CHARACTERIZATION OF SOMATOSTATIN RECEPTOR IN RAT/MICE BRAIN:

IMPLICATION IN HUNTINGTON'S DISEASE

by

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Abstract

Somatostatin (SST) is a multifunctional peptide present in most brain regions as well as in peripheral organs. In QUIN/NMDA-induced excitotoxicity, an experimental model of Huntington's disease (HD), SST positive interneuron coexpressing NPY/NADPH-d/bNOS are selectively spared whereas, projection neurons expressing NMDA receptors and DARPP-32 are vulnerable. SST plays neuroprotective role in excitotoxicity, however, which SSTR subtypes mediate the neuroprotective role is not known. Accordingly, as a first step, we describe the colocaliztion of SSTR subtypes with DARPP-32 to determine the percentage of receptor subtypes in projection neurons. We further extended our study and compared HD transgenic mice (R6/2) with SSTR1 and 5 double knock out mice. In both strains we compared the expression pattern of NMDARs, DARPP-32, SST, bNOS and SSTRs and key downstream signaling pathways linked to the neuronal loss in HD such as PI3K, ERK1/2 PKC-α, synapsin-IIa, enkephalin and calpain. Our data shows that SSTR1/5 double knock out mice mimic the neurochemical changes of HD transgenic mice indicating a key neuroprotective role of SSTR1 and 5 in HD. To derive direct physiological implications and mechanistic explanations for the role of SSTR subtypes in excitotoxicity we used striatal brain slices and determined the effect of SSTR1 and 5 agonist, alone or in combination with NMDA on key proteins such as DARPP-32, calpain, PSD-95 and signaling pathways associated with NMDA induced neurotoxicity. Our results here show significant decrease in NMDA currents and dissociation of NMDARs heterodimerization upon treatment with SSTR1 and 5-specific agonist. Our data further demonstrates significant decrease in NMDARs expression and upregulation of SSTR1 and 5 upon agonist treatment. Unlike NMDA, activation of SSTR1 or 5 in striatal slices induced DARPP-32 phosphorylation at Thr34 and Thr75 enhanced CREB phosphorylation and inhibits expression of calpain and PSD-95. The data presented in this thesis provides a new insight for the role of SSTR subtypes in excitotoxicity with relevance to neurological disorders.

Preface

A version of Chapter 2 has been published. **Rajput PS**, Kharmate G, Somvanshi RK, Kumar U. Colocalization of Dopamine Receptor Subtypes with Dopamine and cAMP-regulated phosphoprotein (DARPP-32) in rat brain Neurosci Res. 2009; 65(1):53-63. I designed and performed all the immunohistochemistry experiment under the supervision of Dr. Kumar. Geetanjali Kharmate helped with the western blot experiments. Rishi Somvanshi helped with the quantification of neurons, I wrote the manuscript under the guidance of Dr. Kumar.

A version of Chapter 3 has been published. **Rajput PS**, Kharmate G, Kumar U. J Mol Neurosci. 2011 Nov 25. Colocalization of Somatostatin Receptors with DARPP-32 in Cortex and Striatum of Rat Brain. I designed and performed all the immunohistochemistry experiments under the supervision of Dr. Kumar Geetanjali Kharmate helped with western blot experiments I wrote the manuscript under the guidance of Dr. Kumar.

A version of Chapter 4 has been published. **Rajput PS**, Kharmate G, Norman M, Liu SH, Sastry BR, Brunicardi CF, Kumar U. Somatostatin Receptor 1 and 5 Double Knockout Mice Mimic Neurochemical Changes of Huntington's Disease Transgenic Mice PLoS One. 2011; 6(9): e24467. I designed and performed the experiments under the supervision of Dr. Kumar. Dr. BR Sastry and Dr. CF Brunicardi helped with designing the experiment. Dr. CF Brunicardi, Dr. M Norman and Dr. SH Liu provided the SSTR1/5-/- mice. Geetanjali Kharmate helped in analyzing the data. I wrote the manuscript under the guidance of Dr. Kumar.

A version of Chapter 5 will be submitted **Rajput PS**, Yang B, Sastry BR, Kumar U. Somatostatin Receptors Mediated Inhibition of NMDA Currents and Dissociation of NMDARs Complex in Striatum: Implication in Excitotoxicity. I designed, performed the experiments and analyzed the data under the supervision of Dr. Kumar. Dr. BR Sastry helped with designing the experiment. Bo Yang performed the electrophysiology experiments. I wrote the manuscript under the guidance of Dr. Kumar.

All experiments and procedures involving animals were performed in accordance to the guidelines of the Canadian Council on Animal Care and were reviewed and approved by the University of British Columbia committee on Animal Care (Protocol #A06-0419).

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List of symbols and abbreviations

ACSF-Artificial cerebrospinal fluid

AD- Alzheimer's disease

AIDS- Acquired immune deficiency syndrome

ALS- Amyotrophic lateral sclerosis

APV- 2-amino-5-phosphonovaleric acid

Aβ- Beta-amyloid

bNOS- Brain nitric oxide synthase

Ca^{2+}- Calcium

cAMP- Cyclic adenosine monophosphate

CaMK- Calmodulin kinases

CREB- cAMP response element binding protein

CSF- Cerebrospinal fluid

CB-1- Cannabinoid receptor 1

CNS- Central nervous system

CO-IP- Co-immunoprecipitation

Cu/Zn SOD- Copper/Zinc superoxide dismutase

DA- Dopamine

DARPP-32- Dopamine and cAMP-regulated phosphoprotein

p-DARPP-32^{Thr-34} – Phosphorylated DARPP-32 at Threonine 34 position

p-DARPP-32^{Thr-75} - Phosphorylated DARPP-32 at Threonine 75 position

DNQX- 6,7-dinitroquinoxaline-2,3-dione

DR- Dopamine receptor

EAE- Experimental allergic encephalomyelitis

EPSCs- Excitatory postsynaptic currents

ERK1/2- Extracellular-signal-regulated kinases 1/2

GABA- γ-Aminobutryic Acid

GH- Growth hormone

GHRH- Growth hormone-releasing hormone

GPCR- G-protein coupled receptors

HEK-293- Humam embryonic kidney cells

***Htt*-** Huntingtin

HIV- Human immunodeficiency virus

HD- Huntington's disease

L-797,591- SSTR1 specific agonist

L-817,818- SSTR5 specific agonist

MSN- Medium-sized spiny neurons

MAPK- Mitogen activated protein kinases

MS- Multiple sclerosis

NPY- Neuropeptide Y

NADPH-D- Nicotinamide adenine dinucleotide phosphate-diaphorase

NGS- Normal goat serum

NMDA- N-Methyl-D-Asparate

NMDAR- N-methyl D-aspartate receptor

PBS- Phosphate buffer saline

PD- Parkinson's disease

PI3K- Phosphatidylinositol 3-kinases

PKA- Protein kinase A

PKC-α- Protein kinase C-alpha

PPtase1- Protein phosphatase-1

PPtase2- Protein phosphatase-2

PSD-95- Postsynaptic density protein-95

PSST- Preprosomatostatin

PTX- Pertussis toxin

QUIN- Quinolinic acid

RIPA- Radioimmune precipitation assay buffer

SST- Somatostatin

SRIF- Somatotropin release-inhibiting peptide

SSTR- Somatostatin receptors

SSTR1/5 $^{-/-}$ mice- Somatostatin receptors 1/5 double knock down mice

TBS- Tris-buffered saline

TSH- Thyroid –stimulating hormone

wt- Wild type

YAC- Yeast artificial chromosome.

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Finally it is inevitable in this research that omission or errors will have escaped the detection. I hope any such mistakes are minor or minimal, I accept the full and exclusive responsibility for them.

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"I would like to dedicate my thesis to my Dad

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pursue my goals with hard work and perseverance."

Chapter 1: Introduction

1.1 Thesis overview

The gradual and progressive neuronal degeneration in region specific manner underlies the pathology of several neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) as well as other neuropsychological conditions. Apoptosis and necrosis are the two distinct-and/or interconnected mechanisms responsible for neuronal death and have been under constant investigation over three decades. In addition to genetic disposition of neurological diseases, abnormal functions in several proteins may also contribute to the pathology of neurological disorder. Amongst them neuropeptides and neurotransmitter receptor proteins are the prominent players. Previous studies have established that disturbances in the somatostatinergic system has profound implications in several human neurodegenerative diseases including AD, HD, hypoxic-ischemic neuronal injury, neoplasia, prion disease and acquired immune deficiency syndrome (AIDS) encephalopathy (Aguila, 1994; Barnea et al., 1999; Beal, 1990; Beal et al., 1984; Da Cunha et al., 1995; Gemignani et al., 2000; Martin and Gusella, 1986; Patel, 1996; Patel et al., 1995b; Riekkinen and Pitkanen, 1990). Age dependent decrease in the expression of SST is well established in human brain. Beal et al., for the first time described that cortical and cerebrospinal fluid (CSF) concentrations of somatostatin (SST) are selectively and specifically reduced level of SST expression in AD (Beal et al., 1988). Conversely, in HD, SST level is increased in basal ganglia i.e., striatum and a subset of SST producing neurons are selectively resistant to neurodegeneration (Dawbarn et al., 1985; Ferrante et al., 1985). This process of selective sparing of SST-producing neurons appears to be a feature of several

neurodegenerative conditions. Furthermore, supporting these *in vivo* observations, *in vitro* models of N-methyl-D-aspartate (NMDA)-induced neurotoxicity using cultured cortical neurons exhibit the upregulation of SST gene expression and SST release in response to NMDA receptors (NMDARs) agonist Quinolinic Acid (QUIN) in time and concentration dependent manner (Patel et al., 1991; Patel et al., 1995b). In parallel, it was also shown that exogenous addition of SST exerts neuroprotective effect against NMDA-mediated toxicity (Forloni et al., 1997). Importantly, a recent study from our lab demonstrated that knocking down SST in cultured striatal neurons resulted in more neuronal loss upon NMDA treatment in comparison to control (Kumar, 2008). These results anticipate that SST functions as neuroprotective peptide against NMDA induced toxicity.

In excitotoxicity, while the activation of NMDARs is a prerequisite to induce neuronal cell death, cell death is also associated with increased calcium, oxidative stress and modulation in several other proteins. Furthermore, changes in many of the downstream signaling pathways play detrimental role in neuronal survival and loss. Most importantly, the clinical and pathological manifestation of several neurological diseases is linked with over activation of NMDARs (Choi and Rothman, 1990; Coyle and Puttfarcken, 1993). The value of *in vitro* NMDA-induced model of excitotoxicity is not only to explore the mechanisms of glutamate toxicity but also mimic an experimental model of human neuronal disease HD *in vitro*. Despite the fact that SST plays an important role in various neurodegenerative diseases, very little is known about the contribution of somatostatin receptors (SSTRs), which are responsible for most of the biological roles of this peptide. Whether SSTR subtypes involve in the pathophysiology of excitotoxicity and exert any possible role in NMDA induced neurotoxicity remains largely illusive. Therefore, this thesis focuses to study

the roles of SSTRs and establish mechanistic relationship between SSTRs and neurodegenerative disease. Prior to delineate functional relevance and interaction of certain key proteins with the role of SSTR subtypes at the molecular level in central nervous system (CNS), it was essential to determine the expression and colocalization of SSTRs in brain with key proteins which are intimately associated with neuronal loss in excitotoxicity. Accordingly, in this thesis we first determined, specifically the expression of the somatostatinergic system (somatostatin receptors) in rat brain (cortex and striatum) with other proteins, which are well known for the crucial role in the process of neurodegeneration. We focused mainly on cortex and striatum region because these brain regions are affected most in neurodegenerative diseases. Striatum is composed of projection (80%) and interneurons (10%) and dopamine- and cAMP-regulated phosphoprotein (DARPP-32) is highly expressed in projection neurons and significantly decreased in excitotoxicity whereas, interneurons are devoid of DARPP-32 and selectively spared (Torres-Peraza et al., 2007). Since DARPP-32 is regulated by dopamine receptors (DR), which are well expressed in striatum, therefore we first accomplished colocalization of DARPP-32 with DR subtypes and followed by colocalization between SSTR subtypes and DARPP-32 in rat brain striatum and cortex. Secondly, to establish any pathophysiological relevance of SSTRs with neurological diseases we compared the neurochemical phenotype and signaling pathways in SSTR1/5 knockout (*ko*) and HD transgenic (R6/2) mice. Previous studies have established that heterodimerization of NMDA receptors is detrimental for receptor functionality and critical for the status and degree of neuronal vulnerability. NMDARs also constitute functional heteromeric complex with other GPCRs mainly the members of dopamine receptor family (Lee et al., 2002). Accordingly, using a combination of morphological, biochemical and

electrophysiological approaches we characterized the role of SSTR1 and SSTR5 in brain slices to determine whether SSTR activation impede NMDAR heteromeric complex formation and consequently altered associated protein and signaling pathways responsible for neuronal loss or survival in excitotoxicity.

