RASA3, a Key Player in Dopamine D2S Receptor-mediated MAPK Signaling

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Abstract

The short form of dopamine D2 receptor (D2S) functions as a presynaptic autoreceptor on dopamine neurons and has an inhibitory effect on dopaminergic tone. D2-MAPKs pathway is involved in many physiological events like production of prolactin and tyrosine hydroxylase (TH) expression. However, the effect of D2S receptor signalling on MAPKs is cell type specific, and is not fully understood.

A recent study in our lab has identified a Gαi-interacting ras-MAPK inhibitor RASA3. Here, we showed that RASA3 is the key effector in D2-induced inhibition of MAPK by knockdown of endogenous RASA3 in the GH4 cell using RASA3 siRNA. We have also transfected a dominant negative RASA3 to compete with the endogenous RASA3 for the binding site on Ras. Both RASA3-siRNA and dominant negative RASA3 blocked D2S-induced inhibition of MAPK activation, clearly implicating that RASA3 is a key effector in Gαi3-dependent D2S mediated MAPKs inhibition

To determine whether RASA3's inhibitory effect could be reconstituted in fibroblast cells, the effect of RASA3 on D2-mediated ERK1/2 activation in COS7 cells was tested. Our results show that both active Gαi2 (or Gαi3) and active RASA3 are required for optimal inhibition of ERK1/2 activation in fibroblast COS7 cells.

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List of abbreviations

Abbreviation	Full name
aa	Amino acid
AC	Adenylyl cyclase
Btk	Bruton's tyrosine kinase
cAMP	Cyclic adenosine monophophate
CHO	Chinese hamster ovary cell line
CNS	Central nervous system
C3G	Crk7 SH3 domain Guanine nucleotide exchanger
DA	Dopamine
DAG	Diacylglycerol
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32
DAT	Dopamine active transporter
Dopa	3, 4-dihydroxyphenylacetic acid
DR	Dopamine receptor
EGF	Epidermal growth factor
EL	Extracellular loop
EPAC	Exchange protein directly activated by cAMP
ERK	Extracellular-signal regulated kinase
G protein	Guanine nucleotide binding protein
GAP	GTPase activating protein

GAP1IP4BP	GAP1 inositol 1,3,4,5 tetrakisphosphate bindin
GDP	Guanosine diphophate
GEF	Guanosine nucleotide exchange factor
GH4ZR7	Rat GH4 lactotroph cells stabely transfected with
GPCR	D2S
GRK	G protein-coupled receptor
GSK	G protein-coupled receptor kinase
GTP	Glycogen synthase kinase
HEK	Guanosine triphosphate
IP3	Human embryonic kidney cell line
IP4	Inositol 1, 4, 5-trisphophate
IL	Inositol 1, 3, 4, 5-tetrakisphophate
JNK	Intracellular loop
KDa	C-Jun-N-terminal kinase
LPA	Kilodalton
MAO	Lysophosphatidic acid
MAPK	Monoamine oxidase
MEK	Mitogen-activated protein kinase
7-OH-DPAT	MAPK/ERK kinase
PH	7-hydroxy-N,N-dipropyl-2-aminotetralin
PI3K	Pleckstrin homology
PIP2	Phosphoinositide 3-kinase

	Phosphatidylinositol 4, 5-biphosphate
PIP3	Phosphatidylinositol 3, 4, 5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein phosphatase 2
PRL	Prolactin
PTX	Pertussis toxin
RIPA	Radio ImmunoPreciptation Assay
SNc	Substantia nigra compacta
Ser	Serine
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
TH	Tyrosine hydroxylase
Thr	Threonine
TM	Transmembrane
TNF	Tumor necrosis factor
TRH	Thyrotropin releasing hormone
VTA	Ventral tegmental area

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CHAPTER I

GENERAL INTRODUCTION

1.1 Dopaminergic system

1.1.1 Brief History of Dopamine

Dopamine was first synthesized in 1910 by George Barger and James Ewens at Wellcome Laboratories in London, England (Fahn et al, 2006). Dopamine was initially considered only a precursor of norepinephrine and epinephrine. In 1957, Dopamine was first discovered as an independent neurotransmitter by the pharmacologist Arvid Carlsson (Benes et al, 2001). This discovery, in combination with his later work, rendered Arvid Carlsson the Nobel Prize for Physiology and Medicine in 2000. For the first decade after its discovery, dopamine drew remarkably little attention. In 1968, George Cotzias made a breakthrough in L-Dopa treatment, which converted the L-Dopa into a practical medication used in large scale treatment of Parkinson's disease (Cotzias et al, 1968). Meanwhile, with the introduction of a range of new neurochemical and pharmacological tool for the study of dopamine neurons and their function in the brain, dopamine has remained at the forefront of psychopharmacological research since the 1970s.

1.1.2 Physiological function of dopamine

Dopamine (DA) has been implicated in various physiological functions including control of movement, cognition, endocrine responses, and reward. Disorders of the DA system have been implicated in multiple brain disorders, such as Parkinson's disease, schizophrenia, Tourette's syndrome, attention deficit hyperactivity disorder, addiction, and affective disorders. For instance, highly addictive compounds such as cocaine and amphetamines directly increase the amount of dopamine by inhibiting its reuptake into the synaptic terminals (Zahniser and Sorkin, 2004), and eventually change the synaptic plasticity (Shen et al, 2008).

1.1.3 Dopamine pathways in the brain

From 1963, central monoamine neurons began to be discovered using the formaldehyde-induced fluorescence method, which uses the reaction between amines and formaldehyde to produce a fluorescent reaction product that labels monoaminergic neurons (Fuxe et al, 1963, 1964; Fuxe and Jonsson, 1973). Using this method, four dopamine pathways were identified in the brain, and three of these pathways originate from the midbrain. The first is the mesolimbic pathway, which is also called the reward circuit. This pathway originates in the ventral tegmental area (VTA) and innervates several structures of the limbic system, including the nucleus accumbens. The mesocortical pathway also originates in the ventral tegmental area, and projects to the frontal cortex (Berger et al, 1976). The third pathway is the nigrostriatal pathway. It projects from the substantia nigra compacta (SNc) to the striatum, where it controls

movement (Anden et al， 1964). A fourth dopaminergic pathway is the tuberoinfundibular pathway, which originates in the median eminence of hypothalamus and secretes dopamine into the portal blood stream to regulate the pituitary gland, inhibiting the secretion of hormones such as prolactin (Fuxe et al, 1963).

1.1.4 Dopamine receptor

1.1.4 a Dopamine receptor classification

In 1979, Kebabian and Calne proposed the concept that dopamine receptors can be divided into two main groups based on their effect on cAMP formation: D1 receptor and D2 receptor. However a few years later, three additional novel DA receptors had been identified by molecular cloning: D3, D4 and D5. All the five DA receptors are still classified into two families: the D_1-like receptor family (D_1, D_5) and the D_2-like receptor family (D_{2L}, D_{2S}, D_3, D_4). Functionally, the D_1-like family receptors shows excitatory effect on target neuron and their activation is coupled to $G\square_s$ or $G\square_{olf}$ to increase the activity of adenylyl cyclase (AC), whereas the D_2 receptor family is shown mainly to inhibit AC activity through both Gi and Go signal transduction pathways. Structurally, D1 and D5 have a high homology in the 7 transmembrane domains (7-TM), and D2-like receptors also share similar 7-TM (Missale et al, 1998). Pharmacologically, D1-like and D2-like receptors show quite different profiles, but within each subfamily, receptors

exhibit similar affinities for a variety of ligands and antipsychotic compounds (Seeman et al, 1994). This is discussed in detail in the following section.

The D_1 and D_5 receptor genes do not contain introns in their coding regions, in contrast, D_2, D_3, and D_4 receptor gene coding regions are interrupted by 6, 5, 3 introns respectively, which allows the generation of receptor variants (Civelli et al, 1993; O'Dowd et al, 1993). Indeed, the D_2 receptor has two main variants (D2S and D2L), and the D_3 receptor has nonfunctional splice variants identified in humans (Fishburn et al, 1993).

The D2 receptor gene is composed of 8 exons and 7 introns. The alternative splicing of the sixth exon generates the two receptor isoforms: D2S and D2L (Missale, et al 1998). D2S is the short form of D2 receptor, which differs from D2L by lacking the 29 amino acids located in the third intracellular loop of D2L. Despite the similarity in structure, there is evidence that these two isoforms have very different location and physiological functions in brain. D2S functions primarily as an autoreceptor to inhibit dopamine release in the presynaptic dopamine neuron in VTA and substantia nigra , while D2L is localized on the dendritic spines of non-dopaminergic neurons and mediates the post-synaptic actions of dopamine (Khan et al, 1998; Usiello et al, 2000).

1.1.4 b Pharmacological profiles

The affinity of D1 and D5 receptors for a variety of antagonists is similar. Nevertheless, antagonist butaclamol shows a slightly higher affinity for the D1 than for

the D5 receptor (Sunahara et al, 1991). D5 receptor has a 10-fold higher affinity for dopamine than D1 receptor. With respect to the D2-like receptors, D3 has higher affinity for some ligands than D2. For example, dopamine itself has 20-fold higher affinity at the D3 than the D2 receptor. Quinpirole and 7-OH-DPAT have affinities 100- and 10-fold higher at D3 than at the D2 receptor, respectively (Sokoloff et al, 1990). Compared with D2 and D3, the D4 receptor has lower affinity for chlorpromazine and nemonapride, but higher affinity for the atypical antipsychotic clozapine (Seeman et al，1993, 1994; Van Tol et al, 1991)(Table 1).

1.1.4 c Structure

The dopamine receptors belong to metabotropic G-protein coupled receptor (GPCR) family. GPCRs are characterized by an extracellular N-terminus, seven transmembrane domains (7-TM) that form α-helices (TM-1 to TM-7) and are connected by three intracellular (IL-1 to IL-3) and three extracellular loops (EL-1 to EL-3), and finally an intracellular C-terminus. The third intracellular loop is an important structure for dopamine receptor signaling, because it is the main binding site for the G protein. The major structural differences of D1-like and D2-like receptor are in intracellular domains i.e. the D2-like receptors have shorter C-terminal tail and longer third intracellular loop. This structural feature is common in several Gαi-selective receptors, while long C-terminal tail and short third intracellular loop is common in Gαs-coupling receptor like D1 receptor (Civelli et al, 1993; Gingrich and Caron, 1993; Missale et al, 1998).

The transmembrane domains of dopamine receptors are the binding site for several

Table 1 Pharmacological profiles of Dopamine receptor

	D_1-Like		D_2-Like		
	D_1	D_5	D_2	D_3	D_4
			Antagonists		
(+)-Butaclamol	+++	++	+++	ND	++
Chlorpromazine	+	+	+++	++	++
Clozapine	+	+	+	+	++
Eticlopride	−	−	++++	ND	+++
Haloperidol	+	+	++++	++	+++
Nafadotride	ND	ND	+++	++++	+/−
Nemonapride	ND	ND	++++	++++	++++
Raclopride	−	ND	+++	+++	+/−
SCH-23390	++++	++++	+/−	+/−	+/−
(−)-Sulpiride	−	−	++	++	++
Spiperone	+	+/−	++++	+++	++++
			Agonists		
Apomorphine	+/−	+	+++	++	+++
Bromocriptine	+	+	+++	+++	+
Dopamine	+/−	+	+	++	++
Fenoldopam	+++	+++	++	ND	+
7-OH-DPAT	+/−	ND	++	+++	+/−
Quinpirole	−	ND	+/−	++	++
SKF-38393	+++	++++	+	+/−	+/−

