/2. The cholinergic system plays a major role in reducing ERK1/2 activation by D_1R within the striatum and PFC. Indeed, non-specific antagonism of muscarinic acetylcholine receptors (mAChRs) with scopolamine was initially shown to enhance D_1R-mediated ERK1/2 activation in the dorsal striatum of 6-OHDA-lesioned

rats[47]. More recently, it was found that scopolamine treatment selectively potentiates D_1R-mediated activation of the synaptic ERK1/2 pool in the dorsal striatum, nucleus accumbens and medial PFC (mPFC) of unlesioned rats[181]. The M_4 mAChR (M_4R) appears to be specifically responsible for cholinergic-induced inhibition of D_1R-mediated ERK1/2 activation **(Fig. 5)**. One study[182] demonstrates that amphetamine-induced ERK1/2 activation downstream of D_1R within the dorsal striatum, nucleus accumbens and mPFC is significantly enhanced by blockade of M_4R, but remains unaffected by blockade of M_1 mAChR. In agreement with this finding, positive allosteric modulation of M_4R inhibits D_1R-mediated ERK1/2 activation within these same brain regions[183].

3.3.7.2. Histaminergic and Sigmaergic Transmission

An important interplay exists between histaminergic and sigmaergic transmission that modulates D_1R-mediated ERK1/2 activation within the striatum[147]. This modulatory effect is exerted through the action of heterotrimeric complexes composed of D_1R, sigma-1 receptor (σ_1R) and H_3R **(Fig. 5)**. Under conditions in which σ_1R is not activated, D_1R-mediated ERK1/2 activation from σ_1R-D_1R-H_3R complexes occurs in a G protein-independent fashion that instead requires β-arr function. Additionally, within these signaling complexes, either co-activation of D_1R and H_3R or selective blockade of H_3R results in greatly reduced D_1R-mediated ERK1/2 activation. Intriguingly, when σ_1R within this trimeric complex is activated concurrently with D_1R, the ability of H_3R to antagonize D_1R-mediated ERK1/2 activation as stated above is abolished, and moreover, the mechanism of D_1R-mediated ERK1/2 activation is switched to a G protein dependent process. Thus, the H_3R interaction with D_1R imparts D_1R-mediated ERK1/2 activation with a β-arr-dependent, G protein-independent mechanism, and can act as a brake

on this D_1R-mediated signaling pathway. While in contrast, σ_1R abrogates the ability of H_3R to modulate the ERK1/2 cascade downstream of D_1R and additionally promotes G protein-dependent D_1R-mediated ERK1/2 activation.

3.3.7.3. Adenosinergic Transmission

One study shows that adenosine A_1 receptor (A_1R) facilitates tonic inhibition of D_1R-mediated ERK1/2 activation within the dorsal striatum, nucleus accumbens and mPFC[184] **(Fig. 5)**. Given the characterization of endogenous D_1R-A_1R heteromers[185–187], such complexes may be important for the inhibitory effect of A_1R on D_1R-mediated ERK1/2 activation in these brain regions.

3.3.7.4. Cannabinergic Transmission

D_1R-mediated ERK1/2 activation is oppositely regulated by signaling of the presynaptic cannabinoid-1 receptor (CB_1R) within striatum versus hippocampus **(Fig. 5)**. Tetrahydrocannabinol (THC)- and cocaine-induced ERK1/2 activation by D_1R in the dorsal striatum and nucleus accumbens require the presence of CB_1R[128,188]. While in direct contrast, D_1R-mediated activation of ERK1/2 is antagonized by CB_1R transmission in hippocampal dentate gyrus granule cells[125].

3.3.7.5. Serotonergic Transmission

The serotonergic system exerts a trans-synaptic modulation of D_1R-mediated ERK1/2 activation. Namely, 5-hydroxytryptamine (5-HT) 2B receptor (5-HT_{2B}R)-facilitated potentiation of DA release into the nucleus accumbens appears to be required for 3,4-methylenedioxymethamphetamine (MDMA)-induced D_1R-dependent accumbal ERK1/2 activation[189] **(Fig. 5)**.

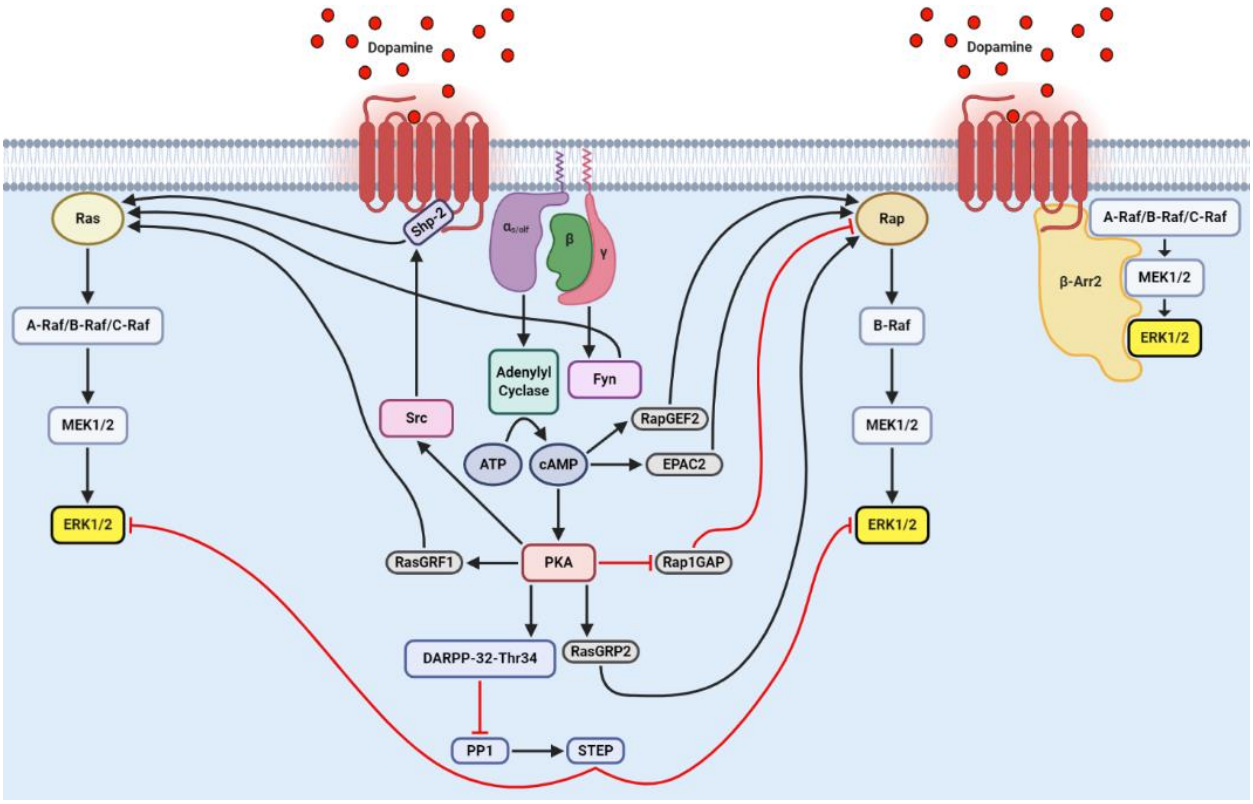
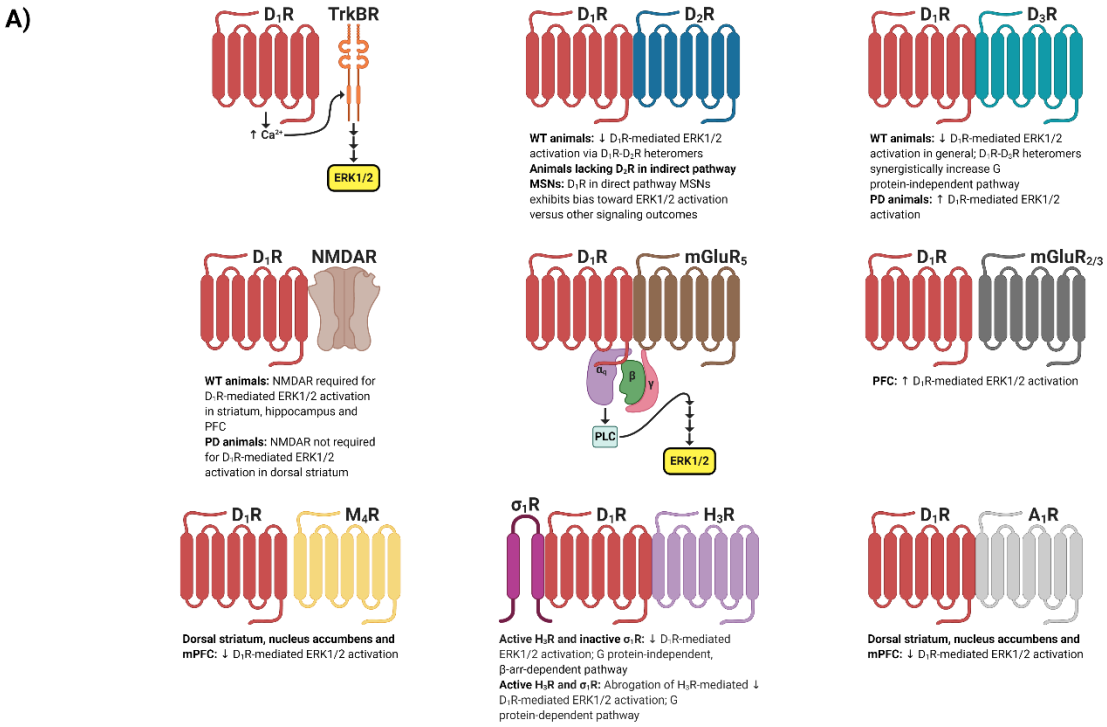


Figure 4. Mechanisms of D_1R-Mediated ERK1/2 Activation

Black lines denote activation; red lines denote inhibition. Created with BioRender.com.



Protein	Interaction with D_1R	Effect
PSD-95	Physical interaction with D_1R CT	Increases D_1R-mediated ERK1/2 activation in rat model of LID
Cav-1	Physical interaction with D_1R transmembrane domain 7	Concomitantly reduces D_1R surface expression and D_1R-mediated ERK1/2 activation
PKD1	Phosphorylation of Ser421 within D_1R CT	Concomitantly increases D_1R surface expression and D_1R-mediated ERK1/2 activation

Figure 5. Additional Modulators of D_1R-Mediated ERK1/2 Activation

A) Postsynaptic receptors modulating D_1R-mediated ERK1/2 activation. **B)** Presynaptic receptors modulating D_1R-mediated ERK1/2 activation. **C)** Other proteins modulating D_1R-mediated ERK1/2 activation. Created with BioRender.com.

3.4. Physiological Roles of D_1R-Mediated ERK1/2 Activation

Dopaminergic activation of ERK1/2 specifically through D_1R is a crucial signaling module for basic physiological processes enabling normal post-natal development and survival. This is supported by findings from a study assessing the phenotypic properties of mice with both ERK1 and ERK2 genes deleted selectively in either D_1R- or D_2R-expressing cells. In particular, mice lacking ERK1/2 within D_1R-expressing cells experience early postnatal death, whereas mice without ERK1/2 in D_2R-expressing cells are viable, although they display hyperlocomotor activity and altered D_2R-expressing MSN function[190]. The critical role of ERK1/2 in D_1R-MSNs during development strongly agrees with the physiological relevance of D_1R-mediated ERK1/2 activation in developed animals. Specifically, D_1R-mediated activation of ERK1/2 plays an important role in the regulation of gene expression, protein synthesis, and excitatory synaptic transmission in the CNS, and significantly modulates normal learning and memory processes, all of which is discussed further in the following sections and depicted visually in **Figure 6**.

3.4.1. Gene Expression and Protein Synthesis

One important event leading to D_1R-dependent regulation of gene expression is phosphorylation of the constitutively expressed transcription factors cAMP-responsive element-binding protein (CREB) and ELK1. CREB phosphorylation downstream of D_1R activation occurs in an ERK1/2-dependent fashion within the striatum[118,127,130,160,166,169,191,192], hippocampus[120] and PFC[122] **(Fig. 6)**. Similarly, ELK1 phosphorylation downstream of D_1R-mediated ERK1/2 activation is reported within the striatum[54,123,128,131,170,193] and PFC[131] **(Fig. 6)**.

A second important event involved in regulation of gene expression by D_1R is phosphorylation of histone H3. D_1R-mediated ERK1/2 activation leading to histone H3

phosphorylation-dependent enhancement of transcription is demonstrated within the hippocampus[51,125] and striatum[48,52,132,161,192,194,195] **(Fig. 6)**.

Ultimately, the conjunctive phosphorylation of CREB, ELK1 and histone H3 downstream of ERK1/2 activation by D_1R leads to induction of various immediate early genes (IEGs), including Fos family members[44,47,70,132,159,161,188,192–199], Zif268[125,128,194,196], early growth response protein 2 (EGR2)[196], activity-regulated cytoskeleton-associated protein (Arc)[47,125], Homer1a[200], Nur77 and neuron-derived orphan receptor 1 (Nor-1)[50] **(Fig. 6)**.

D_1R-mediated activation of ERK1/2 is also implicated in an event with known importance for increasing the rate of protein synthesis. Namely, dorsal striatal[52,201,202] and hippocampal[51,125] D_1R-mediated ERK1/2 activation promotes phosphorylation of ribosomal protein S6 (rpS6) **(Fig. 6)**.

3.4.2. Excitatory Synaptic Transmission

A major role for D_1R-mediated ERK1/2 activation in modulation of excitatory synaptic transmission is demonstrated in several studies. D_1R-mediated phosphorylation of AMPAR and NMDAR within rat striatum, hippocampus and PFC depends on ERK1/2 activation[45]. Specifically, phosphorylation of AMPAR subunit 1 (GluA1)-Ser831, GluA1-Ser845, GluN1-Ser897 and GluN2B-Ser1303 are regulated in an ERK1/2-dependent fashion downstream of D_1R activation **(Fig. 6)**. D_1R-mediated ERK1/2 activation is also implicated in the removal of AMPAR subunit 2 (GluA2) from the synaptic membrane in cortical pyramidal neurons[71] **(Fig. 6)**.

Indeed, important roles for D_1R-mediated ERK1/2 activation in plasticity of glutamatergic synapses have been revealed in several brain regions **(Fig. 6)**. D_1R-mediated ERK1/2 activation can facilitate the conversion of transient early long-term potentiation (LTP)

into persistent late LTP within the CA1 region of the hippocampus[124]. Interestingly, D_1R-mediated ERK1/2 activation may also modulate long-term depression (LTD) induction in the hippocampal CA1 region[203,204]. Similarly, within prefrontal cortical slices, the LTD at synapses between layer I-II afferents and layer V pyramidal neurons depends in part on ERK1/2 signaling via D_1R activation[121]. Furthermore, D_1R-mediated ERK1/2 activation is important for both the early and late phase of LTP within the dorsal striatum[205] and nucleus accumbens[57,165,200]. One mechanism through which activated ERK1/2 downstream of D_1R contributes to accumbal LTP maintenance appears to involve the facilitation of $mGluR_5$-mediated potentiation of slow inward current through GluN2B-containing NMDAR[200].

In addition to playing a role in functional plasticity of excitatory synapses, D_1R-mediated ERK1/2 activation regulates structural plasticity of dendritic spines within the PFC and striatum **(Fig. 6)**. Specifically, D_1R-mediated ERK1/2 activation facilitates shrinkage of dendritic spines within cortical pyramidal neurons[71], while along a similar line, within striatal MSNs is implicated in preventing spine head width increases[72].

Finally, a study using the lateral pyloric neuron from *Panilirus interruptus*[206] suggests that D_1R-mediated ERK1/2 activation also plays a role in the regulation of non-glutamatergic ion channels. This study demonstrates that ERK1/2 activation is partially required for a D_1R-mediated persistent increase in transient K^+ current. Importantly, these results are likely relevant to mammalian D_1R-mediated ERK1/2 signaling because the endogenous DARs in this invertebrate model have been previously shown to signal via the canonical GPCR signaling pathways in their native system[207,208] and have been demonstrated to behave exactly the same

as mammalian DARs when expressed in HEK293 cells[209,210]. Nonetheless, it remains to be shown that D_1R-mediated ERK1/2 activation can regulate mammalian K^+ current.

3.4.3. Learning and Memory

Several studies suggest an important role of D_1R-mediated ERK1/2 activation in certain aspects of learning and memory **(Fig. 6)**. Exposure of mice to an appetitive conditioned stimulus elicits D_1R-mediated ERK1/2 activation in the nucleus accumbens[166]. Similarly, ERK1/2 signaling specifically in D_1R-MSNs of nucleus accumbens core through D_1R activation plays an essential part in stimulus reward learning[211].

D_1R-mediated ERK1/2 activation within the nucleus accumbens core is also involved in motivation for natural rewards[211]. The role of striatal D_1R-mediated ERK1/2 activation in natural motivational states is additionally supported by the finding that food-restricted rats have increased D_1R-mediated ERK1/2 activation in dorsal striatum and nucleus accumbens relative to ad libitum-fed rats[169,191]. Furthermore, motivation of male mice to engage in mating behavior is positively regulated by dorsal striatal D_1R-mediated ERK1/2 activation[212].

Finally, D_1R-mediated ERK1/2 activation in the PFC regulates long-term recognition memory[44] and long-term memory storage for fear conditioning[126].

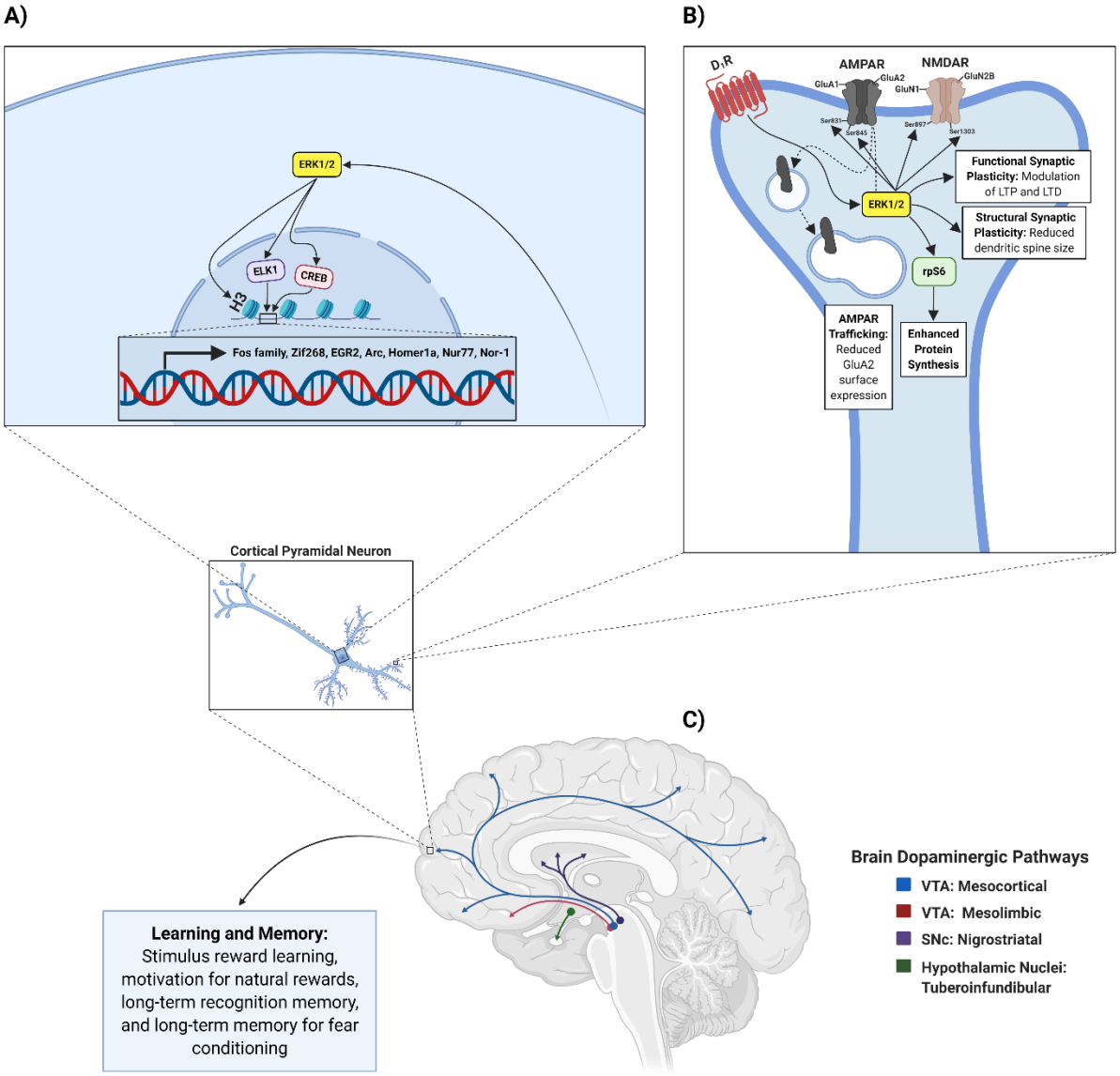


Figure 6. Physiological Roles of D_1R-Mediated ERK1/2 Activation

A) Nuclear functions of D_1R-mediated ERK1/2 activation. **B)** Postsynaptic functions of D_1R-mediated ERK1/2 activation. Dashed lines denote ERK1/2-dependent GluA2 trafficking. **C)** Functions of D_1R-mediated ERK1/2 activation in normal cognition and behavior. Created with BioRender.com.