1.2 Somatostatin

The tetradecapeptide somatostatin (SST), also known as somatotropin release-inhibiting hormone (SRIF) was first isolated from sheep hypothalamus as growth inhibitory peptide with a low molecular weight (Krulich et al., 1968). No more than five years later, Brazeau et al., examined hypothalamic extracts for potency in releasing growth hormone (GH) from long-term cultured anterior pituitary cells and found a factor that surprisingly inhibited even the basal expression of hormone release in minuscule amounts (Brazeau et al., 1973). Since then several studies have shown that SST is not only synthesized in the hypothalamus, but also present in other brain regions and major peripheral organs (Patel, 1999). SST exists in two bioactive isoforms, SST-14 and SST-28 that are synthesized in different amounts in various SST-producing cells in different parts of body (Hobart et al., 1980; Montminy et al., 1984; Patel, 1999; Shen and Rutter, 1984). SST-28 consists of SST-14 sequence at the carboxy terminal, which is preceded by basic double pair of amino acids. Trypsin-like conversion allows direct cleavage from SST-28 to SST-14 (Brazeau et al., 1973; Srikant and Patel, 1981). SST is derived from a common precursor known as preprosomatostatin (PSST). PSST is processed into SST-14 in neuronal tissue as the main product by endoproteolytic action of prohormone convertase1 (Brakch et al., 1995). It is believed that SST-28 is released from the hypothalamus and has longer half-life in the portal blood system when compared to SST-14 (Dahms and Mentlein, 1992).

1.3 Anatomical distribution of somatostatin

SST is a phylogenetically ancient peptide and SST-like immunoreactivity has been reported in microorganisms such as *Tetrahymena pyriformis, Bacillus subtilis and Plasmodium falciparum* (Lenard, 1992). By using radioimmunoassay and immunohistochemistry, the wide spread anatomical distribution of SST-producing cells has been detected in central and peripheral nervous systems along with gastrointestinal tract, the gut, the thyroid, the adrenals, the kidneys, the prostate, the placenta, the lymphoid cells and the δ cells of the pancreatic islets (Brownstein et al., 1975; Finley et al., 1981; Palkovits et al., 1976; Patel et al., 1990). In CNS, SST is highly expressed in hypothalamus, amygdala, and hippocampus, cortex, striatum, brain stem and the periaqueductal gray and all levels of major sensory pathways (Epelbaum et al., 1994; Johansson et al., 1984; Oliva et al., 2000). SST positive neurons in CNS are often co-expressed with several other neuroendocrine peptides including noradrenaline, γ-Aminobutryic Acid (GABA) or neuropeptide Y (NPY), glycine and enzymes such as brain nitric oxide synthase (bNOS) as well as other peptides such as met-enkephalin, substance P and neurotensin (Epelbaum, 1986). These observations anticipate that SST might involve in regulation of secretion and function of these peptides in brain.

SST positive neurons can be characterized in three different groups: interneurons, neurons projecting in single anatomical region and neurons connecting various regions of CNS (Morrison et al., 1983; Morrison et al., 1982). SST positive neurons can also be divided in two categories; long-projecting neurons and short GABAergic neurons (interneurons) (Gulyas et al., 2003; Oliva et al., 2000; Tomioka et al., 2005; Zappone and Sloviter, 2001). In addition to brain, SST-14 is predominantly expressed in peripheral tissues including stomach, pancreatic islets, nervous tissue, retina, peripheral nerves and enteric neurons (Epelbaum et

al., 1994; Patel, 1999). In comparison, SST-28 is also well expressed in brain pancreas and gut and synthesized mainly in intestinal mucosal cells (Patel, 1999). Although, SST-28 represents 20-30% of total SST immunoreactivity in the brain, whether SST-14 and SST-28 are expressed within the same neuron is not known (Patel, 1999; Reichlin, 1983a). Furthermore, the expression of SST has been detected during embryonic development as early as 10 week of gestation in fetal hypothalamus (Bugnon et al., 1978).

In comparison to normal SST release from hypothalamic nerve terminals, the electrical stimulation of preoptic area and median eminence induces 3-7 fold increase of SST release into the blood portal system (Chihara et al., 1979a; Millar et al., 1983). Release of SST from median eminence is also due to nociceptive stress (Epelbaum et al., 1994). Furthermore, studies have described that low doses of dopamine (DA), acetylcholine and norepinephrine also increase SST release (Chihara et al., 1979b). Moreover, ionic dependency and calcium channel play critical role on SST release and secretion (Arancibia et al., 1984; Berelowitz et al., 1978; Iversen et al., 1978).

In CNS, the large population of SST positive neurons in basal ganglia are confined in striatum (Johansson et al., 1984). Striatum is primarily composed of two different types of neuronal population including medium size projection neurons and medium sized aspiny interneurons. Interestingly, projection neurons are devoid of SST like immunoreactivity, whereas SST positive medium sized aspiny interneurons colocalize with NPY, nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)/bNOS (Kowall et al., 1987; Rushlow et al., 1995). In infragranular layer and the subcortical white matter of cerebral cortex, SST colocalizes with NPY in non-pyramidal neurons (Hornung et al., 1992). SST positive interneurons, a subset of GABAergic neurons also colocalizes with NPY in cortex

and hippocampus of rat brain. It is believed that the coexistence of SST with inhibitory GABAergic neurons indicates the inhibitory role of SST in local circuits (Esclapez and Houser, 1995; Hendry et al., 1984; Kohler et al., 1987; Kowall and Beal, 1988).

1.4 Biological roles of somatostatin

SST is known to exert diverse effects like inhibition of endocrine secretions, modulation of neurotransmission and regulation of cell proliferation (Patel, 1999). With this wide spread distribution and several endocrine and exocrine functions, SST is now considered as a multifunctional peptide (**Fig 1.1**). In addition to neurotransmitter the role of some neuropeptides has also been associated with cognitive function. Relatively high expression of SST in hippocampus and cortex suggest the role of SST in learning and memory (Rubinow et al., 1995). In CNS, SST acts as a neuromodulator and neurotransmitter and specifically as a physiological regulator involving in locomotor, cognitive, sensory and other autonomic functions (Epelbaum et al., 1994; Reichlin, 1983a; Reichlin, 1983b). SST released in hypothalamus regulates the secretion of GH from pituitary, which is counter balanced by growth hormone–releasing hormone (GHRH) (Bresson et al., 1984). One of the most important roles of SST is the tonic inhibition of basal and GHRH stimulated secretion of GH from the pituitary (Barinaga et al., 1985). DA, substance P, glucagon, acetylcholine and cholesystokinin further augment SST secretion, however, it is inhibited by glucose (Chihara et al., 1979b; Reichlin, 1983b). Conversely, SST inhibits the secretion of dopamine, norepinephrine, thyroid releasing hormone and corticotrophin releasing hormone from hypothalamus in addition to its prominent role in inhibition of GH (Patel, 1997). Similarly, SST also regulates the secretion of thyroid-stimulating hormone (TSH) (Ferland et al., 1976; Siler et al., 1974). Most importantly, SST *ko* mouse revealed that SST is necessary for

masculinization of GH (Low et al., 2001). Importantly, this study also describes that male SST *ko* was feminized and displayed similar expression of GH like its counterpart female. Furthermore, in SST *ko* mice the expression levels of SSTR1 decreased while SSTR2 and SSTR5 increased, however SSTR3 and SSTR4 were unchanged (Low et al., 2001). Further in support binding analysis in brain extract of SST *ko* mice reveals significant increase in its cognate receptor subtypes (Ramirez et al., 2002).

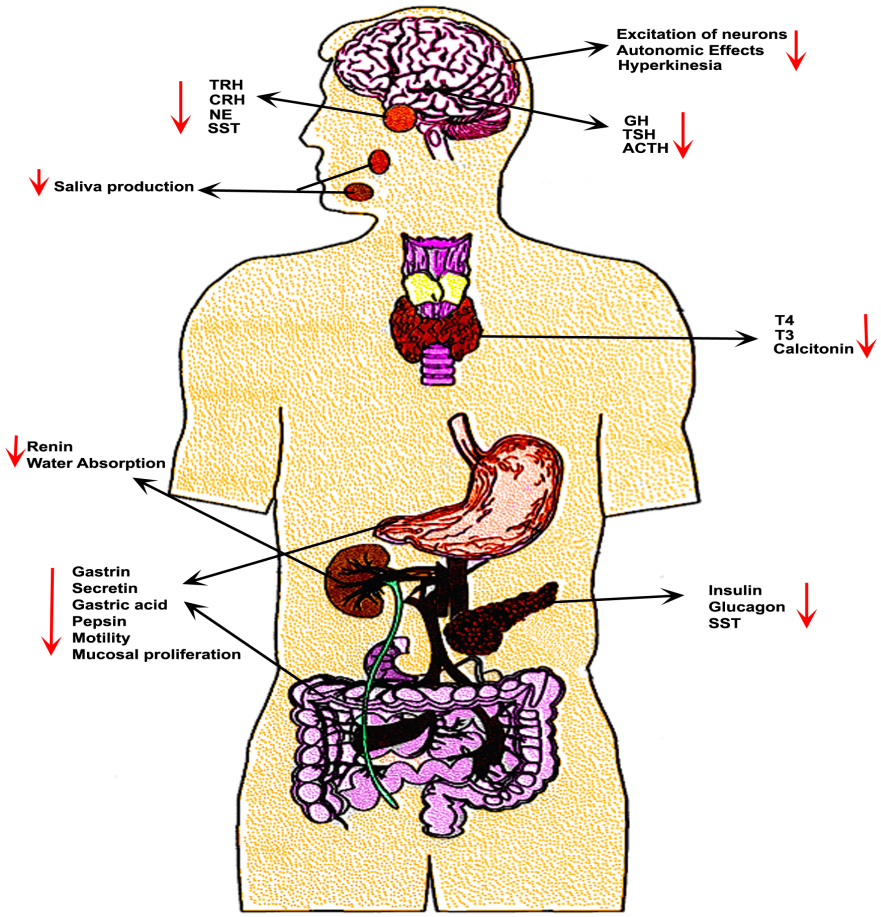

Figure 1.1Schematic illustration depicting the different biological roles of SST in various organs in human body

SST directly affects the release of T4, T3 and calcitonin, inhibit angiotensin II in the adrenal glands, rennin in kidneys and inhibition of hormones in gastrointestinal tract (Patel,

1997). Furthermore, SST suppresses synthesis and secretion of insulin, glucagon and pancreatic polypeptide from δ cells of pancreatic islets (Ballian et al., 2006; Kendall et al., 1995; Redmon et al., 1994). One of the most prominent roles of SST described recently in CNS is associated with the beta amyloid accumulation and aggregation upon down regulation of SST expression (Saito et al., 2005).

1.5 Somatostatin receptors

The diverse biological effects of SST in target tissues are mediated by five different cell surface receptors namely somatostatin receptors 1-5 (SSTR1-5). SSTR subtypes were first studied and identified in the pituitary GH4C1 cell line by whole-cell binding analysis (Schonbrunn and Tashjian, 1978). All five SSTR subtypes have been cloned and pharmacologically characterized and belong to a family of a hepatahelical transmembrane guanine nucleotide-binding proteins coupled receptors (GPCRs) (Patel, 1999). SSTR subtypes bind to SST-14 and SST-28 with equal high affinity. SSTR1-4 shows a weak selectivity for SST-28 while SSTR5 has several fold high binding affinity for SST-28 when compared to SST-14 and appears to be SST-28 selective (Patel, 1997; Patel et al., 1995a; Patel and Srikant, 1994). SSTR subtypes are divided into two subclasses based on their structural similarities and ligand binding affinities for SST analogs, octapeptides (octreotide) and hexapeptides (lanreotide). SSTR2, 3 and 5 comprises one subgroup which binds with similar binding affinity and SSTR1 and 4 constitute members of the other subgroup that bind to SST analogs with low affinity (Patel, 1997).

