Functional Studies of Dopamine-D2S Receptor Signaling through the RASA3 Pathway

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Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

In partial fulfillment of the requirements

For the MSc degree in Neuroscience

Department of Cellular and Molecular Medicine

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Abstract

RASA3 (Ras p21 GTPase Activating Protein 3) is required for D2SR (Dopamine D2 Short Receptor) induced ERK1/2 inhibition in pituitary lactotroph GH4ZR7 cells. We hypothesized that RASA3 may be important for D2SR signaling to inhibit ERK1/2 in dopamine neurons, and thus negatively regulate TH (Tyrosine Hydroxylase) expression and activity. We designed and made shRASA3 lentivirus and showed that it inhibits RASA3 expression. Lentivirus mediated RASA3 knockdown can partially reverse the D2SR mediated ERK1/2 inactivation in GH4ZR7 cells. We then showed that knockdown of RASA3 in dopamine-secreting PC12 cells increased NGF-stimulated ERK1/2 in cells expressing D2SR, but not in cells lacking D2SR, thus implicating RASA3 plays a role in D2SR-mediated inhibition of ERK1/2 signaling. We also found that knockdown of RASA3 increased TH protein levels in cells expressing D2R receptors but not those without D2SR, suggesting that D2SR tonically inhibits the synthesis of TH. We also found preliminary indication that mutant RASA3 mice show increased level of TH in SN compared to WT mice. RASA3 mutant mice showed no striking changes in basal locomotion, anxiety or depression phenotypes, but further studies are needed to specifically address dopamine-driven behaviors. In summary, our data support the role of RASA3 in mediating D2SR-induced inhibition of ERK1/2 in dopamine neurons to negatively regulate TH expression and activity.

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List of Abbreviations

Abbreviation	Full name
aa	Amino Acid
AC	Adenylyl Cyclase
BBK	Beam Break
BTK	Bruton's Tyrosine Kinase
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
CNS	Central Nervous System
DA	Dopamine
DAG	Diacylglycerol
DARPP-32	Dopamine and c-AMP regulated protein phospho-protein of 32Kd
DAT	Dopamine Active Transporter
DOPA	Dihydroxy Phynylalanine
DR	Dopamine Receptor
D2LR	D2 Long Receptor
D2SR	D2 Short Receptor
EPM	Elevated Plus Maze
SPSP	Excitatory Postsynaptic Potential
ERK	Extracellular-Signal Regulated Kinase
FS	Forced Swim
G Protein	Guanine Nucleotide Binding Protein

GPCR	G Protein Coupled Receptor
GABA	Gamma-Aminobutyric Acid
GAP	GTPase Activating Protein
GAP1IP4BP	GTPase Activating Protein 1 (Inositol 1,3,4,5-Tetrakis Phosphate Binding Protein)
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
GRK	G Protein Coupled Receptor Kinase
HEK	Human Embryonic Kidney Cell Line
HLB	RASA3 H794L mutant Black mice
IP3	Inositol 1,4,5-Trisphosphate
IP4	Inositol 1,3,4,5-Tetrakisphosphate
KDa	KiloDalton
MAO	Monoamin Oxidase
MAPK	Mitogen Activated Protein Kinase
MEK	MAPK/ERK Kinase
MSN	Medium Spiny Neuron
NAcc	Nucleus Accumbens
NMDA	N-Methyl-D-Aspartate
OF	Open Field
ORF	Open Reading Frame
OT	Olfactory Tubercle
PH	Pleckstrin Homology

PI3K	Phosphoinositol 3’ Kinase
PIP2	Phosphotidylinositol 4,5-bisphosphate
PIP3	Phosphotidylinositol 1,4,5-trisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2 A type
PRL	Prolactin
PTX	Pertussis Toxin
RASA3	Ras p21 Protein Activator 3
RASAL	Ras GTPase Activating Like Protein
RTK	Receptor Tyrosine Kinase
TH	Tyrosine Hydroxylase
TM	Transmembrane
TRH	Thyrotropin Releasing Hormone
TS	Tail Suspension
Scat	Severe Combined Anemia and Thrombocytopenia
SN	Substantial Nigra
VTA	Ventral Tegmental Area

Acknowledgments

I am forever indebted to my mentor Dr. Paul Albert, for guiding me through my study and research, through ups and downs, for his patience and motivation, for providing encouragement and constructive suggestions at all times. He pays attention to big pictures and details, has a terrific understanding of his research subjects and even other fields. Thank you.

I would like to thank my committee members, Dr. Mario Tiberi and Dr. Johnny Ngsee, for their valuable advices and continuous help along the way.

I would also like to thank all my friends and colleagues in the lab. Mireille Daigle, thank you for helping me initiate and finish my project, for the constructs and the animals, for the techniques you introduced to me. Brice Le François, Laura Fiori, Anne Millar, Faranak Vahid-Ansari, Chris Luckhart, Irshaad Hashim, and other people of the lab I didn't write down their names, thank you all. I owe my special thanks to Dr. Sara Schock and Dr. William Staines, for their kind help more than I expected. I would also like to thank Megan, Mirela and Dr. Lagace, for their advices to my behavior tests. I would also thank the department, for providing Seminars, Journal Clubs and many chances to learn, to think. It has been a memorable and rewarding experience.

Finally, I thank my family. My parents, in-laws and my wife, who never failed to understand and support me on my way beside me. And my baby, your sweet smile is always the best gift.

Chapter I Introduction

1.1 Dopamine System

1.1.1 Brief History of the dopamine system

Dopamine (DA, 3,4-dihydroxyphenethylamine), a member of the catecholamine family, plays a number of important physiological roles (Missale et al., 1998). In the brain, dopamine regulates a variety of processes such as locomotion, reward, emotion, cognition and endocrine function. Apart from roles in the central nervous system, dopamine also plays roles in the immune system, kidney and pancreas, modulating immune activation level, blood pressure, vascular tone and hormone release (Harris et al., 2012; Basu et al., 2000).

Dopamine was first discovered in 1952 as an intermediate of norepinephrine production while at that time people believed that dopamine itself has no signaling properties. In 1957, dopamine was identified as a neurotransmitter by Arvid Carlsson in Sweden (Carlsson et al., 1957). In 1967, George Cotzias showed that high dose oral L-DOPA treatment could completely or partially reduce the manifestations of Parkinsonism (Cotzias et al., 1967). In 1972, Paul Greengard reported that Adenylate Cyclase (now termed adenylyl cyclase) was activated by a low concentration of dopamine and apomorphine, while the activation was blocked by low concentration of haloperidol or chlorpromazine (Kebabian et al., 1972). Along with the identification of dopamine receptors as targets of antipsychotic drugs in 1970s, dopamine and dopamine system have been intensively studied for more than half a century (Clement et al., 1974; Beaulieu et al., 2011).

1.1.2 Synthesis, Storage and Degradation of Dopamine

The starting point of the biosynthesis of dopamine is L-tyrosine, an essential amino acid which is abundant in dietary proteins. Tyrosine from blood is first transferred to the brain by a low affinity amino acid transport system, then to the dopaminergic neurons from brain extracellular fluid. In addition, phenylalanine hydroxylase can convert phenylalanine to tyrosine, this pathway can be considered upstream of tyrosine. In dopamine neurons, tyrosine is converted to L-DOPA by tyrosine hydroxylase, which is the rate limiting enzyme of dopamine and other catecholamine production (norepinephrine and epinephrine) (Molinoff et al., 1971). L-DOPA can be converted to dopamine by Aromatic L-amino Acid Decarboxylase (AADC). L-DOPA is an amino acid that can cross the Blood Brain Barrier, while dopamine can not, and this characteristic makes L-DOPA a potent drug to treat diseases with reduced brain dopamine levels like Parkinson's Disease and Dopamine Responsive Dystonia (Cotzias et al., 1969; Keegan et al., 1973).

The synthesized dopamine is transported to synaptic vesicles from the cytosol by Vesicular Monoamine Transporter 2 (VMAT2) (Allard et al., 1994). Like any other neurotransmitters, dopamine stays in the vesicles until an action potential occurs and opens the voltage gated calcium channel, which allows calcium to enter into the presynaptic dopamine neuron. Calcium triggers the fusion of synaptic vesicles to the membrane, which releases dopamine to the synaptic cleft.

Once in the cleft, dopamine interacts with different kinds of dopamine receptors on the postsynaptic neurons, which might exert excitatory or inhibitory functions, depending on receptor type and cell type. To avoid constant stimulation and excessive firing of the postsynaptic cells, dopamine has to be removed from the cleft. There are three mechanisms dopamine can be inactivated: 1) Reuptake. Reabsorption of dopamine into the presynaptic

neuron. 2) Enzymatic breakdown. 3) Diffusion. Dopamine Transporter is the protein embedded in the presynaptic membrane, which pumps dopamine back to the dopaminergic neurons. This process is called reuptake. A few enzymes play roles in the degradation of dopamine, including MAO (Monoamine Oxidase), ALDH (Aldehyde Dehydrogenase) and COMT (Catechol-O-Methyl Transferase) (Hsu et al., 1989； Meiser et al., 2013). DOPAC, HVA (Homovanillic Acid) and MOPET (Homovanillyl Alcohol) are the metabolic end-products which will be excreted in the urine.

1.1.3 Dopamine pathways

In the brain, dopamine is produced in different groups of dopamine neurons that project to different brain areas, shaping brain function. The presynaptic, postsynaptic neurons, the clefts, cell bodies, terminals and dendrites, all together build the dopamine circuits. There are four major dopamine pathways in the brain: Nigrostriatal, Mesocortical, Mesolimbic and Tuberoinfundibular Pathways (Kruk et al., 1993). The Nigrostriatal pathway transmits dopamine from the Substantial Nigra (SN) to the Neostriatum. This pathway is particularly involved in locomotion, as part of the basal ganglia motor system. Continual loss of dopamine neurons in SN is one of main causes and features of Parkinson's Disease (PD). Dopamine is produced in neuron of the Ventral Tegmental Area (VTA) in midbrain, which project to frontal cortex and to the Nucleus Accumbens (NAcc) located in the ventral striatum. The former pathway is Mesocortical pathway and the latter one is Mesolimbic pathway. These two pathways are highly involved in reward, cognition, emotion, learning and memory, disruption of these pathways would cause disorders such as schizophrenia and addiction. The tuberoinfundibular pathway projects dopamine from mediobasal hypothalamus to the portal blood stream to regulate the pituitary gland. This pathway

negatively regulates prolactin synthesis and secretion by lactotrophs in the anterior pituitary gland.

1.1.4 Dopamine Receptors

All the physiological functions of dopamine are mediated through dopamine receptors directly or indirectly. Not only dopamine, pharmacological agents used clinically targeting dopamine pathways to treat disorders including Parkinson's, Schizophrenia, Attention Deficit Hyperactivity Disorder, Bipolar Disorder and Tourette's Syndrome also work through dopamine receptors directly or indirectly. Dopamine receptors are a class of G protein coupled receptors divided into two groups: the D1 like and D2 like receptors. D1 and D5 receptors are classified as D1-like while D2, D3, D4 receptors are classified as D2-like receptors (Missale et al., 1998). This classification was based on pharmacological characterization of their abilities to activate Adenylyl Cyclase (AC), in other words, to produce cyclic AMP. Upon stimulation, D1 and D5 receptors activate $G\alpha_{s/olf}$ to produce cAMP by activating AC, whereas D2, D3 and D4 receptors couple to $G\alpha_{i/o}$ to inhibit cAMP production. Upon cloning and sequence analysis, these classes were based on the primary amino acid sequence homology, with D1- and D2-like receptors having the most similarity within each family, but less similarity between families.

D1- and D2-like receptors are also different at genetic structure level. D1 and D5 receptors do not contain introns in their coding sequences, while D2 receptor has six introns in its gene, D3 receptor has five, D4 receptor has three (Gingrich et al., 1993). These introns give D2 receptors opportunities to produce isoforms based on the same gene, thus shaping the dopamine pathways with potentially greater diversity. For instance, D2 short receptor (D2SR) and D2 long receptor (D2LR) are two isoforms of D2 gene. D2SR lacks the 29 amino

acids in the third intracellular loop comparing to the D2LR, which contributes to the different localization and functions of these two isoforms (Usiello et al., 2000). D1-like receptors have short third intracellular loops and long C-terminal tails while D2 receptors have long third intracellular loops and short C terminal tails (Missale et al., 1998). D1-like and D2-like receptors also differ in their brain region localization. (Bergson et al., 1995; Levey et al., 1993; Diaz et al., 1995; Seeman et al., 2006).

1.1.5 D1 like Receptors

D1 and D5 receptors are the two members of D1 like receptor group. They are characterized by inducing cAMP production in cell lines and brain tissues (Clement-Cormier et al., 1974; Dearry et al., 1990). The D1 receptor is widely expressed in the brain, especially in striatum, nucleus accumbens, cerebral cortex and olfactory tubercle, also in limbic system such as thalamus and hypothalamus (Boyson et al., 1986). D1 subtype is the most abundant dopamine receptor in the central nervous system. Comparing to D1 receptor, D5 receptor is more weakly expressed with a restricted pattern, mainly found in hippocampus, lateral mammillary nucleus and the parafascicular nucleus of the thalamus (Tiberi et al., 1991; Meador-Woodruff et al., 1992).