3.5. Pathological Roles of D_1R-Mediated ERK1/2 Activation

3.5.1. Drug Addiction

3.5.1.1. Cognitive and Behavioral Processes

A common feature of nearly all drugs of abuse is the facilitation of increased dopaminergic transmission within several brain regions, including the nucleus accumbens, dorsal striatum, PFC and amygdala[213]. Within each of these brain regions, there is a concomitant drug-induced increase in D_1R-mediated activation of synaptic ERK1/2[53,214] linked to behavioral and cognitive effects elicited by said drugs which are thought to represent aspects of drug addiction. Indeed, locomotor sensitization in response to repeated cocaine or amphetamine administration requires D_1R-mediated activation of ERK1/2 within the striatum[57,70,101,119,131,157,158,165,192,200,215,216]. A similar necessity of striatal D_1R-mediated ERK1/2 activation is reported for morphine-induced locomotor sensitization[106].

D_1R-mediated ERK1/2 activation also plays a role in the cognitive processes underlying drug reward, as measured indirectly using the conditioned place preference (CPP) test[217]. Several studies demonstrate that D_1R-mediated ERK1/2 activation within the dorsal striatum and nucleus accumbens is critical in CPP for the psychostimulants cocaine, amphetamine and methamphetamine[46,56,70,101,119,123,157,192,215,218,219]. Indeed, D_1R-mediated ERK1/2 activation specifically within the core region of the nucleus accumbens may be especially important for this process[219]. THC-induced CPP also necessitates striatal D_1R-mediated ERK1/2 activation[128]. Additionally, one study identifies a contributing role of prefrontal cortical D_1R-mediated ERK1/2 activation for CPP in response to cocaine treatment[56].

Intriguingly, in contrast to the identified importance of accumbal core D_1R-mediated ERK1/2 activation in CPP-measured cue elicited drug seeking mentioned above, D_1R-mediated ERK1/2 activation within the shell region of the nucleus accumbens is critical for recognition of drug-paired contextual cues following a prolonged period of withdrawal. Borgkvist and colleagues[220] show that D_1R-mediated activation of ERK1/2 occurs selectively in the nucleus accumbens shell after a 28-day period of morphine withdrawal when mice are re-exposed to an environment previously paired with morphine administration.

3.5.1.2. Cellular and Molecular Processes

D_1R-mediated ERK1/2 activation exerts its cognitive and behavioral effects central to drug addiction through direct regulation of IEG expression. Specific IEGs induced by phosphorylated ERK1/2 downstream of D_1R within the striatum in response to drugs of abuse include Fos family members[70,129,159,188,192–194,196], Zif268[128,194,196], EGR2[196], Nur77 and Nor-1[50]. This occurs primarily through ERK1/2-mediated activation of CREB- and ELK1-facilitated transcription[123,128,131,160,170,192,193,216]. ERK1/2-induced histone H3 phosphorylation also contributes to this process by enhancing the rate of transcription downstream of CREB and ELK1[192,194]. These initial transient changes in gene expression induced by D_1R-mediated activation of ERK1/2 lead to additional long-lasting changes in the genetic expression program, including induction of the truncated FosB isoform ΔFosB, that ultimately result in the development of addiction[157,213,221,222].

A second major downstream action of ERK1/2 activation by D_1R involved in drug addiction, likely linked with its effects on gene expression described above, is modulation of glutamatergic transmission. Results from one study[223] implicate a role for D_1R-mediated ERK1/2

activation in enhanced accumbal AMPAR expression that appears to underlie cocaine-induced locomotor sensitization. Specifically, repeated non-contingent cocaine injections lead to locomotor sensitization after a 21-day withdrawal while concurrently increasing accumbal synaptic expression of GluN1, GluN2B, and GluA1 at this time point—but not at an earlier assessed time point—as well as increasing accumbal ERK1/2 activation in a similar time course. Moreover, at the 21-day time point, the locomotor sensitization, increased GluA1 expression, and elevated ERK1/2 activation are all blocked by inhibition of GluN2B-containing NMDAR, while the increased GluA1 expression is also prevented by direct inhibition of ERK1/2 activation. These results thus suggest that cocaine-induced locomotor sensitization occurs through a mechanism involving a delayed increase in GluN2B-containing NMDAR within the nucleus accumbens, which facilitates D_1R-mediated ERK1/2 activation-dependent enhancement of accumbal AMPAR expression.

Another study[57] demonstrates that acute cocaine treatment is sufficient to elicit ERK1/2-dependent potentiation of specifically D_1R-MSN cortico-accumbal synapses when assessed one week later. Moreover, the animals in this study express locomotor sensitization after one week that is found to depend directly on their potentiated D_1R-MSN cortico-accumbal synapses. Similarly, chronic cocaine treatment leads to an ERK1/2-dependent process that prevents D_1R-MSNs within corticostriatal slices from undergoing depotentiation[200].

3.5.2. PD and LID

3.5.2.1. Development and Maintenance of LID

Supersensitive D_1R-mediated ERK1/2 activation within the striatum is a hallmark feature of PD animal models[47–49,122,132–139,142,161,162,167,195,197–199,201,202,205,224–230]. A foundational study[47]

examining the role of D_1R-induced ERK1/2 activation in PD using the 6-OHDA-lesioned rat model found that D_1R stimulation promotes robust ERK1/2 phosphorylation in both the dorsal and ventral striatum, while producing very little striatal ERK1/2 phosphorylation in non-lesioned animals. Interestingly, adult rats lesioned with 6-OHDA as neonates (postnatal day 3) show an increase of ERK1/2 activation in the dorsal striatum and nucleus accumbens from repeated D_1R agonist treatment (3 doses of SKF38393—6 mg/kg, 3 mg/kg and 3 mg/kg—each spaced 1 week apart) that lasts for approximately 1 hour[122]. In striking contrast, repeated D_1R stimulation in these lesioned animals also leads to much more sustained ERK1/2 activation in several cortical regions lasting up to 7 days, along with an especially prolonged increase of ERK1/2 activation in mPFC for 36 days.

This hypersensitive D_1R activity promoting increased ERK1/2 activation within the striatum following DA denervation has subsequently been implicated as a crucial component to the development and maintenance of dyskinesia in response to chronic L-DOPA treatment for PD-like motor deficits. Indeed, numerous studies[48,49,132,134,135,137–139,142,161,162,167,195,197–199,201,226–230] utilizing the 6-OHDA model of PD find that the level of D_1R-mediated ERK1/2 activation positively correlates with dyskinesia severity in response to the L-DOPA treatment regimen. In fact, in rodent models of LID, the main signaling dysfunction appears to be an inability to desensitize D_1R-mediated ERK1/2 activation with repeated L-DOPA administration[197]. Interestingly however, one study[202] using an MPTP-lesioned rhesus monkey model of PD found that D_1R-mediated ERK1/2 activation is only important for the initial induction of LID rather than for LID maintenance.

Intrinsic differences in the 6-OHDA- and MPTP-lesioning procedures, along with species differences in rodents and monkeys, could be important factors in this observed discrepancy for the role of D_1R-mediated ERK1/2 activation in LID maintenance. Another potential contributing factor could be the much longer L-DOPA treatment course of three months received by the monkeys relative to the rodents, which receive L-DOPA treatment courses lasting no longer than one month. However, MPTP-lesioned rhesus monkeys that receive a six-month L-DOPA treatment course and develop LID experience a reversion of their symptoms following lentiviral-mediated KD of ERK1/2 signaling pathway in D_1R-MSNs[49]. These findings indicate a clear role for D_1R-mediated activation of ERK1/2 in the maintenance of monkey LID within this experimental paradigm. Therefore, among these two PD animal models, depending on the experimental procedure employed, D_1R-mediated ERK1/2 activation is critical for either LID induction alone, or both induction and maintenance of LID.

Meanwhile, mice lacking tyrosine hydroxylase (TH) selectively within DA neurons—and thus deficient of striatal DA—used as a PD model, also exhibit supersensitive striatal D_1R-mediated ERK1/2 activation[225]. However, chronic L-DOPA treatment of these animals leads to restoration of normo-sensitive D_1R-mediated ERK1/2 activation with no concomitant development of LID. Given that KO of TH in DA neurons leads to depletion of striatal DA, yet does not lead to degeneration of DA neurons and consequent loss of their axonal innervation of striatal neurons[231], this finding thus highlights the apparent importance of DA neuron denervation within the striatum—in addition to simple absence of striatal DA—for the generation of LID through hypersensitive D_1R-mediated ERK1/2 activation. Alternatively, brain adaptations during development could explain the lack of LID in TH-deficient mice. In particular,

unlike 6-OHDA- and MPTP-lesioned PD models, TH-deficient mice lack striatal DA during development of the nigrostriatal DA system. Thus, it is possible that nigrostriatal adaptations may take place during this time that somehow influence the capability of animals to experience LID later in life.

3.5.2.2. Cellular and Molecular Processes Contributing to LID

One common feature of animals with LID is the accumulation of striatal ΔFosB downstream of D_1R-mediated ERK1/2 activation[49,134,138,139,197,198]. Presumably, ΔFosB plays an important role in the expression of LID, reminiscent of its critical involvement in drug addiction mentioned above. Additionally, activation of striatal mammalian target of rapamycin complex 1, downstream of D_1R-mediated ERK1/2 activation, contributes to LID induction[201]. Finally, striatal angiogenic changes associated with LID development also require D_1R-mediated ERK1/2 activation[226].

3.5.3. Other Pathologies

3.5.3.1. Epilepsy

D_1R-mediated ERK1/2 activation may have importance in epilepsy. Indeed, ERK1/2 activation downstream of D_1R in the dorsal striatum and hippocampus contributes to seizure induction in mice[55]. Moreover, seizure-evoking D_1R agonist treatment elicits several further downstream effects within the hippocampus that presumably depend on D_1R-meditated ERK1/2 activation[125]. In particular, phosphorylation of histone H3 and rpS6, as well as Zif268 and Arc induction, all occur in conjunction with ERK1/2 activation selectively in granule cells of the dentate gyrus following D_1R stimulation.

3.5.3.2. Conditions Involving Striatal Neurodegeneration

D_1R-mediated ERK1/2 activation may have direct relevance in conditions that involve striatal neuron degeneration, such as L-DOPA-unresponsive parkinsonism subtype of multiple system atrophy, methamphetamine-induced neurotoxicity, and secondary DA dysfunction in Huntington's Disease[232]. DA-induced cytotoxicity occurs via a pathway that depends on D_1R-mediated ERK1/2 activation in both SK-N-MC neuroblastoma cells and striatal neurons. Within SK-N-MC cells[102], D_1R-mediated ERK1/2 activation promotes oxidative stress and cytotoxicity via a mechanism that presumably involves increased cytoplasmic- and reduced nuclear-ERK1/2 activation via enhanced β-arr2-facilitated D_1R-mediated ERK1/2 activation. Similarly, in cultured striatal neurons[54], a short DA treatment (20 min) promotes selective D_1R-mediated ERK1/2 activation resulting in predominantly cytoplasmic phospho-ERK1/2 localization, which is found in tripartite complexes with D_1R and β-arr2. Furthermore, longer DA treatment (24 h) results in cytotoxicity and subsequent neuronal death partially mediated via D_1R-induced ERK1/2 activation. These studies thus suggest that increased cytoplasmic ERK1/2 signaling plays an important role in ERK1/2-mediated cytotoxic effects downstream of D_1R.

CHAPTER 4: ROLES OF SAP102

4.1. Family of MAGUKs: Overview of DLG Subfamily

4.1.1. MAGUKs

MAGUKs are a large family of scaffolding proteins with critical roles at cellular junctions, including at synapses within the central nervous system[36]. Mutations in genes encoding MAGUKs result in neurological disorders and various forms of cancer[233,234]. There are 22 mammalian MAGUK proteins comprising seven distinct subfamilies[235]. All MAGUKs except the membrane-associated guanylate kinase inverted (MAGI) subfamily contain a common module consisting of a PSD-95/DLG/zonula occludens-1 (PDZ) domain followed by a Src homology 3 (SH3) domain and a catalytically inactive guanylate kinase (GUK) domain located within their C-terminal region[36]. In contrast, MAGI proteins lack the SH3 domain and have an inverted arrangement in which the GUK domain is located first within their N-terminal region followed by a subsequent PDZ domain[36]. Each of these domains partakes in protein-protein interactions, thus imparting MAGUKs with a role in organizing multi-component protein complexes[36]. Moreover, individual MAGUK subfamilies have additional unique domains and structural features that results in significant diversity of MAGUK-organized protein complexes[36].

4.1.2. DLG MAGUKs: Basic Properties

DLG MAGUKs, the best studied MAGUK subfamily, are crucial for normal synapse assembly and function[36]. DLG MAGUKs comprise four members with highly similar structure: postsynaptic density protein 93 (PSD-93), PSD-95, synapse-associated protein 97 (SAP97) and SAP102[36]. Moreover, all DLG members contain alternatively spliced sites, giving rise to multiple variants of each protein[36]. DLG MAGUKs contain two additional PDZ domains along with their

PDZ-SH3-GUK tandem[36]. DLGs are predominantly located within dendritic spines at the PSD of glutamatergic synapses where they primarily function as organizers of synaptic protein complexes[36]. In this regard, DLGs are critical for linking changes in synaptic activity with intracellular signaling pathways and alterations in dendritic spine morphology through their simultaneous interactions with ionotropic glutamate receptors, intracellular signaling proteins and components of the neuronal cytoskeleton[36]. Additionally, DLGs are crucial for the trafficking of various membrane proteins, including NMDAR and AMPAR subunits, from the endoplasmic reticulum to the synaptic compartment[36,38,236,237].

4.1.3. DLG MAGUKs: Developmental Regulation of Expression

DLG subfamily members differ in their levels of expression during neuronal development. SAP102 is the predominant DLG within the early stage of development during synaptogenesis[37,237–242], thus suggesting a critical role for SAP102 in the early development of synapses. However, SAP102 expression levels gradually taper off throughout the early developmental period such that much lower levels are observed in adult than developing animals[37,237,238,240]. In contrast, the other three DLG members display the opposite expression pattern to that of SAP102: they are expressed at low levels during early development, but reach much higher levels in adulthood[237,239–242].

4.1.4. DLG MAGUKs: Divergence in Structure and Function

As suggested by their differing patterns of expression during neuronal development described in the above subsection, important functional differences exist between DLG subfamily proteins. These functional differences are not solely the result of differentially regulated expression patterns, but also depend on the structural divergence observed between

DLG MAGUKs, which is most prominent in their N-terminal region and the region located between their SH3 and GUK domains termed the HOOK region[36].

One important example of functional differences between DLGs involves divergence of sequence within the N-terminal region of these proteins that imparts each DLG member with different properties contributing to their targeting to and stabilization at the synaptic membrane. Synaptic targeting of PSD-95 requires palmitoylation of two cysteine residues within its N-terminus[36]. In contrast, while PSD-93 is also dually palmitoylated on cysteine residues within its N-terminus, its synaptic targeting occurs independent of this modification and instead requires sequences within its first 30 N-terminal residues[36]. Moreover, SAP97 and SAP102 do not undergo N-terminal palmitoylation[36], but their N-terminal regions are also important for their synaptic targeting. SAP97 synaptic targeting depends on its N-terminally located Lin-2/Lin-7 domain[36]. On the other hand, the SH3 and GUK domains of SAP102 are required for its proper localization at the synaptic membrane[243], while phosphorylation of Ser632 within an alternatively spliced insert located between these two domains positively regulates SAP102 targeting to synapses[244]. Nonetheless, PSD-95-SAP102 chimera studies identified a postsynaptic targeting signal in the N-terminal region of SAP102 that is likely also important for SAP102 synaptic delivery[245].

DLG subfamily members additionally exhibit differences in their subcellular localization. Namely, SAP102 has a much greater ratio of dendritic shaft to spine localization than PSD-95[243]. Moreover, within spines, both SAP102 and PSD-95 are predominantly located at the PSD, however PSD-95 is present at the PSD in much greater abundance than SAP102, which in contrast, is more highly concentrated extrasynaptically[243].

Studies on SAP102 and PSD-95 also demonstrate that DLG subfamily members display differential mobility within spines. Approximately 80% of spine-localized SAP102 is found to be mobile, while in contrast, mobility is observed in only 36% of PSD-95 within spines[243].

These differences in subcellular localization and mobility within dendritic spines between SAP102 and PSD-95 likely arise in part from the different mechanisms underlying their synaptic targeting and stabilization noted above. Additionally, different properties underlying their clustering at synapses presumably factor in. In this regard, synaptic clustering of PSD-95 requires its PDZ domains[246] and is also negatively regulated by cyclin-dependent kinase 5-mediated phosphorylation of its N-terminal region[247]. On the contrary, SAP102 synaptic clustering occurs in a PDZ-independent fashion[246].

Furthermore, regulatory effects elicited by cytoskeleton remodeling and changes in synaptic activity levels appear to contribute to the differences in synaptic mobility between SAP102 and PSD-95. Namely, inhibiting actin depolymerization or simultaneously blocking NMDAR and AMPAR enhances stabilization of SAP102 at the PSD but does not affect PSD-95 stabilization[243]. Indeed, phosphorylation of SAP102 on Ser632 decreases its mobility at the synapse, while increased synaptic activity leads to dephosphorylation of SAP102-Ser632 and a concomitant reduction in synaptic SAP102[244].

Overall, it is clear that despite their highly similar structures, DLG subfamily MAGUKs have key differences that impart each member with unique functional properties. However, the literature concerning DLG subfamily proteins is most heavily concentrated on PSD-95, with much fewer studies centered on PSD-93, SAP97 and SAP102. The remainder of chapter 4 focuses on what is currently known about the roles of SAP102 in regulating glutamatergic

transmission, ERK1/2 activation and dopaminergic transmission specifically in the context of D_1R function. Additionally, a subsection primarily dedicated to the described role of PSD-95 in D_1R function is also included due to its particularly high relevance for understanding how D_1R function may be modulated by SAP102.