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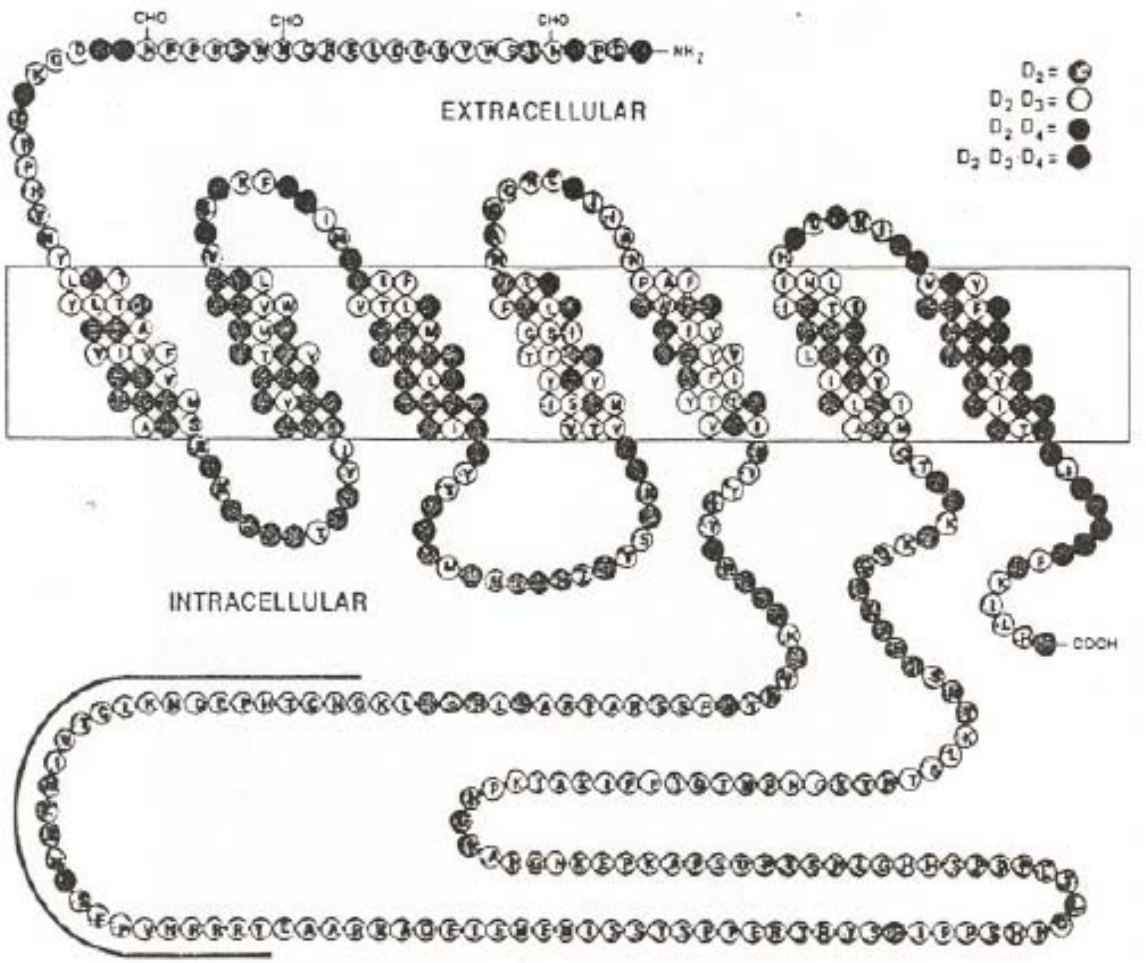
Lignads including dopamine. In a 3-D structural prediction studies, Kalani et al. (2004) proposed that dopamine binds in a site located between TM 3–6. They found 2 main contacts that stabilize dopamine in this docked conformation: a salt bridge to the TM3 (Asp-114) and a network of hydrogen bonds to the TM5 (Ser-193, Ser-194, and Ser-197). Additional studies showed that all 7 antagonists investigated here make a tight contacts with TM3, but not to TM5. In contrast, all agonists studied have strong coupling to both Asp in TM3 and Ser in TM5. This suggests that strong coupling between TM3 and TM5 is essential for dopaminergic transduction (Kalani et al, 2004). The D_1 and D_5/D_{1b} receptors share a very high homology in their transmembrane domains (80%). Similarly, the transmembrane sequences are highly conserved among D_2, D_3, and D_4 receptors. The D_2 and D_3 receptors have a 75% amino acid identity, and the D_2 and D_4 receptors share a 53% identity in the TM domains (Missale et al, 1998).

The C-terminus of GPCRs often contains serine (Ser) or threonine (Thr) residues that can be phosphorylated by G Protein-coupled Receptor Kinase (GRK), increasing the affinity of the intracellular surface for the binding of β-arrestins (β-arr). The C-terminal tail also contains the cysteine residues serving as the palmitoylation site. Palmitoylation facilitates rapid receptor signaling by targeting the receptor to the lipid raft on the plasma membrane, where many downstream effectors stay. Since the C-terminal of the D_2 receptors is very small, it only contains the Cys residue for palmitoylation, and not many Ser/Thr sites; hence phosphorylation of these receptors appears to be primarily at the third intracellular domain.

Fig. 1 Dopamine D2-like receptor structure



Structure of D2-like receptors. D2-like receptors have a short C-terminal tail and a large third intracellular loop. Bar indicates amino acids that are sliced out of the D2S receptor; CHO represents conserved glycosylation sites.

1.1.5 Dopamine-D2 receptor signaling

1.1.5 a Gα-dependent signaling pathway

Various studies showed that D2 receptor coupling to Gi/Go proteins negatively regulates adenylyl cyclase, the enzyme catalyzing the conversion of ATP to cAMP, which then activates protein kinase A (PKA) and catalyzes the phosphorylation of various downstream effectors. In different cells, the D2 receptor couples to different G protein subunits to inhibit cAMP (Liu et al., 1994; Senogles et al., 1994; Ghahremani et al., 1999). Banihashemi and Albert (2002) showed that in pituitary cell line GH4ZR7 cells, activation of the dopamine-D2S receptor inhibited forskolin-induced cAMP production, and this effect was blocked by pretreatment with pertussis toxin (PTX), indicating mediation by Gαi/o proteins. Further experiments showed that PTX blockade of D2S-mediated inhibition of cAMP could be rescued by PTX-insensitive Gαi2 but not Gαo, indicating that D2S-induced inhibition of AC in GH4 cells is through Gαi2.

Activation of cAMP/PKA leads to the phosphorylation of dopamine and adenosine 3'5'-monophosphate-regulated phospho-protein,Mr 32 kD (DARPP-32) at Thr34, a site specifically regulated by PKA (Nishi et al., 1997). Phospho-DARPP-32 is a potent inhibitor of protein phosphatase-1 (PP-1), and thereby controls the phosphorylation of many downstream physiological effectors, including voltage-gated ion channels and ERK1/2. (Greengard et al, 2000).

1.1.5 b Gβγ-dependent signaling pathway

Beside G α, Gβγ subunits also contribute to the D2 signaling. In GH3 pituitary cells transfected with the C-terminal domain of GRK to inhibit G□□ signaling, D2S-mediated L-type calcium channels inhibition was blocked (Banihashemi and Albert, 2002). In HEK293 and COS7 cells, the D2-family receptor D3 activates ERK1/2, and this effect is through Gβγ (Beom et al, 2004).

1.1.5 c G-protein independent signaling pathway

In addition to the cAMP-PKA pathway, the β-arrestin2–Akt–GSK-3 pathway is also implicated in the regulation of dopamine-associated behaviours. Akt is a serine/threonine kinase that is regulated by phosphatidylinositol kinase (PI3K)-mediated signaling. An important substrate of Akt is glycogen synthase kinase 3 (GSK-3), which is constitutively active and can be inactivated by Akt. D2 receptor stimulation in the mouse striatum results in Akt inactivation and concomitant activation of GSK3β, and this effect requires β-arrestin2 and PP2A. The phosphorylation of Akt and both GSK-3 isoforms was markedly reduced in the striatum of DAT-KO mice that have a hyperactive dopamine system, as compared with their WT littermates. In contrast, no difference in Akt phosphorylation was found between βArr2-KO/DAT-KO mice and their βArr2-KO littermates. Overall, the stimulation of D2-class receptors causes the formation of a protein complex that comprises at least Akt, β-arrestin2 and PP2A. The formation of this complex facilitates the dephosphorylation of Akt by PP2A and results in the

activation of GSK3□ (Beaulieu et al., 2004, 2005, 2007a, and 2007b).

1.1.5 d Modulation of D2 signal

The negative modulation of the D2 autoreceptor strongly affects dopaminergic tone in brain. Desensitization of D2 autoreceptors in dopamine neurons increases neuronal firing activity and release of dopamine. D2 receptors can be desensitized by either non agonist (heterologous) or agonist-specific (homologous) desensitization. In non agonist-specific or heterologous desensitization, a GPCR receptor is phosphorylated by PKA or PKC, which is activated by the activation of another type of receptor. D2 receptors can be desensitized by PKC, and the D2S receptor is more sensitive to PKC-induced desensitization than D2L (Liu et al, 1992; Morris et al, 2007). Similarly in agonist-induced D2 desensitization, D2S shows greater sensitivity to GRK2 than D2L (Ito et al. 1999). Activation of D2 caused GRK-dependent receptor phosphorylation and recruitment of beta-arrestin to the cell membrane, followed by receptor internalization. The internalization of the D2 is dynamin-dependent, suggesting that a clathrin-coated endocytic pathway is involved (Vickery et al, 1999; Iwata et al, 1999).

1.2 MAP Kinase

1.2.1 Brief history

In the 1980's, researchers found that incubation of chicken embryo cells with platelet-derived growth factor (PDGF), epidermal growth factor (EGF), or serum

stimulated phosphorylation of two proteins of about 42 kDa on tyrosine (Cooper and Hunter, 1981, 1983). Further studies revealed that these two proteins were structurally similar to each other, and that all mitogens seemed to stimulate the phosphorylation of each protein with the same site specificity (Kohno et al, 1985). The phosphorylation of these proteins contributes to the control of cellular G0-G1 transition. In 1986, Sturgill and Ray detected an insulin-activated protein (Ser/Thr) kinase activity, which was capable of phosphorylating microtubule-associated protein-2 (MAP-2) (Sturgill and Ray, 1986). Partial purification indicated that the MAP-2 kinase is a 35−40 kDa protein (Sturgill and Ray, 1988). Later studies found that this MAP-2 kinase protein was identical to the 42 kDa protein found previously (Rossomando et al, 1989). This realization prompted the redesignation of acronym “MAP” from “microtubule-associated protein” to “mitogen-activated protein". An enormous number of studies since then have featured Mitogen-activated protein kinase (MAPKs) as a Ser/Thr kinase that was activated by insulin and growth factors through tyrosine-specific phosphorylation and controlling many downstream effects (Seger et al, 1995).

1.2.2 MAPK family

The MAPK pathway is one of the most conserved kinase cascades and plays an essential role in connecting cell surface receptors to changes in transcriptional programs. Three major MAPK cascades have been identified in mammalian cells. These include the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-Terminal kinase (JNK), and

p38 kinases (Civelli et al, 1993).

ERK has two major isoforms, ERK1 (p44) and ERK2 (p42). ERK1 and ERK2 are structurally very similar, and are 84% identical at the amino acid level. ERK1 and -2 display the same subcellular localization; both isoforms translocate from the cytosol to the nucleus upon stimulation (Lenormand et al, 1993). However, their relative distributions across tissues differ. Within nervous system, ERK2 is highly expressed in forebrain, while ERKl is expressed more uniformly. Outside of the nervous system, ERK1 is expressed most highly in intestine and placenta and to a lesser extent in the lung. ERK2 mRNA is expressed most highly in muscle, thymus, and heart (Boulton et al, 1991).

The first member of the p38 MAPK family was identified as a 38 kDa protein (p38) that was rapidly phosphorylated on tyrosine in response to lipopolysaccharide (LPS). Until now, four p38 family members have been identified which are encoded by different genes: p38□, p38β (MAPK11), p38γ [SAPK (stress-activated protein kinase) , ERK (extracellular-signal-regulated kinase) 6 or MAPK12] and p38δ (SAPK4 or MAPK13).

The JNKs family have three members; JNK1 (four isoforms), JNK2 (four isoforms), and JNK3 (two isoforms). JNK1 and JNK2 are the products of alternative splicing of a single gene and are expressed in many tissues, but JNK3 is specifically expressed in brain.

1.2.3 Stimulation and Function

ERK1 and ERK2 are preferentially activated in response to growth factors, cytokines and ligands for G protein-coupled receptors. JNK and p38 mediated actions of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, and the actions of stress stimuli. ERK1/2 regulates cell proliferation and cellular differentiation. JNK and p38 are involved in cell differentiation and apoptosis. Many compounds target p38 and/or JNK to attenuate apoptosis induced by stress stimuli (Chang et al 2010). In dopaminergic neurons, p38 mediates the activation of protein 53 (p53) and induces the death of the neuron (Karunakaran et al, 2008).

1.2.4 MAPKs signaling cascade

Each family of MAPKs is composed of a set of three kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (Chang and Karin, 2001). In the ERK1/2 pathway, the series of kinases is from Raf to MAPK/ERK Kinase (MEK) to ERK. Raf is one the MAPKKKs, which are often activated through phosphorylation. C-Raf can be phosphorylated as a result of their interaction with a small GTP-binding protein of the Ras family in response to extracellular stimuli (Zhang et al, 1993; Terai et al, 2005). B-Raf is a second member of Raf family that is primarily activated by the small G-protein Rap (Ohtsuka et al, 1996; Vossler et al, 1997). Raf activation leads to the phosphorylation and activation of the MAPKK MEK1/2, which then stimulates

ERK1/2 activity through dual serine/threonine and tyrosine phosphorylation (Kyriakis et al., 1992; Dent et al., 1992)

1.2.5 G protein regulating ERK1/2 activation

1.2.5 a The dual effect of Gαi/o on ERK

The effect of Gαi/o proteins on MAPK activity is cell-type dependent. In fibroblast HEK293 cells, dopamine treatment at both D2 and D3 receptors increases ERK1/2 phosphorylation (Beom et al, 2004). However, in GH4 cells, a novel pathway of dopamine-D2S receptor induced inhibition of MAPK activation was identified (Banihashemi et al, 2002; Van Ham et al, 2007), that was also observed in lactotrophs (Liu et al., 2002) and striatal neurons (Van Ham et al, 2007). These actions were blocked by pretreatment with pertussis toxin (PTX), which specifically inactivates Gi/Go proteins. This distinct effect of Gαi/o on MAPKs can be explained by its ability to control cAMP-mediated pathways. Gαi/o has an inhibitory effect on AC activity and cAMP formation in all the cells, but cAMP regulates MAPKs activation differently in distinct cells.