D1 and D5 receptors share 80% amino acid identity at the transmembrane (TM) domains, while overall, the identity is only 60%. D5 receptor displays a 10-fold higher affinity for dopamine compared to D1 (Sunahara et al., 1991). It was generally assumed that D1-like receptor stimulation of AC is mediated through Gαs. However in some areas like striatum, nucleus accumbens and olfactory tubercle, where there is very low Gαs expression and still quite strong AC activation is found, suggesting that another Gα protein is exerting this function. G_{olf}, which is highly expressed in these brain areas was identified as another

mediator since it is very closely related to Gs (88% amino acid homology) and can also activate Adenylyl Cyclase (Jones et al., 1989). The role of G_{olf} is in D1 stimulation of AC was further confirmed in G_{olf} knockout mice. In these mice, D1 receptor agonist induced AC activation is greatly reduced, the locomotion stimulation caused by cocaine was also impaired (Corvol et al., 2001). Not only the G-protein α subunit, but also βγ subunits are important in cAMP production (Schwindinger et al., 2003; Ehrlich et al., 2013). cAMP binds to the regulatory subunit of Protein Kinase A (PKA) to activate the catalytic subunit to phosphorylate the Ser or Thr residues of different proteins (Taylor et al., 2013). DARPP-32 (Dopamine and cAMP regulated phosphoprotein, 32kDa) and CREB (cAMP response element binding protein) are the most two important substrates of PKA in dopamine-responsive neurons.

Activated DARPP32 with Thr34 phosphorylated is a potent PP1 (Protein Phosphatase 1) inhibitor, inhibiting its dephosphorylation role on Raf, MEK and PKB (Hemmings et al., 1984; Lindenberg et al., 2007). DARPP32 is a dual-function protein and when it is phosphorylated at Thr75 by Cdk5 (Cyclin Dependent Kinase 5), it is a PKA inhibitor. As both a potent PP1 and PKA inhibitor, DARPP32 is considered a integrator of dopamine neurotransmission (Svenningsson et al., 2004). In dorsolateral prefrontal cortex, DARPP32 is found to be down-regulated in patients with schizophrenia or bipolar disorder (Feldcamp et al., 2008). While the truncated DARPP32 (lacks the N terminal Thr34 to inactivate PP1) mRNA level is increased in dorsolateral prefrontal cortex, hippocampus and caudate of postmortem schizophrenia patients' brain (Kunli et al., 2014), showing a more complicated function of DARPP32 in regulating dopamine signaling.

Upon stimulation, the D1 receptor induces PKA activation, which would phosphorylate

CREB (cAMP response element binding protein) at Ser133, and the activation of CREB with CREB binding protein will initiate the transcription of genes with cAMP response elements (Andersson et al., 2001). The gene expression mediated by CREB is important in synaptic plasticity at cellular level, and in addiction and fear at the behavioral level (Niciu et al., 2013). MAPKs including ERK, p38 and JNK are also downstream effectors of D1 receptors through PKA-dependent or -independent pathways (Gerfen et al., 2002; Zhen et al., 1998).

Upon stimulation, D1-like receptors activate PKA and downstream DARPP32 to inhibit PP1. So the phosphorylation of several types of voltage-gated channels and ligand-gated ion channels is increased, thereby regulating ion channel functions. In vitro and in vivo, D1 dopamine receptor can directly interact with N-type calcium channels to inhibit this channel (Kisilevsky et al., 2008). In the prefrontal cortex and nucleus accumbens (NAcc), D1 receptor helps to enhance the firing and excitation of fast-spiking interneurons and pyramidal neurons by inhibiting the potassium channels (Podda et al., 2010). The sodium channels play a number of essential roles through regulating the firing of neurons. The impact of D1 receptors on sodium channels depends on cell type and the sodium channels expressed. In striatum and hippocampus neurons, sodium channel excitabilities are decreased upon D1 stimulation (Schiffmann et al., 1995). In prefrontal cortex, D1 agonist can attenuate the sodium channel dependent EPSP (Excitatory Postsynaptic Potential) (Rotaru et al., 2007).

D1 receptors can maintain NMDA mediated responses in prefrontal cortical pyramidal neurons through both a Ca^{2+} and PKA dependent pathway (Jian Wang and Patricio O'Donnell, 2001)(Flores-Barrera et al., 2014). In return, NMDA receptor is also required for D1 receptor to exert its function (Glass et al., 2013). It is not surprising that D1 receptor can

also modulate AMPA currents (Han et al., 2009).

D1 receptor gene knockout mice give us the most useful tools to understand the receptor's functions. The D1 receptor knockout mice are smaller, the postnatal development is delayed, the basal activity is modestly increased, while the cocaine induced acute locomotor responses are consistently absent (Xu et al., 1994; Karisson et al., 2008). Mice lacking D5 dopamine receptor have increased sympathetic tone and are hypertensive (Hollon et al., 2002). After amphetamine treatment, D5 receptor knockout mice showed greater locomotor activity and elevated dopamine transporter activity (Hayashizaki et al., 2013).

1.1.6 D2 like receptors

D2, D3 and D4 receptors are the three members of the D2-like receptors. One important property of D2-like receptors is that they have introns. D2 receptors have two variants, D2 long receptor and D2 short receptor, which lacks the 29 aa at the third intracellular domain. Since the 29 aa plays a role in the G protein coupling and phosphorylation site, it leads to the receptor function diversity (Morris et al., 2007; Senogles et al., 1994). In GH4 cells, activation of D2S receptor inhibits TRH (Thyrotropin Releasing Hormone)-induced ERK1/2 signaling, while D2L receptor fails to perform this response (Van-Ham et al., 2007). We will later elucidate the specific characteristics of D2S in the next unit. D3 receptors share 75% protein homology with D2 receptors at the TM domains. D3 mRNA expression is limited to telencephalic areas and limbic areas, such as nucleus accumbens, islands of Calleja, hippocampus and thalamus (Bouthenet et al., 1991). D4 and D2 receptors share a 53% homology in the TM region. D4 receptor is mainly expressed in the frontal cortex, amygdala, olfactory bulb and hippocampus (Jackson et al., 1994). VNTR (Variable Number Tandem Repeat) of D4 receptors is found associated with ADHD (Attention Deficit Hyperactivity

Disorder), Novelty Seeking and Cognitive Development (Faraone et al., 2001; Posner et al., 2012).

In an opposite way to D1 receptors, D2 receptors were first identified as inhibiting AC activity to decrease the cAMP level to affect the downstream signaling. D2-like receptor mediated inhibition was thought to function through $G_{i/o}$ proteins. Gi would directly bind to C1 domain of AC then block the formation of C1-C2 dimer which is important for the activity of AC (Liu et al., 1997). PKA, the downstream effector of cAMP, is attenuated upon D2-like receptor stimulation. Thereby, phosphorylation level of the Thr34 site of DARPP32 is decreased while Thr75 phosphorylation level is enhanced, exactly the opposite of D1 receptor actions. Not surprisingly, PKA regulates Tyrosine Hydroxylase (TH) activity through Ser40 phosphorylation this is reversed by D2 activation. This gives the rationale that D2 receptors can function as inhibitory autoreceptors in dopamine neurons.

D2 receptor reduces the sodium channel discharge rate in striatal cholinergic interneurons through a PKC dependent pathway (Maurice et al., 2004). In pyramidal neurons of prefrontal cortex, activation of D2 like receptors increases axonal potassium, the opposite effect of D1 like receptors(Yang et al., 2013). Upon activation Gα protein dissociates with Gβγ to exert its excitatory or inhibitory function. The dissociated Gβγ has its own signaling properties (Smrcka et al., 2013). Gβγ activated by D2 and D4 receptors strongly increased the AC2 activity by binding to the residues in the less conserved region in ACs (Weitmann et al., 2001). In medium spiny neurons (MSN), Gβγ activated by D2 receptors has been shown to stimulate PLC, thus calcium concentration of the cytoplasm is also increased. As a consequence, the L-type calcium channel currents and excitability is diminished (Hernández-López et al., 2000) For ion channels, the D2 receptors induced modification

through Gβγ subunits is not limited to calcium channels. They also have an impact on G protein-coupled inwardly rectifying potassium channels (GIRKs). Unlike D1 receptors, D2 receptor activation increase the GIRK currents, thereby showed an inhibitory effect on the neuron excitability presumably via Gβγ subunits. Since D1 receptor is usually more abundant and D1 signaling is stronger, D2 induced modulation on sodium channels is usually covered by D1 signaling.

AMPA and NMDA receptors are important for LTD (Long Term Depression) and LTP (Long Term Potentiation), thus playing roles in learning and memory. AMPAR surface expression is significantly decreased after injection of D2 receptor agonist quinpirole in rat nucleus accumbens, while D1 receptor agonist SKF81297 fails to show this effect (Ferrario et al., 2011). In mPFC (medium prefrontal cortex), D2/D3/D4 antagonists block the efflux of glutamate caused by NMDAR agonist MK801, indicating the modulatory role of D2 like receptors on NMDAR function (Xavier et al., 2010).

Like many other GPCRs, activation of D2 receptors may influence MAPK signalling, but whether the effect is activation or inhibition is still not very clear since the effect is dependent on the cell types and receptor types (Cho et al., 2010).

D2R regulates the intracellular signalling in a G protein-dependent and -independent manner. When D2R is phosphorylated by GRK (G Protein Coupled Receptor Kinase), it recruits β-Arrestin2, which would then recruit clathrin, this process triggers clathrin mediated receptor endocytosis. D2R can also form a D2R-βArrestin2-PP2A-AKT complex. PP2A can deactivate AKT, thus stimulate GSK3 mediated signaling (Beaulieu et al., 2011).

D2R knockout mice share pronounced impairments in their ability to coordinate movements and reduction in spontaneous locomotor activity similar to PD (Parkinson's

Disease) (Baik et al., 1995; Tinsley et al., 2009). They also showed reduced response to reinforcers including ethanol and food, indicating that D2 plays a role in reward (Risinger et al., 2000). Other than locomotion, motivation and reward, D2R is also involved in stress and anxiety. Chronic stress treated D2R-/- mice spent much less time in the open arms of the EPM (Elevated Plus Maze) test, and the corticosterone concentration of D2R-/- mice was higher (Sim et al., 2013). Interestingly, none of the above phenomena were found in D2R-/- without stress treatment, indicating D2R's role at responding to stress cues. It was also found that D2R mRNA level is negatively correlated with the time spent in the open arms of EPM and in the center of OF (Open Field), suggesting D2R play a role in anxiety like behaviours (Falco et al., 2014).

1.1.7 D2 Short Receptor

D2 receptors are widely expressed in the brain, localized at postsynaptic neurons and also presynaptic dopaminergic neurons. D2 receptors have two isoforms, D2 short and D2 long receptor, generated by alternative splicing from the same gene. Despite the different structure of the two subtypes caused by the 29aa missing in the third intraloop in D2 short receptor, initially the signaling of these receptors appeared similar. During a long period, there was debate about which receptor subtype functions as the autoreceptor. In the last decade, more and more reports showed that D2 short receptor is the autoreceptor, plays a more important role at regulating dopamine synthesis and release in dopaminergic neurons. By utilizing specific antibodies against D2SR and D2LR, people found that D2SR mainly localizes in the cell bodies and projection axons of the dopaminergic neurons, whereas D2LR was more strongly expressed in the striatum and nucleus accumbens which are targets of dopaminergic fibers, indicating the two isoforms possess distinct functions

(Khan et al., 1998).

Both receptors had been shown to couple to Gi and Go proteins to exert the inhibition function, but the types of Gi/o proteins they bind can be different. By transfecting PTX (Pertussis Toxin) resistant Gi mutants into HEK (Human Embryonic Kidney) cells, people found that D2SR selectively couples to Gi2 while D2LR couples more to Gi3 (Senogles et al., 1994). D2S mutants in the third intracellular loop (R233G and A234T) showed that R233G prefers to bind to Gi3, A234T prefers to bind to Gi1, differ with the D2SR wild type (WT), indicating the essential role of the third intracellular loop in the specificity of Gi protein coupling (Senogles et al., 2004). The coupling is not only isoform-specific and structure-dependent, it's also cell type dependent (Albert et al., 2002). In GH4 cells, a pituitary cell line that is more neuroendocrine, activation of D2SR inhibited forskolin-induced cAMP production, reduced BayK8644-activated calcium influx, and blocked TRH-mediated p42/p44 MAPK (ERK1/2) phosphorylation. The effects were blocked by PTX. PTX resistant Gi2 and Gi3 could partially rescue the inhibition, meaning that in GH4 cells, D2SR preferentially binds to Gi2 and Gi3 upon stimulation (Albert et al., 2002). In addition, the dopamine-induced apoptosis of lactotrophs is mediated by D2SR (Radl et al., 2011; Albert et al., 1990). In AtT20 (ACTH secreting) pituitary cells, by knocking down specific Gi/o proteins to study the Gi/o coupling to inhibit calcium currents, it was found that both D2SR and D2LR were showed to bind to Go proteins, while D2LR couples to Gi3, D2SR couples to Gi2 respectively (Wolfe et al., 1999). By contrast, in GH4ZR7 pituitary lactotroph cells, D2SR coupled mainly to Gi3 to inhibit prolactin secretion, while D2LR coupled to multiple Gi proteins (Albert et al., 2002). The G protein coupling of D2SR is also intracellular function dependent. In Ltk- fibroblast cells D2SR coupled to Gi3

for inhibition of Gs-stimulated cAMP, while it coupled to Gi2 for inhibition of forskolin-stimulated cAMP (Ghahremani et al., 1999). In BALB/c 3T3 fibroblast cells, Gi2 and Gβγ were required for D2S-induced DNA synthesis and MAPK activation and Gi3 was required for cellular transformation (Ghahremani et al., 2000). In general, D2SR utilizes different Gi/o proteins to exert different functions in different cell lines. In neuronal or neuroendocrine cells, D2SR tends to be inhibitory, while in fibroblast cells, D2SR also couples to stimulatory pathways (Albert et al., 2002).

To better understand the distinct functions of the two D2R isoforms, people generated D2R-/- and D2LR-/- mice. Comparing the characteristics of these two knockout mice, the early conclusions about D2SR function were deduced indirectly. Quinpirole, a D2-like receptor agonist with some selectivity for D2 and D3 receptors, was shown to reduce locomotion of rodents at low dose injection through a presynaptic action, which makes it a good tool to study presynaptic D2 receptor. In D2R-/- mice, the locomotion reduction was lost, while in D2L-/- mice, the reduction was more pronounced than in WT mice, indicating the inhibition of dopamine release was mediated through D2SR, meaning that D2SR functions as autoreceptor (Usiello et al., 2000). In the same paper, they also tested the effect of haloperidol on D2L-/- and WT mice. When mice were treated with lose dose haloperidol, dopamine release could be enhanced through blocking presynaptic receptors. Extracellular dopamine concentrations were found increased in both D2L-/- and WT mice, indicating the presynaptic receptor function was retained in D2L-/- mice. This identification further elucidated the postsynaptic function of D2LR, while D2SR is believed to be the autoreceptor (Usiello et al., 2000).