4.2. SAP102 Functions

4.2.1. Regulation of Glutamatergic Transmission

4.2.1.1. Regulation of Glutamate Receptor Trafficking

A crucial role for SAP102 in trafficking NMDAR subunits to the synapse is well-established. SAP102 and PSD-95 both form PDZ-dependent interactions with the CT of GluN2 subunits. Interestingly, differences in GluN2 subtype interaction preference between these two DLG proteins appears to exist, with SAP102 favoring GluN2B interactions and PSD-95 preferring interactions with GluN2A[37,237,238,240,248]. Intriguingly, one contributing factor to the apparent SAP102 preference for GluN2B may involve a recently characterized PDZ-independent interaction selectively with GluN2B that is unique to SAP102. This interaction requires Asp1391 and Asp1392 within the CT of GluN2B along with the alternatively spliced I1 insert located in the N-terminal region of SAP102[40,249].

Indeed, SAP102 is a critical mediator of GluN2B subunit trafficking to the synaptic membrane. Specifically, tripartite complex formation between the exocyst complex subunit Sec8, SAP102, and GluN2B within the endoplasmic reticulum is a requirement for normal levels of synaptic GluN2B[236]. In fact, formation of this complex occurs via independent interactions of Sec8 and GluN2B with the PDZ domains of SAP102. Furthermore, SAP102-mediated GluN2B trafficking also depends on interaction of SAP102 SH3 and GUK domains with the linker region

of mammalian homologue of *Drosophila melanogaster* partner of inscuteable (mPins)[38], a protein originally implicated as a regulator of cell division through physical interactions with both $G\alpha_i$ and nuclear mitotic apparatus protein[250].

Additionally, alternative splicing of SAP102 is a critical modulator of both GluN2A and GluN2B levels at the synaptic membrane. The PDZ-independent interaction between the I1 insert of SAP102 and GluN2B mediates synaptic clearance of GluN2B subunits[40]. In contrast, the I2 insert located within the HOOK region of SAP102 exerts an antagonizing effect on GluN2A synaptic localization[251].

SAP102 also contributes to the regulation of AMPAR localization at the synapse. Specifically, SAP102 influences AMPAR trafficking to the PSD via its effect on EphB2 receptor (EphB2R)-mediated signaling through physical interactions with both EphB2R and the Ras-related C3 botulinum toxin substrate 1 GEF kalirin-7 (Kal7)[237]. Moreover, GluN2B complexes specifically containing SAP102 linked to synaptic Ras GTPase-activating protein (SynGAP) are implicated in promoting AMPAR internalization from the postsynaptic membrane[252–254].

4.2.1.2. Regulation of Glutamate Receptor Function

SAP102 contributes to the regulation of NMDAR kinetics selectively during early development. A recent study[255] using thalamocortical slices from SAP102 KO mice reports that SAP102 is required for normal NMDAR current decay kinetics during the early developmental period known to be critical for experience-dependent plasticity. However, following this period, SAP102 KO mice no longer display different NMDAR decay kinetics than WT mice. Given the differences in decay kinetics between GluN2A-containing NMDAR and GluN2B-containing NMDAR[256], the altered NMDAR kinetics observed in the absence of SAP102 during early

development could reasonably be hypothesized to result from an alteration in the normal ratio of synaptic GluN2A:GluN2B secondary to the loss of SAP102-dependent GluN2B synaptic targeting. Intriguingly, no such alteration was observed, thus implicating a different, yet unknown SAP102-mediated mechanism in the regulation of NMDAR kinetics.

SAP102 is also involved in the regulation of AMPAR activity. When PSD-95 is knocked down in hippocampal slices and replaced with SAP102, SAP102 is capable of alleviating the functional deficit of AMPAR induced by PSD-95 KD[257]. Interestingly, SAP102-mediated rescue of AMPAR activity requires the AMPAR-interacting protein cornichon-2, which in contrast, is not involved in PSD-95-mediated AMPAR regulation. Moreover, these experiments demonstrate that SAP102 and PSD-95 differentially affect AMPAR current decay. Namely, SAP102-regulated AMPAR current exhibits a slower decay rate relative to that of PSD-95-controlled AMPAR current.

4.2.1.3. Regulation of Functional Synaptic Plasticity

SAP102 regulates LTP induction within the hippocampal CA1 area. Indeed, SAP102 KO mice exhibit greatly enhanced expression of two different ERK1/2-dependent forms of LTP, although they display unaltered ERK1/2-independent LTP induction elicited by 100 Hz stimulation[39]. Namely, LTP induced by a 5 Hz stimulation protocol along with LTP induced by a spike timing-dependent plasticity (STDP) protocol are enhanced in SAP102 KO mice relative to WT mice. The enhanced STDP-induced LTP in SAP102 KO mice requires only one postsynaptic action potential. In contrast, WT mice require a train of action potentials to elicit this form of LTP. Therefore, this implicates a role for SAP102 in determining the rules governing how changes in synaptic strength are induced by coincident pre-synaptic and postsynaptic activity.

Interestingly, given that SAP102 KO mice display reduced NMDAR-mediated ERK1/2 activation yet have a concurrent enhancement of ERK1/2-dependent LTP, the authors speculate that the elevated basal ERK1/2 activation also exhibited by these mice is in fact responsible for their increased ERK1/2-dependent LTP through the facilitation of metaplastic effects. However, another conceivable explanation for the apparent dissociation between NMDAR-mediated ERK1/2 activation and ERK1/2-dependent LTP in these mice exists. Indeed, much like PSD-95 KO mice[258], SAP102 KO mice may have altered kinase requirements for LTP induction protocols normally dependent on ERK1/2, such that said ERK1/2-dependency is either reduced or entirely abrogated.

4.2.1.4. Regulation of Structural Synaptic Plasticity

SAP102 is an important regulator of dendritic spine plasticity. Increases in spine length, head width and density are facilitated by SAP102 in a manner that involves its interaction with mPins[38]. Moreover, SAP102 containing the I1 insert is specifically implicated in enhancing dendritic spine length through a GluN2B-containing NMDAR-dependent mechanism[249]. Finally, SAP102-dependent regulation of EphB2R-mediated signaling via its direct interactions with EphB2R and Kal7 promotes actin reorganization within spines through downstream activation of p21-activated kinase[237].

4.2.2. Regulation of ERK1/2

Several lines of evidence point to an important role for SAP102 in controlling ERK1/2 activation. One study[39] identifies a clear role for SAP102 in controlling ERK1/2 signaling within the hippocampus. An unbiased approach assessing the phosphorylation levels of 48 different postsynaptic proteins within the hippocampus of SAP102 KO and WT mice finds that only ERK2

displays an altered basal level of phosphorylation in the absence of SAP102. More specifically, SAP102 KO mice exhibit significantly greater levels of phosphorylated ERK2 under basal conditions. In contrast, experiments with hippocampal slices demonstrate that SAP102 KO mice have greatly reduced NMDAR-mediated ERK1/2 activation. Indeed, a subsequent study[41] shows that the induction of two genes dependent on NMDAR-mediated ERK1/2 activation within the hippocampus of WT mice—Arc and Egr2—is completely abrogated in SAP102 KO mice. This demonstrates that SAP102-dependent facilitation of NMDAR-mediated ERK1/2 activation has functional relevance for controlling gene expression.

Additionally, SAP102 may antagonize NMDAR-mediated ERK1/2 activation under some circumstances via its physical interaction with SynGAP. In particular, the possibility of a specific role for SynGAP-SAP102-GluN2B complexes in this process exists[252,253]. Offering support for this idea, rats exposed to chronic intermittent hypoxia-hypercapnia (CIHH) display elevated GluN2B, SAP102 and SynGAP expression in conjunction with reduced ERK1/2 phosphorylation, all of which requires GluN2B-containing NMDAR activation[254]. Thus, CIHH rats may experience a positive feedback loop of SynGAP-SAP102-linked GluN2B-containing NMDAR activation which antagonizes ERK1/2 phosphorylation.

Finally, SAP102 modulates ERK1/2 activation downstream of the $G_{s/olf}$-linked $A_{2A}R$ through physical interaction of its SH3 and GUK domains with the CT of $A_{2A}R$[43]. In particular, SAP102 alters the time course of $A_{2A}R$-mediated ERK1/2 activation in co-transfected HEK293 cells, specifically causing a shift of peak ERK1/2 activation to an earlier time point.

4.2.3. Regulation of Dopaminergic Transmission: Roles of PSD-95 and SAP102 in D_1R Function

4.2.3.1. PSD-95: D_1R-PSD-95 Signaling Complex

PSD-95 plays an important role in controlling D_1R function. Indeed, the N-terminal region of PSD-95 and CT of D_1R participate in a protein-protein interaction in a D_1R-class agonist-dependent manner[33,259]. Through its interaction with D_1R, PSD-95 exerts regulatory effects on D_1R-mediated trafficking and signaling[33,150,259]; however, these effects remain incompletely understood due to several inter-study discrepancies.

One study[33] demonstrates that PSD-95 reduces D_1R-mediated cAMP production by enhancing dynamin-dependent constitutive D_1R internalization within co-transfected HEK293 cells. Moreover, this study also finds that PSD-95 impairs agonist-mediated internalization of D_1R. In contrast, a subsequent study[259] within HEK293 cells reports no effect of PSD-95 on D_1R-mediated cAMP production under basal conditions; instead, PSD-95 greatly enhances D_1R resensitization following agonist-induced internalization by increasing the rate of D_1R recycling back to the surface membrane. Interestingly, in striatal neurons, D_1R surface expression and lateral mobility are respectively enhanced and reduced through the action of PSD-95[150], thus directly contradicting the purported effect of PSD-95 on D_1R surface localization in HEK293 cells.

4.2.3.2. PSD-95: D_1R Interactions with Glutamate Receptors

PSD-95 plays a key role in regulating the physical interaction between D_1R and GluN1 subunits. PSD-95 and GluN1 interact at distinct but overlapping sequences in the CT of D_1R[260]. Moreover, PSD-95 antagonizes D_1R-GluN1 interactions both through its direct interaction with

D_1R and via an indirect PDZ-dependent mechanism presumably involving an interaction with GluN2 subunits[260]. Nevertheless, tripartite PSD-95-D_1R-GluN1 complexes exist both *in vitro* and *in vivo*[260].

Furthermore, PSD-95 modulates the functional interplay between D_1R and NMDAR. Namely, PSD-95 acts to prevent NMDAR-mediated inhibition of D_1R internalization and enhancement of D_1R-mediated cAMP production observed following co-activation of D_1R and NMDAR[260]. PSD-95 also abrogates the antagonistic effect of NMDAR on D_1R-mediated cAMP production that occurs in the context of activated D_1R and silent NMDAR[260]. Additionally, PSD-95 controls D_1R-mediated regulation of NMDAR function by specifically enabling D_1R-induced enhancement of Ca^{2+} influx through GluN2B-containing NMDAR[261]. Interestingly, this D_1R-mediated effect is also dependent on the activity of PKA and PKC; thus, given the known physical interaction between PSD-95 and A-kinase anchoring protein 5 (AKAP5)[262], and the well-characterized role of AKAP5 in scaffolding PKA and PKC[263], it is possible that D_1R-mediated enhancement of NMDAR function in fact involves a PSD-95-AKAP5 complex.

PSD-95-AKAP5 complex is also implicated in controlling D_1R-mediated regulation of AMPAR. Specifically, D_1R-mediated phosphorylation of GluA1-Ser845 leading to increased AMPAR localization at the surface membrane is dependent on the presence of both PSD-95 and AKAP5[264].

4.2.3.3. PSD-95: D_1R in Drug Addiction

PSD-95 partakes in psychostimulant-induced D_1R-mediated effects contributing to addiction. In mice chronically treated with cocaine there is a striking reduction in PSD-95 mRNA and protein levels within the dorsal striatum and nucleus accumbens that lasts for at least two

months after the final cocaine treatment[265]. Importantly, this reduction in PSD-95 is a crucial underlying factor in two addiction-related processes also observed in these mice which have been previously attributed to hyperactivated D_1R-mediated signaling. Namely, reduced PSD-95 contributes[265] to the enhanced LTP at cortico-accumbal synapses[57,200] and locomotor sensitization[57,70,101,119,157,158,165,192,200,215] exhibited by these mice. In further support of a role for PSD-95 in psychostimulant-induced locomotor sensitization, PSD-95 KO mice display greatly increased locomotor activity in response to acute treatment with both cocaine and a selective D_1R-class agonist[33,265].

Rats chronically treated with amphetamine also display locomotor sensitization in conjunction with reduced PSD-95 in the dorsal striatum and nucleus accumbens. Additionally, these rats exhibit a concomitant reduction in striatal and accumbal GluN2B[266]. Interestingly, the reductions in PSD-95 and GluN2B occur through a mechanism involving increased ubiquitin-induced proteosomal degradation of guanylate kinase-associated protein (GKAP) and SH3 and multiple ankyrin repeat domains (SHANK), proteins known to participate in respective direct[267] and indirect[268] physical interactions with PSD-95. Moreover, the locomotor sensitization observed in these rats appears to result specifically from the reduced GluN2B levels. Together, these results thus suggest that PSD-95—through its physical interactions with D_1R, GKAP and GluN2B—may serve a critical role in amphetamine-induced behavioral sensitization by linking hyperactivated D_1R-mediated signaling pathways with dysfunctional GKAP and SHANK degradative processes that lead to reduced GluN2B-containing NMDAR surface expression.

Finally, maturation of nucleus accumbens silent synapses via insertion of Ca^{2+}-permeable AMPAR (CP-AMPAR) is a signature of drug-context association[269] which may require

contributions from both D_1R and PSD-95. Namely, unsilencing of synapses through CP-AMPAR insertion specifically in D_1R-MSNs within the nucleus accumbens shell is critically involved in cue-induced cocaine seeking[270]. Similarly, PSD-95 is required for CP-AMPAR insertion into accumbal silent synapses induced by a cocaine-CPP procedure[269]. Thus, it is conceivable that functional interactions between PSD-95 and D_1R within the nucleus accumbens shell may partially underlie these observations.

4.2.3.4. PSD-95: D_1R in LID

PSD-95 contributes to the D_1R-mediated signaling alterations known to underlie LID. Indeed, increased PSD-95 expression is observed within the dorsal striatum of a non-human primate model of LID, and KD of striatal PSD-95 within these animals causes LID reversion[150]. Similarly, PSD-95 expression is elevated in rat models of LID[150,271], and lowering PSD-95 levels in these animals with either $mGluR_5$ antagonist treatment[271] or PSD-95 KD[150] reduces existing LID symptoms. Moreover, reversion of symptoms in LID rats is also induced by treatment with an interfering peptide for the D_1R-PSD-95 interaction[150]. Finally, PSD-95 KD in 6-OHDA-lesioned rats prior to starting an L-DOPA treatment regimen is capable of preventing initial development of LID[150]. Together, these results strongly suggest that alterations in D_1R-PSD-95 signaling complexes are crucial to the expression of LID.

Interestingly, altered DLG subfamily binding interactions with GluN2B subunits, including those involving PSD-95, are also critical for the development of LID[272]. Thus, overall it is likely that D_1R-mediated dysfunction resulting in the development of LID involves altered physical and functional interactions among PSD-95, D_1R and NMDAR.

4.2.3.5. PSD-95: D_1R-Mediated ERK1/2 Activation

D_1R-mediated ERK1/2 activation is a critical signaling module affected by PSD-95 in the contexts of LID and drug addiction. In a rat model of LID—which characteristically has elevated D_1R-mediated ERK1/2 activation[134,139,142,162,167,197,198,226,227,229,230] along with altered synaptic DLG subfamily expression levels[272]—PSD-95 KD reduces the level of ERK1/2 phosphorylation[150], thus implicating PSD-95 as a contributing factor to the pathologically increased D_1R-mediated ERK1/2 activation in LID **(Fig. 5)**.

In contrast, reduced PSD-95 in the dorsal striatum and nucleus accumbens of chronically cocaine-treated mice is crucial for expression of two addiction-related processes that are causally linked with D_1R-mediated ERK1/2 activation[265]; specifically, locomotor sensitization[57,70,101,119,157,158,165,192,200,215] and enhanced cortico-striatal LTP[57,200]. This suggests that PSD-95-facilitated modulation of D_1R-mediated ERK1/2 activation could be of central importance for psychostimulant-induced effects underlying the development of addiction.

4.2.3.6. SAP102: D_1R in Drug Addiction

In contrast to PSD-95, no studies have directly assessed a potential role for SAP102 in regulating D_1R function, however a functional interaction between SAP102 and D_1R within the context of cocaine addiction is implicated. In the same manner already described for PSD-95, SAP102 is required for maturation of cocaine-CPP-induced accumbal silent synapses through insertion of CP-AMPARs[269]. Indeed, an additive effect is observed between SAP102 and PSD-95 such that loss of both SAP102 and PSD-95 results in greater reduction of silent synapse maturation than loss of only one of these DLG paralogs[269]. Therefore, conjunctively taking into account these results and the findings demonstrating importance of CP-AMPAR insertion within

nucleus accumbens shell D_1R-MSNs for cue-induced cocaine seeking[270] already detailed above, it is possible that a functional relationship between D_1R, SAP102 and PSD-95 within nucleus accumbens shell D_1R-MSNs regulating maturation of silent synapses plays a crucial role in the development of cocaine addiction.

4.2.3.7. SAP102: D_1R in LID

One important study[272] suggests that SAP102 plays a fundamental part in the development of LID—a condition well-characterized to occur concomitantly with dysfunctional D_1R signaling[48,49,132,134,135,137–139,142,161,162,167,195,197–199,201,202,226–230]—within 6-OHDA-lesioned rats. Greatly reduced SAP102 expression is observed within the striatum of 6-OHDA-lesioned rats relative to sham-lesioned rats. However, subsequent to chronic L-DOPA treatment, SAP102 levels within rats that develop LID return back to levels found in sham-lesioned animals, while rats that do not develop LID experience further increased SAP102 expression above control levels. Interestingly, this appears to be directly linked with a phenomenon critically implicated in LID development within these animals; in particular, failed restoration of DA-depletion-induced changes in SAP102-GluN2B interactions back to control levels. The downstream effect of these persistent alterations in SAP102-GluN2B interactions ultimately leading to the development of dyskinesia seems to be a connected decrease and increase in the respective levels of synaptic and extrasynaptic GluN2B that fail to normalize back to the proper synaptic:extrasynaptic expression ratio.

One other study[271] offers findings pertaining to altered SAP102 expression within LID animals that are somewhat at odds with those detailed above, but nonetheless which further support a role of SAP102 in LID. Huang and colleagues show that SAP102 expression is elevated

in the dorsal striatum of L-DOPA-treated rats with dyskinesia, but is brought back down to a normal level in concomitance with the amelioration of LID symptoms following $mGluR_5$ antagonist treatment.

4.2.3.8. SAP102: Rationale for Investigating D_1R-Mediated ERK1/2 Activation

The interaction that our lab has identified between SAP102 and the IL3 of D_1R suggests that SAP102 could act as a modulator of D_1R function. In particular, results from several studies suggest that investigation into a role for SAP102 in regulating D_1R-mediated activation of ERK1/2 is warranted.

Three lines of evidence offer support for a potential link between SAP102 and D_1R-mediated ERK1/2 activation within the hippocampus. First, the established role of SAP102 in regulating basal and NMDAR-mediated ERK signaling pathway activation within the hippocampus, and the apparent contributions of this regulation to hippocampal CA1 area LTP induction which could involve metaplastic effects[39]. Second, the demonstrated requirement of D_1R-mediated ERK1/2 activation for LTP induction within the hippocampal CA1 area[124]. Third, the characterized D_1R-mediated metaplastic effect involving GluN2B-containing NMDAR within the hippocampal CA1 area acting to modulate the LTP induction threshold[203]. Together, these results suggest that SAP102 may functionally interact with D_1R-mediated regulation of ERK1/2 in a manner that affects the ability of D_1R activation to elicit metaplastic effects leading to a decrease in the LTP induction threshold within the CA1 area of the hippocampus.