Dependent on the cell type, cAMP-PKA can either suppress or induce MAPKs activation. cAMP-mediated MAPK activation is though EPAC (exchange protein directly activated by cAMP). EPAC is a guanine nucleotide exchange factor (GEF), which enhance the GDP to GTP exchange in Rap-1. GTP-bound Rap-1 then activates B-Raf and

the downstream ERK1/2. cAMP-mediated MAPKs inhibition is through the blockade of c-raf. C-raf is the MAPKKK, which activates MEK and ERK1/2. The signaling of c-raf can be blocked by cAMP-dependent phosphorylation by PKA (Häfner et al, 1994). In v-raf-transformed cells, Raf-1 activity was almost completely suppressed after activation of PKA, and this Raf inhibition substantially diminished the activities of ERK (Weissinger et al, 1997).

Gαi-dependent activation of ERK1/2 can be explained by the suppression of cAMP/PKA inhibitory pathway. Receptor-mediated activation of Gi2 led to activation of MAPK and its upstream activator, MAPK/ERK-activating kinase (MEK) in CHO cells (Pace et al, 1995). Using pertussis toxin-insensitive Gi proteins, Quan et al showed that Gi2 is involved in dopamine D2 receptor-mediated ERK activation (Quan et al, 2008). Gαi/o-mediated ERK inhibition is due to the blocking of cAMP-PKA-Epac stimulatory pathway.

Another mechanism involved in Gαi-dependent ERK1/2 activation is through Rap1. Rap1 was a suppressor of Ras and was shown to inhibit Ras-dependent ERK/MAPK activation. Rap1 GTPase activating protein (GAP) isoform rap1GAPII binds specifically to the Gαi of heterotrimeric G-proteins. Stimulation of the Gαi-coupled m2-muscarinic receptor translocates rap1GAPII from the cytosol to the membrane and decreases the amount of GTP-bound Rap1. This translocation of Rap1GAPII to the membrane inhibits Rap1-mediated ras inactivation and enhances the downstream ERK1/2 activation (Mochizuki et al, 1999).

1.2.5 b Gαs-mediated ERK regulation

Three Gαs-mediated pathways of ERK1/2 activation have been identified. One is the cAMP-PKA-Epac pathway. Agonist binding to Gαs-coupled receptors activates AC and promotes the formation of cAMP, which in turn activates EPAC. EPAC then activates Rap-B-Raf-MEK-ERK1/2 pathway (Hepler and Gilman, 1992). The second pathway is also through Epac but is PKA independent (De Rooij et al, 1998, 2000; Rangarajan et al, 2003). Another pathway worth mentioning is an Epac-independent but PKA-dependent pathway. cAMP/PKA induces translocation of the Rap1-GEF C3G (Crk7 SH3 domain Guanine nucleotide exchanger) to the plasma membrane. C3G then activates Rap by converting it from GDP-bound form to GTP-bound active form, leading to activation of the Rap-B-Raf-MEK-ERK1/2 pathway (Wang et al, 2006). However, as described above, Rap can also inhibit ras-mediated c-Raf activation; hence the effect of EPAC/PKA activation on ERK1/2 activity is highly dependent on the ratio of c-Raf to B-Raf.

Like Gαi/o, Gαs also has dual effect on ERK1/2 phosphorylation. It can negatively control ERK1/2 activation via cAMP/PKA-induced inhibition of c-Raf. PKA phosphorylates c-Raf at Ser-259 (Dhillon et al, 2002a, 2002b). Activation of Gαs coupled beta-adrenergic receptors by isoproterenol blocks increases in ERK phosphorylation. This effect can be reproduced by cAMP stimuli, and Raf inhibition is involved in this process (Soltoff and Hedden, 2010).

1.2.5 c Gαq-mediated stimulation of ERK

The prototypical pathway for Gαq-mediated activation of ERK/2 is through the activation of phospholipase C (PLC) (Johnson GL and Dhanasekaran N, 1989). PLC activation results in hydrolysis of phosphatidylinositol (4, 5) bis-phosphate （PIP2) to inositol 1,4,5-trisphosphate（IP3) and 1,2 diacylglycerol (DAG). DAG can activate PKC, which can activate c-Raf directly or indirectly (Kolch, 1993; Ueda, 1996; Schonwasser, 1998). In addition, IP3 mobilizes intracellular calcium stores, signaling to Ca2+-sensitive Ras-GEFs to stimulate the Ras-ERK pathway (Della Rocca, 1997).

1.2.5 d Effect of Gβγ on ERK1/2 phosphorylation

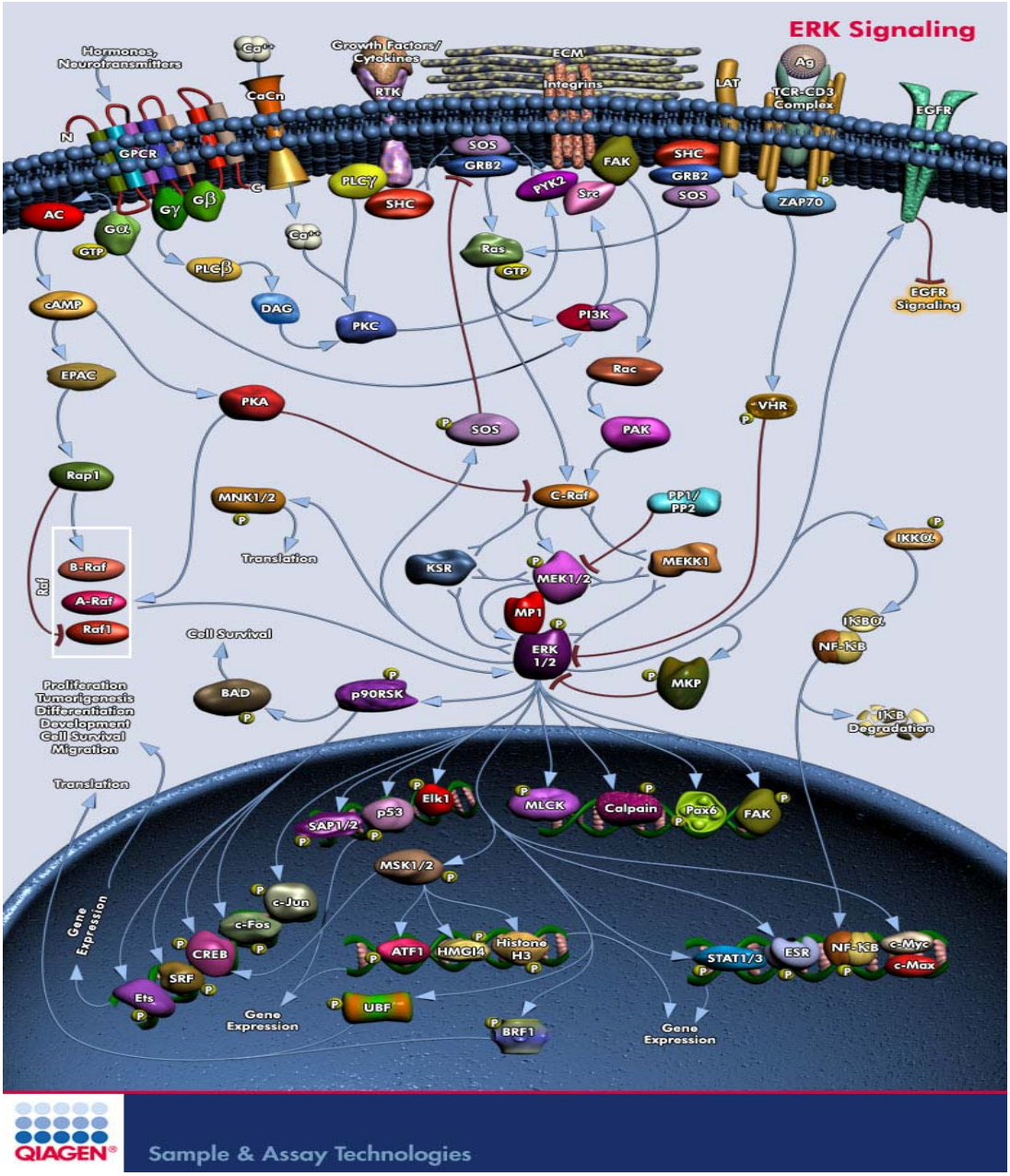
Gβγ-mediated activation of ERK1/2 involves activation of phospholipase-C (PLC) and/or phosphoinositide-3-kinase (PI3K) (Camps *et al.*, 1992; Thomason *et al.*, 1994). In COS7 cells, Gβγ stimulates PLC leading to IP3-mediated increase in intracellular Ca^{2+} and Ca^{2+}-calmodulin-mediated activation of Ras via the Ras-GEF (Luttrell et al, 1996; Della et al, 1997). In other cellular contexts, Gβγ activates ERK1/2 signaling via a pathway involving PI3K and PI3K-mediated activation of a tyrosine kinase. Tyrosine kinase promotes dynamin II-Grb2 complex formation which links the RasGEF SOS to Ras and activates Ras-ERK1/2 pathway (Kranenburg et al., 1999a, 1999b).

1.2.6 Physiological functions of dopamine D2-regulated ERK1/2

1.2.6 a ERK within the VTA regulates responses to stress

Researchers have shown that ERK1/2 is a key modulator of responsiveness to stress

Fig.2 ERK /MAPKs signaling pathway



Network of MAPKs/ ERK signaling pathways in mammalian cells

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in vivo. Acute (1 d) or chronic (4 weeks) exposure to unpredictable stress increases phosphorylation of ERK1/2 and of two downstream targets (ribosomal S6 kinase and mitogen- and stress-activated protein kinase-1) within the ventral tegmental area (VTA). Blocking ERK2 activity in the VTA produces stress-resistant behavioral responses, and this effects induced by ERK2 blockade were accompanied by decreases in the firing frequency of VTA dopamine neurons (Sergio et al, 2010).

1.2.6 b D2 receptor regulates Parkinson' disease via ERK1/2

Parkinson's disease (PD) is a progressive movement disorder that affects about 1 million people in North America. The major motor symptoms are due to the degeneration of midbrain dopamine neurons of the nigrostriatal projection. Mitochondrial dysregulation is reported in Parkinson's and related Parkinsonian disorders. ERK1/2 activation plays an important role in regulating mitochondrial function in PD. In human PD brain, there are significant increases of phospho-ERK1/2 (p-ERK1/2) in the cytoplasm and mitochondria of midbrain dopaminergic neurons (Zhu et al, 2002). In the 6-OHDA dopaminergic lesion model, inhibition of ERK1/2 activation protects neurons from mitochondrial ROS production during the 6-OHDA treatment and rescues them from neuronal injury (Kulich et al, 2007).

1.2.6 c Dopaminergic-ERK1/2 system in schizophrenia

In 1999, researchers first reported ERK1/2 elevation in cerebellar vermis of patients with schizophrenia (Kyosseva et al, 1999). In this study, a significant increase of total

ERK2 level was shown in schizophrenia patients as compared to control, while ERK1 showed a non-significant tendency in the same direction. JNK and p38 remained unchanged. Also in this study, the authors showed that antipsychotic drug did not alter the expression of ERK. Later studies revealed that the atypical antipsychotic drug clozapine and the typical antipsychotic haloperidol acutely decreased cortical pERK1 activation in primary murine prefrontal cortical neurons, but only clozapine stimulated pERK1 and pERK2 with chronic drug exposure via EGF receptor (Pereira et al, 2009). Thus, altered regulation of ERK1/2 levels may have some role of schizophrenia or response to atypical antipsychotics.

1.3 RASA3

1.3.1 RasGap activity of RASA3

Ras GTPase-activating protein 3 (RASA3) has many other names, such as RAS p21 protein activator 3, GAP1^{IP4BP} and GAPIII. It is a RasGap protein that is encoded by the *RASA3* gene. RASA3 is a negative regulator of Ras. Ras cycles between active GTP-bound and inactive GDP-bound states, and it recognizes different effectors when in the active state. Active GTP-bound Ras interacts with Raf kinase and activates MEK-ERK cascade. Ras activation is influenced by the different effect of guanine nucleotide-exchange factors (Ras-GEF) and GTPase-activating proteins (Ras-GAP). Ras-GEFs control Ras activation by stimulating GDP for GTP exchange, while

Ras-GAPs control inactivation by enhancing the intrinsic GTPase activity of Ras. As a member of the RasGAP family, RASA3 promotes GTP hydrolysis, and hence inactivation of Ras (Walker et al, 2002). Mast cells with dominant inhibition of RASA3 showed potentiation of Ras and ERK response (Stokes et al, 2006).