The distinct functions of D2SR and D2LR were further clarified by examining the

phosphorylation level of Tyrosine Hydroxylase (TH) and DARPP32. TH, as the rate-limiting enzyme of dopamine production, is exclusively expressed in the dopaminergic cells. Ser31 and Ser40 site phosphorylation are important for TH stability and enzymatic activity. Dopamine-D2 autoreceptor activation will decrease the phosphorylation level of these two sites to negatively regulate dopamine synthesis and release. In dopaminergic VTA (Ventral tegmental area) and SN (Substantia Nigra) neurons, D2 agonist induced TH phosphorylation reduction is lost in D2R-/- mice, while preserved in D2L-/- mice, indicating a specific D2SR presynaptic effect. DARPP32, a mediator of dopamine downstream signaling, when get phosphorylated at Thr34, is a potent inhibitor of PP1. Blockade of D2 receptor would activate the D1 agonist-cAMP-PKA induced DARPP32 activity. When given D1R agonist SKF81297 and D2R agonist quinpirole, the DARPP32 phosphorylation mediated by SKF81297 is blocked in WT, but not in D2R-/- and D2LR-/-, thereby demonstrating that D2LR is involved in the postsynaptic D2 signaling (Lindgren et al., 2003).

When treated with cocaine, the dopamine release in D2R-/- is much higher than in WT mice (Rouge-Pont et al., 2002). Not surprising that in D2LR-/- mice, the dopamine releasing effect of cocaine was not changed.

It is a good method to elucidate the presynaptic function of D2SR by comparing the properties of D2LR-/- and D2R-/- mice. But a better and more straightforward way is to use the D2SR-/- mice. People used the loxp-loxp-DATCre system to generate the conditional D2SR-/- mice. They found that these mice displayed elevated dopamine synthesis and release, basal locomotion enhancement and hypersensitivity to cocaine administration. The mice also exhibited increased place preference induced by cocaine and enhanced

motivation for food reward (Bello et al., 2011). In temporal hippocampus, after D2SR was depleted, LTP (Long Term Potential) and LTD (Long Term Depression) were both impaired, as well as the spatial learning and memory ability, indicating the role of D2SR at regulating the plasticity of hippocampus (Rocchetti et al., 2014).

It has to be mentioned that the presynaptic effects of D2SR stimulation are not only limited to DA release, but also affect GABA, glutamate and acetylcholine release (Centonze et al., 2004).

In addition to its acute negative feedback regulation, D2SR also showed long term effects. D2SR chronically enhances the dopamine neuron pacemaker activity by regulating potassium channel (Hahn et al., 2006). Chronic activation of the D2SR using quinpirole caused a decrease in the number of dopaminergic and glutamatergic axon terminals in vitro, D2SR function was also impaired after chronic activation, while DA release was not changed (Fasano et al., 2010).

In GH4ZR7 (GH4 cells stably transfected with D2SR) cells, D2SR mediated inhibition of TRH (Thyrotropin Releasing Hormone) induced ERK1/2 signaling was exerted by coupling to Go and Gi3 proteins. The downstream effector of Gi3 was found to be RASA3 (Ras21 p21 Protein Activator 3), which was identified by yeast two hybrid screening. The interaction of Gi3 and RASA3 was greatly enhanced when cells were treated using both TRH and Apo, indicating the essential role of RASA3 in the D2SR induced inhibition of ERK1/2 activity (Nafisi et al., 2008). The D2SR mediated ERK1/2 inactivation is also found in striatal cultures, indicating that this pathway also exists in neurons (Van-Ham et al., 2007). D2SR is known to negatively regulate TH expression in vivo, presumably through ERK1/2-Nurr1 pathway (Lindgren et al., 2001). Hence, RASA3 could be an important link between D2SR

and regulation of TH activity in dopamine neurons.

1.1.8 Dopamine and Schizophrenia

Dys-regulation of the dopamine system is thought to underlie schizophrenia. Schizophrenia is a severe brain disorder characterized by abnormal reality interpretation and social behavior. Symptoms of schizophrenia include delusions, hallucinations, disorganized thinking and negative symptoms refers to reduced ability of lack of ability to function normally (Tandon et al., 2009). It is not clear what causes schizophrenia, but it is very clear that a combination of genetics and environment contributes to this disorder (Mäki et al., 2010).

The dopamine hypothesis of schizophrenia is one of the most enduring ideas about the illness. It emerged from the discovery that antipsychotic drugs increased dopamine metabolism (Carlsson et al., 1963; Seeman et al., 1976). Further studies showed that amphetamine and cocaine, which increase the synaptic dopamine level can induce psychotic symptoms (Curran et al., 2004). Similarly, L-DOPA treated PD patients also experience schizophrenia like side effect. All these above indicate that hyperactive dopamine transduction is highly correlated to schizophrenia.

Almost all antipsychotics have dopamine receptor antagonistic effects. These drugs alleviate psychosis by inhibiting dopamine signaling. D2 receptor is the main target for antipsychotics (Baumeister et al., 2002), and its density in schizophrenia patients is about 50% higher than in control (Seeman et al., 1987). Meta-analysis of PET imaging studies revealed that patients with schizophrenia exhibit higher D2 receptor density, also an increase in DA transmission and DOPA decarboxylase expression after amphetamine challenge, indicating that both pre and postsynaptic alterations of DA transduction in

schizophrenia (Laruelle et al., 1998).

However, there is evidence that the dopamine hypothesis of schizophrenia is not the primary defect. First, these antipsychotic or psychotomimetic drugs can modify dopamine levels in minutes, but it takes several days to be effective in schizophrenia. Second, in some patients, although over 90% of the D2 receptors are blocked by the drugs, some symptoms are only slightly alleviated (Kapur et al., 2000). These findings suggest that other neurotransmitters than dopamine might be involved in the disorder, and is not sufficient to explain or treat the illness.

There is now more and more studies showing that glutamate is associated with schizophrenia, leading to the glutamate hypothesis of schizophrenia (Stahl et al., 2007). The hypothesis arises from the observation that phencyclidine (PCP) can produce schizophrenia like symptoms by blocking NMDA (N-Methyl-D-Aspartate) receptor (Javitt et al., 1991). One important glutamate pathway, Cortical Brainstem Glutamate pathway, originates from cortical pyramidal neurons, projects via interneurons to dopamine neurons in VTA, which results in tonic inhibition of dopamine release from mesolimbic pathway (Goffet al., 2001). If the NMDA receptors are hypoactive in VTA, the dopamine release inhibition ability of NMDA receptors is impaired, which will cause hyperactive dopamine signaling and positive symptoms of psychosis. This glutamate hypothesis is considered to be more proximal to the root causes of schizophrenia.

These two hypotheses are now combined together to better understand and treat schizophrenia.

1.2 RasGAP Family and RASA3

1.2.1 RasGAP Family

The identification of RASA3 as a downstream effector of D2S signaling suggests a role for GAPs in dopamine function. GAPs, the GTPase Activating Proteins, are a group of proteins which can bind to kinds of activated G proteins, GTP bound G protein, to stimulate the weak intrinsic GTPase activities of G proteins, thus deactivate the G protein and terminate the G protein mediated signaling. GAPs are playing a number of important roles through regulating the G protein activity since G proteins are the most essential mediators of extracellular signals and intracellular effectors involved in a variety of key intracellular processes. The balance of GEF (Guanine Nucleotide Exchange Factor) and GAP keeps G protein activities at a proper level, and if any one of these two groups loses its function, the homeostasis of cells might get impaired.

Generally, different small G proteins have their own specific GAPs, while some GAPs might be dual functional (Sot et al., 2010). For p21 Ras, the specific Ras GAP family includes p120GAP, NF1 (neurofibromin1) and GAP1 family constitutes with RASA3 (Ras p21 Protein Activator 3), CAPRI (Calcium Promoted Ras Inactivator), RASAL (Ras GTPase Activating Like Protein), GAP1m (a Mammalian Counterpart of Drosophila GAP1 gene) (Yarwood et al., 2006). There are basically three Ras subtypes, H-, N- and K-Ras. By analyzing the structure and function of p120GAP and NF1, people showed that they have no preferences for the three subtypes (Ahmadian et al., 2003). Although the RasGAPs are widely expressed, they exhibit tissue-specific expression, suggesting that different RasGAPs display roles in different tissues. For example, NF1 only represses Ras activity in neuronal and myeloid cells (Le et al., 2007). GAP1m and RASA3 are both IP4 (1,3,4,5-tetrakis Inositol Phosphate) binding proteins, while GAP1m is highly expressed in brain, spleen and skeletal muscle, RASA3 is more abundant in brain, placenta and pancreas, indicating their different

regulation pattern (McNulty et al., 2001). In addition to tissue specific regulation, at the subcellular level, they also share distinct localizations, RASA3 is exclusively anchored at cell membrane due to its PH (Pleckstrin Homology) domain (Cozier et al., 2000), whereas GAP1m is predominately distributed in cytoplasm, suggesting that RASA3 is a more potent Ras activity suppressor in vivo since Ras is activated when couples to the GPCR at membrane (McNulty et al., 2001).

1.2.2 RASA3 and Ras GAP function

RASA3, Ras p21 protein activator 3, also named GTPase Activating Protein 1 IP4 Binding Protein or GAPIII, a member of the GAP1 family, is a negative regulator of Ras pathway. Human *RASA3* localizes at chromosome 13q34. RASA3 protein is 834 aa long, has two C2 domains, the essential GAP domain, PH and BTK (Bruton's Tyrosine Kinase) domain from N terminal to C terminal. RASA3 was first identified as a Ras inactivator in 1995 (Cullen et al., 1995). In this paper, they showed that RASA3 is a potent GAP on both Ras and Rap, while RASA3 exhibited greater deactivation ability on H-Ras and R-RAS than K-Ras in vitro. To serve its function as a dual GAP on Rap1 and Ras, the GAP domain and the arginine finger motif are required, rather than an asparagine thumb motif, which is different with other common Rap1 GAP proteins (Kupzig et al., 2009). The IP4 and calcium binding site on domain of RASA3 are localized at the C2 domains. IP4 is important for calcium homeostasis (Cullen et al., 1998), indicating RASA3 might play a role regulating calcium concentration. However, the constitutively active or dominant negative forms of RASA3 have no effect on calcium homeostasis, and even when RASA3 was 95% knocked down, there is no impact on calcium stability w/o stimulation (Walker et al., 2002). The PH domain helps RASA3 to localize at the membrane. Since RASA3 is a IP4 binding protein and IP4 is water soluble,

when cells are treated with IP4, RASA3 can be partially recruited to the cytosol, allowing a higher Ras activation. Accordingly, in IP3 kinase mutant mice, RASA3 might be constitutively membrane-associated caused by less IP4 production, suppressing Ras and ERK stimulation (Marechal et al., 2007).

In GH4ZR7 cells, D2SR-mediated inhibition of ERK1/2 signaling requires RASA3, suggesting its role in the dopamine system (Nafisi et al., 2008). The RasGAP1 family member NF1 mutations is reported responsible for the disturbed dopamine signaling in Neurofibromatosis Type 1 disorder (Diggs-Andrews et al., 2013). However, the role of RASA3 in dopamine neurons remains to be tested.

1.2.3 Correlation of RASA3 Down-regulation and PC12 Neurite Growth

As a model of dopamine neurons we have used PC12 cells. PC12 cell, derived from a pheochromocytoma of the rat adrenal medulla, was first cultured at 1976 (Greene et al., 1976). After 1 week of treatment with NGF (Nerve Growth Factor), PC12 cells stop proliferating and begin to extend neurites which is similar to the process found in sympathetic neurons. There are two kinds of NGF receptors, p75 and TrkA (Tyrosine Kinase Receptor A). Upon binding to NGF, the two receptors utilize numbers of pathways including ERK, cAMP, PI3K-AKT, PKC and a variety of other pathways to modulate IEGs (Immediate Early Gene) expression, leading to the PC12 cell differentiation. Acute and prolonged ERK activation is required for NGF-induced differentiation, acute activation is mainly mediated through Ras, while long term activation is mediated through Rap1 (Wu et al., 2001). PC12 cells synthesize and release dopamine (DA) and norepinephrine (NE), DA and NE concentrations can be altered by a number of chemicals. PC12 cells also express high level of Tyrosine Hydroxylase, DAT and Nurr1, a transcription factor for TH production

and synthesize and secrete dopamine, as well as noradrenaline. These above make PC12 cells a good model to study the dopamine signaling. NGF was reported to regulate Tyrosine Hydroxylase (TH) gene transcription through c-Fos, enhance the TH activity by modulating the phosphorylation of Ser31 and Ser40 (Gizang-Ginsberg et al., 1990; Anastasiadis et al., 1996). It was also reported that after 24 hrs of NGF treatment in PC12 cells, TH protein expression is increased (Obara et al., 2009).

In PC12 cells, after the cells were treated with NGF or cAMP, another differentiation inducer, the RASA3 RNA transcript level was decreased about 80%, while EGF (Epidermal Growth Factor), which does not trigger differentiation of these cells, did not have this effect. Since p120GAP was unchanged by NGF, this suggests that inhibition of RASA3 activity is required to facilitate the differentiation process (Iwashita et al., 2007). By overexpressing RASA3 in PC12 cells, the neurite growth was greatly reduced in a RASA3 expression level dependent manner. By replacing the Arginine finger, which is essential for RASA3 activity, a RASA3 loss-of-function mutant mouse was generated. All the homozygous mutant mice died at E12.5-E13.5, indicating the important role of RASA3 and RasGAP activity in development. The homozygous mutant mice dissected at E13.5 exhibited massive subcutaneous and intraparenchymal bleeding, especially in the brain, while heterozygous mutant mice were normal.