As illustrated in **Table 1.1**, all SSTR subtypes inhibit adenylyl cyclase by coupling to pertussis toxin (PTX) sensitive Gαi protein and inhibit the formation of cyclic adenosine

monophosphate (cAMP) (Patel, 1999; Reisine, 1985; Reisine et al., 1985; Reisine et al., 1983). In turn, cAMP binds to and activates protein kinase A (PKA) that phosphorylates specific downstream signaling molecules. The regulatory effects of cAMP on cellular functions include cell division, ion transport, ion channel functions, neuronal excitability and contractile proteins in smooth muscle (Patel, 1999; Patel and Srikant, 1997; Reichlin, 1983a).

Table 1.1 – Summary of various signaling pathways modulated by SSTR subtypes.

Transduction Pathways	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Adenylyl Cyclase	↓	↓	↓	↓	↓
Nitric oxide	↑	↓↑	↓		↓
Ca^{2+}	↓	↓	↓		↓↑
K^+ Conductances		↑			
Phospholipase C		↑	↑		↑
NA^+/H^+ exchanger	↓	↓			↓
CAMK II					↑
MAP Kinase	↓↑	↓↑	↓↑	↓	↓
PI3 kinase	↓	↓↑	↓		↓↑

SSTRs regulate wide range of second messenger systems such as inhibition of voltage –dependent calcium channels and stimulation of potassium ion channels resulting in membrane depolarization (Patel, 1999). SSTRs are involve in activation of phosphotyrosine phosphatase (PTP) and Na^+/H^+ antiporters in PTX independent manner (Patel, 1999). SSTRs couple to several K^+ channels like ATP-sensitive K^+ channels, large conductance Ca^{2+}

activated BK channels delayed rectifier and inward rectifier (White et al., 1991). Receptor activated K^+ channels causes reversible hyper-polarization of the membrane leading to cessation of impulsive action potential activity and reduction in intracellular Ca^{2+} resulting in depolarization induced via voltage-sensitive Ca^{2+} channels. Additionally, SSTRs acts directly on voltage-dependent L-, N- and P/Q type Ca^{2+} channels (Kleuss et al., 1991; Yang et al., 2007). SST mediated inhibition of Ca^{2+} influx through modulation of cGMP-dependent protein kinase is also regulated by SSTR subtypes (Meriney et al., 1994). SSTRs also monitor the expression levels of number of enzymes such as guanylate cyclase, mitogen-activated protein kinase (MAP kinase) as well as serine and threonine phosphatases (Patel, 1999). Taking this in consideration one of the prominent biological effects of SST via SSTRs is inhibition of cell proliferation that has been studied the most with clinical implications in tumor biology (Buscail et al., 2002; Lamberts et al., 1991; Schally, 1988; Watt et al., 2008; Weckbecker et al., 2003).

1.6 Distribution of somatostatin receptor subtypes in central nervous system

Several previous binding studies have shown that SSTRs are expressed in brain, gut, pituitary, pancreas, adrenals, thyroid, kidneys and immune cells (Patel, 1997; Patel et al., 1995b; Patel et al., 1990; Patel and Srikant, 1997; Reisine and Bell, 1995). The development of SSTR subtypes polyclonal antibodies accelerated the understanding and subcellular distribution of SSTR subtypes protein at the cellular levels by using peroxidase and immunofluorescence techniques (Kumar et al., 1997b; Kumar et al., 1999). Using specific antibodies several independent studies have provided somewhat comparable distribution of SSTR subtypes in rat and human brain with some site-specific differences (Cervera et al., 2002; Csaba et al., 2005; Dournaud et al., 1998; Dournaud et al., 1996; Hervieu and Emson,

1998a; Hervieu and Emson, 1998b; Kumar, 2005; Kumar, 2007; Schindler et al., 1998; Schindler et al., 1997; Schreff et al., 2000; Schulz et al., 1998; Selmer et al., 2000a; Selmer et al., 2000b).

The distribution of SSTR1 mRNA has been described in most of the brain regions however, in contrast to mRNA receptor protein expression is confined in selected brain regions (Csaba and Dournaud, 2001; Helboe et al., 1998). SSTR2 like immunoreactivity is widely distributed in the brain. Immunohistochemical examination of SSTR2 distribution clearly revealed cells with a somatodendritic labeling profile in some brain areas (i.e. olfactory tubercle, central amygdala, medial septum, bed nucleus of the stria terminalis, dorsal horn of the spinal cord) (Beaudet et al., 1995; Perez et al., 1994). Moreover, mRNA of SSTR1 and SSTR2 have been found to colocalize in GHRH producing neurons in arcuate nucleus of hypothalamus (Tannenbaum et al., 1998). SSTR3 like immunoreactivity is present most of the brain regions (Csaba and Dournaud, 2001; Handel et al., 1999). SSTR4 is expressed in most brain areas during birth however, receptor expression levels decline at the post-natal periods (Wulfsen et al., 1993). Increased expression of SSTR4 in adult brain has been observed in hippocampus and olfactory lobe (Wulfsen et al., 1993). SSTR5 is the receptor subtype expressed least in rat brain (Ramirez et al., 2004). Previous studies have shown that SSTR1-4 colocalize with SST, suggesting SSTRs also act as autoreceptors however, most regions of hypothalamus lacked SSTR5 like immunoreactivity (Kumar, 2007). Such distribution of SSTR in rat brain hypothalamus is different from mouse brain and opposite to pituitary, where SSTR5 is highly expressed and associated with regulation of GH release. SSTR subtypes are also regulated developmentally in pituitary, hypothalamus

and spinal cord (Goodyer et al., 2004). SSTR1-5 expression has also been shown in normal and AD human brain tissues (Kumar, 2005).

Throughout the CNS, SSTRs display an overlapping pattern of distribution, anticipating the possibility of more than one SSTR subtype in single neuronal cell. Receptor and region specific colocalization of SSTR subtypes with SST is the indication that SSTR might functionally interact with each other (Kumar, 2007; Tannenbaum et al., 1998). Furthermore, receptor specific ko mice which showed compensatory role to each other support this speculation. Most importantly, SSTRs *ko* mice have been instrumental to elucidate the exact physiological role of receptors and its influence on other subtypes. SSTR2 *ko* mice, especially the SSTR2 *ko*/lacZ knock-in strains contributed to the precise anatomical and cellular distribution of the SSTR subtypes (Allen et al., 2003). While SSTR1, SSTR3 and SSTR4 *ko* exhibit similar binding pattern as wild type (*wt*) conversely, specific total binding is dramatically reduced in SSTR2 *ko* brain, suggesting that SSTR2 is the major receptor subtype that might regulate remaining SSTR in CNS (Videau et al., 2003). Importantly, mouse lacking SSTR4 are susceptible to kainic acid induced epilepsy than *wt* and also more susceptible to inflammation and display sustained pain and loss of analgesic effect (Qiu et al., 2008). In addition, SSTR5 *ko* mice exhibited changes in SST and SSTR subtypes in brain and pancreas which are associated with sexual dimorphism (Ramirez et al., 2004). In SSTR5 *ko* mice, increase in SSTR2 and 3 is observed while reduction of SSTR4 in female mice without any significant changes in male mice. Whereas, SST mRNA/immunoreactivity reduced in male *ko* mice without any changes in female mice (Ramirez et al., 2004).

1.7 Role of somatostatin receptors in neurodegenerative diseases

SST and SSTRs play important role in pathophysiology of many neurological diseases including brain tumors (Dutour et al., 1998) and various neurodegenerative diseases such as AD (Davies et al., 1980; Kumar, 2005), PD (Epelbaum et al., 1983) and HD (Beal et al., 1986; Kumar, 2008) as well as in pituitary tumor (Jaquet et al., 1999) by modulating key neuronal signaling pathways. It has been well documented that cultured neurons exposed to various toxins such as NMDA, Aβ, gp-120 HIV-protein or prion protein exhibit neuronal cell death in a concentration and time dependent manner (Barnea et al., 1999; Geci et al., 2007; Gemignani et al., 2000; Patel et al., 1991). However, observations introduced SST as a potential neuroprotective agent and demonstrated that SST-producing neurons are selectively preserved in excitotoxicity (Forloni et al., 1997; Kumar et al., 1997a).

1.7.1 NMDA-induced neurotoxicity

Overactivation of ionotropic NMDARs has been associated with pathophysiology of several neurological diseases. The NMDARs activation resulted in increase Ca^{2+} influx. Forloni et al, examined if SST could afford neuroprotection in NMDA-induced neuronal death and suggested that SST mediated neuroprotective effect is attributed to cGMP-protein kinase action because the cGMP content was also increased in a dose-dependent fashion (Forloni et al., 1997). Therefore, it was argued that intracellular calcium levels play an important role in NMDA induced neurotoxicity. Consistent with these observations NMDA antagonist averts neuronal cell death (Nakamura et al., 1997; Noguchi et al., 1999). Taken all together these studies provided the evidences in support that addition of SST probably by activating SSTR subtypes and inhibition of NMDARs resulted in neuroprotection.

Furthermore, it has been reported that the resistance of SST-containing neurons to NMDA-induced toxicity may result from relatively sparse and less expression of NMDA receptors in comparison surrounding projection neurons (Augood et al., 1994; Kumar et al., 1997a). Furthermore, NMDARs agonist QUIN increases SST secretion and gene expression in cultured cortical and striatal neurons (Patel et al., 1991; Patel et al., 1995b). Blockade of the released SST enhances NMDA-induced cell death whereas exogenously applied SST prevents neuronal cell death, thus suggesting that SST plays a neuroprotective role (Kumar, 2008). Taken together, these observations showed direct implications of SST in pathophysiology of neurological diseases including HD, PD and AD.

1.7.2 Huntington's disease

HD is an inherited autosomal dominant neurodegenerative disorder characterized by progressive chorea and cognitive impairment (de la Monte et al., 1988; Giampa et al., 2006; Wilson et al., 1987). HD is caused by mutation in the huntingtin (*Htt*) gene located at chromosome 4. The cause of mutation is the expansion of CAG repeat >35, in the first exon of the HD gene. The ~350 kDa *Htt* protein, shows no homology to other known proteins (HDCRgroup, 1993). The lengths of CAG repeat sequence is inversely proportional with the onset of HD symptoms (Brinkman et al., 1997; Gusella and MacDonald, 2000). The pathological characteristic of HD is extensive reduction of the striatal mass, affecting caudate putamen. The striatal atrophy is the result of selective degeneration of GABAergic medium-sized projection neurons, while SST/NPY/bNOS positive interneurons are relatively spared (Cicchetti et al., 1996; Cicchetti and Parent, 1996; Ferrante et al., 1987; Ferrante et al., 1985; Vonsattel et al., 1985). Studies have shown that *mHtt* functionally modulate the activation of ionotropic receptors like NMDARs, which are highly permeable to Ca^{2+} causing excessive

influx of ions into the cell leading to neuronal death (Fan and Raymond, 2007; Monaghan et al., 1989). Hence altered NMDARs signaling mediated by the pathogenic effect of *mHtt* result in neuronal degeneration (Fan and Raymond, 2007).

The administration of QUIN and NMDA into striatum of rodents and primates mimic the degeneration of medium sized spiny neurons as well as neuropathological and behavioral changes as seen in HD patients (Beal et al., 1986; Coyle and Schwarcz, 1976; Ferrante et al., 1993). Interestingly, HD is characterized by a reduced number of striatal projection neurons, as well as significant increase of endogenous SST in the basal ganglia (Beal et al., 1988; Palacios et al., 1990; Sperk et al., 1987). Furthermore, appreciable reductions in SST binding sites have also been reported in the striatal tissue obtained from HD post-mortem brain (Palacios et al., 1990). According to Beal et al., SST-like immunoreactivity is increased three fold in caudate, putamen and nucleus accumbens (Beal et al., 1988). Post-mortem human putamen samples from HD patients exhibited 55% increase of SST (Sperk et al., 1987). Furthermore, morphological characterization of striatal-cultured neurons using immunohistochemical analysis revealed that SST co-localizes with NPY and NADPH-d containing neurons (Kumar et al., 1997a). Selective and preferential neurodegeneration associated with HD is mostly confined to the striatum and interneurons those expressing NOS/NPY/SST are relatively spared. Interestingly, it was proposed that the lack of NMDARs presence in these neurons justifies their survival (Koh et al., 1986). However, the relative abundance of NR1 on SST/NPY/NADPH-diaphorase positive neurons varies between a small population of neurons that are NR1 rich and the remainder (~80%) that express only low levels of NR1 (Augood et al., 1994; Kumar et al., 1997a). This important observation may determine the susceptibility to NMDAR-mediated neurotoxicity. In

addition, it has also been proposed that in glutamate-induced neurotoxicity NO released from bNOS-containing neurons may potentially mediate neurodegeneration (Norris et al., 1996). Similar observation has also been confirmed by striatal or systemic injections of 3-nitropropionic acid or malonic acid (inhibitors of mitochondrial complex II) in rodents (Beal et al., 1993; Brouillet et al., 1998; Ferrante et al., 1993). Previous studies have shown that many receptors like SSTRs, NMDARs and DRs and proteins like DARPP-32, calcineurin play a major role in process of neurodegeneration in HD (Fan and Raymond, 2007; Greengard et al., 1999).