Other studies also provide indirect support for a role of SAP102 in modulating D_1R-mediated ERK1/2 activation. SAP102 plays an important role in the development of LID[271,272], a condition predominantly characterized by aberrant ERK1/2 activation downstream of

hyperactivated D_1R[48,49,132,134,135,137–139,142,161,162,167,195,197–199,201,202,226–230]. Furthermore, ERK1/2 activation through another $G_{s/olf}$-linked GPCR, the $A_{2A}R$, is modified by an $A_{2A}R$-SAP102 physical interaction. Namely, SAP102 facilitates a shift in maximal $A_{2A}R$-mediated ERK1/2 activation to an earlier time point[43]. Additionally, PSD-95 modifies the signaling of D_1R, likely including D_1R-mediated ERK1/2 activation, via a direct physical interaction with its CT[33,150,259]. Thus, given our characterized interaction between D_1R and SAP102, a protein highly similar to PSD-95[36], it is reasonable to postulate that SAP102 is another important modulator of D_1R signaling functions, including activation of ERK1/2 pathway.

Finally, due to the significant involvement of D_1R-mediated ERK1/2 activation in basic neurophysiological and neuropathological processes as discussed above, developing a better understanding of the mechanisms underlying D_1R-mediated ERK1/2 activation is of paramount importance. Specifically, detailed knowledge of the processes underlying observed mechanistic differences in ERK1/2 activation by D_1R within distinct brain regions and cell types is lacking. One factor contributing to said differences may be interactions between the intracellular regions of D_1R and unique protein binding partners. As such, investigating the possibility of a role for SAP102 in D_1R-mediated ERK1/2 activation should provide valuable insight.

The central objective of my M.Sc. thesis project is to assess if SAP102 acts to modulate ERK1/2 activation by D_1R *in vitro*, using studies with co-transfected HEK293 cells. I hypothesize that the temporal pattern and/or underlying kinase-dependency of D_1R-mediated ERK1/2 activation is modulated by SAP102.

CHAPTER 5: METHODS

5.1. Materials

Eagle's minimal essential medium (EMEM) and phosphate-buffered saline (PBS) from Wisent Bioproducts (Saint-Jean-Baptiste, QC, Canada). Trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and gentamicin from Gibco (Burlington, ON, Canada). Bio-Rad protein assay dye reagent concentrate from Bio-Rad Laboratories (Mississauga, ON, Canada). Bio-Safe II biodegradable scintillation cocktail from Research Products International (Mount Prospect, IL, USA). [*N*-methyl-^{3}H]-SCH23390 from PerkinElmer (Boston, MA, USA). SCH23390 hydrochloride, H89 dihydrochloride, protein kinase inhibitor 14-22 amide myristoylated (PKI) and U0126 from Tocris Bioscience (Oakville, ON, Canada). Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), *cis*-flupenthixol dihydrochloride, forskolin (FSK), ascorbic acid (AA), DA hydrochloride, aprotinin, benzamidine, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor and rabbit polyclonal anti-HA antibody from Sigma-Aldrich (Oakville, ON, Canada). Rabbit monoclonal anti-α-tubulin antibody, rabbit monoclonal anti-ERK1/2 antibody and rabbit monoclonal anti-p-ERK1/2 antibody from New England Biolabs (Whitby, ON, Canada). Horseradish peroxidase (HRP)-conjugated goat polyclonal anti-rabbit antibody and Amersham ECL prime from GE Healthcare (Mississauga, ON, Canada).

5.2. Cell Culture and Transfection

HEK293 cells were cultured between passages 40-52 in EMEM supplemented with 10% (v/v) heat-inactivated FBS and 40 µg/ml gentamicin (complete EMEM), within a 5% CO_2 atmosphere at 37°C. Cells were seeded in 100mm culture dishes (2.5 million cells/dish) and

transiently transfected with human D_1R and rat HA-SAP102 subcloned in the mammalian expression vector pCMV5 using a modified calcium phosphate method as previously described[273]. Cells were transfected with D_1R or HA-SAP102 alone or in combination, with empty pCMV5 vector used where appropriate to ensure that the total amount of plasmid DNA per dish was 5 µg. Cells transfected with 5 µg of empty pCMV5 vector were used as a mock condition. 18 hours later, transfected cells were washed with PBS, then trypsinized, resuspended in complete EMEM, and reseeded in 6-well culture plates and 100mm culture dishes for respective use in ERK1/2 activation and radioligand binding assay. 24 hours later, cells in 6-well culture plates and 100mm culture dishes were serum starved by replacing complete EMEM with EMEM containing 1% (w/v) BSA and 40 µg/ml gentamicin but lacking FBS. Cells were subsequently grown for an additional 24 hours until the day of the experiment.

5.3. Radioligand Binding Assay

A radioligand binding assay was performed to assess D_1R expression level in transfected HEK293 cells by determining the total number of D_1R binding sites (B_{max}). Cells within 100mm culture dishes were first washed with cold PBS, then ice-cold lysis buffer (comprising: 10 mM Tris-HCl, pH 7.4; and 5 mM EDTA, initial pH 8.0) was added and cells were scraped from the dishes, transferred into centrifuge tubes, and centrifuged for 20 min at 18000 rpm while maintained at 4°C. Next, membrane pellets were homogenized in ice-cold lysis buffer using a Kinematica Brinkmann Polytron 3000 at 17000 rpm for 20 sec, then centrifuged again for 20 min at 18000 rpm and 4°C. The resulting membrane pellet was homogenized at 17000 rpm for 20 sec in resuspension buffer (comprising: 62.5 mM Tris-HCl, pH 7.4; and 1.25 mM EDTA, initial pH 8.0). 100 µl of membrane homogenate was subsequently added to test tubes containing 300

µl of binding buffer (comprising: 62.5 mM Tris–HCl, pH 7.4; 1.25 mM EDTA, initial pH 8.0; 200 mM NaCl; 8.33 mM KCl; 6.7 mM $MgCl_2$; and 2.5 mM $CaCl_2$), 50 µl of [*N*-methyl-^{3}H]-SCH23390 (83.2 Ci/mmol) and 50 µl of either *cis*-flupenthixol or water, such that the final 500 µl reaction mixture contained: 50 mM Tris-HCl, pH 7.4; 1 mM EDTA; 120 mM NaCl; 5 mM KCl; 4 mM $MgCl_2$; 1.5 mM $CaCl_2$; and a saturating [*N*-methyl-^{3}H]-SCH23390 concentration of approximately 9 nM, either in the absence or presence of 10 µM *cis*-flupenthixol. The test tubes without and with *cis*-flupenthixol respectively enabled determination of total and non-specific [*N*-methyl-^{3}H]-SCH23390 binding to membranes. Membranes were incubated within the reaction mixture for 90 min at room temperature, then harvested onto glass fiber filters using a Brandel M-48 Semi-Automated Harvesting System. Filters were subsequently washed three times with cold washing buffer (comprising: 50 mM Tris-HCl, pH 7.4; and 100 mM NaCl). Next, filters were added to scintillation vials containing Bio-Safe II biodegradable scintillation cocktail, and their bound [*N*-methyl-^{3}H]-SCH23390 radioactivity was quantified using a Beckman LS 6500 liquid scintillation counter. A Bradford protein assay was performed to assess the total membrane protein concentration within the reaction mixture using Bio-Rad protein assay dye reagent concentrate in conjunction with the remaining homogenized membrane protein and BSA as a protein standard. Finally, total membrane protein concentration and [*N*-methyl-^{3}H]-SCH23390 radioactivity in dpm were used in an equation to calculate B_{max} in pmol/mg membrane proteins as previously described[274].

5.4. ERK1/2 Activation Assays

5.4.1. FSK Assay

Transfected HEK293 cells within 6-well culture plates first had existing media within each well replaced with 2 ml of incubation buffer (comprising: EMEM without phenol red; 20 mM HEPES; and 10 µg/ml gentamicin). Subsequently, cells were incubated with either 1% (v/v) DMSO or 10 µM FSK prepared in DMSO for 5 min within a 5% CO_2 atmosphere at 37°C. Immediately following DMSO and FSK treatment, cells were put on ice, media was aspirated, and cells were washed with PBS before lysis buffer supplemented with protease inhibitors (20 µg/ml PMSF, 10 µg/ml benzamidine, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 2 µg/ml aprotinin, and 1 µg/ml pepstatin A) was added. Cells were then scraped, and lysates were collected in microcentrifuge tubes and sonicated for 15 sec. Protein concentration of lysates were then determined with a Bradford protein assay using Bio-Rad protein assay dye reagent concentrate in conjunction with BSA as a protein standard. Western blot samples were prepared by combining 40 µl of 2x sample buffer (comprising: 25 mM Tris-HCl, pH 6.5; 10% [v/v] glycerol; 8% [w/v] sodium dodecyl sulfate [SDS]; 5% [v/v] 2-mercaptoethanol; and 0.004% [w/v] bromophenol blue) with the necessary amount of lysates and additional lysis buffer with protease inhibitors to yield samples with 20 µg of protein in a final volume of 80 µl.

5.4.2. DA Assays

Transfected HEK293 cells within 6-well culture plates were first pre-incubated with either 1% (v/v) DMSO or one of four drugs prepared in DMSO (1 µM SCH23390, 1 µM H89, 1 µM PKI or 10 µM U0126) within a 5% CO_2 atmosphere at 37°C. Prior to pre-incubation with

SCH23390, existing media within each well was replaced with 2 ml of incubation buffer, which was to be used for AA and DA incubation in a subsequent step, thus ensuring the presence of SCH23390 during the AA and DA incubation to allow for proper assessment of SCH23390-facilitated antagonism of DA binding to D_1R. In contrast, pre-incubation with H89, PKI and U0126 occurred in the same media from the prior day. H89 pre-incubation was for 15 min, while SCH23390, PKI and UO126 pre-incubation was for 30 min. In order to prevent DA oxidation during the assay, ice-cold 10 mM AA prepared in Milli-Q water was used to prepare ice-cold 1 mM DA. Immediately following pre-incubation with H89, PKI and U0126, media was aspirated and 2 ml of incubation buffer was added, then 100 µM final concentration AA or 10 µM final concentration DA was added to wells, and cells were returned to the 37°C, 5% CO_2 environment for various times (AA treatment for 1 min was performed in all assays for use as a basal condition—denoted as 0 min DA within figures—while DA treatment for 1 min, 2.5 min, 5 min, 10 min, 15 min, 30 min, and 60 min was performed in varying combinations depending on the particular assay). In contrast, following SCH23390 pre-incubation, the identical DA and AA treatment procedure described above was performed without changing the media in wells as already alluded to. Immediately following DA and AA treatment, the identical procedure described for the FSK assay to collect and sonicate cell lysates, determine lysate protein concentrations, and prepare western blot samples was performed.

5.5. Western Blot

Western blot samples containing 20 µg of protein were loaded into 10% (v/v) acrylamide gels for SDS-polyacrylamide gel electrophoresis. Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell was used to transfer proteins onto polyvinylidene difluoride membranes at 15 V

for 20 min. Membranes were cut just below the 75 kD marker into two pieces then incubated with blocking buffer (comprising: 50 mM Tris-HCl, pH 8.0; 80 mM NaCl; 2 mM $CaCl_2$; 5% [w/v] nonfat dry milk; 0.2% [v/v] NP-40 Alternative; and 0.02% [w/v] NaN_3) on a rocking platform shaker at 4°C overnight (~16 hours). Membranes were rinsed with Tris-buffered saline containing Tween 20 (TBS-T) (comprising: 20 mM Tris-HCl, pH 7.4; 137 mM NaCl; and 0.2% [v/v] Tween 20) the following day, then incubated on a rocking platform shaker at 4°C overnight (~16 hours) with polyclonal rabbit anti-HA antibody diluted 1:2000 in TBS-T (used for top half of membrane), or monoclonal rabbit anti-p-ERK1/2 antibody diluted 1:1000 in TBS-T (used for bottom half of membrane). The next day, using a rocking platform shaker, membranes were washed three times with TBS-T for 10 min, incubated for 1 hour with HRP-conjugated goat polyclonal anti-rabbit antibody diluted 1:10000 in TBS-T, then washed four times with TBS-T for 40 min. Finally, protein bands were visualized using Amersham ECL Prime in conjunction with DNR Bio-Imaging Systems MicroChemi 2.0 gel imaging system and DNR Bio-Imaging Systems GelCapture image acquisition software.

To assess total protein levels, bottom membrane halves were first incubated with stripping solution (comprising: 500 mM acetic acid; 100 mM 2-mercaptoethanol; and 0.5 % [w/v] SDS) for 30 min on a rocking platform shaker at room temperature to remove antibody bound to p-ERK1/2, then rinsed with TBS-T. Subsequently, membranes were subjected to the same blocking, antibody incubation, washing, and protein band visualization steps described above, with exception of a mixture containing monoclonal rabbit anti-α-tubulin antibody and monoclonal rabbit anti-ERK1/2 antibody respectively diluted 1:3000 and 1:1000 in TBS-T used in place of the other specified primary antibodies.

Densitometric analysis of protein bands was performed using DNR Bio-Imaging Systems GelQuant image analysis software. To account for the possibility of any differences in the level of total protein factoring in to differences in quantified p-ERK1/2, a normalized p-ERK1/2 value was calculated for each lane using the formula $\frac{(\mathrm{p-ERK1+p-ERK2})}{(\mathrm{ERK1+ERK2})}$.

5.6. Statistical Analysis

GraphPad Prism 8.1.1 was used for all statistical analyses. All data are expressed as mean ± SEM. Unpaired *t*-test and one-sample *t*-test were performed where indicated in the results section, with $p \leq 0.05$ deemed the threshold for statistical significance.

CHAPTER 6: RESULTS

6.1. cAMP-Dependent Processes Activate ERK Signaling Pathway in Mock-Transfected HEK293 Cells

Previous studies have cast doubt on the ability of cAMP-dependent processes to activate ERK1/2 within HEK293 cells[275–280]. To address this issue with our HEK293 cell isolates, one proof-of-principle experiment assessing the effect of the AC activator FSK on ERK1/2 regulation in mock-transfected HEK293 cells was performed. Our lab previously reported that FSK stimulation of mock-transfected HEK293 cells raises intracellular cAMP levels[273]. Herein, 5-min FSK treatment of mock-transfected HEK293 cells led to strong activation of ERK signaling pathway, in contrast to cells treated with DMSO that experienced no ERK1/2 activation **(Fig. 7)**. This proof-of-principle experiment indicates that increased levels of cAMP elicit a large ERK regulatory response, most noticeably for ERK2, in our HEK293 cells. Thus, these results confirm the suitability of our HEK293 cells for studying cAMP-dependent mechanisms potentially involved in D_1R-mediated ERK1/2 activation.

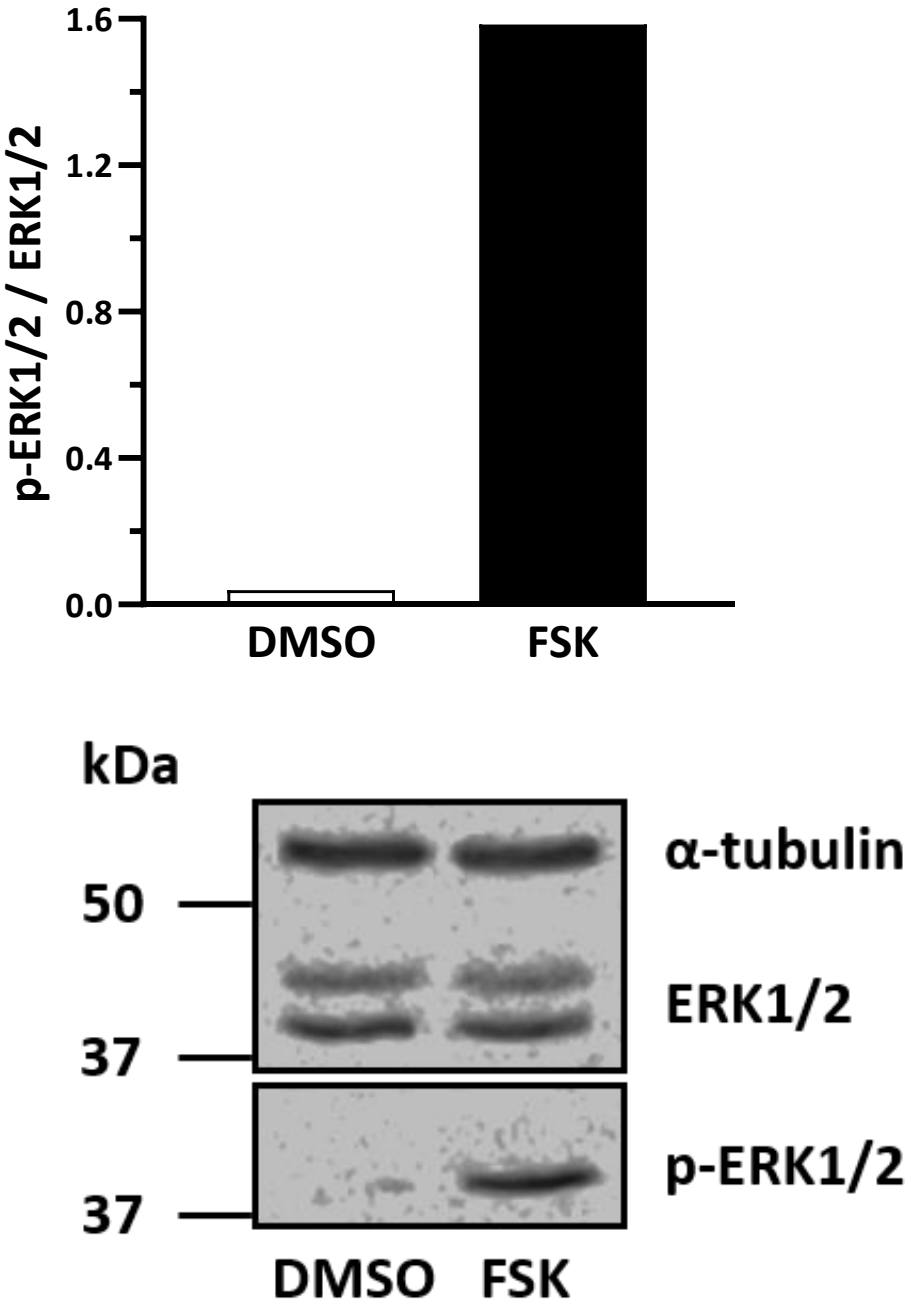


Figure 7. FSK Induces ERK1/2 Activation in Mock-Transfected HEK293 Cells
In this proof-of-principle experiment (n=1), mock-transfected HEK293 cells were treated with DMSO (1% v/v) or FSK (10 µM) for 5 min. Cell lysates were then prepared and subjected to western blotting with the following antibodies: anti-α-tubulin (1:1000, loading control), anti-ERK1/2 (1:1000, total ERK1/2), and anti-p-ERK1/2 (1:1000). **Top**, Data are presented as p-ERK1/2 (p-ERK1+p-ERK2) normalized relative to total ERK1/2 (ERK1+ERK2). **Bottom**, Western blot used to quantify data is shown.

6.2. DA Induces Robust, Temporal ERK1/2 Activation in D_1R-Transfected HEK293 Cells

Only one prior study[35] has investigated ERK1/2 regulation in HEK293 cells by exogenously expressed D_1R, demonstrating a clear role for D_1R in activating ERK1/2 within this experimental system. Consequently, given the reported inconsistencies between different HEK293 cell isolates in ERK1/2 activation downstream of other $G_{s/olf}$-linked GPCRs better studied in this regard, like β_2-adrenergic receptor (β_2AR)[76,78,79,82,85,100,104,279,281–283], an initial assessment of D_1R-mediated regulation of ERK1/2 in our HEK293 cell isolates was made with a single proof-of-principle time course experiment.