1.2.4 RASA3 Mutant Mice

The above studies indicate that RASA3 is an important negative regulator of Ras and Rap and plays roles in cell differentiation and development. It's not surprising that the point mutation of RASA3 might cause very severe diseases. Scat (Severe Combined Anemia and Thrombocytopenia) mice, an autosomal recessive mutation mouse arose on the BALB/c

mouse strain, is characterized by severe bleeding. At birth, the Scat mice are pale with intradermal petechiae and bruises on exposed surfaces. 22% of the Scat mice exhibit CNS bleeding (Peters et al., 1990). Scat mice experience two crises in their lifetime. The first crisis starts in utero, lasts till postnatal day 9 (P9), 10-15% of Scat mice would die after the first crisis. The surviving mice enter a remission phase wherein the disease phenotypes revert to normal. However, the remission period is short-lived, and is followed by a second crisis. Finally, 94% of the Scat mice die after the two crises. The Scat mice were identified two decades ago, only recently people found that the disease was caused by a RASA3 Gly125Val (between the two C2 domain) mutation (Blanc et al., 2012). Scat mice showed a delayed erythropoiesis and megakaryopoiesis in spleen. Knockdown of RASA3 in zebrafish leads to anemia and thrombocytopenia. RASA3 is down-regulated during reticulocyte maturation, which corresponds to the finding in PC12 cells that down-regulation of RASA3 is correlated with PC12 differentiation (Iwashita et al., 2007). By employing immunofluorescence studies of RASA3, it was found that RASA3 G125V was mis-localized in cytosol rather that cell surface, suggesting that RASA3 G125V is a loss-of-function mutation since RASA3 needs to anchor at the cell membrane to exert it Ras GAP function. Accordingly, in Scat red blood cells, more GTP bound Ras was found using Western Blotting, consistent with reduced RASA3 GAP function, which might lead to more active ERK signaling and a series of downstream pathways (Peters et al., 2013).

Another RASA3 mutant mouse, HLB mouse, which carries a RASA3 H794L mutation, was generated using ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis from C57BL/6 mice. His794 sits beside the BTK (Bruton's Tyrosine Kinase) domain, might also be important for RASA3 localization. HLB mice have a blood phenotype too, while they are healthier and survive to

adulthood.

1.3 Hypothesis

Based on the role of RASA3 as a potent Ras-ERK signaling inhibitor, and its essential role in D2SR induced ERK1/2 inactivation in pituitary cells, RASA3 might play a role in the brain dopamine system. We hypothesize that RASA3 plays a role at D2SR mediated negative regulation of dopamine signaling through a RASA3-Ras-ERK-TH pathway in dopamine neurons.

1.4 Approach

GPCR functions through G protein-dependent or -independent signaling. To explain the specificity of RASA3 for presynaptic D2SR, we hypothesize that the D2SR may directly bind to RASA3 in addition to that through Gi/o protein. To test this, we will utilize BRET2 assay. In order to effectively knock down RASA3 to study its function in neurons we will produce shRASA3 lentivirus, test its infection efficiency and RASA3 knockdown ability, then examine if lentivirus mediated RASA3 knockdown in GH4ZR7 cells can reverse the D2SR mediated ERK1/2 inhibition. As a model of dopamine neurons we will examine if RASA3 can regulate ERK1/2 signaling and TH expression in PC12 cells treated with NGF using RT-PCR and Western Blotting. To better understand the function of RASA3 in vivo, we will inject the shRASA3 lentivirus into rat SN or VTA, check the TH expression level using immunofluorescence. We will also examine TH expression in RASA3 mutant mice Scat and HLB mice. We suppose that the locomotor activity of these mice are impaired due to the RASA3 mutation which might lose its function of regulate D2SR signaling. Also, we will test the anxiety and depression level of these mice since D2R is involved in these behaviors. We will utilize BBK to test the locomotion, EPM and OF to test the anxiety level, TS and FS to

test the depression level. These studies will address the role of RASA3 in regulation of dopamine function.

Chapter II Materials and Methods

2.1 Materials

Apomorphine, quinpirole, TRH, ATP, anti-β-Actin, anti-RASA3 antibodies were from Sigma-Aldrich; Anti-pERK1/2, anti-Rabbit IgG HRP, was from Cell Signaling; anti-TH antibody and ECL Kit were from Millipore; Goat Anti-Mouse HRP was from Jackson ImmunoResearch; DMEM, F-10, RPMI1640, FBS, Horse Serum, Donkey and Goat serum were from Wisent; β-Actin, RASA3, DAT and TH PCR primers were synthesized from Integrated DNA Technologies; Trizol, chicken anti-GFP antibody, Goat anti-chicken 488 and Donkey anti-Rabbit 594 were from Life Sciences. DeepBlueC was from Perkin Elmer. M-Mulv, RNase Inhibitor and Taq Polymerase were from New England Biology. Scat and HLB mice were from the Jackson Lab. MagNA Lyser and MagNA Lyser Green Beads tubes were from Roche Applied Science. Victor^{3V} was from Perkin Elmer.

2.2 Methods

2.2.1 Cell Culture and Transfection

HEK293 (Human Embryonic Kidney) cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) with 10% FBS. GH4ZR7 (GH4C1 stably transfected with D2S) cells were cultured in HAM's F10 with 10% FBS. PC12 cells were cultured in RPMI1640 with 10% FBS and 5%HS. A modified calcium phosphate precipitation method is employed for transfection (Cold Spring Harbor, 2005). HEK293T cells were transfected using PEI method to produce lentivirus (Zaric et al., 2004). All the cell lines were cultured in a humidified 5% CO_2 at 37°C.

2.2.2 BRET2 Assay

HEK293 cells were seeded at 4 x 10^5 cells/well in 6-well plates the first day. The second day,

they were cotransfected with 0.2µg RASA3-GFP2 or GFP2-RASA3 with 0.2µg D2S-Rluc (or with 0.2µg Gαi3) per well using a modified calcium phosphate method. On the fourth day, cells were collected and aliquoted to a 96-well opaque white optic plate, agonists and DeepBlueC added, and signals for GFP2 and Rluc read at OD 515nM and 407nM respectively using the Victor ^{3}V optic plate reader. The BRET Ratio is calculated by the following formula:

(Sample GFP2 – Control GFP2) / (Sample Rluc- Control Rluc).

2.2.3 mRNA Extraction and RT-PCR

mRNA was extracted using the Trizol method (Chomczynski et al., 1995). The cells were washed twice with cold 1×PBS, and 1 ml Trizol / 35mm well was added, and pipetted 5 times. Lysates were transferred to a sterilized 1.5ml tube, and 0.2 ml chloroform added, samples vortexed vigorously for 15 seconds, incubated at room temperature for 2 min, centrifuged at 10,000g for 15 min at 4℃. The upper aqueous phase was transferred to a new tube, 0.5 ml isopropanol added, and incubated for 10min at room temperature and spun at 10,000g, 10min at 4℃. The supernatant was removed and the pellet washed with 75% ethanol, centrifuged at 10,000g, 10min at 4℃, ethanol removed, and the tube air dried for 10min at room temperature; the RNA pellet was dissolved in 30 µl ddH_2O. cDNA was synthesized using M-Mulv: the reaction contained 1.5ug RNA, 40 µM random primer, dNTP mix (2.5mM each), then ddH_2O was added to 16 µl, and incubated at 65℃ for 10 min, quick spin and put the tube on ice, add 2ul 10×RT PCR buffer, 1µl RNAse inhibitor and 1 µl M-Mulv Reverse Transcriptase, incubated at 42℃ for 1 hr, inactivated at 85℃ for 10 min, stored at -20℃.

PCR system (25µl system) was, cDNA 1.5ul, 5' Primer 0.5pM, 3' Primer 0.5pM,

10×PCR buffer 2.5μl, Taq 1μl, dNTP 0.2mM, add ddH_2O to 25μl,

PCR condition was,

	94℃	2min
cycles	94℃	20s
	Annealing	20s
	72℃	45s
	72℃	10min

The annealing temperatures for β-Actin, RASA3, DAT and TH were 60℃, 58℃, 58℃, 57℃ respectively, cycle numbers were 25, 29, 29, 29 respectively.

2.2.4 Western Blotting

Cells were lysed in 50 mM Tris pH7.4, 100 mM NaCl, 1 mM EDTA, 1% Triton X100 and protease inhibitor (Aprotinin 2μg/ml; Leupeptin 2μg/ml, PMSF 1mM). 600ul lysate buffer/35mm well. Protein was quantified using the Bradford method. Equal amount of protein sample was loaded for each lane, 200 volt electrophoresis till the dye runs out of the gel. Transfer the protein to PVDF membrane, 30V over night or 150mA 1hr. After transferring, PVDF membrane was blocked with Block Buffer (50mM Tris, pH7.4, 150mM NaCl, 0.1% Tween 20, 5% Low Fat Milk) for 1hr at room temperature or overnight at 4℃. Dilution for primary antibodies were: β-Actin 1:5,000, RASA3 1:500, TH 1:1,000, pERK 1:1,000, antibodies were diluted in Block Buffer, add diluted primary to PVDF membrane, room temperature for 1 hr and washed 3 x 5 min in TBST buffer (Tris 50mM, pH7.4, NaCl 150mM, Tween20 0.1%). For Secondary antibodies, dilution ratios were: anti-Mouse HRP, 1:10,000, anti-Rabbit 1:2,000, secondary antibodies were diluted in Block Buffer, diluted secondary antibody was added to PVDF membrane at room temperature for 1 hr, washed 3 5 min in TBST buffer. The PVDF membrane was developed using the ECL kit and exposed to

film.

2.2.5 Lentivirus Package and Infection

Nonspecific short hairpin sequence and shRASA3 sequence were inserted into pLVX-sh2 vector (Clontech) separately. Human D2SR cDNA was subcloned between MluI and SpeI sites in pWPXLd vector (Addgene).

Cotransfect HEK293T using lentiviral vectors, pMD2.G and psPAX2 (Addgene). After 14 hrs, change the medium to fresh culture medium. After 48 hrs, collect the medium, centrifuge 2000 rpm, 5 min at room temperature to remove cell debris. Filter the supernatant using 0.45μm cellulose acetate (low protein binding) filter. Store the supernatant at 4°C overnight. Place 4 ml 20% sucrose (1 x PBS) into Beckman ultra clear centrifuge tube; transfer 23 ml viral supernatant to the tube. Concentrate by ultracentrifugation at 20,000 rpm for 2 hours at 6°C in a Beckman SW32 Ti swinging rotor. Discard the supernatant and dry the pellet under biosafety hood. Resuspend the pellet using 300 μl 1 x PBS/1%BSA. Aliquot the suspension, 20 μl/tube, store the virus in -80°C. Titer the short hairpin lentiviruses by counting GFP positive cells two days after infection. Titer the hD2SR expression lentivirus using QuickTiter FIV Lentivirus Quantitation Kit (Cell Biolabs).

Seed the cells at a density of $3x10^4/cm^2$. Infect the cells using short hairpin lentiviruses at MOI 10. After 7 days, infect the cells using hD2SR lentivirus at MOI 10. 14 hrs after each infection, change the medium to fresh culture medium.

2.2.6 Microinjection, Perfusion and Immunofluorescence

Male Sprague Dawley rat weighing 180-220 g when ordered were housed in Biohazard room for 1 week prior to injection. On the day of injection, rats were given first dose of Buprenorphine (1mg/Kg)1 hour prior to starting injection. The rat was placed inside the

Plexiglass box, and the IsoFluorane flow rate set to 5%, monitor the rat until it is non-responsive. The rat placed on a platform and IsoFluorane Flow rate set to 2%. The head was shaved, eye ointment and ear gel applied to protect the rat, and 5 ml saline injected subcutaneously for hydration. The shaved region was cleaned using water, soap and water again, swiped dry, a few drops of Chlorhexidine Alcohol applied and swiped. The rat was placed on the stereotaxic instrument, set the Isoflorane to 2% and the nose clamp and ear bars secured. A vertical incision was made to expose the skull and identify Bregma and lambda. The coordinates (AP,-5.8;ML,±2;DV,+8.2mm) were marked and using the drill, slowly the skull was removed at the two marks. The syringe was loaded with lentivirus, slowly lowered until the needle reaches the coordinate, and the pump started at infusion rate set to 0.2ul/min, total amount 1 µl. The needle was left in place for another 5 min, and then withdrawn slowly. The procedure was repeated on the other side of the brain, the skin suture and the rat placed in a cage in the incubator with food and water. After 30 min, when the rat recovered, it was placed in the biohazard room for 2 weeks. The rats were then perfused with 4% PFA and the brains dissected and placed in PFA. The PFA was changed to 20% sucrose the next day, changed again once a day for 3 days. The brain was frozen with methylbutane at around -40℃ and stored at -80℃, then sectioned on microtome to thickness of 25µm for rats, section thickness 20µm for mice.

The sections were washed with 1×PBS once, air-dried, blocked for 1hr at RT using 1×PBS, 1% BSA, 0.1% Triton X100, 3% Donkey Serum, 3% Goat Serum. They were washed 5 min× 3 using 1×PBS. The sections were incubated with primary antibodies, Chicken anti-GFP 1:2,000 or Rabbit anti-TH 1:500, diluted in Blocking buffer, at RT overnight and washed 3 times using 1×PBS. Then the sections were incubated with secondary antibodies, Goat anti

Chicken 488 (1/200) or Donkey anti Rabbit 594 (1/200) at RT for 2hr, washed 3 times using 1×PBS, air dried for 20 min, mounted and placed at RT for 20min, then transferred to -20℃.

2.2.7 Drug Treatment (Apo, TRH for GH4ZR7. NGF for PC12)

For GH4ZR7 cells the culture medium was changed to DMEM without serum, and after 1 hr, 2.5 μM Apo was added for 7 min followed by 0.5 μM TRH for 7min, and the cells were collected. For PC12 cells the culture medium was changed to DMEM+2%FBS, and 24 hrs later NGF was added to a final concentration of 20 ng/ml, and cells collected at different time points.