1.7.3 Parkinson's diseases

HD is not the only isolated example; significant changes in SST expression have also been reported in PD and AD. Reduced (40-60%) SST like immunoreactivity has been shown in different brain regions including cortex in patients who also represent Alzheimer-type cortical pathology. The loss in SST like immunoreactivity in PD patients paralleled intellectual deterioration (Beal et al., 1988; Beal et al., 1985). Previously using 6-hydroxydopamine induced experimental model of PD, SST administration to the lesion showed inhibition of apomorphine mediated rotation (Lu and Stoessl, 2002). Although these existing data suggest that SST is associated with PD however which receptor subtypes involve in pathogenesis of disease is not known.

1.7.4 Alzheimer's disease

Alzheimer's disease is a progressive neurodegenerative disease characterized by the presence of senile plaques, neurofibrillary tangles, and neuronal loss in several brain regions but especially in the cerebral cortex (Yankner, 1996). The abnormal production of β-amyloid

(Aβ) with an increased tendency to form fibrils has been clearly shown to result in neurotoxicity (Yankner, 1996). Oxidative stress and activation of glutamate receptors play an important role in the degenerative process. Cortical and CSF levels of immunoreactive SST immunoreactivity were first reported to be markedly decreased and have since become a reproducible biochemical marker of the disease (Epelbaum et al., 1994; Hyman et al., 1992; Kowall and Beal, 1988; Rossor et al., 1980). Previous studies have shown that SST positive neurons in deeper cortical layers are selectively preserved and the loss of SST positive neurons in superficial upper cortical layers is due to functional depletion of SST from these cells (Gaspar, 1989; Hyman et al., 1992). Studies have shown that higher concentrations of Aβ increased SST expression and mRNA synthesis at early times points in cultured cortical neurons that correlate with pathophysiology of AD (Geci et al., 2007). Increased accumulation of SST, in presence of Aβ was linked to impaired membrane permeability (Geci et al., 2007). Like SST, SSTRs measured by binding analysis in brain homogenates, show marked depletion in AD brain (Beal et al., 1985; Epelbaum et al., 1994; Krantic et al., 1992). However, recent progress in development of SSTR subtypes specific analogs may provide an important tool for exploring the role of each individual SSTR in neurodegeneration. Comparative immunohistochemical analysis of SSTR subtypes in control and AD human brain tissues revealed 70% reduction in SST positive neurons in frontal cortex of AD brain (Kumar, 2005). Cortical AD brain showed reduction in expression of SSTR4 and 5 along with a moderate decrease in SSTR2-like immunoreactivity with no changes in SSTR1 positive neurons. Interestingly, only SSTR3 receptor subtype showed increased expression in AD cortex in comparison to control brains. These data suggests the

loss of SSTR2, 4 and 5 immunoreactivity and increased SSTR3 expression in frontal cortex of AD brain (Kumar, 2005).

1.7.5 Other neurodegenerative diseases

In hypoxia-ischemia a massive loss of striatal efferent neurons with relative sparing of SST/NPY/NOS neurons has been observed. Interestingly, in addition to the preservation of SST positive neurons, increased SST mRNA levels have also been demonstrated (Loudes et al., 2000). The cortical encephalopathy of AIDS in infected monkeys and human patients are associated with an increase in SST mRNA levels in the cortex (Reisine and Bell, 1995).

Studies in patients suffering from schizophrenia have shown no significant changes of SST concentrations in CSF in comparison to controls (Banki et al., 1992a; Banki et al., 1992b; Heikkila, 1993). However, in contrast studies have also shown increased and decreased levels of SST in CSF of drug free schizophrenia patients (Bissette et al., 1986). Studies in postmortem brains of schizophrenia patients with predominant negative symptoms have shown decrease in expression levels of SST in hippocampus, cerebral cortex and lateral thalamus (Ferrier et al., 1983; Gabriel et al., 1996; Roberts et al., 1983). Interestingly, plasma concentrations and serum SST autoantibodies are found to increase in schizophrenia patients (Rogaeva et al., 1990; Roy et al., 1994; Saiz-Ruiz et al., 1992).

Multiple sclerosis (MS) is the demyelinating disease characterized by myelin damage with inflammation in the CNS (Compston and Coles, 2002; Steinman, 1996). In experimental allergic encephalomyelitis (EAE) rodent model of MS, reduced SSTR2 expression was detected with loss in inhibition of adenylyl cyclase activity of SST in EAE rats in comparison to forskolin treatment (Aguado-Llera et al., 2007). The physiological significance of impaired somatostatinergic system is correlated with the loss of SSTR2, that has been

associated with locomotors activity and spatial learning (Dournaud et al., 1998). Somatostatinergic system plays a key role in the inhibitory modulation of hippocampal neuronal circuitry. Significant changes in SST and SSTR subtypes have been observed in the experimental models of temporal lobe epilepsy especially in kindling and kainate models of epilepsy (Perez et al., 1995; Piwko et al., 1996; Vezzani and Hoyer, 1999). In contrast, SST biosynthesis and release have been shown to increase in kindling model of epilepsy. However, pharmacological activation of SSTR2 upon agonist treatment resulted in antiepileptic effects (Binaschi et al., 2003).

1.8 Somatostatin receptor homo-and heterodimerization

SSTR subtypes upon ligand binding have shown great diversity in pharmacological properties and receptor signaling efficiency in cell and receptor specific manner. The changes in receptor functional properties in large are attributed to the formation of homo-and heterooligomers. Concept of SSTRs dimerization was first supported due to the presence of more than one receptor in single cells. The pharmacological, biochemical and biophysical studies provided the direct evidence in favor of SSTR homodimerization with an example of SSTR5 in heterologous system using human embryonic kidney (HEK 293) and Chinese hamster ovary (CHO-K1) transfected cells (Grant et al., 2004b; Rocheville et al., 2000b). In this study, authors described that SSTR5 which otherwise exist as monomer in basal condition exhibited dimerization upon agonist treatment. SSTR2 that exist as preformed dimers in basal condition and dissociated to monomer in response to ligand, a step essential for receptor internalization (Grant et al., 2004a). SSTR3 exist as preformed dimer and homodimerization decreased upon ligand treatment. Interestingly, the C-tail of SSTR3 has no significant role in the process of dimerization however C-tail mutant SSTR3 lost its effect on

cAMP inhibition (War et al., 2011). SSTR4 exists as homodimers in basal condition and the dimerization is enhanced upon ligand treatment (Somvanshi et al., 2009). C-tail of SSTR4 plays significant role in receptor dimerization as C-tail deficient SSTR4 failed to form homodimers as well as looses ability of membrane expression. Replacing C-tail of SSTR4 with C-tail of SSTR1 displayed SSTR1 like properties, as the chimeric receptor is not capable to form dimers. However, swapping SSTR4 C-tail with the C-tail of SSTR5 resulted in receptor dimerization. SSTR4 constituted a heterodimeric complex with SSTR5 but not with SSTR1 (Somvanshi et al., 2009).

In addition to homo-and/or heterodimerization within the family, SSTR subtypes also forms functional heteromeric complex with other related GPCR subtypes and attributed to distinct pharmacology and signaling properties (Baragli et al., 2007; Rocheville et al., 2000a). Further, in extension SSTR subtypes also interact functionally with β–adrenergic receptors (Somvanshi et al., 2011a; Somvanshi et al., 2011b). Recent studies have also shown that SSTR subtypes functionally interact with the members of receptor tyrosine kinase family such as epidermal growth factor receptor (EGFR/ErbBs) in breast cancer cells and HEK 293 and modulate tumor promoting signaling pathway and lead to the dissociation of ErbBs homo-and heterodimers (Kharmate et al., 2011a; Kharmate et al., 2011b).

1.9 Evidence supporting neurotrophic effect of somatostatin and somatostatin receptors

Role of SST as multifunctional peptide and its wide distribution in different parts of body has increased clinical interest and is linked to pathophysiology of several diseases (Cervia and Bagnoli, 2007; Panteris and Karamanolis, 2005). SST analogs such as octreotide and lanreotide are used in treatment of acromegaly (Hofland et al., 2005; Newman et al.,

1998; Tolis et al., 2006), various neuroendocrine/pancreatic or gastroenterological tumors (Weckbecker et al., 2003). Studies have shown that direct blockade of autocrine/paracrine secretions and indirect antitumor effects of SSTRs like antiangiogenic actions and modulation of immune cell functions are the rationale behind therapeutic use of SST analogs and role of SSTRs in tumors (Susini and Buscail, 2006).

In CNS a subset of neuronal population in striatum known as medium sized aspiny interneurons coexpressed SST/NPY/NADPH-d (Beal et al., 1986; Vincent and Johansson, 1983). Although controversies exist, several previous studies have shown that these subclasses of neurons survive the neurodegeneration induced by NMDA receptor agonist QUIN injection and patients with HD (Aronin et al., 1983; Beal et al., 1984; Dawbarn et al., 1985; Nemeroff et al., 1983). Previous studies have shown that QUIN induced excitotoxicity not only leads to the survival of SST/NPY neurons it also increases SST secretion and SST gene expression without any discernable changes in NPY expression (Patel et al., 1991; Patel et al., 1995b). Selective preservation of aspiny interneurons in excitotoxicity was supported due to the lack of NMDA receptors on these neurons and presence of NADPH-diaphorase (Koh et al., 1986). Later studies argued the lack of NMDA receptor and demonstrated mRNA and protein expression of NMDA receptor in these neurons however expression levels was relatively lower than those of projection neuron (Augood et al., 1994; Chen et al., 1996; Kumar et al., 1997a). Additionally, Landwehrmeyer et al. suggested that relative resistance of these neurons in excitotoxicity is because of NMDA channels present on aspiny interneurons that might exhibit distinct pharmacological and physiological properties than the projection neurons (Landwehrmeyer et al., 1995). Furthermore, studies have also shown that the selective survival of SST/NPY/bNOS-positive neurons after QUIN and NMDA treatment

might be due to the presence of NADPH-d and Cu/Zn superoxide dismutase (Cu/Zn SOD) (Kumar, 2004). However, one of the recent study from our laboratory has shown that knocking down of SST by using antisense oligonucleotides and immunoadsorption of released SST with SST specific antibodies potentiates neuronal cell death in QUIN and NMDA induced excitotoxicity including NDAPH-d positive interneurons which are selectively preserved in excitotoxicity (Kumar, 2008). Dawson et al., by using NOS deficient mice have shown that there is a selective preservation of SST positive neurons in QUIN/NMDA induced toxicity indicating NOS is not playing a neurotrophic role in excitotoxicity (Dawson et al., 1996). These studies provide a strong rationale for the neuroprotective role of SST in excitotoxicity as well as in the pathophysiology of HD. SST manifests its neurotrophic and neuroprotective role via SSTRs. To determine the role of SSTRs in excitotoxicity with specific reference to HD, characterization of SSTR subtypes with specific protein associated with HD will help in identifying the role of specific receptors. Elucidating the interactions between SSTRs, DARPP-32 and NMDARs its inference in signaling pathways involved in HD will help in understanding the neuroprotective role of SST and SSTRs. Most importantly, blockade of the released SST enhances NMDA-induced cell death whereas exogenously applied SST prevents death, thus suggesting that SST plays a neuroprotective role (Forloni et al., 1997).