Mock- and D_1R-transfected HEK293 cells were treated with DA for 1, 5, 10, 30 or 60 min, or left unstimulated. Mock-transfected cells did not display activation of ERK1/2 following DA treatment at any time point **(Fig. 8)**. Our lab previously reported that D_1R-tranfected HEK293 cells show increased cAMP levels under basal conditions relative to mock-transfected cells[273]. In contrast to these cAMP studies, D_1R-transfected cells showed no increase in ERK1/2 activation relative to mock-transfected cells when left unstimulated **(Fig. 8)**. However, DA treatment led to ERK1/2 activation selectively within D_1R-transfected cells that was detected after 1 min of stimulation, reached a robust peak level of activation following 5 min of stimulation, sharply declined to near baseline levels after 10 min of stimulation, and then gradually decreased over the next 50 min until completely returning to baseline after 60 min of stimulation **(Fig. 8)**. These results thus indicate that DA-induced stimulation of D_1R leads to a robust but transient activation of ERK1/2 in our HEK293 cell isolates.

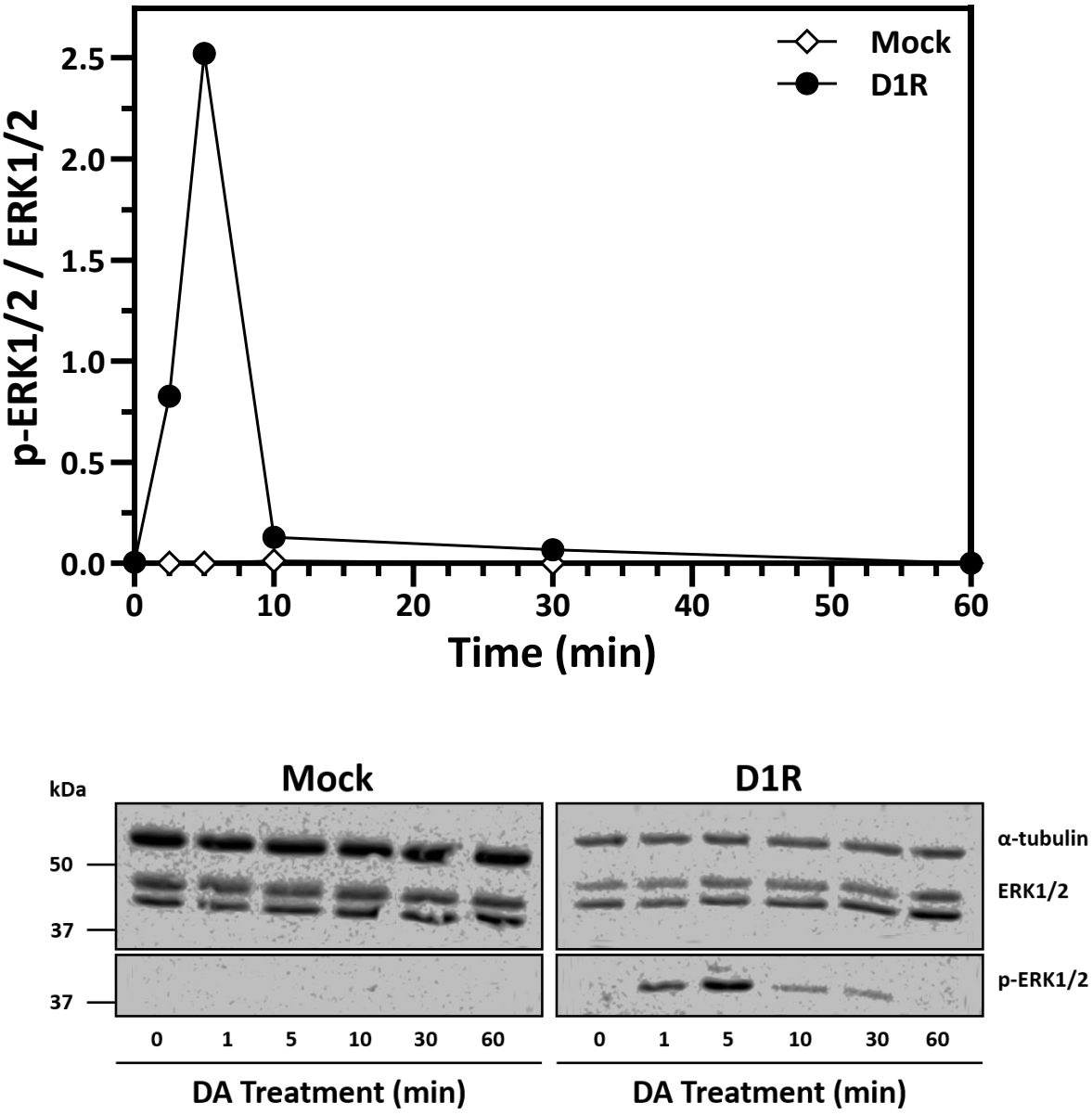


Figure 8. DA Induces Robust, Temporal ERK1/2 Activation in D_1R-Transfected HEK293 Cells
In this proof-of-principle experiment (n=1), mock- and D_1R-transfected HEK293 cells were treated with DA (10 µM) for 1, 5, 10, 30, or 60 min, or left untreated. Cell lysates were then prepared and subjected to western blotting with the following antibodies: anti-α-tubulin (1:1000, loading control), anti-ERK1/2 (1:1000, total ERK1/2), and anti-p-ERK1/2 (1:1000). **Top**, Data are presented as p-ERK1/2 (p-ERK1+p-ERK2) normalized relative to total ERK1/2 (ERK1+ERK2). **Bottom**, Western blot used to quantify data is shown. B_{max} (pmol/mg membrane proteins) value for [^{3}H]-SCH23390 in D_1R-transfected cells was 1.18.

6.3. SAP102 Reduces Basal ERK1/2 Activation in HEK293 Cells

A pilot experiment was performed to assess the effect of SAP102 expression alone on ERK1/2 activation following 1 min, 2.5 min and 5 min DA treatment of our HEK293 cell isolates, which do not natively express SAP102 (data not shown). All DA treatment times failed to promote ERK1/2 activation in mock- and SAP102-transfected cells, while in contrast, robust ERK1/2 activation was observed in D_1R-transfected cells following 2.5 min and 5 min DA treatment **(Fig. 9)**. These results thus indicate that expression of SAP102 by itself does not enable DA-induced ERK1/2 activation in our HEK293 cells.

These findings also suggest that β_2AR, which is endogenously expressed at low levels in HEK293 cells[284,285], does not evoke detectable ERK1/2 activation following DA treatment in the absence or presence of SAP102. DA-induced β_2AR-mediated ERK1/2 activation might be expected because of the partial agonist activity exhibited by DA at β_2AR[286]. Moreover, based on the ability of β_2AR to engage in physical interactions with several PDZ domain-containing proteins, one of which appears to involve the DLG subfamily member PSD-95[287,288], there is a chance that β_2AR may interact with SAP102. Thus, confirming a lack of DA-induced ERK1/2 activation by β_2AR within this experiment importantly eliminates β_2AR as a potential confounding factor in the next series of experiments investigating the effect of SAP102 on D_1R-mediated ERK1/2 activation.

During these preliminary studies, I also observed a variability in the detection of basal p-ERK1/2 levels within mock-transfected cells. Interestingly, when basal p-ERK1/2 levels were detected in mock-transfected HEK293 cells (n=3), matched SAP102-transfected cells exhibited a

significant reduction of basally phosphorylated ERK1/2, while D_1R-transfected cells displayed unchanged basal p-ERK1/2 levels relative to the mock condition **(Fig. 10)**.

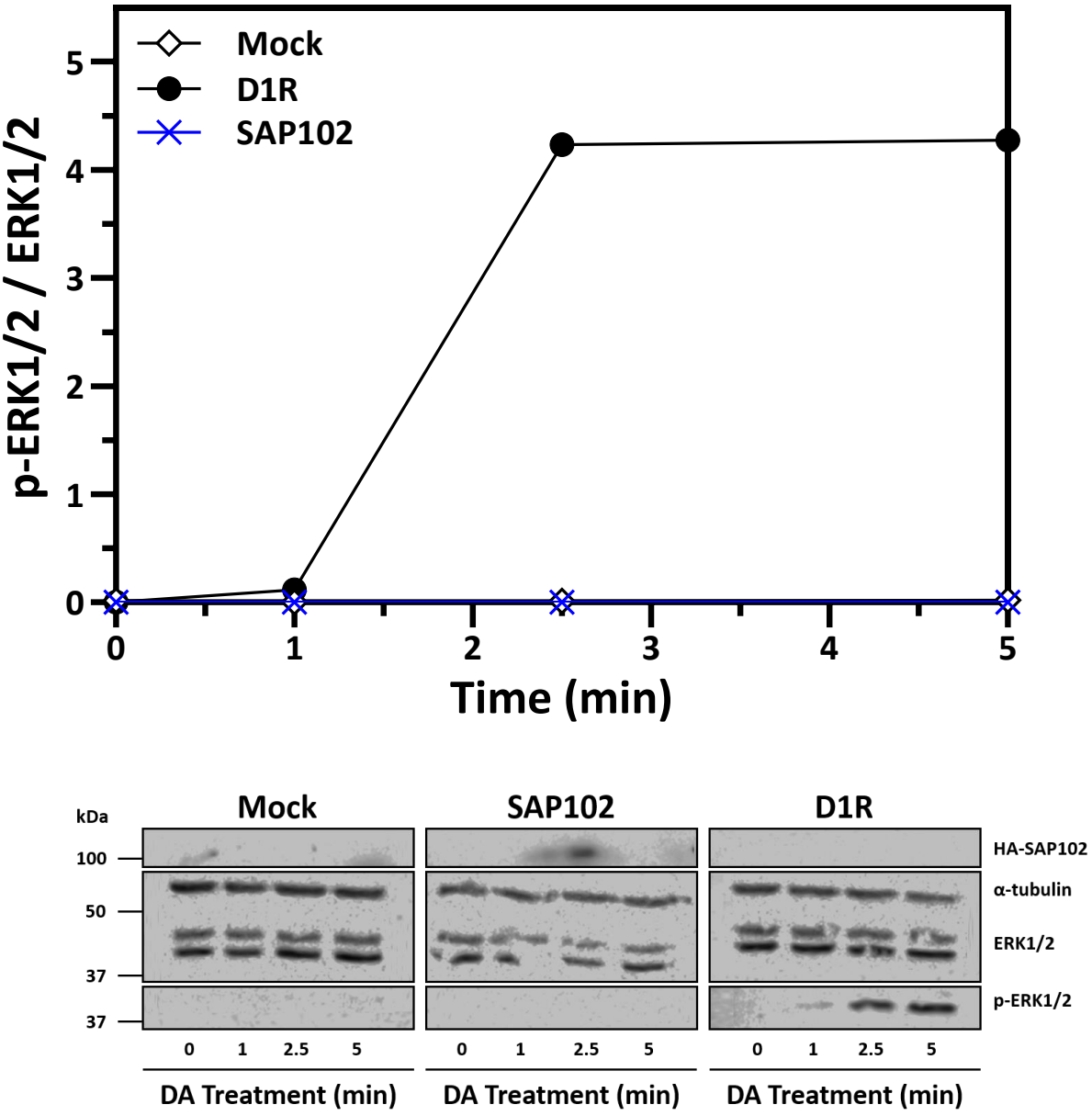


Figure 9. Expression of SAP102 Alone Does Not Impart DA-induced ERK1/2 Activation to HEK293 Cells

In this proof-of-principle experiment (n=1), mock-, D_1R-, and SAP102-transfected HEK293 cells were treated with DA (10 µM) for 1, 2.5, or 5 min, or left untreated. Cell lysates were then prepared and subjected to western blotting with the following antibodies: anti-HA (1:2000, SAP102 expression), anti-α-tubulin (1:1000, loading control), anti-ERK1/2 (1:1000, total ERK1/2), and anti-p-ERK1/2 (1:1000). **Top**, Data are presented as p-ERK1/2 (p-ERK1+p-ERK2) normalized relative to total ERK1/2 (ERK1+ERK2). **Bottom**, Western blot used to quantify data is shown. B_{max} (pmol/mg membrane proteins) value for [^{3}H]-SCH23390 in D_1R-transfected cells was 0.71.

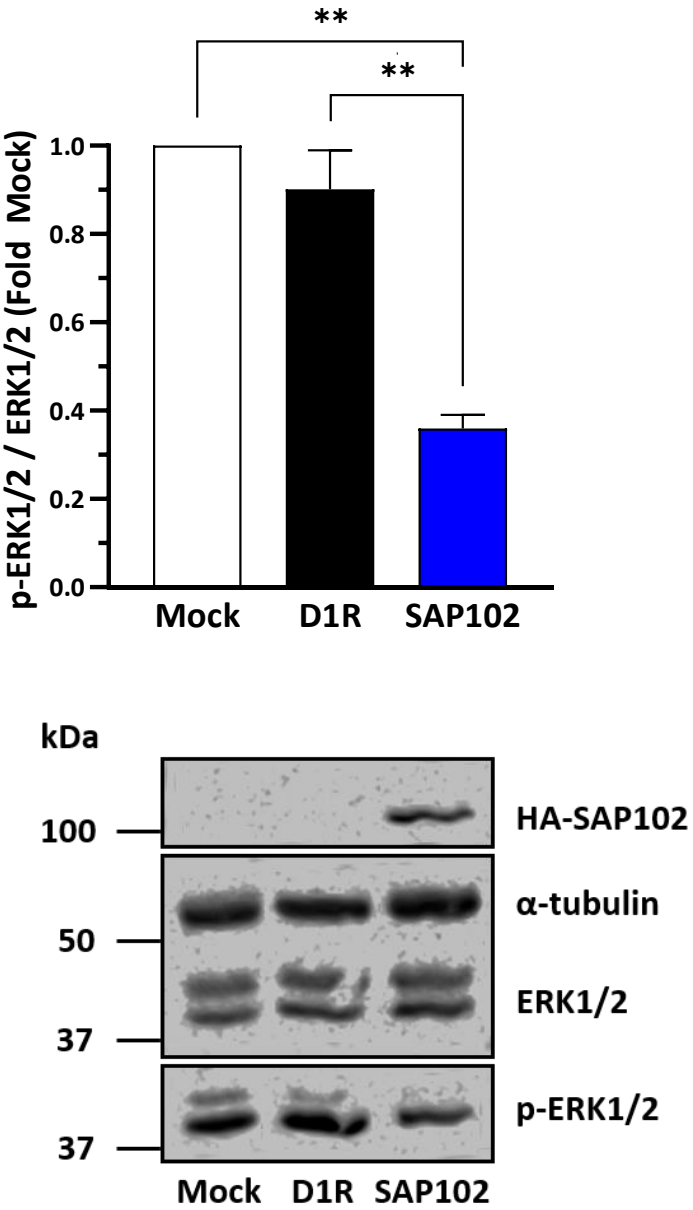


Figure 10. SAP102 Reduces Basal ERK1/2 Activation in HEK293 Cells

Mock-, D_1R-, and SAP102-transfected HEK293 cells were left untreated. Cell lysates were then prepared and subjected to western blotting with the following antibodies: anti-HA (1:2000, SAP102 expression), anti-α-tubulin (1:1000, loading control), anti-ERK1/2 (1:1000, total ERK1/2), and anti-p-ERK1/2 (1:1000). **Top**, Data are presented as arithmetic mean ± SEM (n=3) of p-ERK1/2 (p-ERK1+p-ERK2) normalized relative to total ERK1/2 (ERK1+ERK2) and then expressed as a ratio of the normalized p-ERK1/2 value for mock-transfected cells. **p<0.01 when compared to mock using a one-sample *t*-test and when compared to D_1R using an unpaired *t*-test. **Bottom**, A representative western blot is shown. B_{max} (pmol/mg membrane proteins) value expressed as arithmetic mean ± SEM for [^{3}H]-SCH23390 in D_1R-transfected cells was 4.05 ± 1.42.

6.4. SAP102 Alters the Temporal ERK1/2 Activation by D_1R

Time course experiments (n=9) were next performed to assess the ability of SAP102 to modulate temporal ERK1/2 activation by D_1R. HEK293 cells transfected with D_1R alone or D_1R and SAP102 were treated with DA for 1, 2.5, 5, 10, 15, 30 or 60 min, or left unstimulated. SAP102 promoted a significant enhancement of early phase (1 min) DA-induced ERK1/2 activation by D_1R **(Fig. 11)**. In contrast, D_1R-expressing cells lacking SAP102 exhibited greater ERK1/2 activation beyond 1 min until reaching the time point of peak activation at 5 min **(Fig. 11)**. Subsequently, SAP102 reduced the rate of decline in D_1R-mediated ERK1/2 activation up to the 30 min mark, a time point beyond which SAP102 maintained ERK1/2 activation by D_1R at detectable, albeit lower than baseline levels until 60 min **(Fig. 11)**. This contrasts with the absence of observable p-ERK1/2 found in cells expressing D_1R alone from the 30 min time point onward **(Fig. 11)**. D_1R dependency of DA-induced temporal activation of ERK1/2 was confirmed within both SAP102-lacking and SAP102-expressing HEK293 cells using the selective D_1R-class antagonist SCH23390 **(Fig. 12)**. These results thus demonstrate a role for SAP102 in the regulation of temporal D_1R-mediated ERK1/2 activation within HEK293 cells. Moreover, these findings support the possibility of a difference in the signaling pathway utilized by D_1R to activate ERK1/2 in cells expressing versus lacking SAP102.

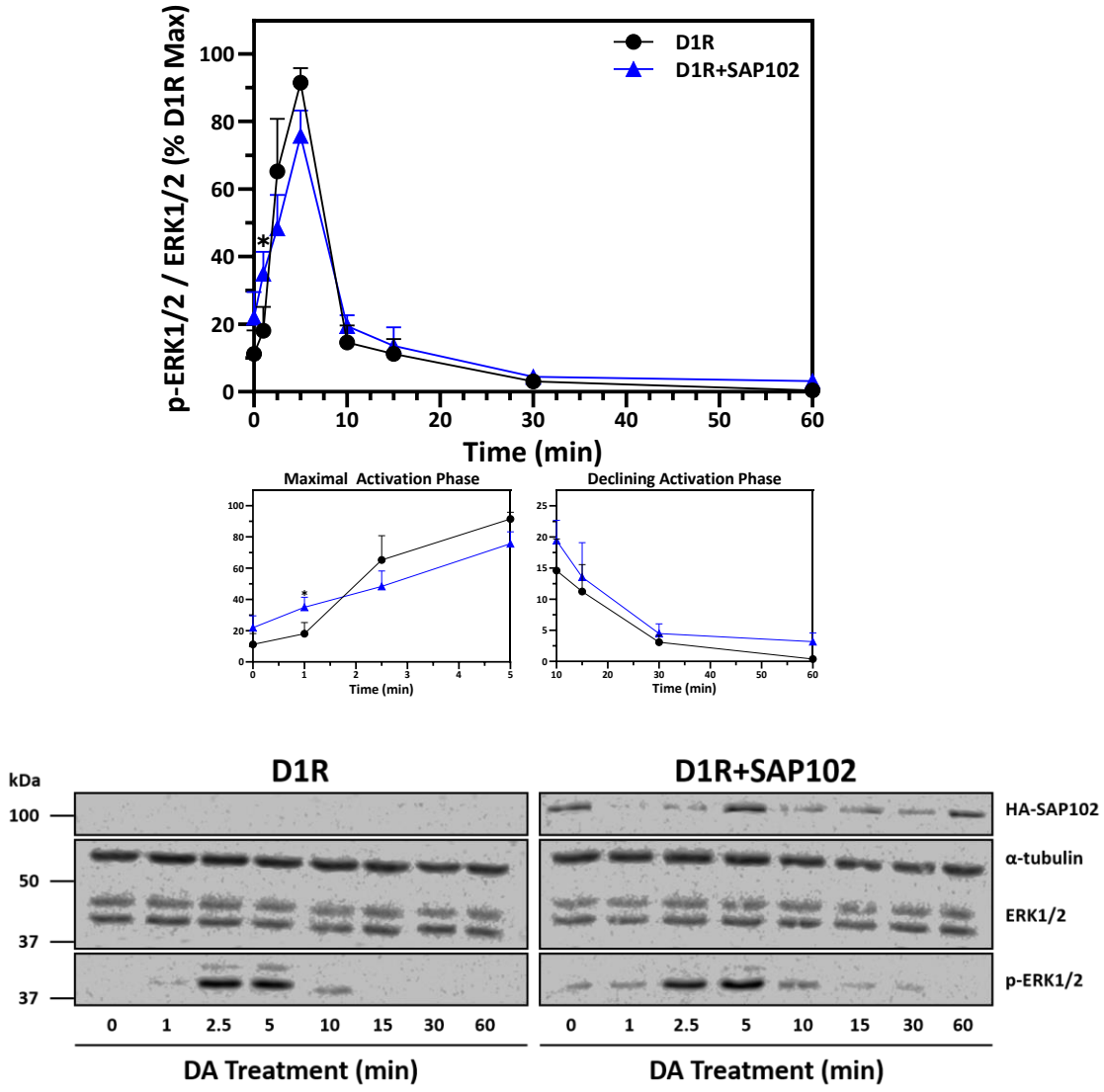


Figure 11. SAP102 Alters the Temporal ERK1/2 Activation by D_1R

D_1R- and D_1R+SAP102-transfected HEK293 cells were treated with DA (10 µM) for 1, 2.5, 5, 10, 15, 30, or 60 min, or left untreated. Cell lysates were then prepared and subjected to western blotting with the following antibodies: anti-HA (1:2000, SAP102 expression), anti-α-tubulin (1:1000, loading control), anti-ERK1/2 (1:1000, total ERK1/2), and anti-p-ERK1/2 (1:1000). **Top**, Data are presented as arithmetic mean ± SEM (n=9) of p-ERK1/2 (p-ERK1+p-ERK2) normalized relative to total ERK1/2 (ERK1+ERK2) and then expressed as a percent of the maximum normalized p-ERK1/2 value for D_1R-transfected cells. *p=0.05 when compared to matched treatment time of D_1R-transfected cells using an unpaired *t*-test. **Bottom**, Representative western blots are shown. B_{max} (pmol/mg membrane proteins) values expressed as arithmetic mean ± SEM for [^{3}H]-SCH23390 in D_1R-transfected cells (1.81 ± 0.69) and D_1R+SAP102-transfected cells (1.58 ± 0.57) were not significantly different (p>0.05) as determined using an unpaired *t*-test.