2.2.8 Substantial Nigra dissection and protein extraction

On a bed of wet ice, rodent brain matrix (coronal sections segregated 1 mm apart) was chilled, and in a separate container labeled MagNA Lyser Green tubes were place onto dry ice. After positioning the brain into the matrix, insert cold razor blades into the cooled brain, beginning from bregma area, continue inserting cold razor blades with each 1 mm section until arriving midway through the pons, staggering the placement of each subsequent razor blade so as to facilitate easier removal of each individual blade. When pulling out the razors, we looked for the start of the hippocampus. When it has completely wrapped around the midbrain, begin to take sections, cut the darker area and place the dissected tissue into the pre-cooled green tube (Salvator et al., 2012). RIPA buffer (Tris-HCl: 50 mM pH 7.4, NP-40: 1%, Na-deoxycholate: 0.25% , NaCl: 150 mM, EDTA: 1 mM, PMSF: 1 mM, Aprotinin, leupeptin 2μg/ml each, Na3VO4: 1 mM, NaF: 1 mM) was added to MagNA Lyser green tubes (1ml/200mg tissue). Tubes were centrifuged 6,500 rpm, 30 sec in the MagNA Lyser, cooled 10 sec on ice, then another 6,500 rpm, 30 sec. Centrifuged 1hr at 10,000g at 4℃. The

supernatant was recovered, quantified with Bradford method, and 5×SDS loading buffer added to 1x.

2.2.9 Behavior tests

Mice were housed with a 12-hr light/dark cycle. Tests started when the mice were around 8 weeks. 2 days before test, tags were placed on the cages to ensure that no bedding change occurred during the behavior test period. 1 hr before tests, the mice were taken to the test room, the equipment set up, the light to illumination set to 100Lux, the noise level set at 70dB. Ethovision8 monitoring system was used to track and record the behaviors. BBK was always the first test, then EPM, OF, TS and FS was done at least every the other day.

2.2.9.1 Beam Break

The mice were placed into the new cages, the cages were covered with a metal grate and plastic lid to mimic the original cage and tracking begun. Sample duration was set at 5 min and 24 samples collected, totaling 2 hrs.

2.2.9.2 Elevated Plus Maze

The maze was 74 cm high off the floor, arms were 75 cm long and 6 cm wide, walls were 20cm high. The mouse was placed at the center of the maze and tracked for 10 min.

2.2.9.3 Open Field

The box measured 45cm×45cm×45cm, and the mouse was placed at the corner of the box and tracked for 10 min.

2.2.9.4 Tail Suspension

The resolution was set to 200 msec and graph history to 40 sec, movement upper threshold was 50, lower threshold was 3, start trigger was 95. Each block was set at 30 sec, with 12 blocks, total 6min. The tail was taped to the metal bar, making sure that the ventral side of

the mouse was facing the wall.

2.2.9.5 Forced Swim

The plastic cylinder is 22cm diameter×37cm high, filled with about 4 liters of water pre-warmed to 25℃-30℃. The water was changed between mice and the test was performed under red lighting. The test was 6 min long.

2.2.10 Statistical Analysis

The mean and standard errors of experiments were calculated, and statistical significance was determined using T test (two tailed, paired).

Chapter III Results

3.1 Weak interaction between D2SR and RASA3 using BRET2 assay

BRET2 assay was used to test the interaction between D2SR and RASA3 since this assay has been used to detect interactions between GPCRs and G-proteins (Audet et al., 2008; Marulloet al., 2007). BRET2 occurs when light generated by metabolism of Deep Blue C by renilla luciferase (**rLuc**) is transferred to GFP2 when the proteins localized within 10 nm of each other. HEK-293 cells were cotransfected with D2SR-Rluc and RASA3-GFP2, and after adding DeepBlueC no direct interaction detected by reading BRET2 signal (Figure 1). The interaction of RASA3 and Gi3 is greatly enhanced after Apo/TRH treatment in GH4ZR7 cells, indicating that receptor activation might have an impact on the RASA3 and D2SR interaction (Nafisi et al., 2008). Since HEK-293 cells do not have TRHR, we used ATP instead to activate the ERK1/2 pathway in HEK293 cells through activation of P2Y purinergic receptors. When treated with ATP and Apo, still no interaction was detected. We next examined if Gi3 would help to form a tricomplex D2SR-Gi3-RASA3 by cotransfecting pcDNAGi3, data showed that with drug treatment or not, there was no positive BRET2 signal detected. GFP2-TNFAIP8 and RlucGi1wt91, an interaction previously identified in the lab, was used as a positive control (Laliberté et al., 2010). D2S-Rluc single transfection was used a negative control. The functionalities of RASA3-GFP2 and D2SR-Rluc were confirmed by the positive signals between RASA3-GFP2 and RlucGi3QL, D2SR-Rluc and 5HT1A-GFP2. It is possible that the positive interaction between RASA3 and D2SR might be covered by the GFP2-tagged to the C-terminal of RASA3. To avoid that, GFP2-RASA3 with the GFP2 tagged at the N-terminal was constructed, and a very weak positive signal was detected (Figure 2).

Fig 1

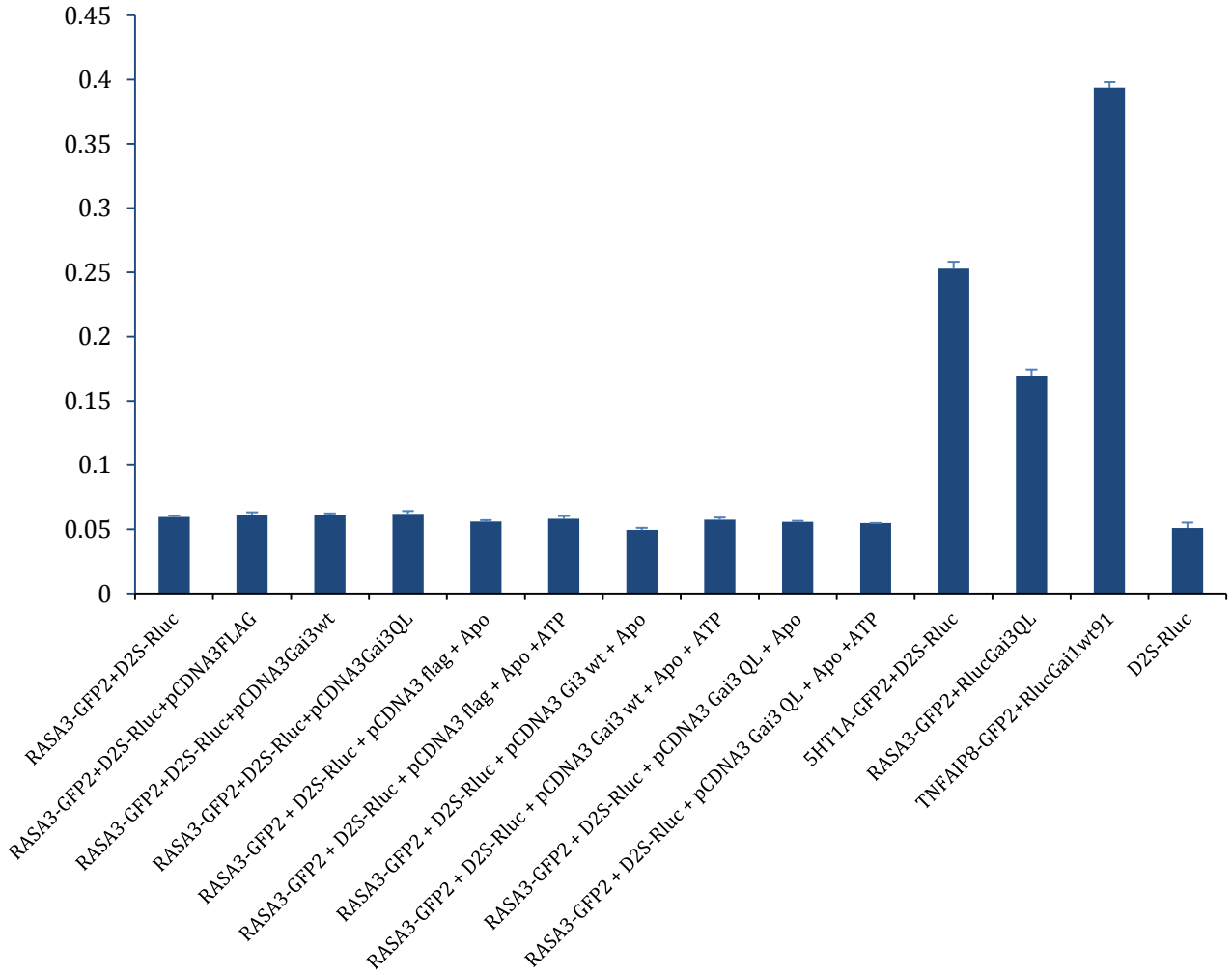


Figure 1. No direct interaction between D2SR and RASA3 when GFP was tagged at the C terminal of RASA3. HEK293 cells were cotransfected with 0.2ug RASA3-GFP2, 0.2ug D2S-Rluc and 0.2ug Gαi3 constructs. Cells were treated with 1uM Apo and 100 uM ATP. Data show that with or without Gαi3 construct or drug treatment, there is no positive signal detected compared to control. Results are representative of three independent experiments.

Fig 2

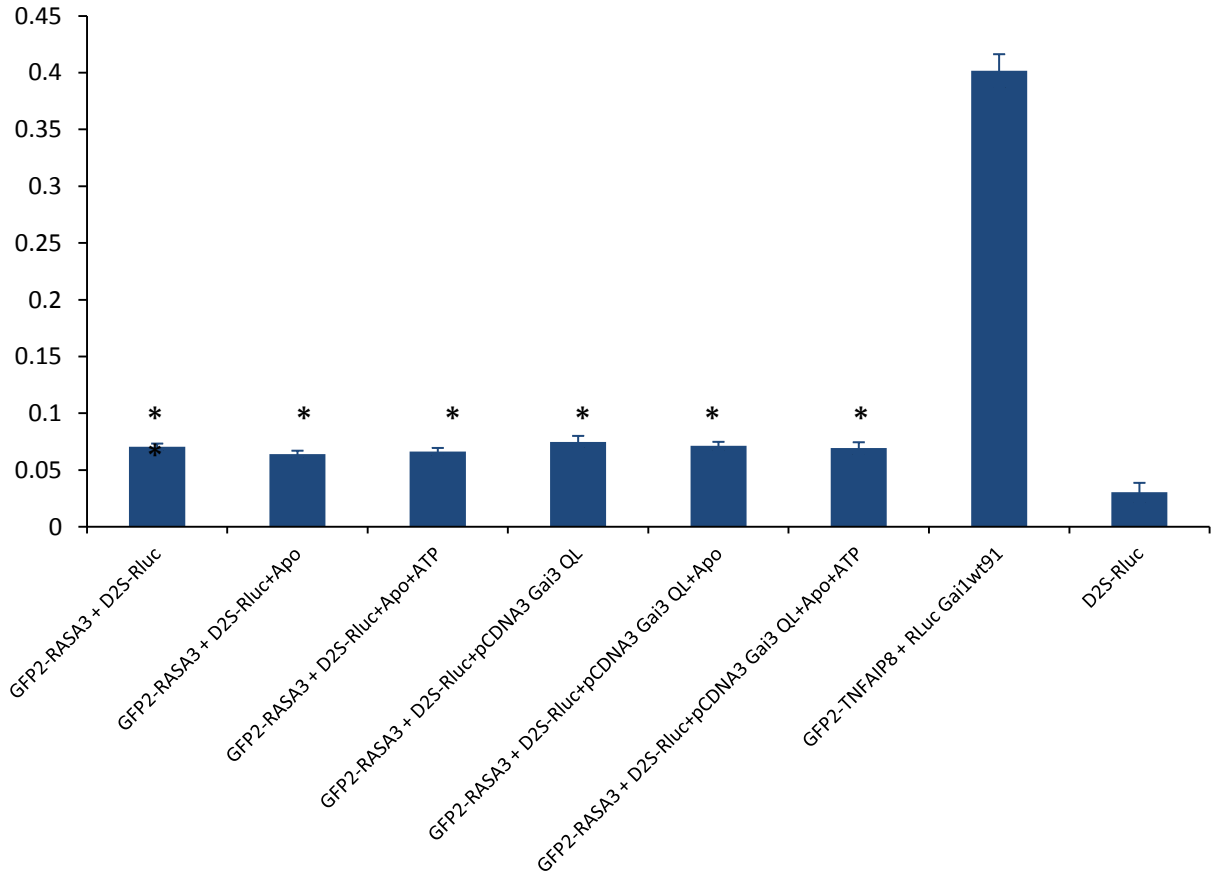


Figure 2. Direct interaction detected between D2SR and RASA3 when GFP was tagged at the N terminal of RASA3. HEK cells were cotransfected with 0.2ug GFP2-RASA3, 0.2ug D2S-Rluc and 0.2ug Gαi3 construct. Cells were treated with 1 uM Apo and 100 uM ATP. Data shows that w/o Gαi3 construct, w/o drug treatment, there were positive signals detected compared to control (lane 8), while the signals were very weak (<0.1). Results are representative of three independent experiments; $p < 0.05$ compared to control (D2S-luc) by ANOVA with Dunnett's post-test.