1.10 Role of NMDARs in neurodegeneration

Glutamate is the major excitatory neurotransmitter in the CNS, which activates NMDARs resulting in significant increase in Ca^{2+} influx and neuronal cell death, also known as glutamate excitotoxicity. Glutamate is known to play an important role in many neurodegenerative diseases like cerebral ischemia, epilepsy and HD (Coyle and Puttfarcken,

1993; Greenamyre, 1986). NMDARs are involved in biological functions such as learning, memory processing, feeding behaviors and play a role in a variety of disease processes in addition to HD, AD, PD, hypoxia/ischemia, seizure disorders as well as neuropsychological disorders including schizophrenia (Loftis and Janowsky, 2003; Waxman and Lynch, 2005). Studies in non-human primates and rodents have shown that over activation of NMDARs in striatum mimics the pathological, neurochemical and behavioral changes of HD (Li and Li, 2004). Autopsy brain tissues from HD patients revealed degeneration of NMDARs positive neurons in striatum (Albin et al., 1990; Young et al., 1988). NMDARs are tetrameric structures composed of two subunits of NR1 and two subunits of NR2A, NR2B or NR2C (Monyer et al., 1994; Ozawa et al., 1998). NR 2A and 2B subunits are highly expressed in the adult forebrain (Kew and Kemp, 2005). Moreover, NMDAR subunit composition and distribution in striatum, hippocampus and cortex may determine the role in synaptic plasticity and neurodegeneration (Hardingham and Bading, 2003; Liu et al., 2004; Morishita et al., 2007). Previous studies have shown that neurons in striatum display more NR2B expression in comparison to other NR2 subunits (Ghasemzadeh et al., 1996; Landwehrmeyer et al., 1995; Li et al., 2003; Rigby et al., 1996). NMDARs activated by glutamate are highly permeable to calcium and modulated by variety of cations including Zn^{2+}, H^{+} and Mg^{2+} (Cull-Candy et al., 2001; McBain and Mayer, 1994). As discussed earlier stereotaxic injections of NMDARs agonist like NMDA/QUIN replicate the neurophathological features of HD and have been used as experimental models of HD pathology (Beal et al., 1991; Beal et al., 1986; Schwarcz et al., 1984).

Receptor specific heterodimerization between NMDARs is associated with diversity in receptor function and a critical determinant in neurodegeneration (Cull-Candy et al., 2001;

Ishii et al., 1993; Monyer et al., 1994). Post-synaptic density protein 95kDa (PSD-95) has been shown to bind with C-terminal of NMDARs and plays a stabilizing role for cell surface expression of NMDARs (Kornau et al., 1995; Roche et al., 2001). Sun et al., have shown that *mHtt* binds to PSD-95, hence PSD-95 might act as a link between *mHtt* and NMDARs (Sun et al., 2001).

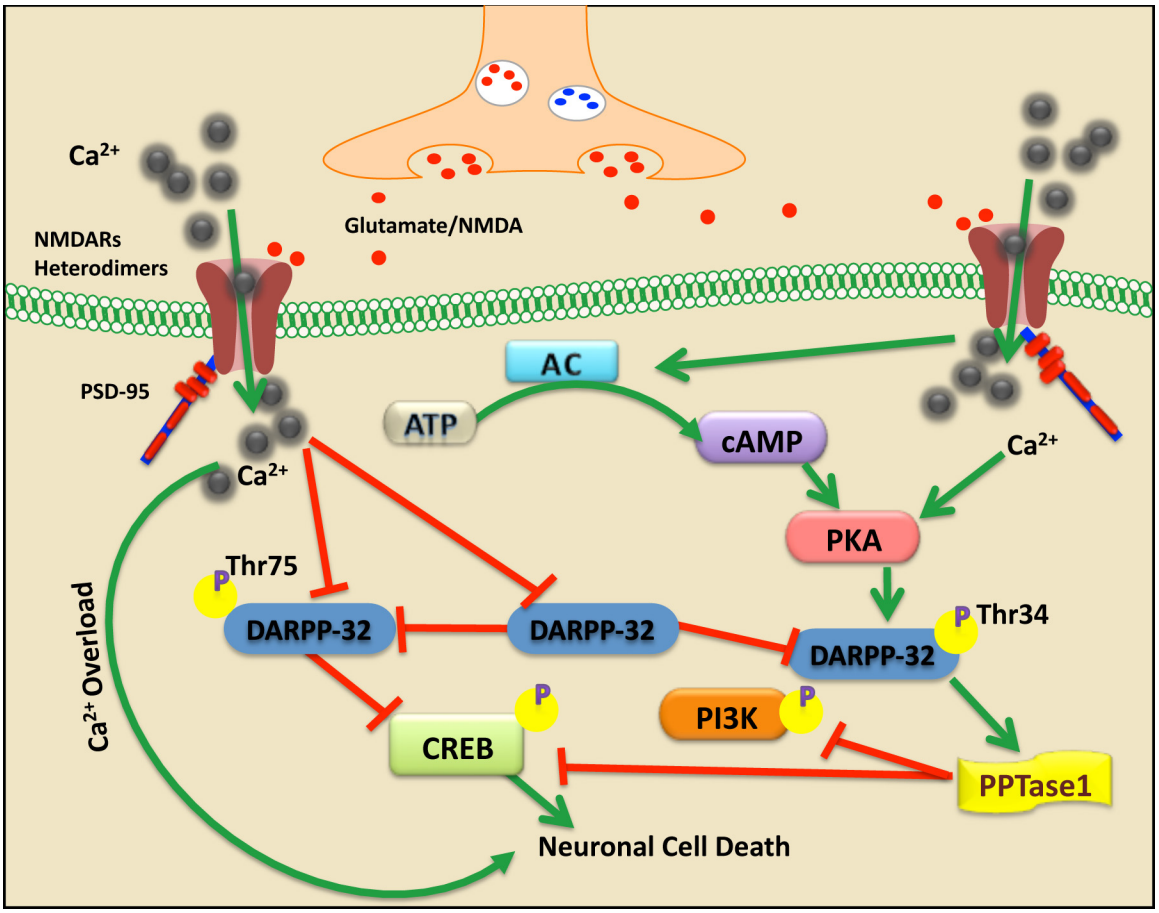

Figure 1.2 Schematic representation illustrating NMDARs mediated signaling pathways associated with excitotoxicity

Chen et al., for the first time described functional interaction between NMDARs and polyQ expansion in *htt* (Chen et al., 1999). HEK293 cells expressing NR1/NR2B exhibit larger NMDAR-mediated currents in presence of *htt*-138Q in comparison to *htt*-15Q. Interestingly, similar effect was not observed in cells expressing NR1/NR2A (Chen et al.,

1999). Increased apoptotic cell death was observed in HEK293 cells transfected with NR1/NR2B-subtype by *mhtt* (Zeron et al., 2001). Furthermore, studies in YAC transgenic mouse model indicate a major role of NR2B in mediating neuronal degeneration (Zeron et al., 2002). Previous studies in pre-symptomatic R6/2 mice have shown no significant changes in mRNA levels of NR1 or NR2B however, a decrease in mRNA levels of NR2A was observed (Ali and Levine, 2006). These data indicate NR2B subunit as a key receptor subtype modulating the excitotoxic damage induced via *mhtt* (Christie et al., 2000; Ghasemzadeh et al., 1996; Kuppenbender et al., 2000; Landwehrmeyer et al., 1995; Li et al., 2003; Rigby et al., 1996).

Recently, it has been described that the specific and selective role of NMDARs is confined in synaptic and extrasynaptic location in CNS (Hardingham, 2009; Hardingham and Bading, 2003; Raymond et al., 2011). Studies have also reported the functional consequences of activation of synaptic and extrasynaptic NMDARs in neuronal death and survival. Most importantly, activation of extra synaptic NMDARs leads to neuronal loss, whereas activation of synaptic NMDARs is linked to survival of neurons (Hardingham and Bading, 2003; Hardingham et al., 2002; Soriano et al., 2006).

1.11 Dopamine and cAMP-regulated phosphoprotein (DARPP-32)

In addition to NMDAR, in striatum, projection neurons are highly rich with DARPP-32 which constitute >80% of medium-sized spiny projection neurons (Ouimet et al., 1998; Walaas and Greengard, 1984). The loss of DARPP-32 like immunoreactivity has been used as an index of neuronal loss in excitotoxicity as well as in several neurological diseases, specifically in HD. DARPP-32 is involved in regulation of signal transduction pathway mediated by DR subtypes (Greengard, 2001; Greengard et al., 1999; Svenningsson et al.,

2004; Walaas et al., 1983). Dopamine induced activation of D1R resulted in DARPP-32 phosphorylation and thus forms a potent inhibitor of protein phosphatase-1 (PPtase1) leads to the prevention of dephosphorylation DARPP-32 (Nishi et al., 2000; Nishi et al., 1997; Svenningsson et al., 2000). DR subtypes specifically D1 subtypes activate adenylyl cyclase converting ATP to cAMP and increasing the formation of cAMP and consequent activation of PKA. Further activated PKA phosphorylates DARPP-32 at Thr34 converting it into a potent inhibitor of PPtase1. PPtase1is known to inhibit the phosphorylated form of cAMP response element-binding protein (CREB), cAMP response element modulator (CREM) and activating transcription Factor-1 (ATF-1) (Andersson et al., 2005; Bastia and Schwarzschild, 2003). Phosphorylation of CREB is essential for survival of CNS neurons (Mantamadiotis et al., 2002). Furthermore, the activation of postsynaptic NMDAR generates calcium signals, which once decoded by Ca^{2+} sensitive proteins have an impact on cell survival. Ca^{2+} modulates phosphorylation of DARPP-32 at Thr75 by activating calcium-dependent proteins like calretinin, calcineurin and calbindin. Phosphorylated DARPP-32 at Thr75 modulates protein phosphatase-2B (PP2B), which in turn also modulates PKA inhibition and plays important role in cell survival (Jay, 2003; Zhang et al., 2005). Phosphorylation of DARPP-32 play an important role in modulating PKA and CREB which are involved in neuronal death due to over influx of Ca^{2+} upon NMDARs activation. With this existing information the association of receptor protein in CNS with DARPP-32 might serve as a crucial indicator for neuronal survival and loss in the process of neurodegeneration. Accordingly the characterization of receptors in striatum with colocalization with DARPP-32 might help to establish first the distribution in projection neurons and second their fate in excitotoxicity.

1.12 Hypothesis

As discussed above SST positive neurons in brain respond to a variety of neurotoxins by inducing gene expression, peptide production and secretion. There is a large body of evidence supporting that SST is induced in the cortex and in the striatum in response to NMDA-mediated neurotoxicity as well as in certain pathological conditions. Furthermore, exogenous addition of SST provides neuroprotection against NMDA-induced toxicity. ***Accordingly, we hypothesize that the loss of the protective effect of SST facilitates the neurotoxic effect of NMDA agonist and changes in SST and SSTR subtypes may be critical determinant of neuronal changes in neurological diseases and transgenic models of disease.*** To accomplish my aforesaid hypothesis I proposed the following four specific aims

1. **Colocalization of dopamine receptor subtypes with dopamine and cAMP-regulated phosphoprotein (DARPP-32) in rat brain.**

2. **Colocalization of somatostatin receptors with DARPP-32 in cortex and striatum of rat brain.**

3. **Somatostatin receptor 1 and 5 double knock out mice mimic neurochemical changes of Huntington's disease transgenic mice.**

4. **Somatostatin receptors-mediated inhibition of NMDA currents and dissociation of NMDARs complex in striatum: Implication in excitotoxicity.**

Chapter 2: [1]Colocalization of dopamine receptor subtypes with dopamine and cAMP-regulated phosphoprotein (DARPP-32) in rat brain

DARPP-32 is highly expressed in projection neurons of striatum and protein expression is significantly diminished in excitotoxicity and many neurological diseases. Since Dopamine Receptors are well expressed in striatum and other brain regions and play major role in regulation of DARPP-32. In excitotoxicity, like DARPP-32, DRs positive neurons are also highly vulnerable. Although, SSTR subtypes share almost >30-40% structural homology and functionally interact with each other however, whether SSTR subtypes are involved in regulation of DARPP-32 in striatum is still not known. Therefore in this chapter we first established the relation between DRs and DARPP-32 using immunohistochemistry and co-immunoprecipitation assay.