1.3.2 Distribution

RASA3 expression is wildly spread among species and body parts. The RASA3 mRNA was expressed at highest levels in the brain, where its expression increased with development. Lower levels of the mRNA were expressed in the spleen, skeleton muscles and peripheral blood leukocytes. In the brain, immunoreactivity for RASA3 is highest in the CA1 of the hippocampus, amygdala, cerebellum, and pyriform cortex (Signore et al, 1999). Subcellular RASA3 is located to the plasma membrane both endogenously in RBL-2H3 cells and under conditions of over-expression in COS7 cells (Lockyeret al, 1999). In brain tissue, RASA3 mRNA is expressed in neurons and oligodendrocytes, but not in astrocytes (Baba et al, 1995; McNulty TJ, 2001).

1.3.3 Structure

RASA3 is a 834-amino acid protein with a predicted molecular weight of 96 kDa. It contains three structural domains, an N-terminal tandem C2 domain, a RasGAP domain, and a C-terminal pleckstrin homology/Bruton's tyrosine kinase (PH/Btk) domain. The

C2 domain is a calcium-dependent phospholipid binding domain, and RasGAP domain contains the catalytic activity required to enhance the intrinsic GTPase activity of Ras. PH/BTK domain is the binding domain for phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 3,4,5-trisphosphate (PIP3) and inositol (1,3,4,5) tetrakisphosphate (IP4), which is required for the constitutive plasma membrane association of the RASA3 (Cullen et al, 1995, Cozier et al, 2000). Within PH/BTK domain, PIP2 and PIP3 binding site overlaps with the IP_4-binding site. One model argues that binding of IP_4 to the PH domain serves to remove the inhibitory influence of PIP_2, thereby allowing activation of the Ras-GAP activity (Cozier et al, 2000).

1.3.4 RASA3 and G alpha interaction

The previous work in our lab identified RASA3 as a novel Gαi3 target in the two-hybrid screen with constitutively active Gαi3-QL mutant. The interaction between RASA3 and activated Gαi proteins was validated by several different approaches, including yeast mating assay, *in vitro* pull down assay and immunoprecipitation, which suggest that RASA3 can directly interact with both wild type Gαi3 and Gαi2 protein and preferentially interacted with constitutively-active Gαi3 or Gαi2. Interestingly, despite the evidence of preferential interaction of RASA3 with activated Gαi3, we did not observe an increase in endogenous RASA3-Gαi3 interaction upon stimulation with D2 agonist apomorphine. However, treatment with Thyrotropin-releasing hormone (TRH) and apomorphine together induced a robust increase in RASA3-Gαi3 interaction, while

TRH alone did not affect RASA3-Gαi3 interaction. Functional ERK1/2 phosphorylation assay showed that antisense knockdown of RASA3 in GH4 cells put a brake on D2S-mediated ERK1/2 inhibition (Nafisi et al., 2008).

1.3.5 RASA3 in D2-mediated ERK1/2 signaling

The interaction between Gαi and RASA3 suggests a role of RASA3 in G-protein coupled receptor signal and its control over MAPK pathways. Dopamine D2S receptor regulates many physiological events via MAPKs, such as the expression of rate limiting enzyme for dopamine synthesis, tyrosine hydroxylase (TH). MAPK is known to activate the dopaminergic transcription factor Nurr1. Nurr1 is an orphan nuclear receptor that heterodimerizes with RXR to regulate transcription of TH (Kim et al, 2006). A group of researchers showed *in vitro* Nurr1 is phosphorylated by ERK2 on S126 and T132 and its phosphorylation upregulates tyrosine hydroxylase expression in SH-SY5Y cells (Zhang et al, 2007). Jacobsen et al. identified a Parkinson's disease-related Ser125Cys NURR1 mutation, which blocks dopamine-D2 agonist quinpirole stimulated ERK1/2 phosphorylation and transcriptional activation of TH promoter, in SK-N-AS cells (Jacobsen et al, 2008).

The widespread distribution of RASA3 and Gαi proteins suggests an important role for this coupling in a variety of physiological processes. This complex could affect the production of dopamine via ERK pathway and Nurr1. However, the inhibitory effect of this D2S-Gαi3-RASA3 pathway on ERK1/2 activation still needs to be validated. Future

experiments should be performed to confirm the initial results obtained in GH4 cells.

1.4 Hypothesis

We hypothesize RASA3 is the key candidate that link D2S/ Gαi3 to Ras-Raf-MEK-ERK1/2 in pituitary GH4ZR7 cells. Depletion of RASA3 will suppress the inhibitory effect of D2S on TRH-induced ERK1/2 activation.

Given the fact that RASA3 can directly interact with Gαi2 and Gαi3, we hypothesize that overexpressing RASA3 and Gαi will inhibit D2S-induced ERK1/2 in COS7 cells.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials—Apomorphine, puromycin, TRH, anti-β-actin, anti-FLAG antibody were from Sigma-Aldrich; polyvinylidene difluoride membrane was from PerkinElmer Life Sciences; enhanced chemiluminescence detection kits were from Roche Applied Sciences; sera and media were obtained from Wisent, Inc. Endonucleases were purchased from New England Biolabs, Inc; anti-phospho-p42/44 ERK1/2, anti-p42/44 ERK1/2, anti-myc and anti-rabbit IgG, horseradish peroxidase-linked antibody were from Cell Signaling Technology, Inc; peroxidase-conjugated AffiniPure goat anti-mouse IgG antibody was obtained from Jackson ImmunoResearch. Lipofectamine was purchased from Invitrogen. Coomassie (Bradford) protein assay kit was obtained from Pierce.

2.2 Methods

2.2.1 Cell culture and transient transfection

COS7 green monkey epithelial cells and HEK293 human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium (DMEM)(Invitrogen) supplemented with 10% fetal bovine serum (FBS)(Invitrogen) in a humidified 5% CO_2 atmosphere at 37°C. GH4ZR7 cells were grown in HAM's F-10 medium (Invitrogen) with 10% FBS. Twenty-four hours before transfection, the cells were plated in 6-well plates at density of $3x10^5$ cells/well to obtain 50% confluence at the time of transfection. HEK293 cells were transfected using the calcium phosphate co-precipitation method. COS7 cells and GH4 cells were transfected with the lipidophilic transfection reagent lipofectamine 2000 (Invitrogen).

2.2.2 Stable transfection

GH4ZR7 cells were co-transfected with plasmids for myc-tagged dominant negative RASA3 (5 μg) and pGKpuro (0.5 μg) per 10 mm dish using Lipofectamine 2000 (Invitrogen). The transfected cells were selected using 20 μg/ml puromycin-containing media for two weeks. Isolated puromycin-resistant clones were picked and transfered to new plates. These selected clones were then tested for expression of the corresponding RASA3 protein by Western blot using anti-myc antibody (1/1000 dilution).

2.2.3 Western Blot

The cells were lysed using lysis buffer containing equal volumes of 2.5x SDS loading and RIPA lysis buffers (50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl) and sonicated for 5 sec. The samples were then centrifuged at 14,000 rpm for 5 min at 4°C to obtain a clear cell lysate. The proteins were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked at room temperature for 1 hr in 5% skimmed milk in TBS buffer. The blots were probed with various antibodies (1/1000 dilution) overnight, washed three times with cold TBS with tween-20 (TBST), probed with secondary antibodies for 1 hr at room temperature and washed another three times with TBST before the blots were subjected to chemiluminescent detection.

2.2.4 MAPK assay

Cells ($3x10^5$ cells/well) plated in six-well plates were transfected 24 hrs later with the indicated plasmids using lipofectamine 2000. Phospho-ERK1/2 was measured 48 hrs after transfection using the following protocol: Cells were placed in serum-free DMEM medium for 1 h at 37 °C and treated with apomorphine (1 μM) for 6 min at 37°C. After drug treatment, the plates were transferred on ice and washed two times with cold PBS. The cells were lysed in 120 μl of lysis buffer (see above), stored on ice, sonicated for 6 sec, and centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant (30 μl) was boiled for 5 min and rapidly cooled on ice. Samples were separated by SDS-PAGE, and subjected to Western blot analysis. Phosphorylation was detected using (1:1000) antiphospho-p42/44 ERK1/2; parallel blots were probed for total ERK1/2 (1:1000). The corresponding bands for ERK1 and ERK2 were digitally quantified using Adobe Photoshop. The results were normalized to the Total ERK1/2 control.

2.2.5 Design of shRNA

The RASA3 shRNA is designed based on the siRNA (GAAGUAUGCAGAUGCCGU). In general, the DNA oligonucleotides consist of the 19-nucleotide sense siRNA sequence followed by a short spacer (TTCAAGAGA), then the antisense strand of the siRNA sequence. 5 T's are added to the 3' end of the oligonucleotide as a pol III transcriptional termination signal. BamHI and HindIII restriction enzyme sites are added to the 5' and 3' end in order to insert the oligonucleotides into the pSilencer 3.0 vector. The resulting RNA transcript is expected to fold back and form a stem-loop structure. The oligonucleotides were annealed and then

ligated into psilencer 3.0 vector which contains the H1 promoter. The pSilencer sh-RASA3 efficiency to inhibit Flag-RASA3 expression was tested in COS7 cells.

2.2.6 Data analysis

All the western blot data were quantified by measuring the band intensity of digitally scanned images by densitometry analysis using Photoshop software. Results were analyzed using the one-way analysis of variance (ANOVA) followed by Dunett post hoc test (multiple comparison tests) to assess statistical significance. The difference was considered statistically significant with a p value either less than 0.05. Figures were generated and analyzed statistically using GraphPad Prism 3.0 software.

CHAPTER III

RESULTS

3.1. RASA3 is required in dopamine D2 inhibition of TRH-induced ERK1/2 activation in GH4ZR7 cells

3.1.1 Inhibitory effect of D2S on TRH-induced ERK1/2 activation is suppressed in GH4ZR7 stably transfected dominant negative RASA3

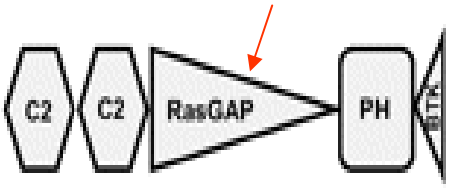
Previous studies from our laboratory have shown that Thyrotropin-releasing hormone (TRH) resulted in a significant increase in ERK1/2 phosphorylation in GH4ZR7 cells, whereas the dopamine receptor agonist apomorphine completely suppressed TRH-induced ERK1/2 phosphorylation (Banihashemi and Albert, 2002). Given the fact that GH4ZR7 cells are rat pituitary GH4 cells stably transfected with the dopamine-D2S receptor cDNA, the previous results suggested a D2S-mediated inhibition of TRH-induced ERK1/2 phosphorylation.

The present study addresses whether the endogenous RASA3 in GH4ZR7 cells contributes to this D2S-induced inhibition of ERK1/2 activation. To inhibit endogenous RASA3, GH4ZR7 cells were stably transfected with a dominant negative myc-tagged RASA3 construct. The dominant negative RASA3 (dnRASA3) construct contains a Leucine to Alanine substitution at position 481, which inhibits its RasGAP activity (Fig 3A)(Walker et al., 2002). Stable transfectant clones were screened for expression of dnRASA3 by Western blot with the anti-myc antibody, and two myc-dnRASA3 expressing clones were identified: DN5 and DN4 (Fig 3B). We performed experiments on both normal GH4ZR7 and DN5 cells to check the change in ERK1/2 phosphorylation.

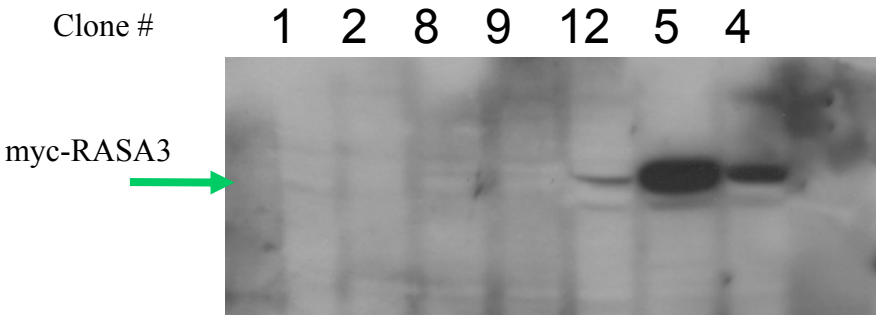
Fig 3. Suppression of dopamine D2S-induced ERK1/2 inhibition by dominant negative RASA3.

A. Schematic representation of the functional domains of RASA3. Dominant negative RASA3 (dnRASA3) has an a Leucine to Alanine substitution in the RasGap domain, which abrogates the RasGap function of RASA3. **B**. Screen of RASA3 GH4ZR7 clones stably transfected with dominant negative RASA3**.** GH4ZR7 cells were stably transfected with pcDNA encoding myc-tagged dominant negative RASA3 and puromycin-resistance gene, and stable clones were selected by puromycin selection and screened for myc-tag, as detected by Western blot using anti-myc antibody (1/1000). **C**. Suppression of dopamine D2S-induced ERK1/2 inhibition in DN5 stable clone. Parental GH4ZR7 or DN5 cells were pretreated or not with 1 μM apomorphine (Apo) for 15 min followed by the addition of 1 μM TRH or control for 6 min, as indicated; duplicate samples were done for TRH/Apo. Western blot analysis was done using specific antibody anti-phospho-p42/44MAPK (1/1000). The membranes were striped and reprobed with anti-p42/44MAPK antibody (1/1000) as a loading control.