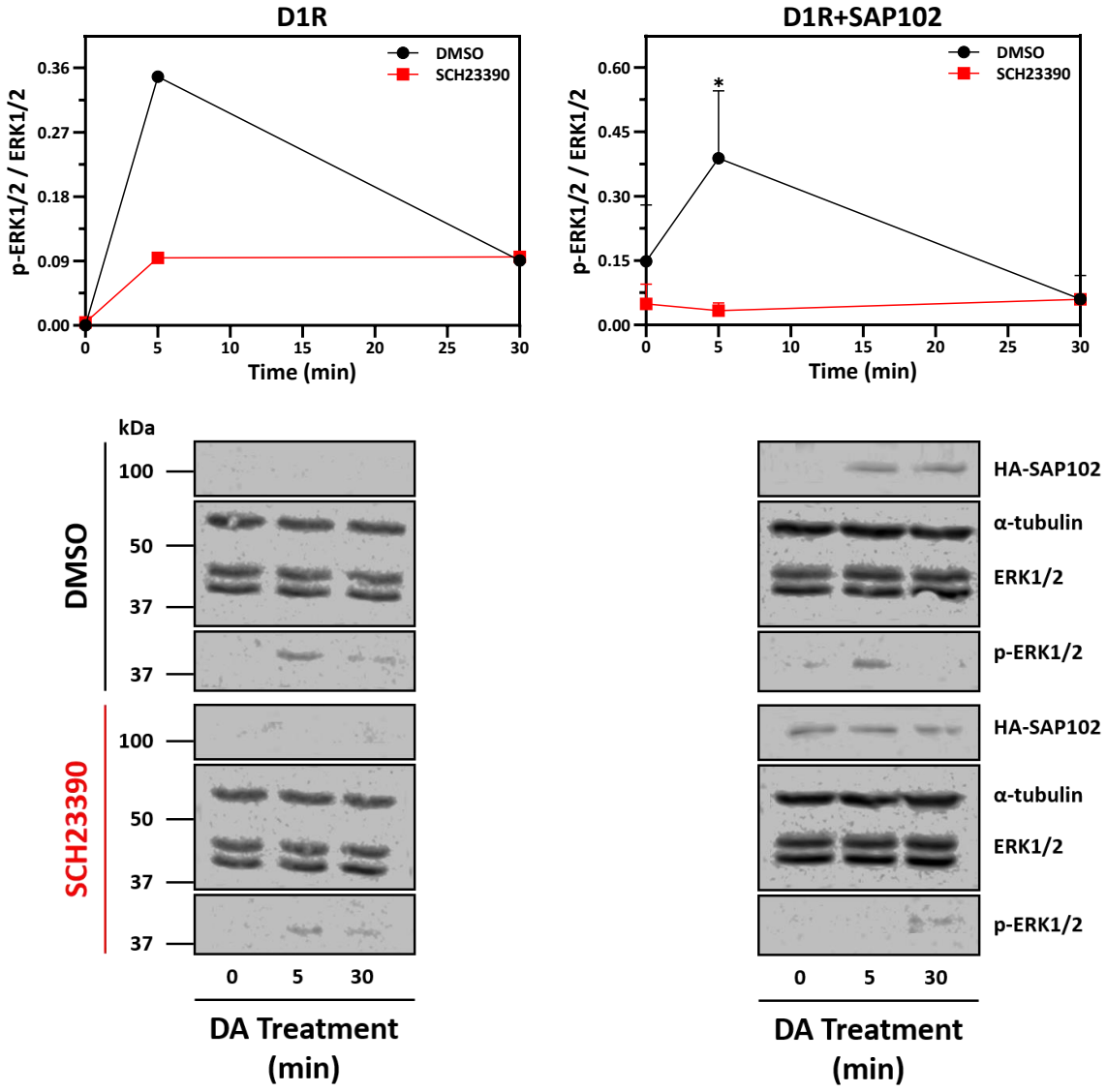


Figure 12. SCH23390 Blocks DA-Induced ERK1/2 Activation in HEK293 Cells Expressing D_1R Alone or D_1R and SAP102

D_1R- and D_1R+SAP102-transfected HEK293 cells were pre-incubated with DMSO (1% v/v) or the selective D_1R-class antagonist SCH23390 (1 µM) for 30 min, then treated with DA (10 µM) for 5 or 30 min, or left untreated. Cell lysates were then prepared and subjected to western blotting with the following antibodies: anti-HA (1:2000, SAP102 expression), anti-α-tubulin (1:1000, loading control), anti-ERK1/2 (1:1000, total ERK1/2), and anti-p-ERK1/2 (1:1000). **Top**, Data for D_1R-transfected cells (n=1) and D_1R+SAP102-transfected cells (n=3) are presented as arithmetic mean ± SEM of p-ERK1/2 (p-ERK1+p-ERK2) normalized relative to total ERK1/2 (ERK1+ERK2). *p<0.05 when compared to DMSO condition using an unpaired *t*-test. **Bottom**, Representative western blots are shown. B_{max} (pmol/mg membrane proteins) value expressed as arithmetic mean ± SEM for [^{3}H]-SCH23390 in D_1R+SAP102-transfected cells was 2.00 ± 1.13, while B_{max} value in D_1R-transfected cells was indeterminate.

6.5. SAP102 Switches the Mechanism of D_1R-Mediated ERK1/2 Activation from a PKA-Independent to PKA-Dependent Pathway

Investigation into a potential mechanism underlying the SAP102-dependent temporal pattern of D_1R-mediated ERK1/2 activation within HEK293 cells was subsequently undertaken. Two pieces of evidence prompted an inquiry into the role of PKA in this process. Firstly, the characterized interaction between SAP102 and neurobeachin (Nbea)[289–291], an AKAP capable of scaffolding PKA. Secondly, the demonstration that HEK293 cells have two distinct $G_{s/olf}$-cAMP-mediated pathways leading to ERK1/2 activation, corresponding to a PKA-independent K-Ras-facilitated pathway and a PKA-dependent Rap1-facilitated pathway[76]. Accordingly, time course studies using the small-molecule PKA inhibitor H89 were performed (n=6). Strikingly, H89 (1 μM) pre-treatment did not affect D_1R-mediated ERK1/2 activation in cells expressing D_1R alone, but led to a significant reduction in SAP102-dependent ERK1/2 activation by D_1R **(Fig. 13)**. Similar results were obtained in experiments using another inhibitor of PKA, PKI (n=3 for D_1R; n=4 for D_1R+SAP102) **(Fig. 14)**. These results thus reveal that D_1R-mediated ERK1/2 activation in HEK293 cells lacking SAP102 occurs in a PKA-independent fashion. However, the presence of SAP102 in these cells clearly switches D_1R-mediated ERK1/2 activation to a PKA-dependent process.

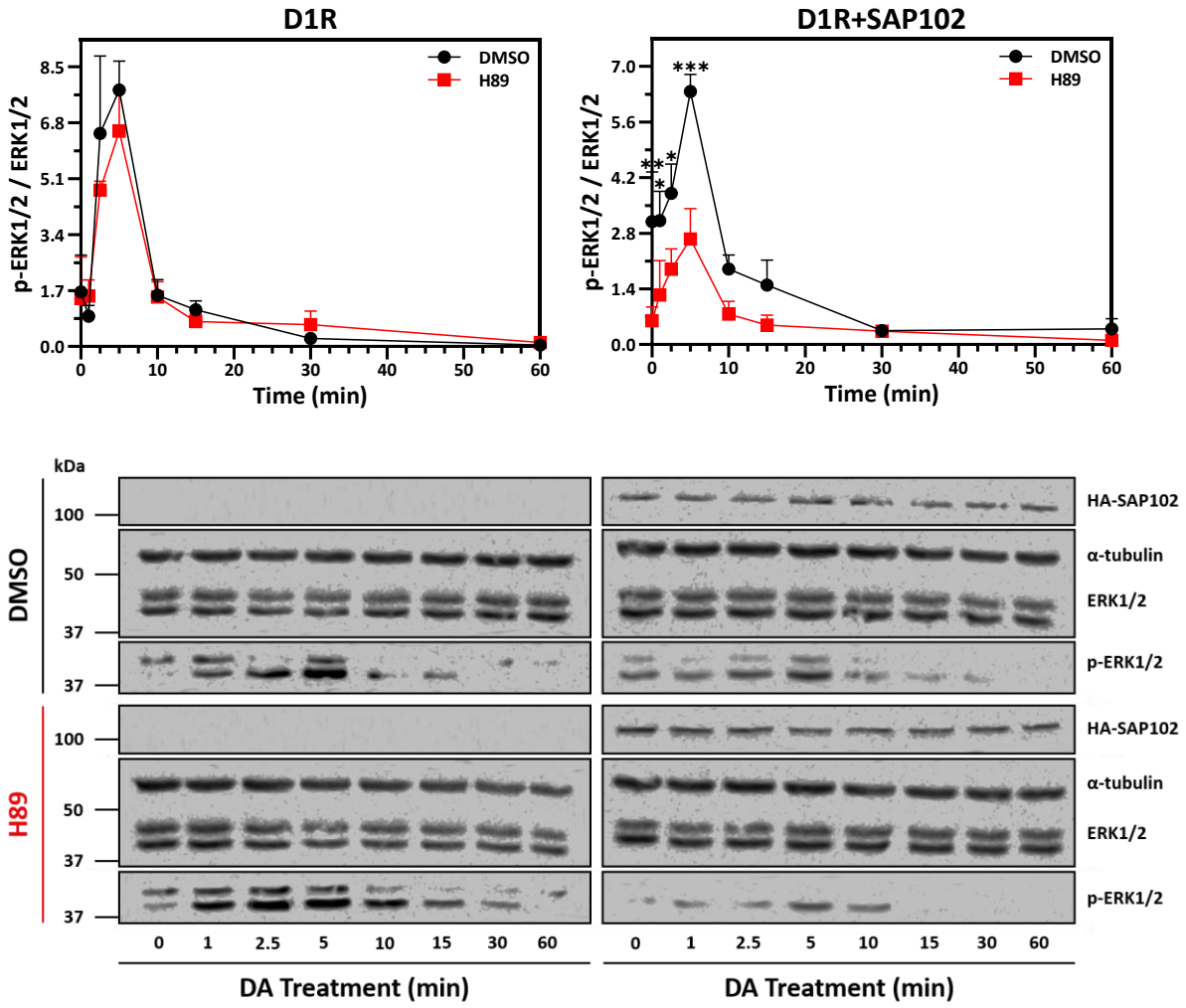


Figure 13. H89 Selectively Inhibits SAP102-Dependent D_1R-Mediated ERK1/2 Activation
D_1R- and D_1R+SAP102-transfected HEK293 cells were pre-incubated with DMSO (1% v/v) or the PKA inhibitor H89 (1 µM) for 15 min, then treated with DA (10 µM) for 1, 2.5, 5, 10, 15, 30, or 60 min, or left untreated. Cell lysates were then prepared and subjected to western blotting with the following antibodies: anti-HA (1:2000, SAP102 expression), anti-α-tubulin (1:1000, loading control), anti-ERK1/2 (1:1000, total ERK), and anti-p-ERK1/2 (1:1000). **Top**, Data are presented as arithmetic mean ± SEM (n=6) of p-ERK1/2 (p-ERK1+p-ERK2) normalized relative to total ERK1/2 (ERK1+ERK2). *p<0.05, **p<0.01, ***p<0.001 when compared to DMSO condition using an unpaired *t*-test. **Bottom**, Representative western blots are shown. B_{max} (pmol/mg membrane proteins) values expressed as arithmetic mean ± SEM for [^{3}H]-SCH23390 in D_1R-transfected cells (1.73 ± 0.97) and D_1R+SAP102-transfected cells (1.47 ± 0.68) were not significantly different (p>0.05) as determined using an unpaired *t*-test.

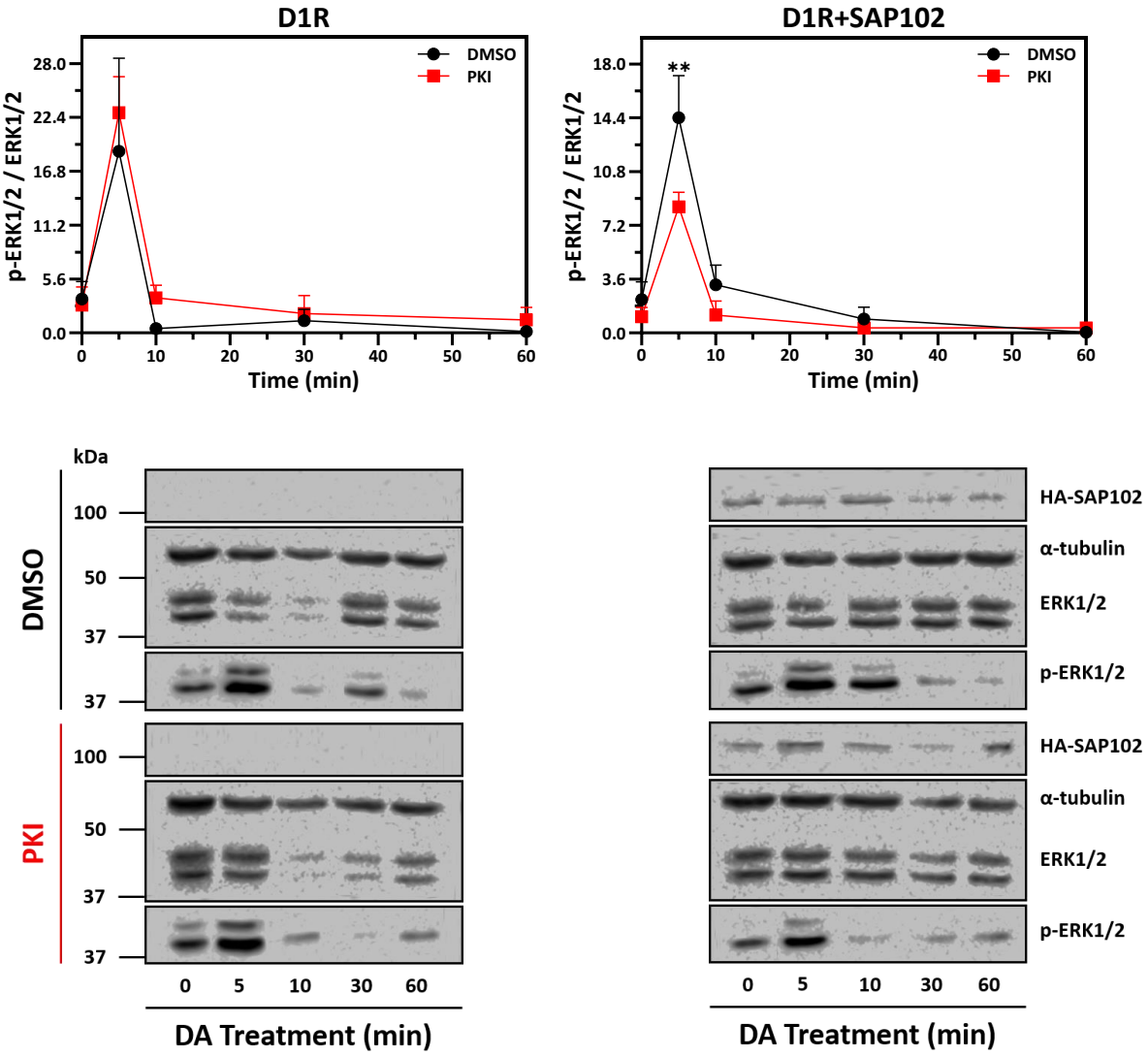


Figure 14. PKI Selectively Inhibits SAP102-Dependent D_1R-Mediated ERK1/2 Activation
D_1R- and D_1R+SAP102-transfected HEK293 cells were pre-incubated with DMSO (1% v/v) or the PKA inhibitor PKI (1 µM) for 30 min, then treated with DA (10 µM) for 5, 10, 30, or 60 min, or left untreated. Cell lysates were then prepared and subjected to western blotting with the following antibodies: anti-HA (1:2000, SAP102 expression), anti-α-tubulin (1:1000; loading control), anti-ERK1/2 (1:1000, total ERK1/2), and anti-p-ERK1/2 (1:1000). **Top**, Data for D_1R-transfected cells (n=3) and D_1R+SAP102-transfected cells (n=4) are presented as arithmetic mean ± SEM of p-ERK1/2 (p-ERK1+p-ERK2) normalized relative to total ERK1/2 (ERK1+ERK2). **p<0.01 when compared to DMSO condition using an unpaired *t*-test. **Bottom**, Representative western blots are shown. B_{max} (pmol/mg membrane proteins) values expressed as arithmetic mean ± SEM for [^{3}H]-SCH23390 in D_1R-transfected cells (1.80 ± 1.09) and D_1R+SAP102-transfected cells (1.71 ± 0.85) were not significantly different (p>0.05) as determined using an unpaired *t*-test.

6.6. SAP102 Does Not Alter the MEK-Dependency of D_1R-Mediated ERK1/2 Activation

Prior studies[292–314] show that ERK1/2 activation can occur independently of the canonical MAPK cascade, in some cases involving the PDZ-domain interacting kinase, PDZ-binding kinase (PBK)[306,307]. Moreover, $G_{s/olf}$-mediated PKA-dependent ERK1/2 activation in particular has been found to occur in a MEK-independent fashion in at least one instance[305]. Thus, an experiment was performed to assess if SAP102 imparts D_1R-mediated ERK1/2 activation with MEK-independence by pre-treating HEK293 cells with the MEK 1 and 2 (MEK1/2) inhibitor U0126 before applying DA. MEK1/2 inhibition with U0126 completely abrogated DA-induced ERK1/2 activation by D_1R both in cells lacking and expressing SAP102 **(Fig. 15)**. This finding thus indicates that SAP102-independent and -dependent D_1R-mediated ERK1/2 activation within HEK293 cells proceeds canonically through MEK1/2.