2.1 Background

In the central nervous system (CNS) dopamine (DA) via five different receptor subtypes namely dopamine receptors (D1R-D5R) function as one of the most prominent neurotransmitter (Lloyd and Hornykiewicz, 1970). Dopamine receptors (DRs) belong to G-protein coupled receptors (GPCRs) family and have been divided into two major sub classes, D1 receptor subtypes (D1R and D5R) preferentially couple to G_s protein and stimulate adenylyl cyclase as well as protein kinase A dependent pathway (Sunahara et al., 1991). D2 receptor subclass (D2R, D3R and D4R) couple to G_i protein and inhibit adenylyl cyclase (Bunzow et al., 1988; Missale et al., 1998; Sunahara et al., 1991; Van Tol et al., 1991). The

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role of dopaminergic system in several neurological disorders such as Alzheimer's disease (AD), Huntington's disease (HD) and Parkinson's disease (PD) and neuropsychiatric disorders like Tourette's syndrome, attention deficit hyperactivity disorder, schizophrenia as well as in drugs of abuse is well established (Koob and Le Moal, 1997; Seeman et al., 1987; Seeman and Van Tol, 1993; Self et al., 1996). Amongst all five DR subtypes, D1R and D2R are the major receptor subtypes that are expressed predominantly in various regions of the brain (Amara et al., 1998; Sotnikova et al., 2006). D1 and D5 subtypes primarily regulate the regulation of motor control, cognitive function and emotions. D2R, D3R and D4R have been shown to play considerable roles on the regulation of antipsychotic drugs. Furthermore, increased expression levels of D3R have also been shown in schizophrenia (Gurevich et al., 1997; Joyce, 2001). Immunohistochemical and *in situ* hybridization studies have shown that the two sub classes of DRs are present in the projection neurons in the striatum and also expressed in receptor specific manner in other brain regions (Le Moine and Bloch, 1995; Surmeier et al., 1993). DR subtypes alter the permeability of ion channels or act on various signaling pathways that mediate the effects of neurotransmitters in receptor specific manner. One of the major effects of DRs is to modulate the cyclic adenosine monophosphate (cAMP) levels in receptor specific manner and thus causing complicated phosphorylation cascades which in turn resulted in modifications of synaptic transmission (Sotnikova et al., 2006; Sunahara et al., 1991).

In addition to the major neurotransmitters, there are some regulatory proteins which are activated by GPCRs. Amongst them Dopamine and cAMP-regulated phosphoprotein (DARPP-32) is one of the most studied protein which predominantly involves in the regulation of signal transduction pathway mediated by DR subtypes (Greengard, 2001;

Greengard et al., 1999; Svenningsson et al., 2004; Walaas et al., 1983). DARPP-32 is expressed in >80% of medium-sized spiny neurons (MSN) in striatum (Ouimet et al., 1998; Walaas and Greengard, 1984). The presence of DARPP-32 in striatal neurons has shown to be associated with modulation of cAMP (Andersson et al., 2005). In comparison to the striatum, other brain regions exhibited rather low abundance of DARPP-32 in region specific manner and associated with different array of functions in selective fashion. Since its discovery, DARPP-32 is proven to be a fundamental protein to modulate DA mediated central effects in the brain (Walaas et al., 1983). DARPP-32 is phosphorylated in response to DA upon activation of D1R and thus forms a potent inhibitor of protein phosphatase-1 causing further prevention of the dephosphorylation (Nishi et al., 2000; Nishi et al., 1997; Svenningsson et al., 2000). It has also been shown that via D2R activation, DA inhibits the dephosphorylation of DARPP-32. Hence due to actions of different DRs, DA bidirectionally regulates the phosphorylation of DARPP-32 (Lindskog et al., 1999b; Nishi et al., 1997).

Several previous studies have shown the differential distributional pattern of DARPP-32 in different parts of the brain in many species (Durstewitz et al., 1998; Ouimet et al., 1992; Schnabel et al., 1997). DARPP-32 is the major protein which plays pivotal role in neurological diseases, drugs addiction and schizophrenia (Nairn et al., 2004; Svenningsson et al., 2005). Like DARPP-32, DR subtypes also exert critical role in similar condition in receptor specific manner and dopaminergic projections are the potential target for several drugs of abuse and anti-psychotics drugs (Hyman and Malenka, 2001; Nestler, 2001). The role of DARPP-32 has greatly been appreciated in pathophysiology of HD (Torres-Peraza et al., 2007). Whether DRs mediated effects on DARPP-32 are direct or indirect is not well understood yet. Accordingly, colocalization studies are essential to understand any potential

interaction between DR subtypes and DARPP-32 in brain. Although previous studies have shown the colocalization of DARPP-32 with D1R and D2R, whether remaining receptors D3R, D4R and D5R colocalize with DARPP-32 is not known (Aizman et al., 2000; Langley et al., 1997). Therefore, the present study was undertaken to determine the comparative colocalization of DR subtypes with DARPP-32 in the cortex and striatum of the rat brain. Further to confirm our colocalization studies we also determined the expression of DARPP-32 in immunoprecipitate prepared by using DR subtypes antibodies from the tissue lysate of rat brain cortex and striatum. Our data indicates the colocalization of DARPP-32 and DR subtypes in a receptor and region specific manner in rat brain cortex and striatum.

2.2 Materials and methods

2.2.1 Animals

Sprague-Dawley rats (males) weighing 150-200g were obtained from UBC animal care unit and used for immunohistochemistry and colocalization studies. The protocols regarding animal care were followed in compliance with the principles of the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee.

2.2.2 Materials

Rabbit polyclonal antibodies directed against D1R-D5R were generated as described earlier (Kumar and Patel, 2006). Mouse anti-DARPP-32 monoclonal antibody was purchased from BD Biosciences (Pharmingen, San Diego, CA, USA). Goat anti-mouse Alexa-488 and goat anti- rabbit Alexa-594 secondary antibodies were purchased from Invitrogen

(Burlington, ON, Canada). Protein A/G agarose beads were purchased from Calbiochem (Mississauga, ON, Canada). Nitrocellulose Hy-Bond membrane and Enhanced chemiluminescence (ECL) detection system was purchased from Amersham Ltd. (Oakdale, ON, Canada). All other general chemicals were of analytical grade and were obtained from various commercial sources.

2.2.3 Immunohistochemistry

The animals were sacrificed using intravenous pentobarbital (100mg/kg) followed by perfusion with 5% heaprinized saline and 4% paraformaldehyde with an infusion pump (KD Scientific Model 200 Series, Holliston, MA). Brains were then removed and postfixed in 4% paraformaldehyde at 4°C for 24 h. 30 μm thick coronal brain sections were cut using a vibratome and free floating sections were collected in phosphate buffer saline (PBS) and incubated in 0.2% Triton X-100 for 10 min. Following three subsequent washes in PBS, sections were incubated in 5% normal goat serum (NGS) for 1 h at room temperature (RT). The brain sections were incubated with a cocktail of polyclonal DR subtype 1-5 antibodies (1:300) and monoclonal DARPP-32 antibody (1:250) for 24 h at 4°C in a humid atmosphere. The sections were washed with PBS and then incubated in the presence of goat anti-mouse Alexa-488 (green) and goat anti-rabbit Alexa-594 (red) secondary antibodies for 1 h at RT to visualize DARPP-32 and DR subtypes respectively. Following three washes in PBS, sections were mounted on the slides and were examined under Leica DMLB microscope attached with the Retiga 2000R camera. All the photographs composites were made using Adobe Photoshop (San Jose, CA) and merged images showing colocalization where generated by using NIH, ImageJ software.

2.2.4 Quantitative analysis of DR subtypes colocalization with DARPP-32

D1R-D5R colocalizations with DARPP-32 were analyzed quantitatively to determine receptor specific colocalization variation in cortex and striatum. Serial brain sections (30μm) double labeled with DR subtypes and DARPP-32 were processed for quantitative analysis as previously described (Kumar, 2007). Briefly, from individual rat three to four brain sections were taken for each receptor subtypes from five different rats and 10-15 fields were selected from cortex and striatum, following stereotaxic coordinates from rat brain atlas of Paxinos and Watson (1998). Total numbers of neurons displaying colocalization were counted as described previously (Kumar, 2007). Neurons with the intact morphology and immunoreactivity higher then background were only included for quantification purpose. Numbers of neurons positive for individual proteins and colocalized were pooled and then mean was taken to determine percent colocalization. Data are presented as percentage mean ± SD. Blind study was also performed to support our quantitative analysis. Background staining referred to the staining observed in absence of primary antibodies. In order to verify the specificity of the immunofluorescence and to eliminate the possibility of fluorescent bleed through in colocalization, tissues stained for single staining were also photographed in green and red channels, respectively.

2.2.5 Western blot analysis and co-immunoprecipitation

Total tissue protein from rat brain cortex and striatum were obtained by using glass homogenizer in homogenization buffer containing (62.5 mM Tris-HCl, 50 mM dithiothretiol [DTT], 2% SDS, 10% glycerol). The total tissue protein concentration was then estimated by Bradford protein assay. 20 μg of total protein was then solubilized in Laemmli sample buffer

with 5% 2-mercaptoethanol (Bio-Rad) and denatured at 99° C for 5 min. The samples were subsequently fractionated by electrophoresis on 10% SDS polyacrylamide gel and then transferred onto 0.2μm nitrocellulose membrane in transfer buffer (20 mM Tris, 192 mM glycine and 20% methanol). The membrane was blocked with 5% non-fat dried milk at room temperature for 1 h and subsequently incubated overnight at 4°C with specific primary antibodies for DRs 1-5 (1:600) and DARPP-32 (1:250) diluted in 5% bovine serum albumin. The membranes were later incubated with goat anti-rabbit or a goat anti-mouse secondary antibody at room temperature for 1 h to detect DR subtypes and DARPP-32 respectively. The bands were detected with chemiluminescence reagent and images were taken using an Alpha Innotech FluorChem 8800 (Alpha Innotech Co., San Leandro, CA) gel box imager. Beta-actin was used as the housekeeping protein.

To perform co-immunoprecipitation whole tissue lysates were solubilized in 1 ml of radioimmune precipitation assay buffer (RIPA) consisting (150 mM NaCl, 50 mM Tris–HCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, pH 8.0) for 1 h at 4°C. The lysates were then incubated with anti-D1-D5R antibodies (1:300) overnight at 4°C. The antibodies were then immunoprecipitated using protein A/G agarose beads for 2 h at 4°C. The agarose beads were then washed three times in RIPA buffer and then the pellet was solubilized in Laemmli sample buffer with 5% 2-mercaptoethanol (Bio-Rad). The samples were heated at 99°C for 5 min and fractionated on a 10% SDS polyacrylamide gel by electrophoresis. The fractionated proteins were then transferred electrophoretically onto 0.2 μm nitrocellulose membrane and processed for DARPP-32 as described in western blot analysis above.

2.3 Results

2.3.1 Specificity of dopamine receptors (D1R- D5R) antibodies

We have previously characterized the specificity of DR subtypes antibodies by using immunohistochemistry and western blot analysis in various tissues (Kumar and Patel, 2007; Shin et al., 2003; Sidhu et al., 1998). In the absence of primary antibodies or in the presence of preimmune serum we observed only the background staining.

2.3.2 Immunohistochemical expression of dopamine receptor subtypes and DARPP-32 in rat brain cortical and striatal regions

Consistent with several previous studies, we found wide spread distribution of DRs and DARPP-32 of rat brain cortex and striatum (**Figures 2.1 – 2.8**). DRs like immunoreactivity was seen in different layers of cortex and expressed as membrane and cytoplasmic protein. In striatum, DRs are well expressed in MSNs along with medium sized aspiny interneurons (**Figure 2.7 red**). Distributional pattern of DARPP-32 like immunoreactivity in cortex and striatum was consistent with previous studies (Ouimet et al., 1998; Ouimet et al., 1984). In cortex, DARPP-32 positive neurons were mainly restricted to the deep layer of cortex whereas in upper cortical layer DARPP-32 positive neurons were sparsely distributed. Most strong DARPP-32 staining was observed in cingulate cortex in comparison to other cortical regions. In striatum, DARPP-32 like immunoreactivity was confined to MSNs and restricted to cytoplasm, neuronal processes, dendrites and densely innervated nerve fibers (**Figure 2.7 green**). We next determined the pattern of colocalization of DARPP-32 and DR subtypes in cortex and striatum. In cortical brain regions, DARPP-32 positive neurons are most intense in cingulate, deep layer of temporal/frontal cortex as well

as in amygdaloid complex of enterohinal cortex. Therefore, the colocalization studies were only focused on cingulate, enterohinal and temporal/frontal cortical regions.