A.

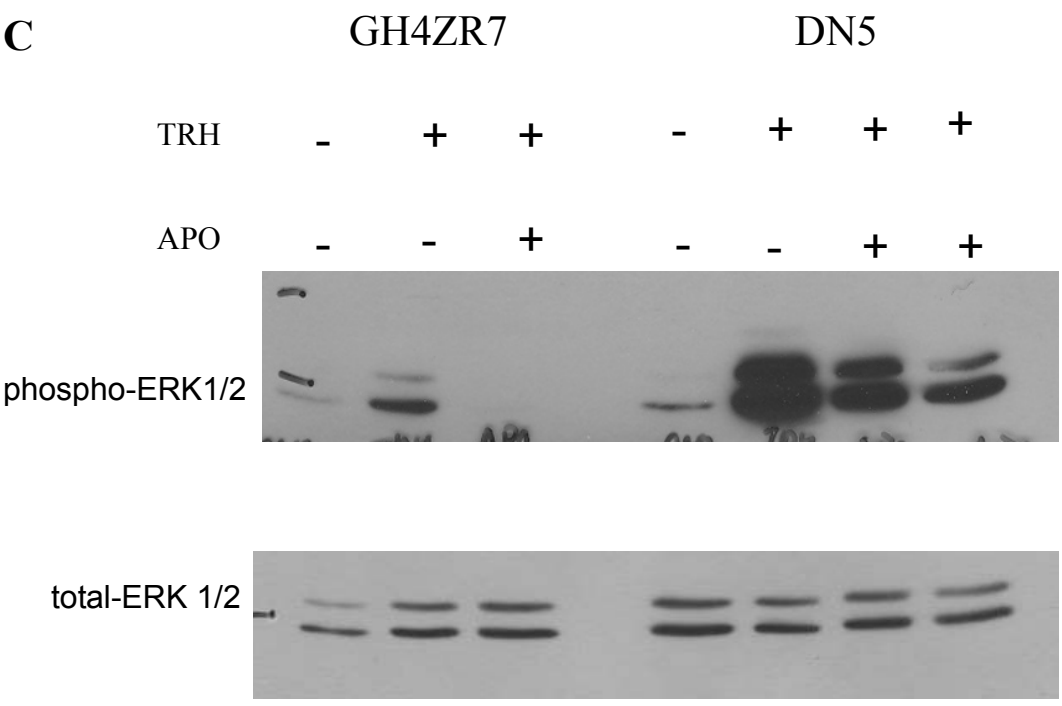

DN: Leucin 481 Alanine
C2
C2
RasGAP
PH

B.


Clone #
1 2 8 9 12 5 4
myc-RASA3

.

C


GH4ZR7
DN5
TRH
- + + - + + +
APO
- - + - - + +
phospho-ERK1/2
total-ERK 1/2

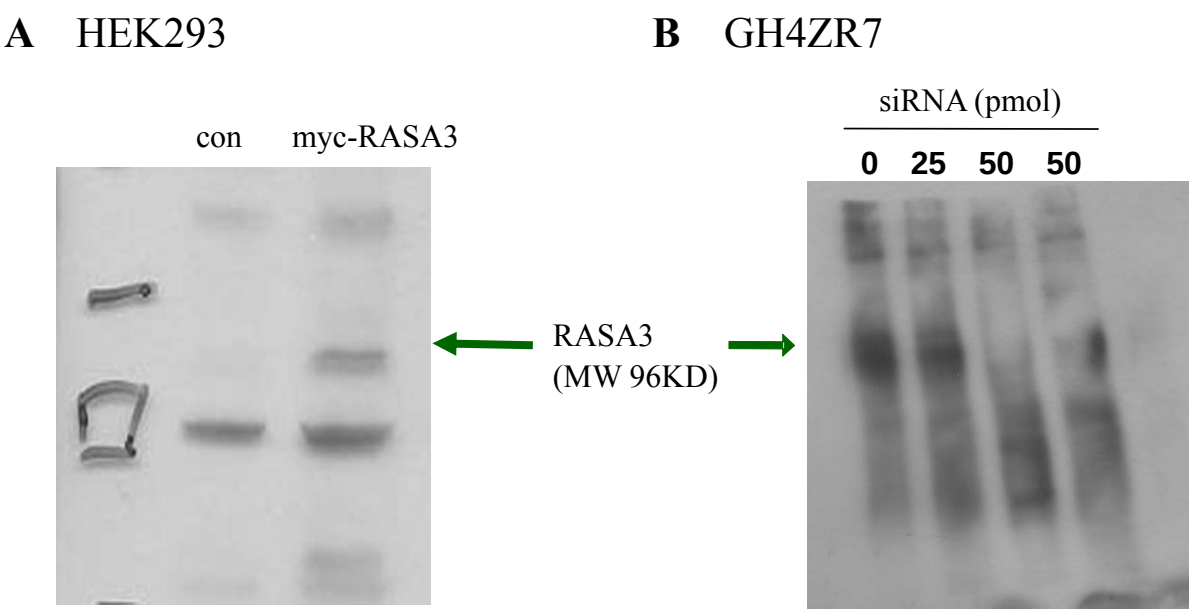
As shown in Fig 3C, treatment with TRH alone activated pERK1/2, but when TRH and dopamine agonist apomorphine were added together, the ERK1/2 activation was blocked in parental GH4ZR7 cells. In the DN5 stable cell line, TRH-induced ERK1/2 phosphorylation was greater than in GH4ZR7 cells, and adding apomorphine with TRH did not block this ERK1/2 activation. This result suggests that RASA3 RasGAP activity is required for dopamine-induced inhibition of ERK1/2 activation.

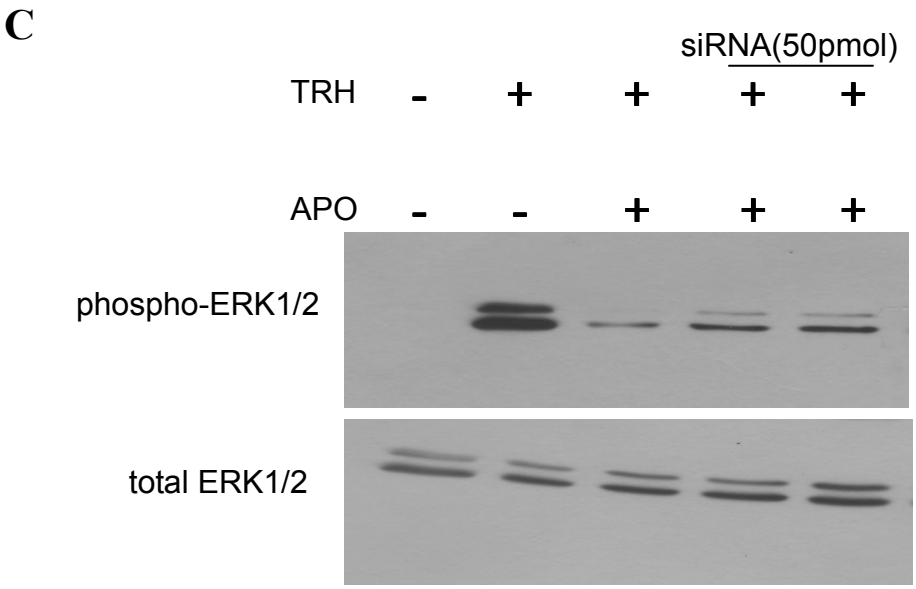
3.1.2 Inhibitory effect of D2S on TRH-induced ERK1/2 activation is reduced in GH4ZR7 cells treated with RASA3 siRNA.

To verify the role of endogenous RASA3 in D2S-induced inhibition of ERK1/2 activation in GH4 cells, siRNA knockdown of RASA3 was also performed in GH4ZR7 cells. Figure 4 shows that siRNA (5'-UACGGCAUCUGCAUACUUC-3') to rat RASA3 was effective to reduce endogenous RASA3 levels in GH4ZR7 cells at a concentration of 50 pmol/ml. The level of endogenous RASA3 protein was revealed using a chicken antibody raised previously against a 13-aa peptide (QYRRNKFKKTRYG) of human RASA3. The specificity of this antibody was verified by detecting myc-RASA3 in transfected HEK293 cells compared to non-transfected cells in which RASA3 was not detected (Fig 4A). Three days after siRNA transfection of GH4ZR7 cells, Western blot for pERK1/2 showed that upon depletion of RASA3 using RASA3-siRNA but not scrambled control siRNA, D2S signaling to inhibit TRH-induced ERK1/2 activation was partially inhibited. This result further supports our hypothesis that RASA3 is a key effector that mediates D2S receptor coupling to inhibition of MAPK. The partial

Fig. 4 RASA3 siRNA reduces D2S-induced inhibition of ERK1/2

A. Specificity of anti-RASA3 antibody**.** Anti-RASA3 antibody (1:10,000) was used to detect RASA3 protein in Western blot of nontransfected (lane 1) vs. myc-RASA3 transfected (lane 2) HEK293 cell extracts. A single specific RASA3 band of 90 kDa was detected in the myc-RASA3 cells. **B.** Depletion of RASA3 in GH4ZR7 by RASA3 siRNA**.** GH4ZR7 cells were transfected with increasing amounts of RASA3 siRNA using lipofectamine 2000. The expression of endogenous RASA3 protein (arrow, 90 kDa) was detected by Western blot using anti-peptide RASA3 antibody (1:10,000). **C.** siRNA knockdown of RASA3 reduces D2S-induced ERK1/2 inhibition in GH4ZR7 cells. Cells were non-transfected or transfected with 50 pmol siRNA three days before assay. Cells were treated with TRH and/or apomorphine for 6 min., and measurement of phospho-ERK1/2 and total ERK1/2 (as loading control) was conducted as described in Fig 1.


A HEK293
B GH4ZR7
con
myc-RASA3
siRNA (pmol)
0 25 50 50
RASA3
(MW 96KD)


C
siRNA(50pmol)
TRH - + + + +
APO - - + + +
phospho-ERK1/2
total ERK1/2

D2S-induced inhibition of ERK1/2 activation in RASA3-siRNA treated cells may be due to residual RASA3.

3.2. RASA3 and Gαi inhibit D2S-induced MAPKs activation in fibroblast COS7 cells.

3.2.1 Gαi2 and RASA3 mediate the inhibition of D2S-induced MAPKs.

It has been reported that the D2S receptor regulates the ERK1/2 differently in fibroblast cells and endocrine cells (Albert and Robillard, 2002). Opposite to its inhibitory effect on ERK1/2 activation in endocrine cells like GH4ZR7 cells, apomorphine-induced D2S activation stimulates the phosphorylation of ERK1/2 in fibroblast cells (Ghahremani et al., 2000). In endocrine GH4ZR7 cells, I have successfully shown that RASA3 is the key effector in D2S inhibition of ERK1/2 activation. However, it remained unclear whether RASA3's inhibitory effect could be reconstituted in fibroblast cells. In this study, I tested how RASA3 affects D2S-mediated ERK1/2 activation in COS7 cells. Because of the interaction between RASA3 and G alpha subunits, we also tested whether co-expression of G alpha proteins was needed to enhance RASA3 function. To achieve these goals, we transfected the D2S receptor plasmid in COS7 cells, together with constructs for wild-type or active mutants of Gαi and/or RASA3, and measured the level of phospho-ERK1/2 after apomorphine treatment. COS7 cells transfected with empty vector, pcDNA3, were used as control.

We first checked whether Gαi2 and RASA3 affect D2-induced ERK activation (Fig 5). Compared with control COS7 cells, the level of phospho-ERK1/2 was increased significantly by apomorphine in cells expressing D2S receptors, while D2S-expressing cells not treated with apomorphine were not different from control (data not shown). Cotransfection of RASA3 did not affect D2S-induced enhancement of phospho-ERK1/2. Cotransfection of wild-type Gαi2 (Gαi2), constitutively active Gαi2 (Gαi2QL), or Gαi2 plus RASA3 slightly lowered ERK1/2 phosphorylation, but this was not statistically significant. However, when D2S receptor, Gαi2QL and RASA3 were cotransfected, apomorphine treatment failed to increase phospho-ERK1/2 levels, suggesting that the active form of Gαi2 and RASA3 work together to inhibit D2S-induced ERK1/2 phosphorylation.