3.2 shRASA3 lentivirus effectively knock down RASA3 in GH4ZR7 and PC12 cells

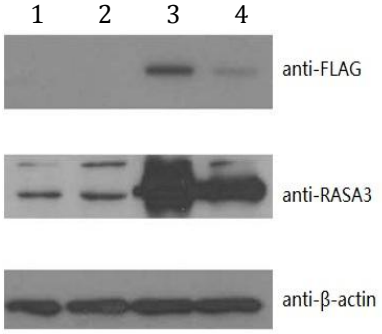
To test the role of RASA3 in vivo, we designed the siRNA sequence based on the rat RASA3 sequence, the sense and antisense were as follows:

RASA3shS 5' GATCC **AGTATGCAGATGCCGTAAA** TTCAAGAGA TTTACGGCATCTGCATACTTC TTTTTT ACGCGT G 3'

RASA3shAS 5' AATTC ACGCGT AAAAAA GTATGCAGATGCCGTAAA TCTCTTGAA **TTTACGGCATCTGCATACT** G 3'

Annealed sense and antisense sequence was inserted into the pLVX-sh2 vector from Clontech. Flag-RASA3 with pLVX-RASA3shRNA or pLVX-NonSpecific plasmids were cotransfected at (0.5μg+0.5μg)/well in a 6-well plate. Cells were lysed after 48 hrs of transfection. We did observe a strong knockdown of Flag-RASA3 and total RASA3 in cells transfected with Flag-RASA3 (Figure 3.a). There was not much change in endogenous RASA3 (Figure 3.a, lanes 1 and 2), as the HEK293 cells express human RASA3 that may be resistant to rat RASA3 shRNA (12/19 mismatches in the rat siRNA compared to the human RASA3 sequence). After the lentivirus was packaged, we tested the infection efficiency by adding the lentivirus to GH4ZR7 cells at an MOI of 10. The infection rate is nearly 100% by checking the GFP signal, which is coexpressed from the pLVX-sh2 vector (Figure 3.b). RASA3 protein expression level in rat GH4ZR7 after 7 days lentivirus treatment and rat PC12 cells after 14 days lentivirus treatment were greatly reduced (Figures 3.c and 3.d).

Fig 3.a



1, pLVX-NS; 2, pLVX-shRASA3; 3, Flag-RASA3+pLVX-NS; 4, Flag-RASA3+pLVX-shRASA3

Fig 3.b

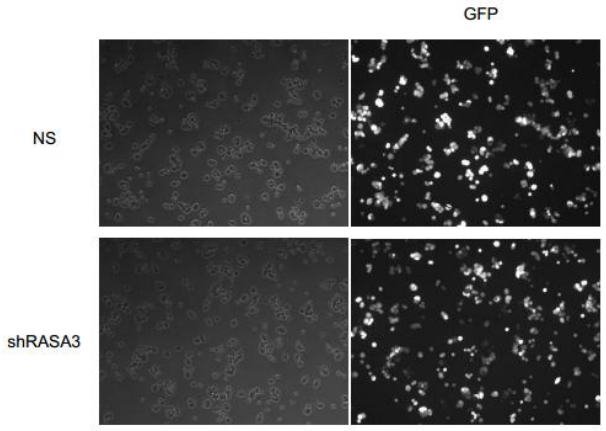


Fig 3.c

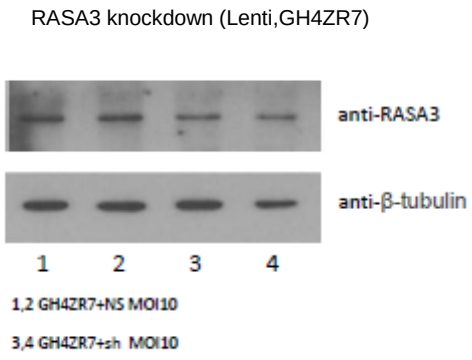


Fig 3.d

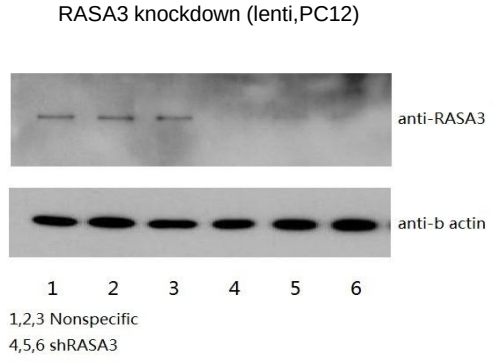


Figure 3. Lentivirus mediated RASA3 knockdown. a. Plasmid-based RASA3 knockdown in HEK293 cells. Flag-RASA3 expression was efficiently reduced in HEK293 cells cotransfected with shRASA3 construct compared to siRNA control (NS); b, Lentivirus efficiently infects GH4ZR7 cells; GH4ZR7 cells were infected with MOI 10 of siRASA3 or control (NS) virus for 7 days. c,d, in GH4ZR7 and PC12 cells, endogeneous RASA3 was reduced when treated with siRASA3 lentivirus compared to control (NS), MOI 10 (30% RASA3 reduction for GH4ZR7 cells, nearly 100% reduction for PC12 cells). 7 days for GH4ZR7, 14 days for PC12. Results are representative of three independent experiments.

3.3 Knockdown of RASA3 can partially reverse the D2SR induced ERK1/2 inhibition

By stimulating D2SR receptor in GH4ZR7 cells, the TRH-induced ERK1/2 signaling can be inhibited. The intracellular downstream effector, RASA3, was identified by yeast two hybridization and pull-down assay. Initial data showed that RASA3 antisense can reverse the inhibition(Nafisi et al., 2008). Here we showed that by partially knocking down RASA3 using shRASA3 lentivirus, the inhibited ERK1/2 activation can be partially reversed.

Figure 4.

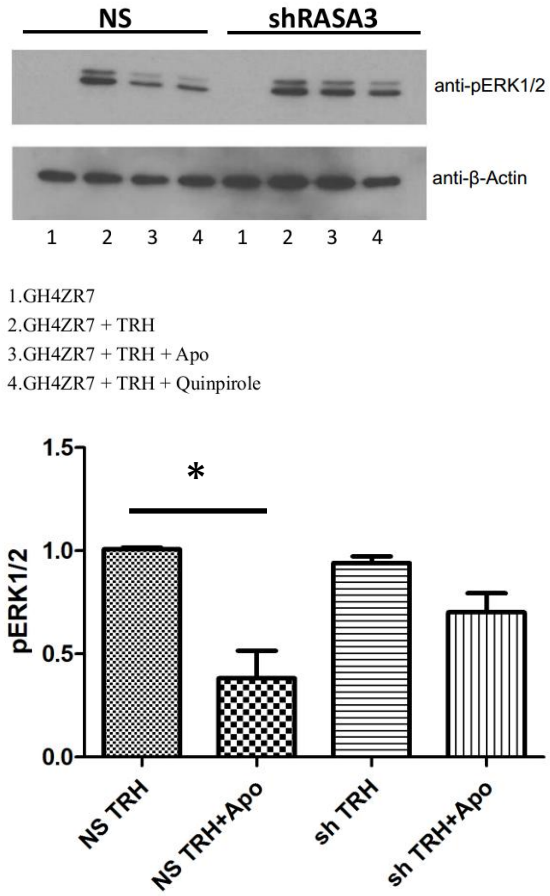


Figure 4. Knockdown of RASA3 in GH4ZR7 can partially reverse the D2SR induced ERK1/2 inhibition. Cells were pretreated with 2.5 μM apomorphine (Apo) or 10 μM quinpirole for 7 min followed by addition of 0.5 μM TRH for 7 minutes, as indicated. Western blot analysis of lysates was done using specific antibody against phospho-ERK1/2 (1/1000). Membranes were reprobed with β-actin antibody (1/5000) as a loading control. Representative blots from GH4ZR7 infected with Nonspecific and shRASA3 lentivirus were shown. Statistical data from three independent experiments were presented as mean±S.E. *,$p<0.05$

3.4 RASA3 knockdown has no effect on basal TH, Nurr1, DAT mRNA level in PC12 cells

Since RASA3 overexpression in PC12 cells can reduce NGF-induced ERK-mediated neurite outgrowth, we tested whether knockdown of RASA3 might enhance expression of dopamine- and ERK1/2-regulated genes. We examined the mRNA levels of RASA3, TH, Nurr1, and DAT using RT-PCR after RASA3 knockdown. Whereas RASA3 RNA levels was reduced, TH, Nurr1, DAT mRNA did not change after RASA3 knockdown (Figure 5.a). From the previous data, we know that RASA3 can be recruited when D2SR was activated. We reasoned that upon expression of D2SR and its activation by endogenous dopamine secreted from PC12 cells, RASA3 may have greater basal activity. However, upon coinfection of PC12 cells with D2SR expression lentivirus, there was still no change in TH and DAT mRNA levels (Figure 5.b).

Fig5.a

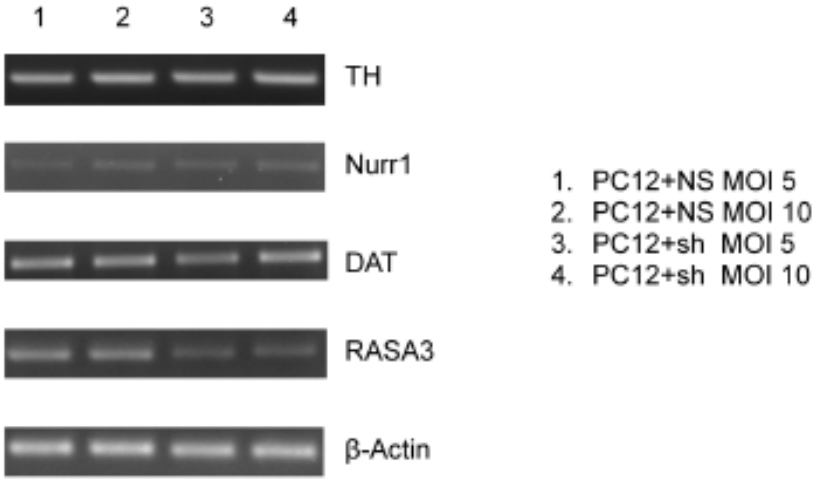


Fig5.b

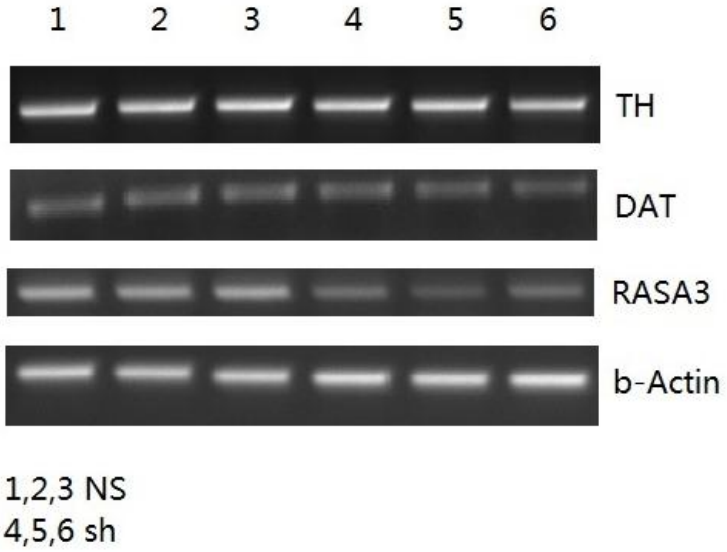


Figure 5. Effect of knockdown of RASA3 in PC12 cells on RNA levels. a, Lentivirus knockdown of RASA3 (sh) in PC12 cells had no effect on TH, Nurr1, DAT mRNA level compared to control (NS). b, Lentivirus knockdown of RASA3 in PC12 cells co-infected with D2SR lentivirus (MOI 10). RASA3 knockdown had no effect on TH, DAT mRNA level when D2SR was present. Cells were infected for 14 d, representative of 3 independent experiments.

3.5 ERK1/2 signaling is enhanced, TH expression is increased after RASA3 knockdown when D2SR is present in PC12 cells treated with NGF

We predicted that, based on its role in GH4ZR7 cells, RASA3 may be important for inhibition of ERK1/2 activation in PC12 cells, and this might be more sensitive to RASA3 depletion than changes in RNA levels. We then tested whether ERK1/2 signaling was enhanced in PC12 cells treated with 20 ng/ml NGF at different time points when D2SR was expressed. After 1 hr and 24 hr of NGF treatment, ERK1/2 signaling was greatly enhanced. After 24 hr NGF treatment, TH expression was increased (Figure 6.a). Conversely, when D2SR was not expressed, these effects were lost (Figure 6.b). These results show that RASA3 mediates inhibition of NGF-stimulated ERK1/2 activation only when RASA3 is activated in the presence of D2SR. Interestingly, TH expression in control cells was increased by NGF when D2SR was absent but not when it was present, suggesting that D2SR exerts tonic inhibition on TH expression. Similarly in control cells, basal and NGF-induced ERK1/2 appeared much weaker in cells expressing D2SR, also consistent with tonic activation of the D2SR-RASA3 pathway.