2.3.3 Colocalization of dopamine receptor subtypes with DARPP-32 in rat brain cortex

As shown in **Figure 2.1 A-L**, DARPP-32 (green) displayed differential distributional pattern of colocalization with D1R in cingulate cortex (**Figure 2.1 A, C** and **D**). We identified three different populations of neurons; exhibiting colocalization and DARPP-32 or D1R immunoreactivity. DARPP-32 positive dendrites were devoid of colocalization with D1R. The colocalization was limited to the apical ending of neuronal cell bodies. In frontal cortex, colocalization between D1R and DARPP-32 was prominent in layer V and VI. Despite the significant expression of D1R in layer I-III, less colocalization between D1R and DARPP-32 was observed. In addition few neurons in upper layer of cortex expressing strong DARPP-32 immunoreactivity were also lacking colocalization. Enterohinal cortex displayed distinct pattern of colocalization, only few neurons expressed D1R and DARPP-32 whereas majority of neurons were positive for D1R like immunoreactivity (**Figure 2.1 I-L**). Nerve fibers expressing moderate immunoreactivity either for D1R or DARPP-32 were also observed in enterohinal cortex (**Figure 2.1 K and L**).

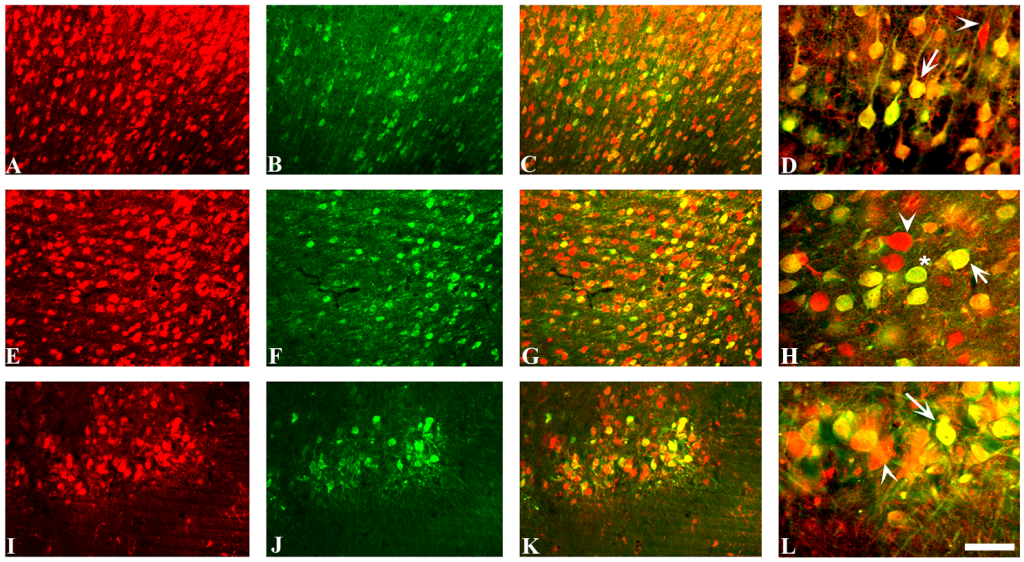

Figure 2.1 Indirect immunofluorescence photomicrographs illustrating the colocalization of D1R and DARPP-32 in rat brain cortex.

30µm coronal brain sections were incubated with polyclonal D1R and monoclonal DARPP-32 antibodies for 24 h and followed by incubation in goat anti rabbit Alexa-594 and goat anti mouse Alexa-488 secondary antibodies to visualize D1R and DARPP-32 respectively (please see Material and Methods section for details). In cingulate cortex (Panel A-D**),** frontal cortex (panel E-H**)** and enterohinal cortex (panel I-L**)** represent the expression of D1R (red) DARPP-32 (green) and colocalization is identified in orange yellow color. Note, neurons either expressing only D1R or DARPP-32 were also present in all three cortical regions. Arrows indicate colocalization, arrowheads indicate neurons expressing D1R, whereas neurons positive for only DARPP-32 are indicated by an asterisk (*). Panels A-C, E-G and I-K scale bar = 100µm and for panel D, H and L scale bar = 20µm.

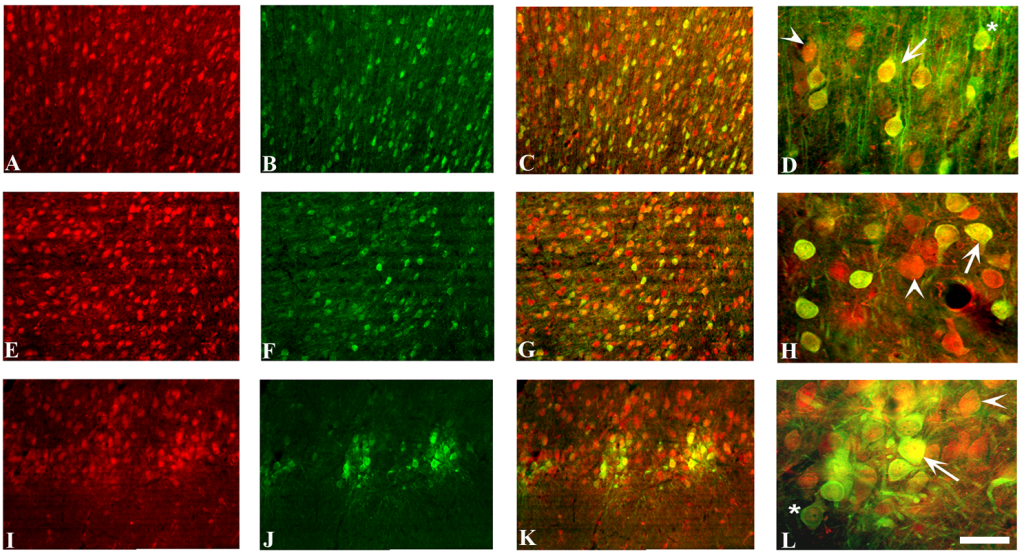

Figure 2.2 Photomicrographs illustrating the colocalization of D2R and DARPP-32 in cortical rat brain.

Colocalization of D2R and DARPP-32 is identified in cingulate cortex (A-D), in temporal cortex (E-H) whereas colocalization in enterohinal region is represented in panels I-L**.** In addition to the neurons expressing colocalization, D2R or DARPP-32 positive neurons are also present in the same region. In all representative panels arrows indicate colocalization, arrowheads indicate neurons expressing D2R, whereas neurons positive for DARPP-32 like immunoreactivity are indicated by an asterisk (*). Scale bar represent 100 µm for Panels A-C, E-G and I-K and 20µm for panel D, H and L.

In rat brain cortex, the colocalization between DARPP-32 and D2R was strikingly different in all three cortical regions with significant changes in colocalization within neuronal cell bodies, dendrites and innervated nerve fibers (**Figure 2.2 A-L**). As shown in **Figure 2.2 A-D**, D2R (red) and DARPP-32 (green) cingulate cortex displayed different patterns of colocalization. Selective neurons in cortical region showed strong colocalization between DARPP-32 and D2R but were comparatively less than D1R. Neuronal dendrites positive to DARPP-32 were devoid of D2R like immunoreactivity. As shown in **Figure 2.2 E-H**, in frontal cortex three different populations of neurons was observed neurons expressing only D2R or DARPP-32 and neurons expressing both. DARPP-32 like immunoreactivity was also observed in innervated nerve fibers throughout the frontal cortex

without any expression of D2R. In enterohinal cortex similar pattern of colocalization was observed showing three different populations like in frontal cortex (**Figure 2.2 I - L**). Strikingly, in enterohinal cortex dense innervated nerve fibers positive to DARPP-32 were devoid of colocalization.

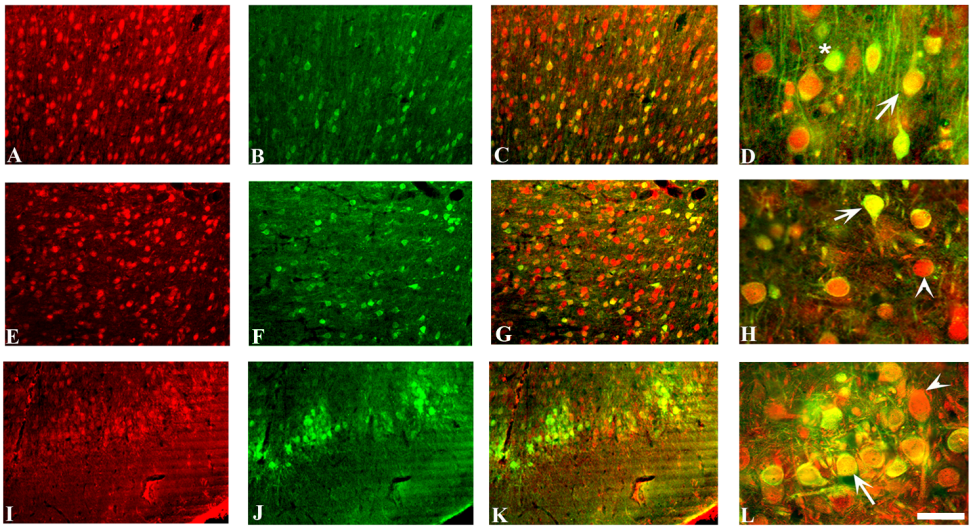

Figure 2.3 Photomicrographs illustrating colocalization of D3R and DARPP-32 in rat brain cortex.

Panel A-D represents colocalization in cingulate cortex; panel E-H represents colocalization in temporal cortex and panel I-L represent colocalization in enterohinal cortex. D3R colocalized with DARPP-32 in cortex in a region specific manner. Neurons either expressing D3R or DARPP-32 were also present in the same region in addition to colocalization. In all representative panels arrows indicate colocalization, arrowheads indicate neurons expressing D3R, whereas neurons positive for DARPP-32 like immunoreactivity are indicated by an asterisk (*). Scale bar represent 100 µm for Panels A-C, E-G and I-K and 20µm for panel D, H and L.

As shown in **Figure 2.3 A-L**, colocalization of DARPP-32 and D3R revealed striking differences in cortical brain regions. D3R (red) and DARPP-32 (green) positive neurons constitute three different populations of cortical cells, first expressing only D3R, second exhibiting DARPP-32 like immunoreactivity and third population expressing colocalization. Interestingly, DARPP-32 like immunoreactivity was observed in dendrites and nerve fibers in cingulate cortex were devoid of colocalization with D3R (**Figure 2.3 A-D**).

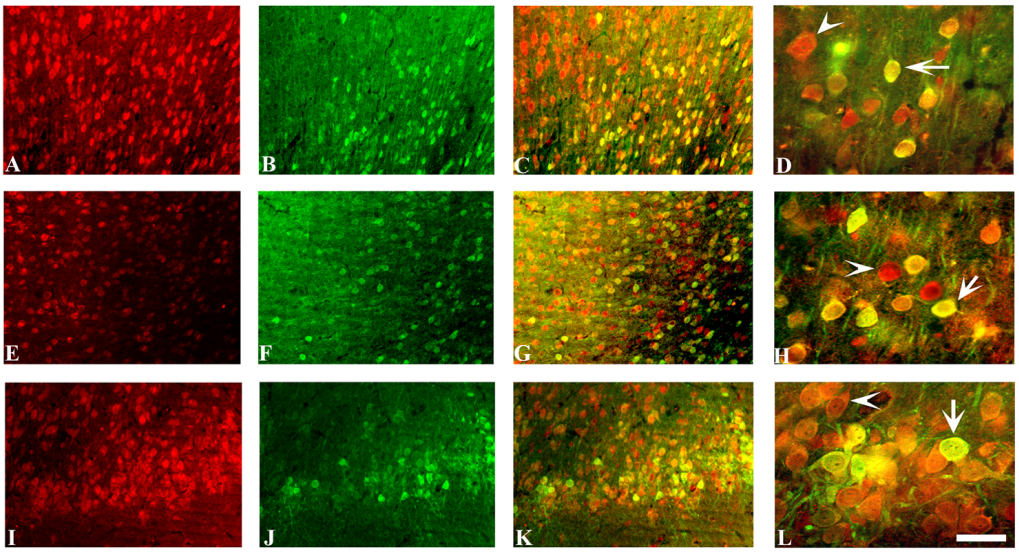

Figure 2.4 Photomicrographs illustrating colocalization of D4R and DARPP-32 in rat brain cortex.