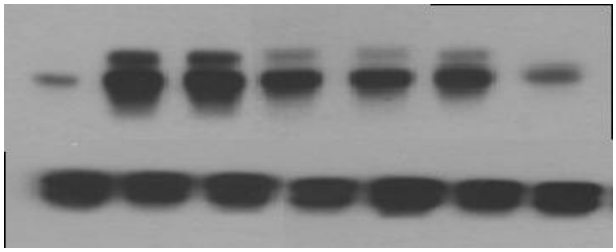
To further clarify the role of Gαi2, we conducted additional studies of the effect of RASA3 and Gαi2 on D2S-induced ERK1/2 phosphorylation (Fig. 6). Two RASA3 mutants were tested: dominant negative RASA3 (dnRASA3) and constitutively active RASA3 (caRASA3). Activation of D2S by apomorphine enhanced ERK1/2 phosphorylation. When compared with control, the three Gαi2QL/RASA3 (wild-type or mutant) groups do not exhibit significant increased phospho-ERK1/2 level. When compared with D2S group, only Gαi2QL/RASA3 and Gαi2QL/caRASA3 mutants groups show a significant reduction of D2S-induced ERK activation, whereas Gαi2QL/dnRASA3 did not show a significant difference from the D2S group. Taken together, our results suggest that RASA3 is capable to inhibit D2S-induced ERK activation in COS7 cells, but this inhibition requires the active form of Gαi2. Although

Fig. 5 Effect of Gαi2-RASA3 on D2S-induced ERK1/2 activation in COS7 cells.

COS7 cells were transiently transfected with plasmid DNA encoding D2S receptor, together with plasmid DNA encoding RASA3, wild-type Gαi2 (Gαi2) or constitutively active Gαi2 (Gαi2QL). Serum-starved cells were incubated for 6 min with apomorphine (1 μM) and phosphorylated ERK1/2 was detected by immunoblotting with anti-phospho-p42/44. Membrane were striped and reprobed with anti-p42/44 ERK1/2. **A** is shown a representative Western blot. **B** is the quantification of phospho-ERK1/2 level normalized to total ERK1/2. One-way analysis of variance (ANOVA) with Dunnett's post-test was used for the statistical comparison between control and transfected groups, N = 3-6. *$p < 0.05$ compared with the control group.

A

D2S	-	+	+	+	+	+	+
RASA3	-	-	+	-	-	+	+
Gαi2	-	-	-	+	-	+	-
Gαi2QL	-	-	-	-	+	-	+



phospho- ERK1/2

total ERK1/2

B

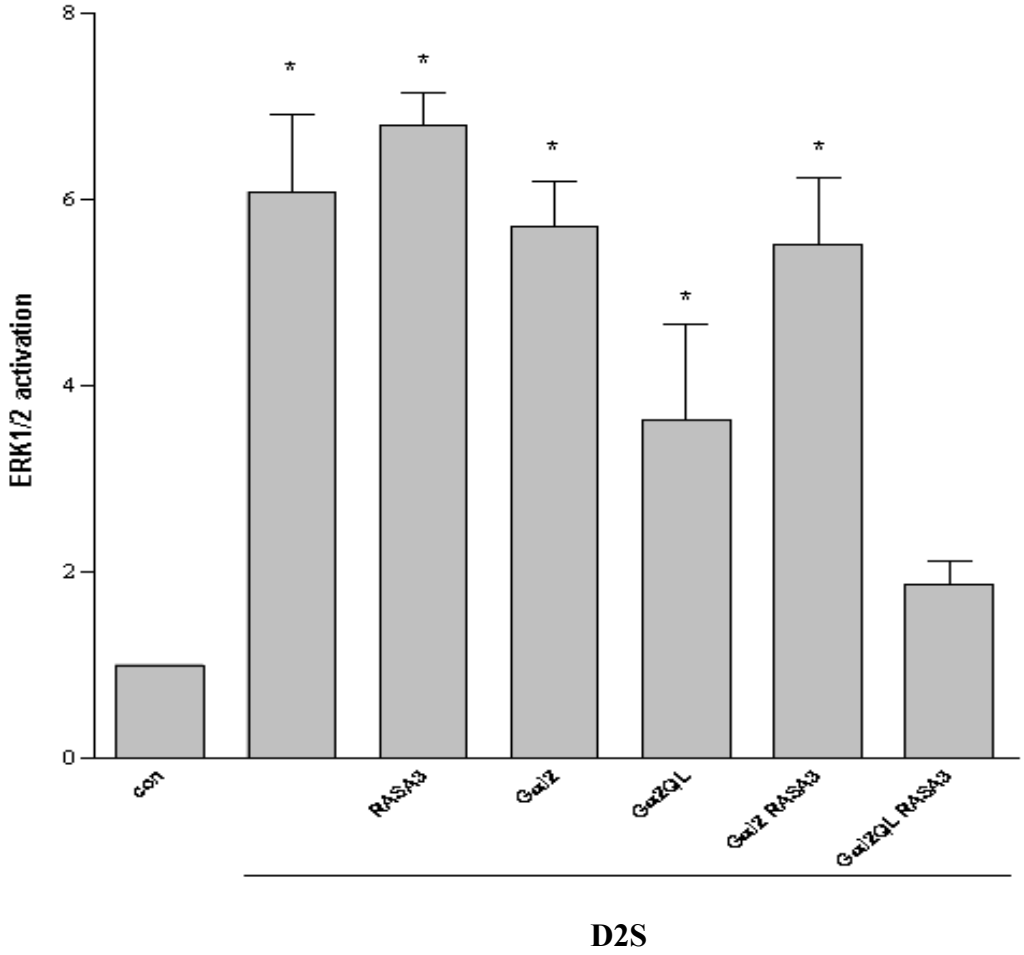
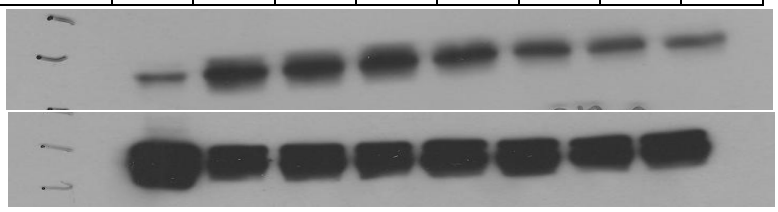


Fig.6 Summary of effects of RASA and Gαi2 on D2S-induced ERK1/2 activation.

COS7 cells were not transfected (control) or transfected with D2S receptor and RASA3 (wild type RASA3, dominant negative dnRASA3, or constitutively active caRASA3) and Gαi2 (wild type Gαi2 or constitutively active Gαi2QL). ERK1/2 phosphorylation after 1 μM apomorphine treatment (except control) was measured as described in Fig 3. **A** is shown a representative Western blot; **B** is summary of the phospho-ERK1/2 level from multiple experiments. Phospho-ERK1/2 levels from each set of experiments were normalized to total ERK1/2 and expressed as –fold control. All data were then compiled and subjected to Tukey's test. *, $p < 0.05$ compared with the control group. **, $p < 0.05$ compared with the D2S group.

A

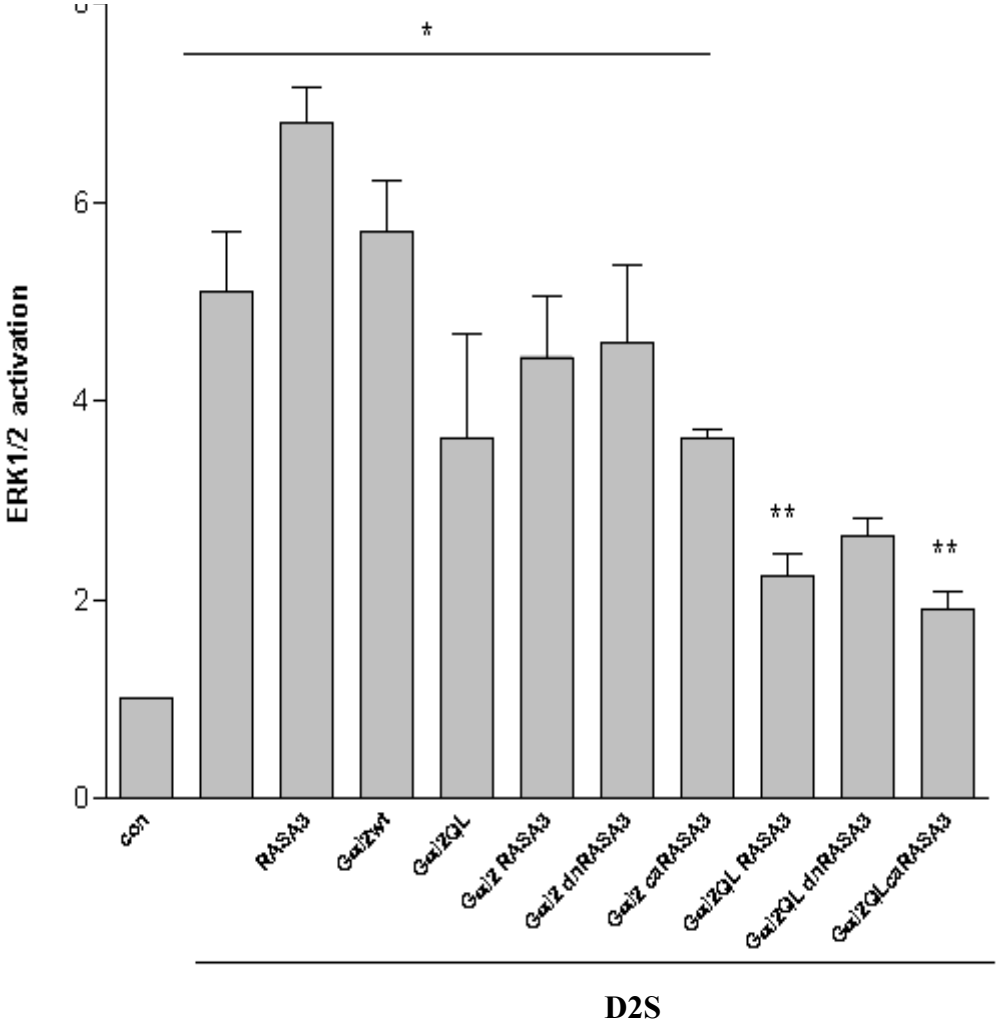
D2S	-	+	+	+	+	+	+	+
RASA3	-	-	+	-	-	+	-	-
dnRASA3	-	-	-	+	-	-	+	-
caRASA3	-	-	-	-	+	-	-	+
Gαi2wt	-	-	+	+	+	-	-	-
Gαi2QL	-	-	-	-	-	+	+	+



phospho- ERK1/2

total- ERK1/2

B



n=3-6

Gαi2 or Gαi2QL alone did not significantly reduce D2S-induced ERK1/2, the combination of Gαi2QL with caRASA3 or wild type RASA3 showed significant inhibition, while no additional effect was seen with dnRASA3. The Gαi2QL/caRASA3 group showed an even lower level of phosphor-ERK1/2 than Gαi2QL/RASA3 group. This means that the combination of active Gαi2 and active RASA3 leads to greatest inhibition of ERK1/2.

3.2.2 Gαi3 and RASA3 mediate the inhibition of D2S-induced MAPKs.

Besides Gαi2, the previous protein interaction experiments showed that Gαi3, especially activated Gαi3, interacts with activated RASA3 (Nafisi et al, 2008). Therefore, I also checked whether RASA3 can work with Gαi3 to affect D2S-induced phospho-ERK1/2 levels in COS7 cells. Both wild-type Gαi3 (Gαi3) and constitutively active Gαi3 (Gαi3QL) were cotransfected with wild type RASA3, dnRASA3 or caRASA3 (Fig 7). The results are similar those obtained with Gαi2: With Gαi3QL, RASA3, dnRASA3 and caRASA3 all reduced D2S-induced ERK1/2 activation. However, with wild-type Gαi3 only caRASA3 significantly reduced D2S-induced ERK1/2 stimulation. Interestingly, while caRASA3/Gαi3 reduced ERK1/2 activation, caRASA3/Gαi2 did not, suggesting that RASA3/Gαi3 may have a stronger inhibitory effect on D2S-induced ERK1/2 activation.

3.2.3 Basal phospho-ERK1/2 is not affected by RASA3 and Gai2

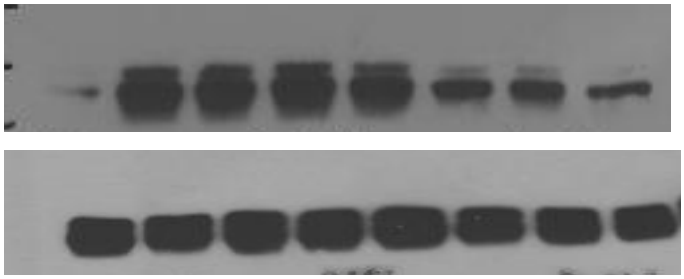
As a control for the inhibitory effect of RASA and Gαi on D2S-induced ERK1/2,

Fig. 7 Effect of Gαi3 and RASA3 on D2S-induced ERK1/2 activation.