Fig 6.a

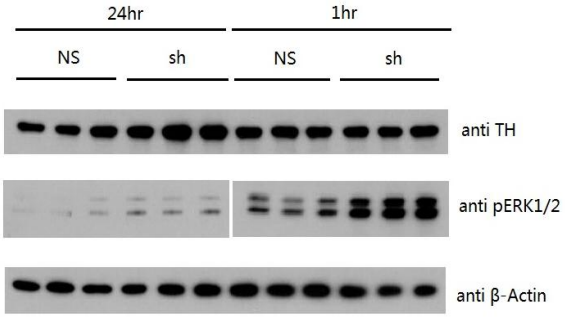


Fig 6.b

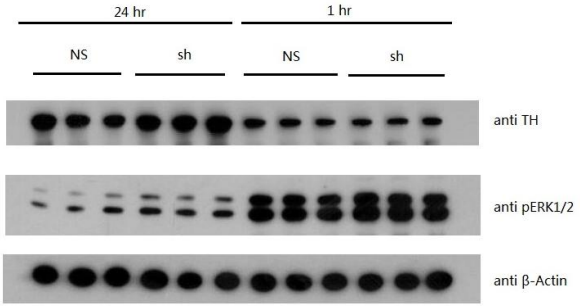


Fig 6.c

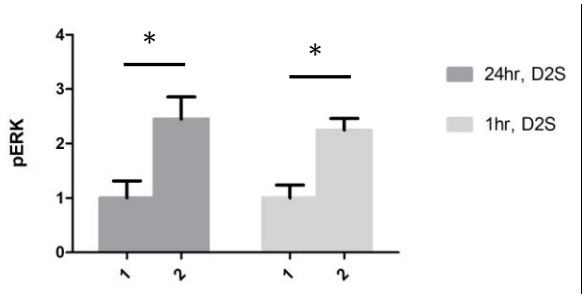


Fig 6.d

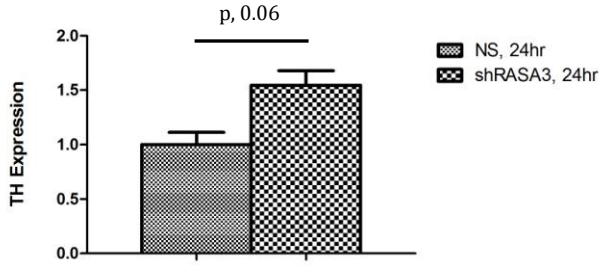


Figure 6 ERK1/2 signaling was enhanced after 1hr and 24hr NGF treatment when D2SR was present. TH expression was increased after 24hrs NGF treatment, while no difference after 1 hr (6.a). When there was no D2SR, no ERK1/2 signaling or TH expression was enhanced after 1hr or 24hr NGF treatment (6.b.).

Fig 6.c, statistical results of pERK activation after 24hr and 1hr when D2SR is present (1,NS; 2,shRASA3); *p<0.05 by t-test.

Fig 6.d, statistical results of TH expression after 24hrs when D2SR is present. Representative of 3 independent experiments.

3.6 Lentivirus infection in vivo

To test whether RASA3 knockdown can regulate TH expression in vivo, we microinjected shRASA3 lentivirus into the rat SN (Figure 7). Unfortunately, we found that the lentivirus seldom enters the TH-positive neurons but largely enters what appear to be astrocytes, which may be due to the lentivirus infection preference.

3.7 TH expression level is higher in SN of Scat and HLB mice

Since RASA3 G125V is a loss-of-function mutation, and RASA3 is involved in dopamine signaling we predicted that TH expression level in Scat mice might be different from that in wild type mice. So far, we don't have Scat homozygotes for tissue extraction since most die before birth, so we used Scat heterozygote mice for tissue extraction. TH expression level of HLB mice (wild-type and homozygous), which does not have the anemia disorder of Scat mice, was also tested. SN tissues from wild type Balb/c and Scat Hetero mice, wild type C57Bl/6 and HLB mice were extracted using MagNA Lyzer. Western Blotting result shows that TH expression level in SN of Scat and HLB mice were slightly higher (Figure 8). These data need to be further confirmed.

Fig.7

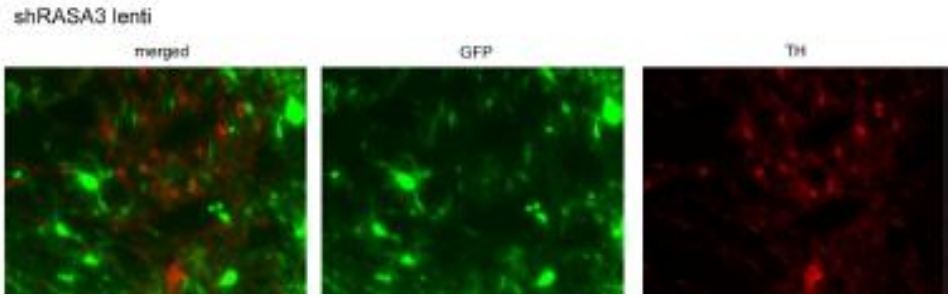


Figure 7. shRASA3 lentivirus infection in rat SN. Rats were sacrificed 14 days after microinjection into SN. GFP (488, green) was used to identify lentivirus-infected cells, TH (594,red) was used to identify dopaminergic neurons. Colocalization (yellow) was seen in very few cells.

Fig.8

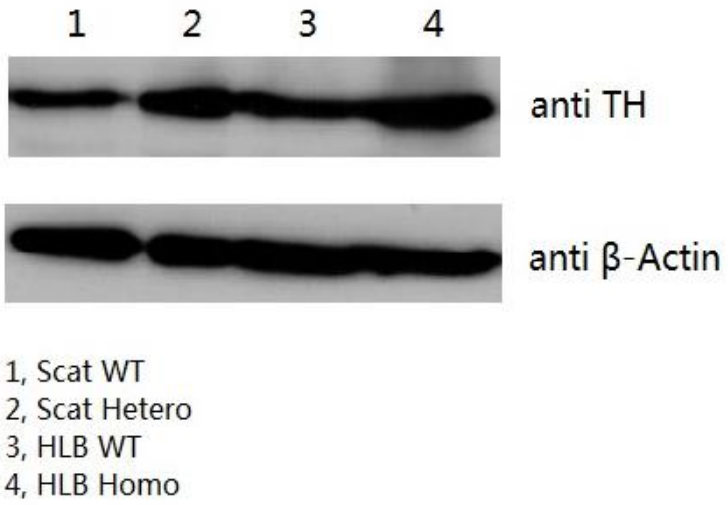


Figure 8. TH level in SN of RASA3 mutant mice is higher. SN was dissected from one mouse each and protein extracted by MagNA lyzer. Western Blot analysis of lysates was done using specific TH antibody (1/1000), β-Actin staining (1/5,000) was used as control. Comparing to wild type, the TH levels in RASA3 mutant mice were higher.

3.8 No locomotion impairment in Scat or HLB mice

BBK behavior test was utilized to test the locomotion activities of Scat and HLB mice. Over the 2-h test, locomotion activity decreased for both wild-type and mutant mice reflecting adaptation to the new environment (Figure 9). Neither Scat nor HLB mice showed any difference in locomotion activity compared to wild type (Figure 9.a and 9.b).

3.9 No anxiety level difference detected in Scat or HLB mice

EPM and OF behavior tests were utilized to test the anxiety level of Scat and HLB mice. The tests last 10 min. For EPM test, time spent in the open arms was calculated. For OF test, the time HLB mice spent in the larger center was accumulated, the time Scat mice spent in the 4 corners was determined since they seldom enter the center. Neither Scat nor HLB mice showed anxiety level differences comparing to wild type (Figures 10 a,b,c,d). However, there appeared to be some increase in anxiety in the EPM for HLB homozygous mice that may be statistically significant if more mice are tested.

3.10 No depression level difference detected in Scat or HLB mice

TS and FS behavior tests were utilized to test the depression level of Scat and HLB mice. The tests last 6 min. For the two tests, immobility time of the mice was measured to examine the depression level. Neither Scat nor HLB mice showed depression level differences comparing to wild type (Figures 11 a,b,c,d). Again since the number of mice was low, it may be that increased number would reveal a significant effect, particularly in the HLB mice, suggesting increased immobility in FST.

Fig 9.a

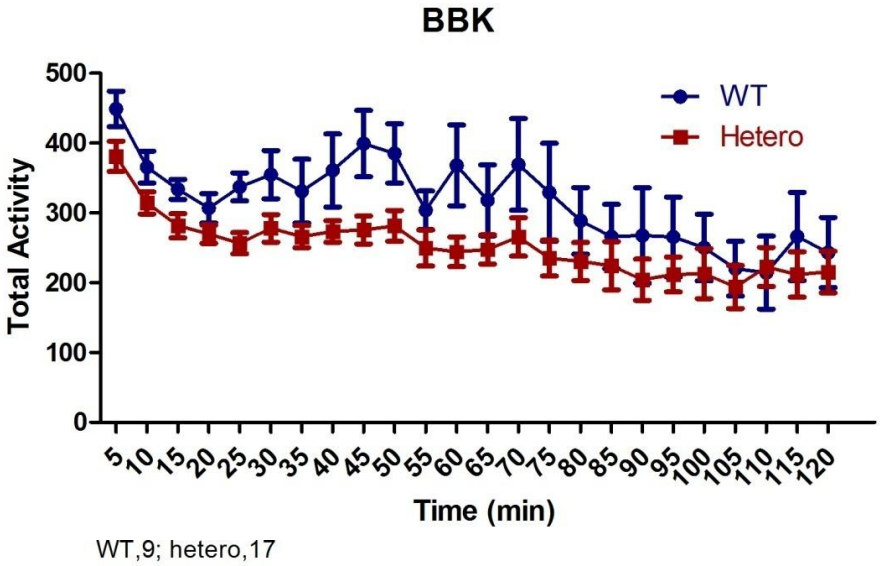


Fig 9.b

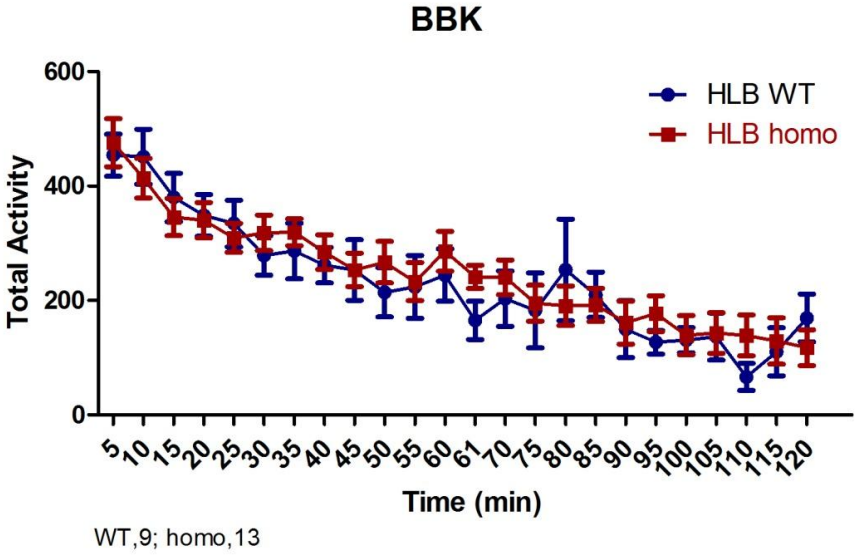


Figure 9. No locomotion impairment in BBK test. a, Scat heterozygous (Hetero, n=17) vs. wild-type (WT, n=9); b. HLB homozygous (Homo, n=13) vs. wild-type (WT, n=9).

Fig 10.a

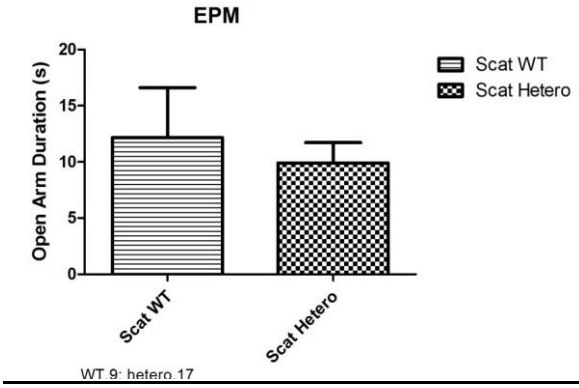


Fig 10.b

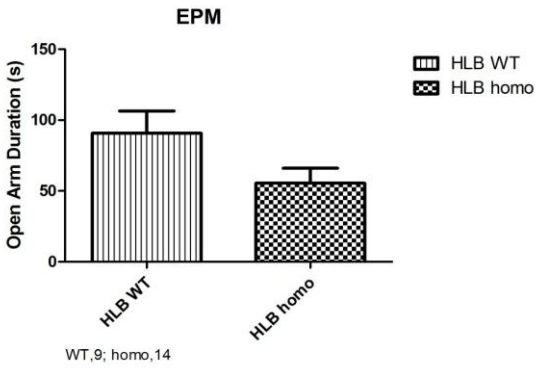


Fig 10.c

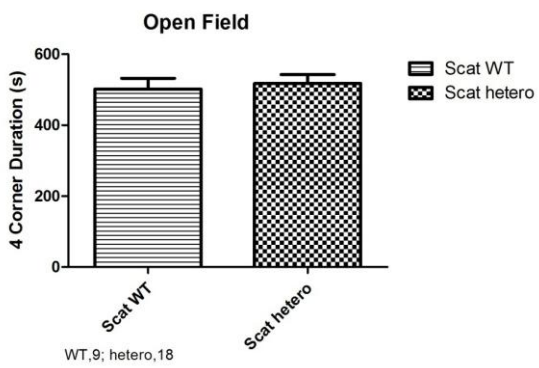


Fig 10.d

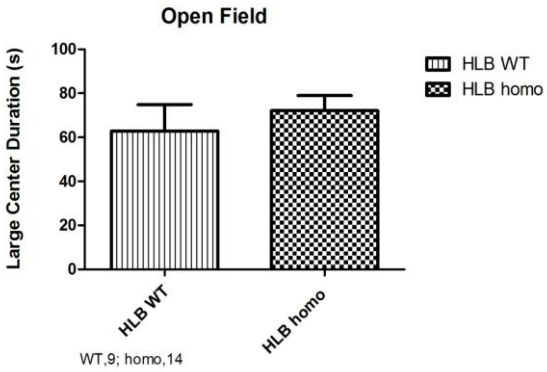


Figure 10 a,b. EPM test. No significant difference in anxiety was found. a. Scat Hetero vs. WT; b. HLB homo vs. WT

Figure 10 c,d. Open Field Test. No anxiety level difference was found. c. Scat Hetero vs. WT; d. HLB homo vs. WT