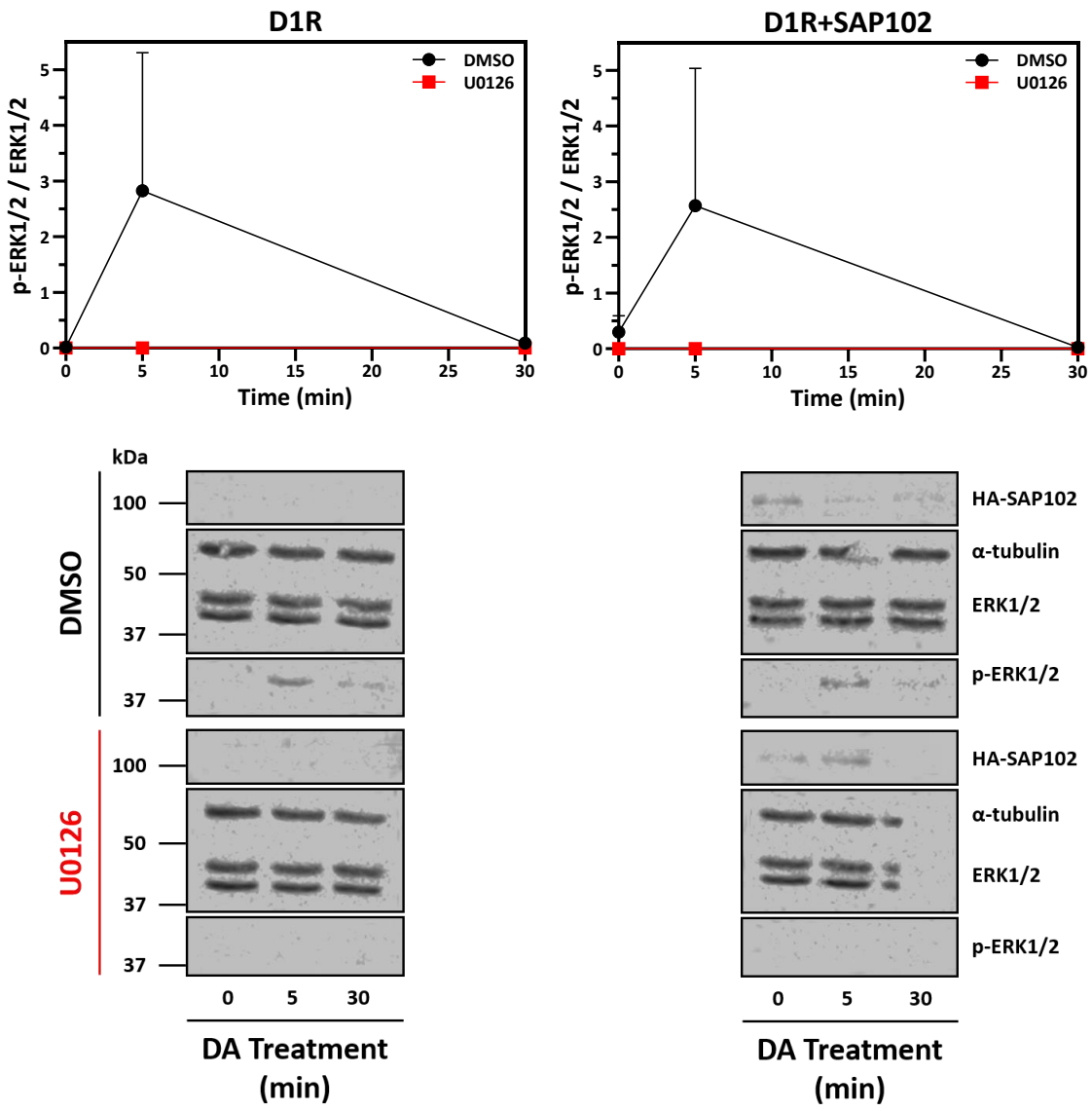


Figure 15. SAP102-Independent and -Dependent D_1R-Mediated ERK1/2 Activation Both Require MEK Stimulation

D_1R- and D_1R+SAP102-transfected HEK293 cells were pre-incubated with DMSO (1% v/v) or the MEK1/2 inhibitor U0126 (10 µM) for 30 min, then treated with DA (10 µM) for 5 or 30 min, or left untreated. Cell lysates were then prepared and subjected to western blotting with the following antibodies: anti-HA (1:2000, SAP102 expression), anti-α-tubulin (1:1000, loading control), anti-ERK1/2 (1:1000, total ERK1/2), and anti-p-ERK1/2 (1:1000). **Top**, Data are presented as arithmetic mean ± SEM (n=2) of p-ERK1/2 (p-ERK1+p-ERK2) normalized relative to total ERK1/2 (ERK1+ERK2). **Bottom**, Representative western blots are shown. B_{max} (pmol/mg membrane proteins) values for [^{3}H]-SCH23390 in both D_1R- and D_1R+SAP102-transfected cells were indeterminate for one experiment, while respective B_{max} values for these conditions in the other experiment were 4.59 and 3.94.

CHAPTER 7: DISCUSSION

My thesis studies have highlighted distinct roles for SAP102 in modulation of the ERK1/2 pathway within HEK293 cells depending on the cellular context. SAP102 expressed alone in the absence of D_1R reduces the level of basally activated ERK1/2 relative to mock- and D_1R-transfected cells. In contrast, SAP102 expressed in the presence of D_1R does not reduce basal ERK1/2 activation relative to cells transfected with D_1R alone, but instead alters the mechanism and temporality of D_1R-mediated ERK1/2 activation.

Below, I discuss possible processes underlying the effect of SAP102 on basal ERK1/2 activation, hypothesized signaling pathways utilized by SAP102-free D_1R versus SAP102-linked D_1R to activate ERK1/2, and potential mechanisms underlying SAP102-dependent D_1R-mediated ERK1/2 activation. To conclude this thesis, I discuss the physiological and pathological relevance of my findings, along with future studies to expand on the work presented herein.

7.1. Effect of SAP102 on Basal ERK1/2 Activation

Presumably, when D_1R is absent, SAP102 is largely free within the cytosol to interact with and disrupt the function of some unknown protein involved in regulating the ERK1/2 cascade. However, when D_1R is present, SAP102 is likely anchored at the plasma membrane through its interaction with D_1R, thus rendering it unable to interfere with the cytosolic target protein controlling activation of the ERK1/2 pathway **(Fig. 16)**. These differential effects of SAP102 on HEK293 cell basal ERK1/2 activation purportedly arising secondarily to different SAP102 subcellular localization thus underscore the importance of spatial protein control for determining a protein's overall cellular function.

One conceivable target involved in SAP102's effect of antagonizing basal ERK1/2 activation could be SAP97. Indeed, several pieces of evidence strongly implicate a role of

endogenous SAP97 in controlling HEK293 cell basal ERK1/2 activation. Firstly, SAP97 is capable of scaffolding different proteins involved in the ERK1/2 cascade. SAP97 physically interacts with both MEK2[315,316] and KSR1[317]. Moreover, the presence of a consensus ERK1/2 docking site within SAP97[315] suggests the possibility of direct physical linkage between SAP97 and ERK1/2. Thus, given that KSR1 is known to facilitate Raf activation[95], it is possible that a SAP97-KSR1 complex is somehow involved in spatially constraining Raf in a manner that makes activation of SAP97-scaffolded MEK2-ERK more favorable under basal conditions.

SAP97 also physically interacts with PBK[318,319], also known as T-lymphokine-activated killer cell-originated protein kinase[306], which is a member of the MAPKK family[306], and has been found capable of directly activating ERK1/2 in a MEK-independent fashion[306,307]. PBK expression is elevated in several cancerous cell lines and contributes to unregulated, stochastic ERK1/2 activation[306,307]. Thus, given the high expression level of PBK within HEK293 cells[306,320], it is also possible that SAP97 could contribute to PBK-dependent processes facilitating activation of ERK1/2 under basal conditions.

Therefore, given the known endogenous expression of SAP97 within HEK293 cells[321,322], as well as the characterized physical interaction between SAP97 and SAP102[323], it is possible that overexpressing SAP102 in HEK293 cells could result in aberrant formation of SAP102-SAP97 complexes that disrupt SAP97 interactions with components of the ERK1/2 cascade and thereby antagonize basal ERK1/2 activation. Alternatively, SAP102 may mediate this effect by interacting with the ERK1/2 cascade binding partners of SAP97.

Secondly, a recent study shows that overexpressed G protein-coupled receptor 30 (GPR30) within HEK293 cells mediates constitutive activation of ERK1/2 in a manner that is

dependent on physical interaction between GPR30 and endogenous SAP97[324]. Moreover, given that GPR30 is endogenously expressed within HEK293 cells[285], it is possible that some of the basal ERK1/2 activation within HEK293 cells may arise from endogenous GPR30-SAP97-dependent processes. Thus, a second mode by which SAP102 may contribute to disrupting SAP97-dependent regulation of basal ERK1/2 activation could involve altering SAP97 physical interactions with GPR30 or other membrane proteins potentially contributing to SAP97-dependent activation of ERK1/2 under basal conditions, through either direct SAP102-SAP97 interaction or SAP102 interaction with the associated membrane protein involved.

Thirdly, SAP97 also regulates agonist-induced ERK1/2 activation downstream of other exogenously expressed GPCRs within HEK293 cells. Namely, a requirement for endogenous SAP97 is reported for ERK1/2 activation downstream of 5-HT 2A receptor[325], corticotropin-releasing factor receptor 1 ($CRFR_1$), and corticotropin-releasing factor receptor 2[326], which in all of these cases is completely independent of any physical interaction between SAP97 and the receptor. It is thus possible that SAP97 may also affect constitutive ERK1/2 activation by some endogenously expressed receptors in a manner that does not require direct SAP97-receptor physical interaction. Therefore, a third potential mechanism by which SAP102 may impede SAP97-dependent basal ERK1/2 activation could involve SAP102 interaction with SAP97 or any of its binding partners in a manner that disrupts SAP97-facilitated processes contributing to basal ERK1/2 activation initiated by GPCRs or other membrane proteins which occur independently of SAP97 interactions with said membrane proteins.

Interestingly, a study using SAP102 KO mice has characterized a role for SAP102 in suppressing basal ERK2 activation within the hippocampus[39]. Thus, investigation into the

mechanism underlying SAP102-mediated antagonism of basal ERK1/2 activation within HEK293 cells found in my studies could yield physiologically relevant information on how DLG family members might cooperate to regulate basal levels of ERK1/2 signaling pathway activation.

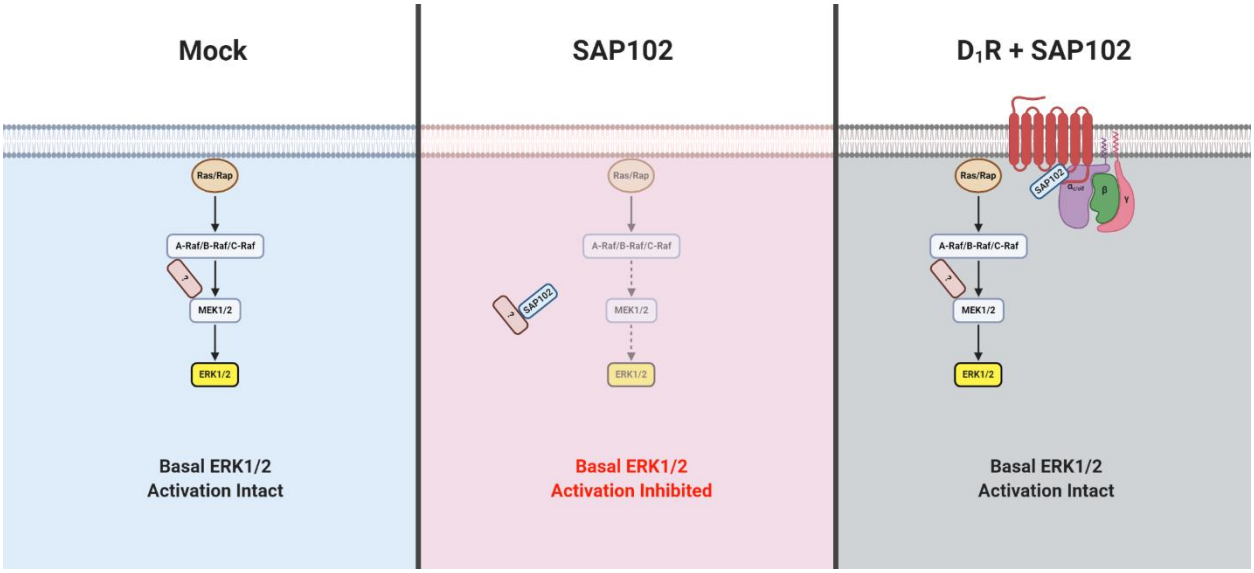


Figure 16. Hypothetical Mechanism of SAP102-Mediated Basal ERK1/2 Inhibition in HEK293 Cells

Black lines denote normal activation; dashed, faded lines denote reduced activation. Created with BioRender.com.

7.2. Effect of SAP102 on D_1R-Mediated ERK1/2 Activation

It was recently demonstrated that ERK1/2 activation downstream of the endogenously expressed $G_{s/olf}$-coupled β_2AR within HEK293 cells is mediated by the combined action of two distinct, parallel pathways through Ras and Rap[76]. ERK1/2 activation through Ras occurs in a PKA-independent fashion through direct cAMP binding to and activation of RapGEF2 that initiates K-Ras activation of C-Raf and B-Raf. ERK1/2 activation through this pathway initiates rapidly and is of large magnitude, but exhibits a relatively transient temporal pattern. In contrast, ERK1/2 activation through Rap occurs in a PKA-dependent fashion via SFK-mediated RapGEF1 activation that promotes Rap1 activation of B-Raf. ERK1/2 activation by this pathway also initiates fairly quickly but is of slightly lesser magnitude and displays a prolonged time course.

Intriguingly, the temporal patterns and PKA-dependency for ERK1/2 activation by SAP102-free D_1R and SAP102-linked D_1R respectively mirror that of β_2AR-facilitated Ras- and Rap-dependent pathways[76]. Indeed, D_1R in the absence of SAP102 exhibits essentially complete PKA-independent ERK1/2 activation with a trend of greater magnitude and lesser duration relative to the PKA-dependent ERK1/2 pathway mediated by D_1R in the presence of SAP102. In light of this, it is tempting to speculate that ERK1/2 activation by SAP102-free D_1R within HEK293 cells occurs principally through the aforementioned RapGEF2-K-Ras pathway, while in contrast, SAP102 facilitates a shift in the mechanism of ERK1/2 activation by D_1R toward primary utilization of the RapGEF1-Rap1 pathway. However, based on the lack of complete inhibition of ERK1/2 activation by D_1R-SAP102 signaling complex in the presence of the PKA inhibitors H89 and PKI used in my experiments, it is possible that both the RapGEF2-K-Ras

pathway and RapGEF1-Rap1 pathway are still being employed to some extent by D_1R-SAP102 signaling complex rather than SAP102 completely biasing ERK1/2 activation toward utilization of the RapGEF1-Rap1 pathway. Alternatively, cells expressing D_1R in the presence of SAP102 may contain one population of D_1R that is linked to SAP102 and facilitates activation of ERK1/2 through the PKA-RapGEF1-Rap1 pathway, along with a second population of D_1R that is free of SAP102 and promotes ERK1/2 activation through the PKA-independent RapGEF2-K-Ras pathway. In contrast, the almost completely unaffected D_1R-mediated ERK1/2 activation by H89 and PKI in the absence of SAP102 suggests a possibility that the RapGEF1-Rap1 pathway either plays only a minor contributing role or is entirely uninvolved in ERK1/2 activation by D_1R that is not bound to SAP102.

D_1R-mediated ERK1/2 activation after 1 min DA treatment represents a particularly interesting time point, with divergent results observed between SAP102-free D_1R and SAP102-linked D_1R. Much like previously demonstrated with β_2AR in HEK293 cells[76], SAP102-free D_1R exhibits almost no ERK1/2 activation at the 1 min time point. In contrast, in the presence of SAP102, D_1R facilitates noticeable ERK1/2 activation at the 1 min time point which is significantly greater than that elicited by SAP102-free D_1R, despite the fact that maximal magnitude of ERK1/2 activation reached within the overall time course is lesser for SAP102-linked D_1R than SAP102-free D_1R. This suggests that SAP102 is specifically acting to increase the rapidity of initiation of ERK1/2 activation, potentially through biasing D_1R toward utilization of the RapGEF1-Rap1 pathway.

Indeed, results from a prior study[43] assessing the effect of SAP102 on ERK1/2 activation by the $G_{s/olf}$-coupled $A_{2A}R$ within HEK293 cells offer potential support for this finding. Within

this study, a somewhat related effect of SAP102 on temporal A_{2A}R-mediated ERK1/2 activation is found, with SAP102 facilitating a shift in peak ERK1/2 activation toward an earlier time point. These results thus suggest the possibility that SAP102 may generally act to accelerate the initiation of ERK1/2 activation by $G_{s/olf}$-coupled GPCRs, possibly by altering the ratio of Ras:Rap dependent signaling utilized by receptors through increasing the rate of or degree to which Rap-mediated signaling pathways are activated.

However, there is a possibility that pathways other than the purported RapGEF2-K-Ras and RapGEF1-Rap1 pathways could contribute in parallel with or in place of these two pathways to ERK1/2 activation by SAP102-free D_1R and SAP102-linked D_1R, with SAP102 possibly imparting altered utilization of them to D_1R. Notably, HEK293 cell isolates display great variability in expression of relevant proteins for $G_{s/olf}$-cAMP-mediated regulation of the ERK1/2 pathway—perhaps best exemplified in the multiple different mechanisms reported for HEK293 cell β_2AR-mediated ERK1/2 activation between studies[76,78,79,82,85,100,104,279,281–283]. Therefore, additional cAMP-mediated ERK1/2 activation signaling components could conceivably factor in to the modulatory effect of SAP102 on D_1R-mediated activation of ERK1/2. EPAC1, EPAC2 and RasGRF1, three other GEFs implicated in $G_{s/olf}$-cAMP-mediated ERK1/2 activation through Ras and/or Rap1[70,71,75,80,82,83,85] **(Fig. 1)**, are endogenously expressed within HEK293 cells[71,75,82,83,85,327] and could thus also partake in the processes underlying ERK1/2 activation by SAP102-free D_1R and SAP102-linked D_1R. Additionally, HEK293 cells endogenously express Rap1GAP[321,322], which D_1R inactivates to elicit ERK1/2 activation within striatal neurons[72] **(Fig. 4)**. Thus, modulation of Rap1GAP activity could also play a role in D_1R-mediated ERK1/2 activation measured in my studies.

Moreover, the possibility of Shp-2 and/or TrkBR factoring in to D_1R-mediated ERK1/2 activation observed in my experiments in the absence and/or presence of SAP102 should also be considered. This is supported by the demonstrated importance of Shp-2 in D_1R-mediated ERK1/2 activation within HEK293 cells[35] **(Fig. 4)**, along with the report of D_1R-mediated ERK1/2 activation through transactivation of TrkBR within striatal neurons[114] **(Fig. 5)**, and known expression of a TrkBR isoform within HEK293 cells[328]. In particular, Shp-2 is known to physically interact with D_1R-IL3[35], and phosphorylation of a C-terminal-Tyr within Shp-2 creates a docking site for the SH2 domain of GRB2, which in turn facilitates the activation and recruitment of the Ras GEF Sos[143]. Thus, it is possible that Shp-2 physical interaction with D_1R could recruit a Sos-dependent Ras-ERK1/2 activation pathway directly to D_1R that occurs differently in the presence versus absence of SAP102. Additionally, Shp-2 contributes to TrkBR-mediated ERK1/2 activation[144], suggesting the possibility that Shp-2 could also be involved in activating a pool of ERK1/2 downstream of D_1R or D_1R-SAP102 complex that involves TrkBR transactivation.