Gαi3 (wild-type or constitutively active Gαi3QL) was co-transfected with D2S and RASA3 or mutants as indicated into COS7 cells. Cells were untreated (control) or treated with 1 μM apomorphine (D2S) and ERK1/2 phosphorylation determined by Western blot as described above in Fig 3. Above is shown a representative Western blot; below is shown quantification of phospho-ERK1/2 normalized to total ERK1/2. One-way analysis of variance (ANOVA) was used for the statistical comparison between control and transfected groups (N=3). *$p < 0.05$ compared with the control group. **, $p < 0.05$, compared with D2S except for D2/RASA3 transfected cells (n=2).

A

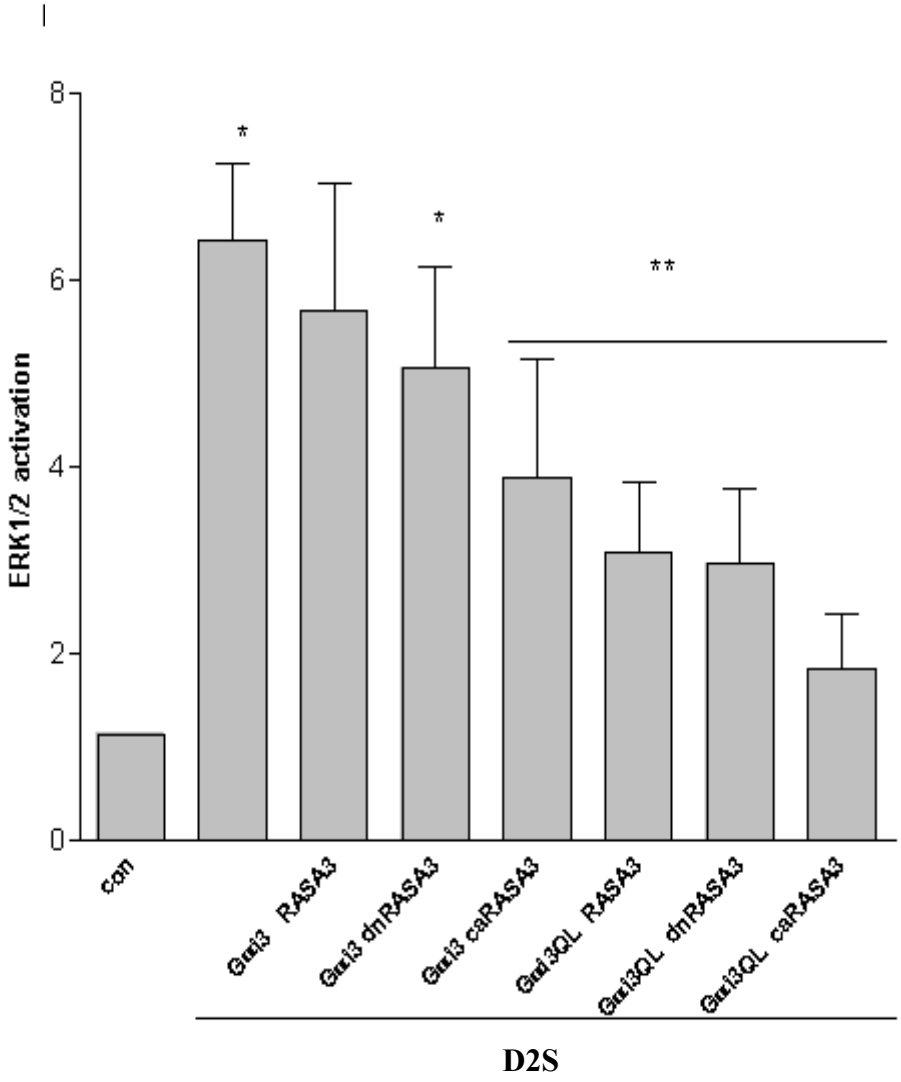
D2S		+	+	+	+	+	+	+
RASA3			+			+		
dnRASA3				+			+	
caRASA3					+			+
Gαi3wt			+	+	+			
Gαi3QL						+	+	+



phospho-ERK1/2

total ERK1/2

B



their effect on basal ERK1/2 activity was examined in COS7 cells. In Fig 8, basal phospho-ERK1/2 was measured 48 hrs after transfection, the same duration as for D2S-induced ERK phosphorylation experiments. The results showed no striking changes in the basal ERK1/2 phosphorylation. Thus the effect of Gαi2/RASA3 was due to inhibition of D2S-induced ERK1/2 phosphorylation, and not of basal ERK1/2 phosphorylation.

3.3. RASA3 shRNA-mediated knockdown of RASA3

In GH4ZR7 cells, the efficiency of RASA3 siRNA has been shown and siRNA successfully reduced the inhibitory of D2 on TRH-induced MAPKs activation. However, siRNAs are generally rapidly degraded by serum RNases, and hence the block of D2S inhibition obtained was only partial. Furthermore, SiRNAs are negatively charged and therefore do not easily penetrate hydrophobic cellular membranes. In contrast, intracellularly expressed short hairpin RNAs can mediate long-term knock-down of target RNA transcripts. Moreover, delivery of shRNAs into target cells via lentiviral vectors is very efficient, and is commonly used in regulation of target gene *in vivo*.

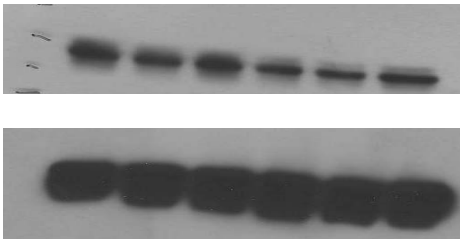
In order to develop a high efficiency RASA3-shRNA lentivirus, we constructed an shRNA plasmid based on the siRNA sequence tested above, which was inserted into the pSilencer3.0 vector that contains the histone-H1 promoter to drive shRNA transcription. In COS7 cells transfected with Flag-RASA3, the efficacy of the pSilencer-RASA3 construct to inhibit Flag-RASA3 protein levels was detected by Western blot for the Flag

Fig. 8 Effect of Gαi2 / Gαi3 and RASA3 on basal phospho-ERK1/2.

A. Effect of Gαi2-RASA3 on basal ERK phosphorylation. COS7 cells were co-transfected with RASA3 with or without Gαi2 or Gαi2QL, as indicated. Cells were collected 48 hrs after transfection, prepared for Western blot, and phospho-ERK1/2 level was detected with anti-phospho-p42/44MAPK antibody. Membranes were stripped and reprobed with anti-p42/44 ERK1/2. **B**. Effect of Gαi3-RASA3 on basal ERK phosphorylation. Gαi3 or Gαi3QL and RASA3 were co-transfected into COS7 cells and phospho-ERK1/2 was measured as described above.

A

RASA3		+			+	+
Gαi2wt			+		+	
Gαi2QL				+		+

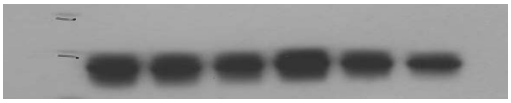


phospho- ERK1/2

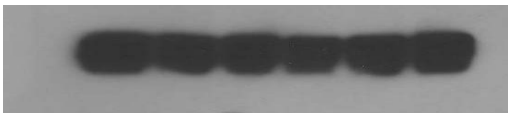
total ERK1/2

B

RASA3		+			+	+
Gαi3wt			+		+	
Gαi3QL				+		+



phospho- ERK1/2



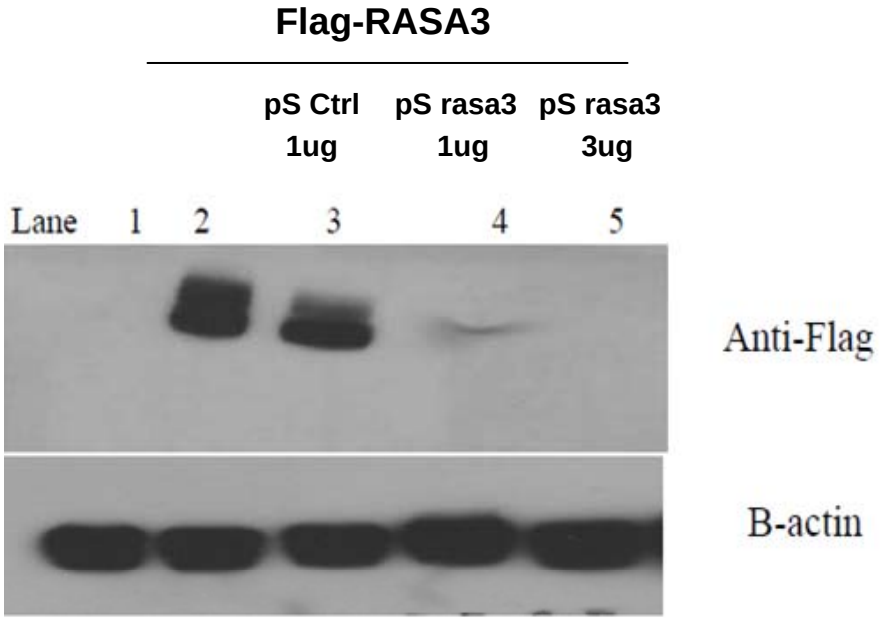
total ERK1/2

epitope (Fig 9). Transfection of either 1ug and 3 ug of pSilencer RASA3 effectively blocked the expression of Flag-RASA3, while control pSilencer shRNA had no effect on Flag-RASA3 expression.

This result verified RASA3 shRNA-mediated target gene knockdown. The ongoing project now is to ligate the H1 promoter-shRASA3 fragment into the lentiviral vector and produce lentivirus to completely knockdown the endogenous RASA3 in GH4ZR7 cells and neurons.

Fig. 9 shRNA knockdown of Flag-RASA3 expression in COS7 cells.

COS7 cells were transfected with Flag-RASA3 (1 μg), together with control pSilencer (1 μg) or pSilencer RASA3 (1 μg or 3 μg). The level of expressed Flag-RASA3 was detected by Western blot using anti-Flag antibody.


Flag-RASA3
pS Ctrl 1ug
pS rasa3 1ug
pS rasa3 3ug
Lane 1 2 3 4 5
Anti-Flag
B-actin

CHAPTER IV

DISCUSSION

4.1 RASA3-mediated D2 inhibition of MAPKs in pituitary cells

Dysregulation of the dopmine D2 receptor is associated with multiple psychiatric and neurological conditions. DRD2 gene has been regarded as one of the top candidate genes for schizophrenia [Monakhov et al, 2007, 2008; Fan et al, 2009]. Dysfunction of the D2 dopamine receptors also leads to substance seeking behaviour (alcohol, drug) and other related behaviours such as pathological gambling [Zack et al,2009]. At the pituitary level, the dopamine-D2 receptor regulates prolactin synthesis, secretion and lactotroph cell proliferation [Iaccarino et al, 2008]. For example, D2R activation mediates the inhibitory action of dopamine on the release of prolactin (PRL) from the amphibian pituitary. In an experiment, TRH stimulated the release of PRL from pituitaries. Quinpirole (a D2 receptor agonist) suppressed the stimulatory effect of TRH on the release of PRL, whereas SKF-38393 (a D1 receptor agonist) exhibited no such an effect (Nakano et al, 2010). Mice with a disrupted D2 dopamine receptor gene had chronic hyperprolactinemia and prolonged lactotroph hyperplasia which eventually resulted in pituitary lactotroph adenomas (Kelly et al, 1997; Asa et al, 1999). DRD2 agonist cabergoline is the first choice for treatment of prolactin-secreting pituitary adenomas, and it can reduce tumor size in 85 – 95% of patients [Filopanti et al, 2010]. Thus the D2 receptor is a key negative regulator of lactrotroph secretion and proliferation in vivo.

Various studies have shown that D2R's inhibitory effect on prolactin synthesis,

release and pituitary cell proliferation is mediated through its regulation of MAPK. Unlike most non-neuronal cells, in endocrine pituitary cells, D2S leads to the reduction of MAPKs. Ohmichi and colleagues first showed that TRH activated ERK1/2 in GH3 pituitary cell line, and dopamine could block this activation of ERK1/2 (Ohmishi et al., 1994). This D2-mediated inhibition of ERK1/2 has been confirmed in GH4ZR7 cells (Banihashemi et al, 2002; Liu et al, 2002). GH4ZR7 cells are from rat pituitary tumors and stably transfected with D2S. It is worth mentioning here that only D2S but not D2L reduced TRH-induced MAPK phosphorylation (Van Ham et al., 2008). In the normal situation, the effect of dopamine on ERK1/2 activity is determined by the ratio of D2L and D2S. Van Ham et al. (2008) successfully measured the decrease in potassium-stimulated ERK1/2 phosphorylation following quinpirole (D2 selective agonist) treatment in striatal cultures, indicating the existence of D2 inhibition of MAPK in neurons.