Fig11.a

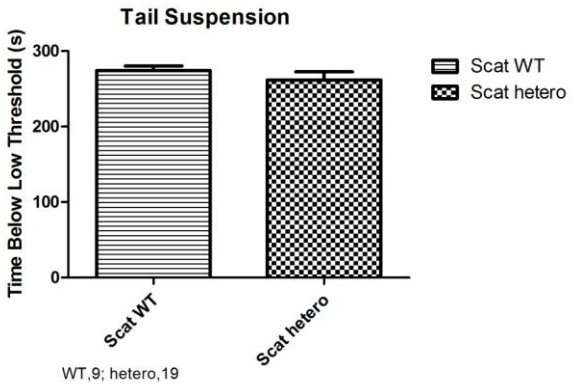


Fig11.b

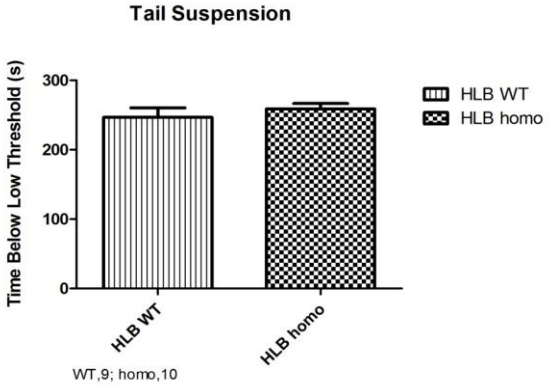


Fig 11.c

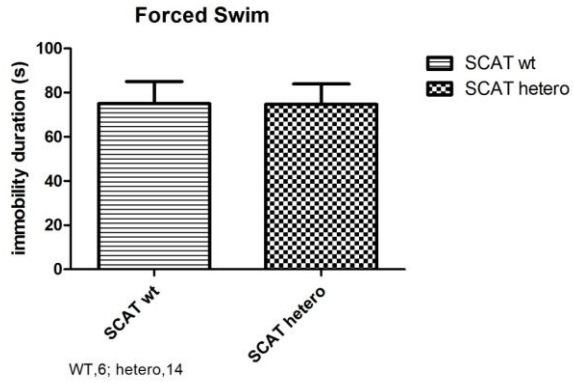


Fig 11.d

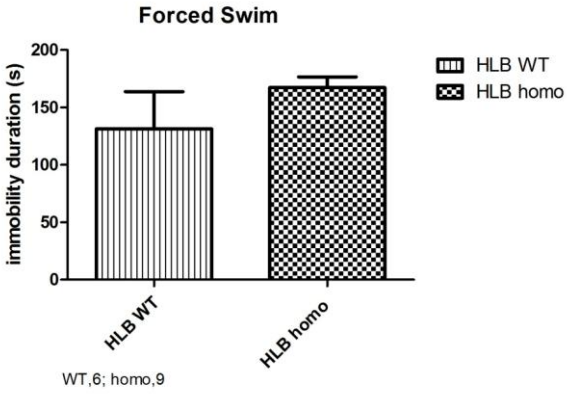


Figure 11 a,b. Tail suspension test. No depression level difference detected. a. Scat Hetero vs. WT; b. HLB homo vs. WT.

Figure 11 c,d. Forced swim test. No depression level difference were detected. c. Scat Hetero vs. WT; d. HLB homo vs. WT.

Chapter IV Discussion and Conclusion

4.1 D2SR as a regulator of dopamine synthesis

Dopamine is a major neuromodulator of the central nervous system. Dysfunction of dopamine signaling leads to neurological and psychotic disorders like PD, schizophrenia and drug abuse. Thereby the understanding of dopamine synthesis and release will facilitate drug design for future treatment. Most neurotransmitters inhibit their own release through autoreceptors through negative feedback. Dopamine system also utilizes this autoreceptor pathway to regulate dopamine transduction. The D2SR is now believed to be the main presynaptic receptor expressed on dopamine neuron dendrites, cell body and terminals (Khan et al., 1998; Usiello et al., 2000). However, the physiological function and mechanism of this presynaptic inhibition is still not well understood.

D2SR regulate dopamine synthesis and reduces the expression of Tyrosine Hydroxylase, the rate limiting enzyme of dopamine production (Harada et al., 1996). Because ERK1/2 activates tyrosine hydroxylase directly by phosphorylation on Ser31 and can increase TH transcription by phosphorylation of its transcriptional activator Nurr1 (Jacobsen et al., 2008; Lindgren et al., 2002), it is logical to hypothesize that ERK1/2 signaling can regulate dopamine synthesis in vivo. In neuroendocrine pituitary cells, D2SR but not D2LR inhibits TRH (Thyrotropin Releasing Hormone) induced ERK1/2 signaling, the similar ERK1/2 inhibition was also observed in striatum neurons (Banihashemi et al., 2002; Van-Ham et al., 2007). RASA3, a potent Ras and Rap inhibitor, was required for this inhibition (Nafisi et al., 2008), indicating that D2SR regulation of TH might be mediated through RASA3-Ras-ERK1/2-TH pathway.

4.2 D2SR coupling to RASA3

In order to address how RASA3 can be activated by D2SR but not D2LR, we examined whether RASA3 might directly interact with D2SR. RASA3 is constitutively anchored at cell membrane, giving a possibility that D2SR can directly recruit RASA3 to Giα3 to induce its activation. Using the N-terminal but not with C-terminal GFP-tagged RASA3, a very weak but consistent interaction with the D2S receptor was detected compared to control. In BRET2 assay, GFP and Rluc were tagged to the proteins, while GFP and Rluc are both around 30KDa, which might bury the domains required for interaction. This result suggests that the C-terminal GFP tag interferes with the D2S receptor interaction. However, there was strong interaction between RASA3-GFP and Gai3, and D2SR-rLuc and 5-HT1AR suggesting that these proteins remain functional with the tagged domains. Thus there may be a complex of D2S receptor-RASA3-Gai3. While there is only very weak signal detected using BRET assay, it could be that under optimal conditions there is a strong interaction between D2S receptor and RASA3. First, the BRET test was done in HEK cells, while the D2SR-ERK1/2 inhibition was found in GH4ZR7 cells. Second, the stimulation was different. To mimic the condition in GH4ZR7 cells, we treated HEK cells with ATP to activate ERK1/2, thereby the intracellular signaling might be different, leading to a weak RASA3-D2SR interaction. It was not possible test BRET in GH4ZR7 cells since the transfection efficiency is too low for a detectable BRET signal. To explain the G-protein specificity of D2S-RASA3 signaling to ERK1/2, it may be that D2SR is more efficient to activate Gai3 signaling and recruit RASA3 to activate it. There is some evidence for this since in Ltk- cells D2S couples to Gi3 to inhibit prostaglandin E1-stimulated AC (Ghahremani et al., 1999), and in GH4ZR7 cells also preferentially requires Gi3 to inhibit

prolactin secretion (Albert et al., 2002). Several other GPCRs show specificity for different Gi proteins depending on the signaling pathway (Albert et al., 2002).

4.3 Lentiviral Knockdown of RASA3 to assess its function

The shRASA3 lentivirus showed high infection and knockdown efficiency. By knocking down RASA3 in GH4ZR7 cells, the D2SR-induced ERK inhibition was partially reversed. The partial reverse might due to the incomplete knockdown of RASA3, which appears to be less than 50% in these experiments (Even though infection efficiency was close to 100%, Fig 3.b). However RASA3 appears to be very stable, and requires a long infection to reduce its protein level, and this may have been a factor. Using stable expression of antisense RASA3 persisting for months, Nafisi obtained about 70% knockdown of RASA3, which was sufficient to block D2S-induced inhibition of RASA3 (Nafisi et al., 2008). Alternately it is possible that RASA3 is required whereas not sufficient for ERK1/2 inhibition. RASA3 is a potent Ras and Rap inhibitor, while D2SR could also potentially inhibit cAMP-EPAC-rap-ERK1/2 indirectly through the D2SR-AC-cAMP inhibitory pathway, and this alternate pathway does not necessarily need RASA3 to exert the inhibition (Adams et al., 2001).

In order to address RASA3 function in dopaminergic cells, we used PC12 cells as a model since PC12 cells express TH, Nurr1, DAT, synthesize and release dopamine. Following lentiviral knockdown of RASA3 in PC12 cells, we found reduced RASA3 RNA but no changes in TH, Nurr1 or DAT mRNA levels, in the presence or absence of co-expressed D2SR. In GH4ZR7 cells, drug treatment was used to stimulate RASA3 to deactivate ERK1/2. We therefore treated PC12 cells with NGF which activate ERK1/2 signaling to see if RASA3

knockdown would have an impact on TH level and ERK1/2 activation. By introducing D2SR to PC12 cells, ERK1/2 was enhanced after RASA3 knockdown, and TH expression was increased after 24hr NGF treatment. The enhancement was lost without D2SR. This further confirms that D2SR can mediate the ERK1/2 inhibition and RASA3 is required for this inhibition. It is known that the acute ERK activation caused by NGF is mediated through Ras, while the sustained ERK1/2 activation is mediated through Rap and Ras, indicating that RASA3 in PC12 might regulate both Ras and Rap activation (York et al., 1998; Zwartkruis et al., 1998). Accordingly, NGF induced TH expression enhancement was improved after RASA3 knockdown. This PC12 model mimics the dopaminergic neurons, indicating the role of RASA3 in regulating TH expression. ERK1/2 mainly regulate TH activity and stability by phosphorylating Ser31 (Haycock et al., 1992; Dunkley et al., 2004). We can then test the TH Ser31 phosphorylation level to further confirm the D2SR mediated TH negative regulation through RASA3. Although we found ERK1/2 signaling and TH expression was both enhanced after RASA3 knockdown, it doesn't necessarily mean that TH higher expression was caused by ERK1/2 activation, we can use ERK1/2 inhibitor PD98059 to prove that (Klesse et al., 1999). While PC12 cells can be used to test TH expression, there are other cell models such as human SH-SY5Y or mouse MN9D, which are more pure dopaminergic cells used as cell models to study dopamine signaling (Balasooriya et al., 2007). While the shRASA3 was designed based on rat RASA3 sequence, there are mismatches between the rat shRASA3 sequence with mouse or human sequences. We can then design human or mouse based shRASA3 to further confirm the effect by using these more dopaminergic models.

In order to begin to address the function of RASA3 in vivo, we first tested the shRASA3

lentivirus in SN of rats. Unfortunately, although we did target the virus to SN as evidenced by the TH-positive cells, very few of these cells expressed the marker GFP. Previous studies showed infection of dopamine neurons with lentivirus expressing trophic factors is neuroprotective, indicating that lentivirus can be taken up by dopamine neurons(Fiord-Larsen et al., 2005). One difference was the lentivirus was injected in the striatum, and taken up at dopamine terminals; hence it may be more effective to try injections of shRASA3 at the striatum. However, infection of striatal neurons could complicate the interpretation.

4.4 RASA3 Mutant Mice to address RASA3 function

Because RASA3 knockout is lethal, and there are no conditional RASA3 knockout lines available, we took advantage of RASA3 mutant mice to further address RASA3 function in vivo (Peters et al., 2013). The findings in PC12 cells indicate that there is a negative correlation between RASA3 and TH expression. We therefore tested TH expression level in SN of Scat and HLB mice. Western Blotting analysis shows that the TH expression is potentially higher in Scat heterozygotes and HLB homozygotes than in WT. This corresponds to the finding in PC12 cells. Since RASA3 GV125 appear to be a loss-of-function mutation, which would cause a higher level of Ras activate form and subsequent ERK1/2 activation, leading to the increased TH expression (Peters et al., 2013). Further studies are required to determine whether ERK1/2 phosphorylation is increased in SN.

If TH expression is increased in Scat and HLB mice, it might affect the dopamine synthesis and release, then regulate the behaviors which dopamine pathways are involved. Additional studies such as HPLC and microdialysis analyses are required to determine whether dopamine levels are increased in SN and whether dopamine release is enhanced in

striatum. Since dopamine is implicated in locomotion, we examined the locomotor activity of Scat and HLB mice in preliminary studies using BBK, but found no movement difference in these mutant mice. For Scat mice, the homozygous mutation may be required to see robust locomotor phenotypes since the Scat heterozygotes perform normally. Because the Scat homozygotes experience two crises with finally less than 5% surviving, we did not have enough homozygous mutants to test their locomotion. These mice are genetic RASA3 mutants, so the locomotion differences caused by dopamine pathways might be hidden by other phenotypes like blood diseases, or other transmitter pathways such as altered glutamate or serotonin transduction (Swanson et al., 2000; Prinssen et al., 2006). Dopamine signaling is also involved in anxiety and depression, but using EPM, OF, TS and FS tests, we found no significant behavior differences in RASA3 mutant mice. Future studies will address alcohol preference, PPI (Prepulse Inhibition) and Quinpirole-induced locomotion impairment test which are more related to dopamine signaling. Since RASA3 global knockout is embryonically lethal, the best way to understand the role of RASA3 in dopamine signaling is to generate SN/VTA conditional RASA3 knockout mouse.

4.5 Conclusion

Based on studies in pituitary lactotroph GH4ZR7 cells, we hypothesized that RASA3 may be important for D2SR signaling to inhibit ERK1/2 in dopamine neurons, and thus negatively regulator TH expression and activity. To inhibit RASA3 in neurons we tested shRNA to RASA3 and showed that it inhibits RASA3 expression. We confirmed that partial knockdown of RASA3 in GH4ZR7 cells using lentiviral infection of shRNA to RASA3 impaired D2SR mediated inhibition of ERK1/2. We then showed that shRASA3 lentivirus

induced knockdown of RASA3 in dopamine-secreting PC12 cells increased NGF-stimulated ERK1/2 in cells expressing D2SR, but not in cells lacking D2SR, thus implicating RASA3 in D2SR-mediated inhibition of ERK1/2 signaling. We also found that knockdown of RASA3 increased TH protein levels in cells expressing D2SR receptors but not those without D2SR, suggesting that D2SR tonically inhibits the synthesis of TH. Consistent with a role for this pathway in vivo, we found preliminary indication that mutant RASA3 mice show increased level of TH in SN compared to WT mice. Preliminary data suggest no striking changes in basal locomotion, anxiety or depression phenotypes, but further studies are need to specifically address dopamine-driven behaviors. In summary, our data support the role of RASA3 in mediating D2SR-induced inhibition of ERK1/2 in dopamine neurons to negatively regulate TH expression and activity.

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