7.3. Potential Mechanisms of SAP102-Dependent D_1R-Mediated ERK1/2 Activation

Based on the finding by Takahashi et al[96] that sustained PKA-dependent ERK1/2 activation by the RapGEF1-Rap1 pathway within HEK293 cells is seemingly dependent on PKA-mediated formation of a novel complex containing Rap1, KSR1 and B-Raf, several mechanisms of how SAP102 may bias D_1R-mediated ERK1/2 activation can be hypothesized. One mechanism involves a potential SAP102 physical interaction with connector enhancer of kinase suppressor of Ras 2 (CNK2). CNK2 physically interacts with the PDZ domains of PSD-95, SAP97 and PSD-93[329], thus there is a strong likelihood that it also interacts with at least one of the SAP102 PDZ

domains. CNK2 is capable of influencing the ERK1/2 pathway through physical interaction with B-Raf[330]. Moreover, studies assessing the *Drosophila* homolog of CNK2 provide evidence that CNK2 may also physically interact with KSR1 in an indirect manner through mutual interaction with the novel sterile α-motif domain-containing protein aveugle[331]. Thus, it is possible that a SAP102 interaction with CNK2 might be acting to promote biased D_1R-mediated activation of ERK1/2 through the RapGEF1-Rap1 pathway in some manner involving physical linkage of the D_1R-SAP102 signaling complex to the tripartite Rap1-KSR1-B-Raf complex **(Fig. 17)**.

Additionally, several pieces of evidence suggest that the mechanism underlying modulation of D_1R-mediated ERK1/2 activation by SAP102 could also involve SAP102-facilitated linkage of the D_1R-SAP102 complex to the AKAP Nbea. It is well-established that DLG family members and AKAPs act in conjunction to modulate ionotropic glutamate receptor and GPCR functions. PSD-95 is required to link AKAP5 to NMDAR via its interaction with GluN2 subunits, while in contrast SAP97 is required to link AKAP5 to AMPAR via its interaction with GluA1 subunits[262]. The AKAP5-PSD-95-GluN2B complex enables NMDAR-mediated AMPAR endocytosis[332], whereas the AKAP5-SAP97-GluA1 complex increases the phosphorylation of GluA1-Ser845, which enables the delivery of GluA1 to the synaptic membrane[242,262,287,317].

The physical interaction between SAP97 and β_1-adrenergic receptor (β_1AR) is also required to tether AKAP5 to β_1AR, which is necessary for normal β_1AR recycling and resensitization[333–336], and may additionally be needed for dampening β_1AR-mediated cAMP signaling through the action of AKAP5-linked phosphodiesterase 4D8[337]. On the other hand, β_2AR appears to physically interact with a PSD-95-AKAP5 complex in a fashion necessary for β_2AR-mediated phosphorylation of GluA1-Ser845, that facilitates increased AMPAR surface

expression[287]. AKAP5-linked PSD-95 is similarly required for GluA1-Ser845 phosphorylation and the associated enhancement of AMPAR surface expression downstream of D_1R activation, potentially in a manner involving D_1R-PSD-95 physical interaction[33,259,264].

Interaction between SAP97 and another GPCR, GPR30, is also required to link AKAP5 to the receptor complex and modulate several receptor functions. Namely, AKAP5-linked SAP97 facilitates enhanced GPR30 surface expression and constitutive GPR30-mediated ERK1/2 activation, while also eliciting GPR30-mediated inhibition of cAMP accumulation downstream of other receptors through an incompletely understood mechanism that does not involve $G_{i/o}$[324,338]. Moreover, the function of several additional GPCRs, including the $G_{s/olf}$-coupled $CRFR_1$, is also modulated through interactions with SAP97 and/or PSD-95[325,326,339–344], however the potential role of AKAP5 in SAP97/PSD-95-dependent functional modulation of these receptors has not yet been investigated. Thus, it is evident that DLG family-AKAP complexes are critical for normal glutamatergic transmission and the function of various GPCRs.

Indeed, an interaction between Nbea and SAP102 has recently been demonstrated as critical for glutamatergic function in hippocampal neurons[289–291]. Given that Nbea is endogenously expressed within HEK293 cells[321,322], it is possible that a contributing mechanism to the effect of SAP102 on imparting PKA-dependency to D_1R-mediated ERK1/2 activation could involve a physical interaction with Nbea. This may act to localize PKA in a favorable position to initiate the RapGEF1-Rap1-ERK1/2 pathway, which would otherwise not occur in cells expressing D_1R alone **(Fig. 17)**.

Moreover, it is also possible that SAP102 interaction with Nbea may be involved in linkage of D_1R-SAP102 complex to the Rap1-KSR1-B-Raf complex in place of or in concert with

the proposed function of CNK2 in this process. Findings pertaining to the role of another AKAP, AKAP-lymphoid blast crisis (AKAP-Lbc), in cAMP-mediated ERK1/2 activation within HEK293 cells support this possibility. AKAP-Lbc that is exogenously expressed in HEK293 cells mediates two necessary functions for enabling maximal cAMP-PKA-mediated ERK1/2 activation that involve direct linkage to MAPK cascade components[345]. First, AKAP-Lbc enables more favorable Raf-mediated MEK activation by directly interacting with C-Raf and B-Raf. Second, AKAP-Lbc facilitates PKA-mediated phosphorylation of KSR1-Ser838 through physical linkage with KSR1. Based on these findings, it is thus conceivable that a SAP102-Nbea complex could in fact be involved in linking D_1R to the purported Rap1-KSR1-B-Raf complex.

Shp-2-dependent processes may also factor in to the hypothetical effect of SAP102 on facilitating a switch in D_1R-mediated ERK1/2 activation to a PKA-RapGEF1-Rap1-dependent pathway. One group previously found that Shp-2-interaction with D_1R-IL3 is critical for D_1R-mediated ERK1/2 activation in HEK293 cells[35] **(Fig. 4)**. Thus, based on our finding that SAP102 interacts with D_1R-IL3, it is possible that in our experimental system SAP102-D_1R-IL3 interaction alters the D_1R-IL3 interaction with Shp-2 which may somehow contribute to biasing D_1R-mediated ERK1/2 activation toward the PKA-dependent RapGEF1-Rap1 pathway.

Finally, it is worth mentioning an entirely different potential explanation for the effect of SAP102 on imparting PKA-dependency to D_1R-mediated ERK1/2 activation that does not involve selectively biasing this process toward the utilization of a PKA-initiated signaling pathway. Hypothetically, upon D_1R activation, PKA-mediated phosphorylation of the known PKA site on Thr268 within D_1R-IL3[346,347] could alter the SAP102-D_1R-IL3 interaction in a manner that permits relatively normal D_1R-mediated ERK1/2 activation. However, under conditions in which PKA-

mediated phosphorylation of D_1R-Thr268 does not occur following D_1R-initiated increase of intracellular cAMP, such as when cells are treated with PKA inhibitors, a potential inhibitory effect of the unaltered SAP102-D_1R-IL3 interaction on D_1R-mediated ERK1/2 activation may be unmasked, which could involve the utilization of similar SAP102-dependent processes as those used by free SAP102 to antagonize basal ERK1/2 activation. Thus, the observed PKA-dependency for SAP102-dependent D_1R-mediated ERK1/2 activation may actually be reflected in the requirement for PKA to alleviate an inhibitory effect of SAP102 on D_1R-mediated ERK1/2 activation that would otherwise occur in the absence of PKA-mediated D_1R-IL3-Thr268 phosphorylation, rather than a requirement per se for PKA to initiate downstream signaling toward ERK1/2 activation.

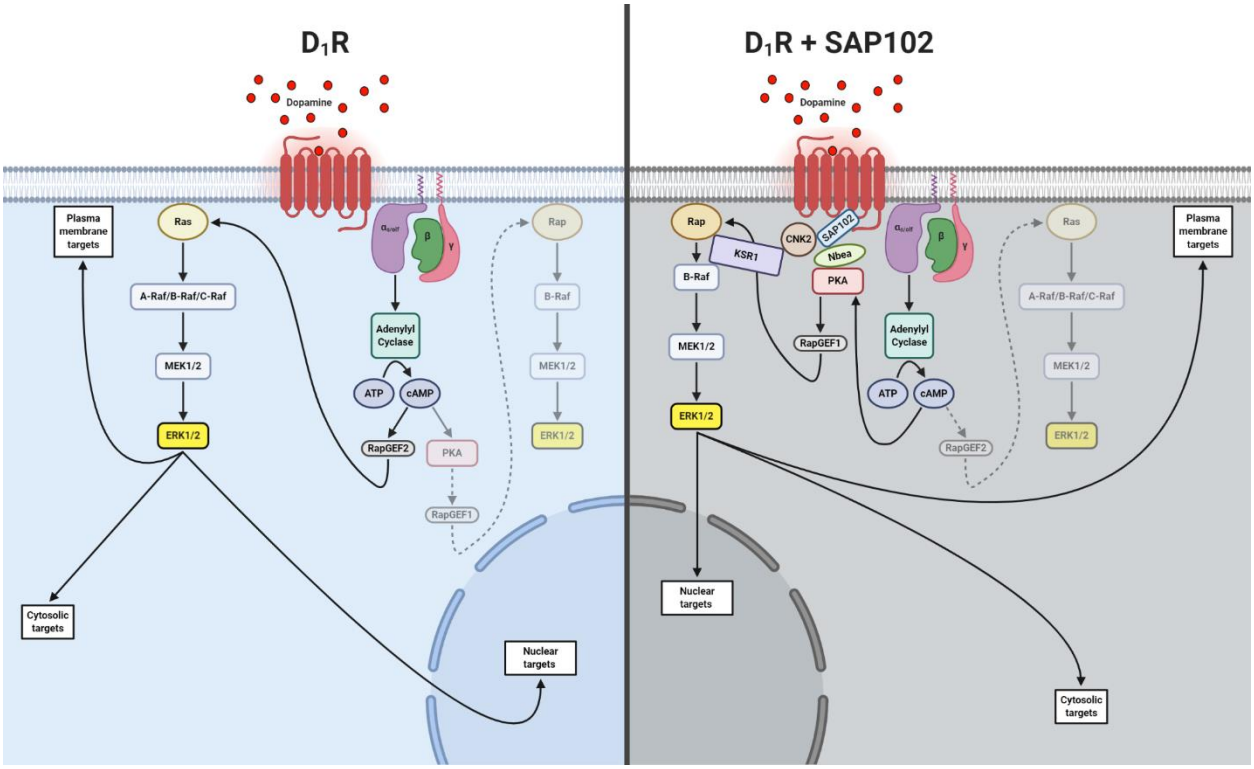


Figure 17. Hypothetical Mechanisms of SAP102-Independent and -Dependent D1R-Mediated ERK1/2 Activation

Black lines denote normal activation; dashed, faded lines denote reduced activation. Created with BioRender.com.

7.4. Conclusions and Future Studies

My thesis studies have yielded two main findings pertaining to the role of SAP102 in ERK1/2 activation. Firstly, they demonstrate an effect of SAP102 on reducing basal ERK1/2 activation when expressed alone within HEK293 cells relative to mock-transfected cells. Secondly, they characterize an effect of SAP102 on facilitating a switch in the mechanism of D_1R-mediated ERK1/2 activation to a PKA-dependent process with a seemingly altered temporal pattern. Interestingly, cells expressing SAP102 in the presence of D_1R do not show altered levels of basal ERK1/2 activation relative to cells expressing only D_1R.

These results support the idea that spatial control of SAP102 is critical for determining its effect on basal activation of the ERK1/2 pathway. Presumably, SAP102 in the absence of D_1R is "free" within the cytosol to interact with proteins controlling the MAPK cascade. While in contrast, the presence of D_1R results in SAP102 anchorage at the plasma membrane, which impedes its ability to reduce basal ERK1/2 activation **(Fig. 16)**. Indeed, together, several studies support the possibility that SAP102 within the cytoplasm could act to inhibit SAP97-dependent basal ERK1/2 activation in particular[306,315–319,323–326]. Future studies using D_1R-IL3 mutants that do not physically interact with SAP102, or an inhibitory peptide for the D_1R-SAP102 interaction, could be undertaken to help determine the veracity of these ideas. These approaches could also be used to confirm the SAP102-D_1R-IL3 interaction-dependency for SAP102's effect on D_1R-mediated ERK1/2 activation. Additionally, KD of SAP97 could be performed to probe for potential SAP97 involvement in the SAP102 effect on basal ERK1/2 activation.

SAP102 also reduces the level of basal ERK2 activation in hippocampal neurons[39]. Thus, better understanding of the mechanism underlying this process in HEK293 cells could provide

important insight on an apparent *in vivo* function of SAP102 in regulating the level of ERK signaling pathway activation under basal conditions. Additionally, based on the potential involvement of SAP97 in the effect of basal ERK1/2 activation within HEK293 cells, further study of this SAP102-mediated effect may yield valuable information on how DLG subfamily members cooperate to regulate activation of ERK1/2.

Similarly, the detailed pathways utilized by D_1R to activate ERK1/2 within HEK293 cells lacking or containing SAP102, and thus the specific factors imparting PKA-dependency of ERK1/2 activation by the D_1R-SAP102 signaling complex, remain up for speculation. However, my thesis studies in conjunction with the recently uncovered details of β_2AR-mediated ERK1/2 activation within HEK293 cells[76] support the idea that ERK1/2 activation by D_1R in the absence of SAP102 may occur almost exclusively through a PKA-independent RapGEF2-K-Ras-C-Raf/B-Raf pathway, while in contrast, ERK1/2 activation by the D_1R-SAP102 complex might occur primarily through a PKA-dependent RapGEF1-Rap1-B-Raf pathway **(Fig. 17)**. Studies utilizing pharmacological inhibition or genetic KD of the RapGEF1-Rap1 and RapGEF2-K-Ras pathway could be undertaken to assess this hypothesis. These studies would also confirm whether residual SAP102-dependent D_1R-mediated ERK1/2 activation observed in the absence of functional PKA involves partial utilization of the RapGEF2-K-Ras pathway. Moreover, I am speculating that the effect of SAP102 on selectively partitioning D_1R-mediated ERK1/2 activation toward the RapGEF1-Rap1 pathway could involve two major contributing mechanisms: SAP102-facilitated tethering of Nbea-linked PKA to D_1R, and linkage of D_1R-SAP102 to the Rap1-KSR1-B-Raf complex—purported to be critical for the PKA-RapGEF1-Rap1-

ERK1/2 pathway within HEK293 cells[96]—through SAP102 interaction with CNK2 and/or Nbea **(Fig. 17)**.

The altered PKA-dependency of D_1R-mediated ERK1/2 activation in the presence versus absence of SAP102 is only associated with slightly different temporal patterns of ERK1/2 activation, which is unlikely to significantly alter the overall ERK1/2-mediated effects. However, it is conceivable that SAP102 has a greater modulatory effect on the spatial dimension of D_1R-mediated ERK1/2 activation that may lead to major differences in downstream ERK1/2 substrate activation. Indeed, the $G_{s/olf}$-coupled vasoactive intestinal polypeptide $VPAC_2$ receptor ($VPAC_2$R) activates ERK1/2 within GH4C1 cells through parallel Ras and Rap1 pathways that promote distinctly different patterns of nuclear and cytosolic phospho-ERK1/2 spatial localization[348]. Moreover, Ras- and Rap1-mediated ERK1/2 activation by $VPAC_2$R within GH4C1 cells clearly elicit different downstream physiological effects, with the Rap1-ERK1/2 pathway shown to promote prolactin gene induction but the Ras-ERK1/2 pathway found to antagonize prolactin gene induction[349]. Thus, if SAP102-free D_1R versus SAP102-linked D_1R differentially utilize Ras- and Rap1-dependent pathways to ERK1/2 activation, it is possible that activated ERK1/2 downstream of SAP102-free D_1R and SAP102-linked D_1R may exhibit different patterns of subcellular localization and thereby result in activation of different downstream ERK1/2 substrates **(Fig. 17)**.

Alternatively, it is also possible that SAP102 alters the spatial pattern of D_1R-mediated ERK1/2 activation in a manner that is independent of the proposed switch in utilization of Ras and Rap1. This idea is supported by the finding that mouse embryonic fibroblasts (MEFs) exhibit different spatial Ras-mediated ERK1/2 activation and downstream ERK1/2 substrate

phosphorylation from the same membrane nanodomain in a fashion dictated by the specific scaffolding protein present within Ras signaling complexes[350]. In particular, Ras-ERK1/2 activation from lipid rafts within the plasma membrane results in phosphorylation of cytosolic phospholipase A2 within the cytoplasm in a mode requiring KSR1, but selectively leads to phosphorylation of epidermal growth factor receptor at the plasma membrane when IQ domain-containing GAP is utilized in the absence of KSR1. Studies using fluorescence resonance energy transfer- or bioluminescence resonance energy transfer-based ERK1/2 biosensors could be employed to investigate the hypothesized effect of SAP102 on spatial D_1R-mediated ERK1/2 activation, and to clarify the existence of a SAP102-dependent effect on temporal ERK1/2 activation by D_1R suggested in my results.

It is interesting to speculate about the potential *in vivo* implications for my finding of SAP102-facilitated modulation of D_1R-mediated ERK1/2 activation within HEK293 cells, should this result first be reconstituted in a more physiologically relevant system. One idea involves the possibility of a differential regulatory effect on D_1R-mediated ERK1/2 activation by D_1R-SAP102 complexes versus D_1R-PSD-95 complexes. Given the known roles of the D_1R-PSD-95 complex[33,259–261] and its implication in D_1R-mediated ERK1/2 activation[150], it is possible that one function of distinct D_1R-SAP102 and D_1R-PSD-95 complexes is to impart different mechanisms of D_1R-mediated ERK1/2 activation, conceivably leading to distinct ERK1/2 spatiotemporal activation patterns in conjunction with differential ERK1/2 substrate phosphorylation. The possibility of this idea is supported by the above mentioned finding that MEF Ras-ERK1/2 activation from the same subcellular nanodomain results in distinct spatial ERK1/2 activation and downstream ERK1/2 substrate phosphorylation in a manner strictly depending on the

particular scaffolding protein involved in the process[350]. Additionally, the idea of different overall downstream signaling outcomes stemming from D_1R-mediated ERK1/2 activation involving either SAP102 or PSD-95 may help explain the findings that animal models of LID and drug addiction exhibit concurrent alterations in D_1R-mediated ERK1/2 activation[46,48,49,53,56,57,70,101,106,119,123,128,131–133,135,138,151,159,194,195,197,200–202,216,219,220,226,227,230] and expression levels of SAP102 and PSD-95[150,265,266,271,272,351]. Indeed, it is possible that disruption of the normal ratio of D_1R-PSD-95 to D_1R-SAP102 complex is a major contributing factor underlying the dysfunctional D_1R-mediated ERK1/2 activation in these conditions.

Overall, to the best of my knowledge, my finding that SAP102 alters the process utilized by D_1R to activate ERK1/2 within HEK293 cells is the first demonstration of a scaffolding protein directly switching the mechanism used by a GPCR to activate a particular signaling pathway. Further studies aimed at understanding more details of this process would thus not only provide valuable mechanistic insight on D_1R-mediated activation of the ERK1/2 cascade, but could also offer clues to how this phenomenon may occur more generally for other GPCRs and their associated GPCR-interacting proteins, potentially including other DLG family members. Finally, such studies may also yield important information for the development of novel therapeutics to treat conditions with D_1R dysfunction, like drug addiction and LID.

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