However, it was not clear by which mechanism D2 receptors inhibit MAPK activation. In GH4ZR7 cells, previous work in the Albert lab showed that D2S-induced inhibition of ERK1/2 was via Gαi3 and Gαo, but not Gαi2 and Gβγ (Banihashemi and Albert, 2002). Gαo is mainly involved in the regulation of basal MAPKs, and Gαi3 is more likely involved with suppression of TRH-induced activation of MAPKs. Although neither Ras- Gαi3 nor Raf- Gαi3 interaction had been found before, a yeast two hybrid screen identified a Gαi3-interacting RasGAP protein, RASA3, which is capable of inhibiting MAPKs via the Ras-Raf-Mek-ERK pathway. This finding suggests a potential

role of RASA3 in D2-mediated inhibition of ERK1/2. In this thesis, the functional importance of RASA3 in D2S-MAPK pathway has been investigated using expression of dominant negative RASA3 and siRNA knockdown of endogenous RASA3. Depletion of the endogenous level of RASA3 by both methods in pituitary cells leads to the suppression of D2S-induced MAPK inhibition, which supported our hypothesis that RASA3 is the key candidate that link D2S/ Gαi3 to Ras-Raf-MEK-ERK1/2 in GH4ZR7 cells.

As described before, the active form Gαi3 has stronger interaction with RASA3 after activation of Gαi3 and RASA3, following D2S and TRH receptor activation, respectively (Nafisi et al. 2008). In our model GH4ZR7 cells, TRH acted on the TRH receptor to activate ERK1/2 via Gαq, which activates PLC leading to breakdown PIP2 into DAG and IP3. IP3 is then converted into IP4, which binds to RASA3 on the PH/BTK domain and removes the inhibitory effect of PIP2 to activate RASA3. Competition with TRH-induced RASA3 could account for the potentiating effect observed for dnRASA3 on TRH-induced ERK1/2 activation. D2 agonist apomorphine acted on D2S receptor to activate Gαi3. Active forms of Gαi3 and RASA3 then formed a strong complex, which worked together to inhibit Ras and the downstream Raf-MEK-ERK. Thus, inhibition of the Ras required simultaneous activation of RASA3 (by TRH) and Gαi3 (by D2S).

4.2 RASA3-G alpha proteins inhibit D2-mediated ERK1/2 activation in COS7 cells

The effect of D2S receptor activation on MAPKs is cell type specific. Both G alpha and G betagamma subunits are reported to play roles in ERK1/2 activation depending on the cell types involved (Luttrell, 2005). In BALB-D2S cells, Gαi2 is crucial for D2S-induced activation of p42/44 MAPK. In addition, blocking Gβγ signaling by ectopic expression of GRK-CT inhibited D2S-induced activation of endogenous p42/44 MAPK in Balb-D2S cells (Ghahremani et al., 2000). Another study showed that D2R-mediated ERK1/2 activation involved Gαi2 but not Gβγ in HEK293 cells (Beom et al, 2004), whereas in Chinese hamster ovary (CHO) cells, Gβγ subunits are required in the activation of ERK1/2 by D_2R (Choi et al, 1999). Hence a variety of signaling pathways can couple D2S receptors to ERK1/2 activation.

We showed in this thesis that RASA3 inhibited D2S-induced ERK1/2 activation, when cotransfected with with Gαi2 or Gαi3, suggesting that the D2S receptor activates ERK1/2 in COS7 cells via a Ras-dependent pathway. It is surprising that RASA3 alone had no effect on ERK1/2 activation. Activated Gαi2 and Gαi3 are required for the inhibitory action of RASA3 on ERK1/2 phosphorylation. Consistent with our previous results that constitutively active G alpha proteins interact in a stronger way with RASA3, only active Gαi2QL or Gαi3QL can act with RASA3 to inhibit D2-induced ERK1/2 activation. Although D2S activation should activate wild-type Gαi2 and lead to a similar inhibitory effect as for Gαi2QL groups, groups with Gαi2 failed to inhibit ERK1/2 activation. That may be explained by the fact that we did not cotransfect Gαi2 with Gβγ, which helps Gαi2 to localize to the plasma membrane for activation by D2S. As mentioned before, experiments in GH4ZR7 cells revealed that D2S-induced

interaction of endogenous RASA3-Gαi3 only existed in TRH/apomorphine dual-treated cells in which both RASA3 and Gαi3 were activated. Here we show that in COS7 cells, transfection of Gαi3 and constitutively active RASA3 lead to a stronger suppression of D2S-induced ERK1/2 activation than wild type RASA3. The active RASA3 that was used has the R601C mutation, overlapping the binding site for both IP4 and PIP2 in PH domain, and results in a constitutively active RasGAP domain which shows greatly increased RasGAP activity *in vitro*. Thus, this constitutively active RASA3 appears to mimic the IP4-activated endogenous RASA3 in vivo. The importance of activating RASA3 for its signaling is consistent with results in GH4ZR7 cells in which IP4 activation induced by TRH was required for RASA3-Gαi3 interaction (Nafisi et al., 2008). Therefore, in our model, active RASA3 combines with receptor-activated Gαi2 or Gαi3 to inhibit D2S-induced ERK1/2 activation. However, in the presence of constitutively active Gαi2 or Gαi3, RASA3 itself does not have to activated, but active form of RASA3 has stronger inhibitory effect.

Although evidence in other cell types indicates Gαi2 as the mediator of D2S-induced ERK1/2 activation (Beom et al, 2004), co-expessing D2S receptor with Gαi2 in COS7 cells did not enhance D2S-induced ERK1/2 phosphorylation. Based on this result, we hypothesize that Gαi2 alone is not sufficient for the activation of ERK1/2 by D2S in COS7 cells. Gβγ may be the link between D2S receptor and ras-ERK1/2 pathways. Further experiments can be performed to investigate the molecular mechanism of D2-induced ERK activation in COS7 cells. The carboxy terminus of the β-adrenergic

receptor kinase 1 (βARK1-CT) contains the binding domain for Gβγ and can be used to study roles of Gβγ in cell signaling (Koch et al, 1994).

Another issue worth testing is what role RASA3 plays in D2L signaling. It has been reported before that D2L receptors fail to inhibit ERK1/2 in GH4 cells (Van Ham et al., 2007). We will test in the future whether RASA3 and different Gαi proteins are specifically associated with the D2S vs. D2L receptor in GH4 cells, and test whether RASA3 inhibits D2L-induced ERK1/2 activation in other cells such as COS7 cells or HEK293 cells. We will also test inhibitors such as PTX (to inhibit Gi/Go), MEK inhibitors, or other signaling inhibitors of PKA, PKC, PI3K or GSK3β inhibitor to test for convergent regulation of this response.

4.3 Role of endogenous RASA3

D2S receptor regulates many physiological events via MAPK in vivo. Understanding RASA3's effect in vivo may help scientists to control many D2S-MAPK related events. RASA3 is widely expressed in the brain in regions like CA1 layer of the hippocampus, amygdala, cerebellum, and pyriform cortex, which suggest a potential to participate in many physiological events. As the major presynaptic receptor, the D2S receptor plays a key role in the inhibitory regulation of dopaminergic activity. For example, D2S receptor regulates the expression of the rate limiting enzyme for dopamine synthesis, tyrosine hydroxylase (TH) via MAPK in substantia nigra neurons. Given the fact that RASA3 is an inhibitor in D2S-mediated MAPK, RASA3 pathway may mediate inhibition of TH

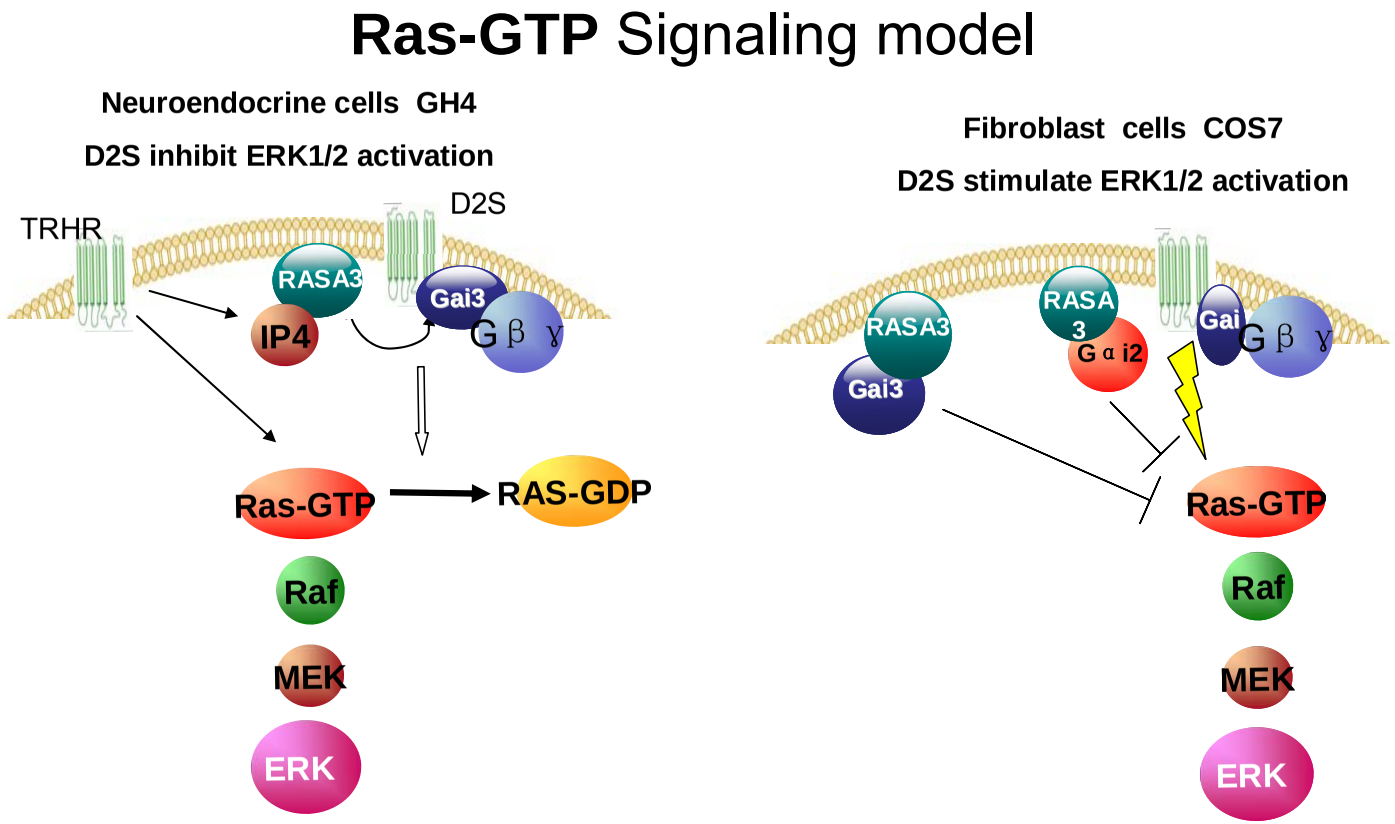
expression to thus regulate dopamine levels. RASA3 may be an important signaling protein in schizophrenia, due to the hyper-dopaminergic phenotype in schizophrenia. Also, RASA3's role in dopamine-induced suppression of ERK1/2 phosphorylation, prolactin synthesis, PRL release and pituitary cell proliferation needs to be verified in primary cultured pituitary tissues.

To examine the importance of the molecular mechanisms of D2- RASA3 signaling and its possible role in etiology or treatment of mental illness, depletion of endogenous RASA3 in neurons is necessary. So far, we verified the sequence and efficiency of a short hairpin RNA-expression construct that targets RASA3 mRNA. The ongoing step is to use lentivirus to delivery shRNA into dopamine neurons. Lentivirus are capable of transducing dividing and non-dividing cells (e.g. neurons), which makes them desirable for nervous system transduction. With the lentivirus tool, future experiments can be performed to elucidate the role of RASA3 in vivo in regulation of prolactin level, TH expression, dopamine levels, and ultimately in dopamine-dependent behaviors.

4.4 Conclusion

In conclusion, I have shown that RASA3 is required to inhibit D2S-induced ERK1/2 activation using dnRASA3 or siRNA approaches to RASA3 to inhibit its signaling in GH4ZR7 cells. This supports previous evidence in GH4ZR7 cells, that the activated RASA3-Gαi3 complex contributes to the D2S inhibition of TRH-induced ERK1/2 activation (Fig.9A). I have also shown that the RASA3-Gαi pathway to inhibit D2S-induced ERK1/2 activation in COS7 cells can be reconstituted by cotransfection of active forms of RASA3 and Gαi2 or Gαi3 (Fig.9B). Hence, the active form of RASA3 (caRASA3 in COS7 cells or IP4-activated RASA3 in GH4ZR7 cells) and the active form of Gαi (GαiQL in COS7 cells or D2S-activated Gαi3 in GH4ZR7 cells) are required to achieve the optimal inhibition of ERK1/2 phosphorylation. Finally, a RASA3 shRNA construct was generated that has good efficiency to reduce RASA3 expression and can be used in the future in the lentivirus-mediated RASA3 knockdown in vivo.

Fig.10 RASA3-Gαi signaling model



A is D2 signaling to RASA3-Gαi3 mediated inhibition of TRH-induced ERK1/2 activation in GH4ZR7 cells. B is the RASA3-Gαi mediated inhibition of D2-induced ERK1/2 activation in COS7 cells.

Appendices

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