D4R positive immunoreactivity was localized by Alexa- 594 (red) and DARPP-32 like immunoreactivity indicated by Alexa-488 (green). Panel A-D represents colocalization of D4R and DARPP-32 indicated by yellow color in cingulate cortex, panel E-H represents colocalization in temporal cortex and panel I-L represent colocalization in enterohinal cortex. D4R colocalized with DARPP-32 in most cortical regions in a region specific manner. In addition to colocalization, neurons either expressing D4R or DARPP-32 like immunoreactivity were also identified in the same region. In all representative panels arrows indicate colocalization, arrowheads indicate neurons expressing D4R, whereas neurons positive for DARPP-32 like immunoreactivity are indicated by an asterisk (*). Scale bar represent 100 µm for Panels A-C, E-G and I-K and 20 µm for panel D, H and L.

The colocalization of DARPP-32 with D4R in cortical brain region was comparable to D1R displaying three different populations showing colocalization or DARPP-32 / D4R like immunoreactivity. Relatively, DARPP-32 positive neurons displayed greater degree of colocalization in cingulate cortex compared to frontal and enterohinal cortex. As illustrated in **Figure 2.4 A-L**, in addition to neuronal population neuronal processes and innervated nerve fibers also exhibit weak colocalization in cortical brain regions. In enterohinal cortex DARPP-32 moderately colocalized with D4R whereas, DARPP-32 positive nerve fibers and dendrites were devoid of colocalization.

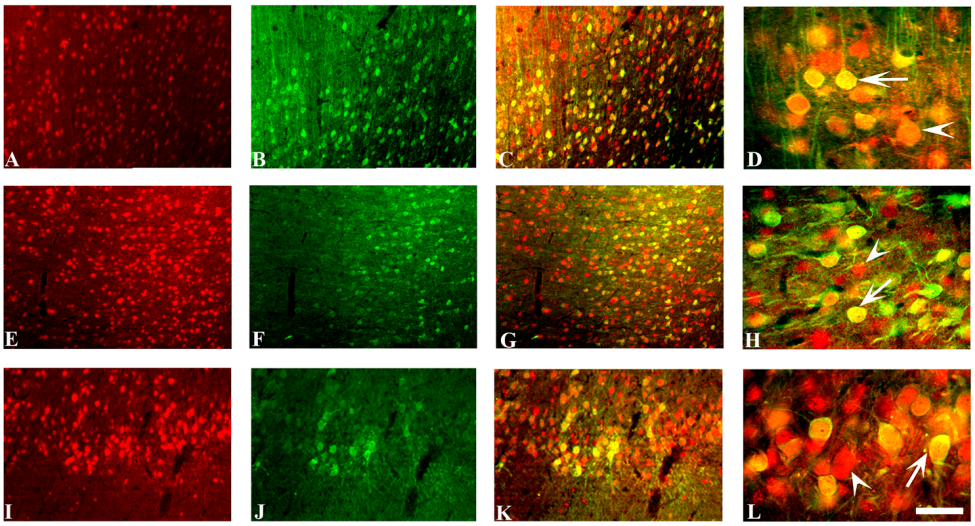

Figure 2.5 Photomicrographs illustrating colocalization of D5R and DARPP-32 in cortical rat brain.

D5R positive immunoreactivity was localized by Alexa- 594 (red) and DARPP-32 like immunoreactivity indicated by Alexa-488 (green). Panel A-D represents colocalization indicated by yellow color in cingulate cortex; panel E-H represents colocalization in temporal cortex and panel I-L represent colocalization in enterohinal cortex. D5R colocalized with DARPP-32 in most cortex regions in a region specific manner. In addition to colocalization, neurons either expressing D5R or DARPP-32 were also present in the same region. In all representative panels arrows indicate colocalization, arrowheads indicate neurons expressing D5R, whereas neurons positive for DARPP-32 like immunoreactivity are indicated by an asterisk (*). Scale bar represent 100µm for Panels A-C, E-G and I-K and 20µm for panel D, H and L.

As shown in **Figure 2.5 A-L**, in cortical brain regions we observed significant variation in colocalization between DARPP-32 and D5R in comparison to D1R-D4R subtypes. In majority the numbers of neurons expressing D5R positive immunoreactivity were negative for DARPP-32 expression in all three regions of cortex without any discernable changes. Only few neurons showed colocalization between D5R and DARPP-32. Neuronal processes and innervated nerve fibers throughout the cortex were strongly positive for DARPP-32 and were lacking colocalization with D5R (**Figure 2.5 A-L**).

2.3.4 Quantitative analysis of DR subtypes colocalization with DARPP-32

We next quantified the percentage distribution of DARPP-32 positive neurons expressing DRs in rat brain cortex. The expression pattern of DR subtypes and DARPP-32 in frontal and temporal cortex was not discernable; accordingly, the quantitative analysis was performed on data accumulated from frontal and temporal cortex. Further DARPP-32 positive neurons expressing DR subtypes were counted from each section, and averaged and are presented in **Figure 2.6**.

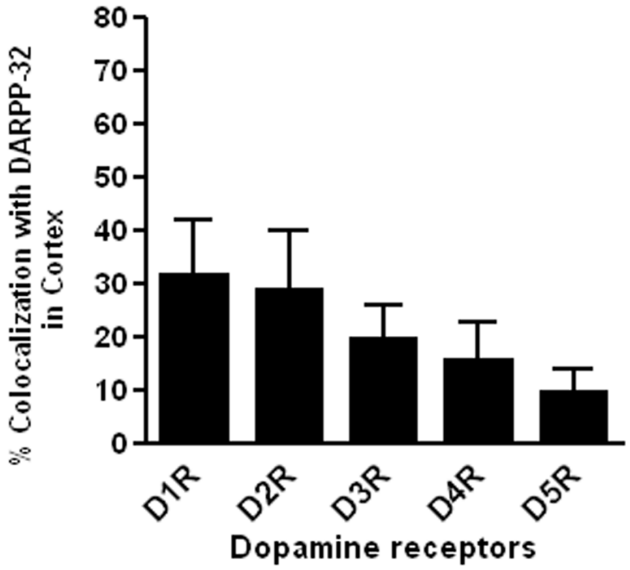

Figure 2.6 Quantitative analysis of DR subtypes positive cortical neurons colocalized with DARPP-32 in temporal/frontal cortex.

D1R and D2R colocalized with DARPP-32 in a comparable manner and constitute 32 % and 29 %. D3R and D4R exhibit similar degree of colocalization i.e., 20 % and 16 % respectively. D5R colocalized with DARPP-32 the least and constitutes 10 % colocalization. Each bar graphs represent mean ± SE from at least three sections from four different rats for each receptor subtype.

As shown D1R was the most prominent receptor subtype expressing DARPP-32 and constituted 32% colocalization followed by D2R (29%), D4R (20%) whereas D3R and D5R

constituted 16% and 10% respectively. The distributional pattern of colocalization of D1 and D2R with DARPP-32 was comparable.

2.3.5 Immunohistochemical expression of DARPP-32 and DR subtypes in rat brain striatum

DARPP-32 and DRs immunopositive neurons are widely distributed throughout the striatum. As shown in **Figure 2.7**, DARPP-32 like immunoreactivity (green) confined in majority of MSNs whereas DR subtypes are selectively distributed in projection as well as in medium sized aspiny neurons in receptor specific manner (**Figure 2.7 red**).

2.3.6 Colocalization of D1R-D5R with DARPP-32 in rat brain striatum

In striatum most of the neurons displaying D1R like immunoreactivity were also positive for DARPP-32. As shown in **Figure 2.7 A-D**, despite the fact that D1R colocalized with DARPP-32 two other populations of neurons were identified either showing D1R or DARPP-32 alone. In addition to the projection neurons, interneurons positive for D1R like immunoreactivity were devoid of colocalization. As shown in **Figure 2.7 D**, the dendrites or neuronal processes of the MSNs showed selective colocalization of D1R with DARPP-32 to some extent.

The colocalization between D2R and DARPP-32 was observed in neuronal cell bodies as well axonal dendrites (**Figure 2.7 E-H**). A sub-population of projection neurons positive for D2R was seen to colocalize with DARPP-32 indicating the colocalization was confined to MSNs. However, some projection neurons were DARPP-32 positive whereas a few large interneurons showed D2R like immunoreactivity. High magnification

photomicrographs revealed heavily innervated nerve fibers positive to DARPP-32 throughout the striatum and displayed lesser degree of colocalization in selective manner.

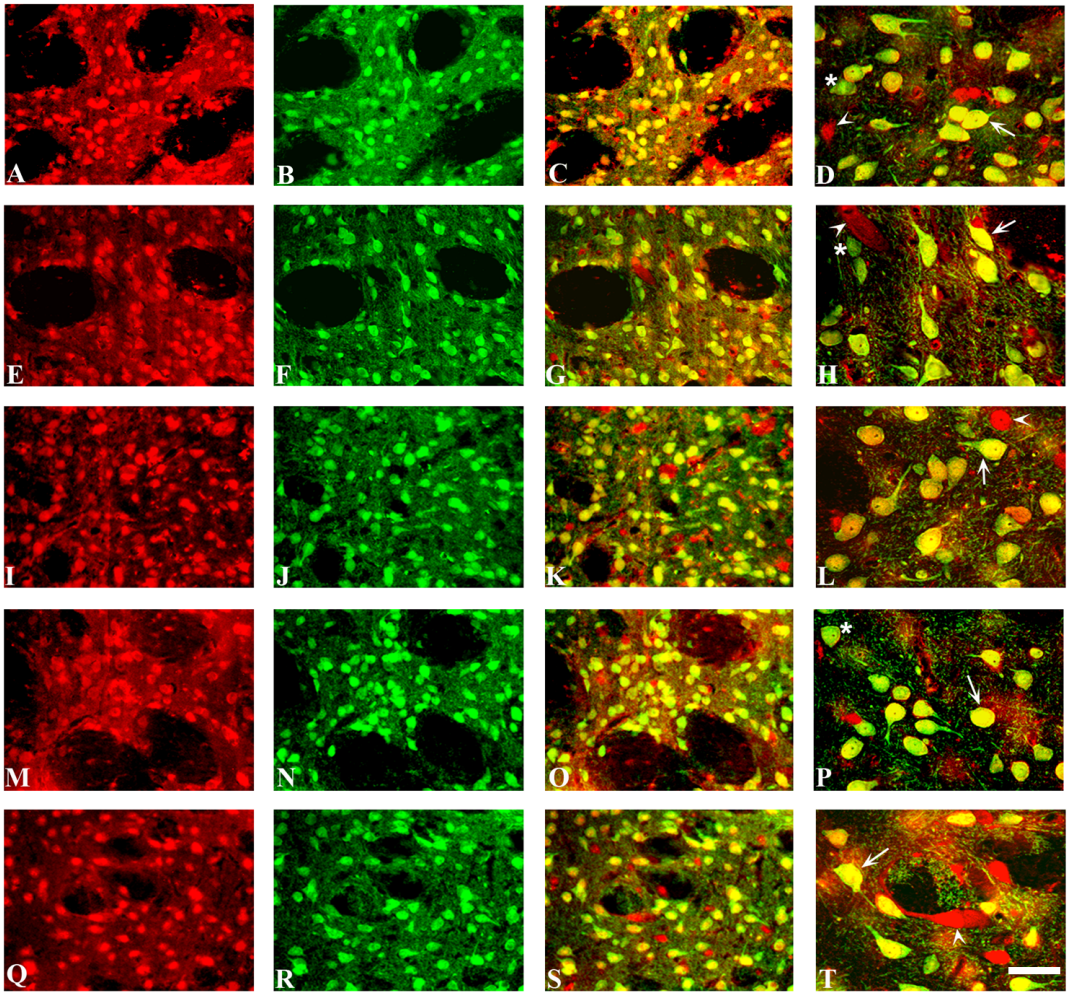

Figure 2.7 Immunofluorescence photomicrographs illustrating colocalization of D1R-D5R and DARPP-32 in rat brain striatum.

The final product of D1R-D5R positive immunoreactivity was developed by Alexa-594 (red) fluorescence (A, E, I, M, and Q). DARPP-32 positive immunoreactivity was identified by using Alexa-488 (green) fluorescence (B, F, J, N, and R). The colocalization of D1R-D5R with DARPP-32 is indicated by a yellow color in the overlapped images (C, D, F, H, K, L, O, P, S and T). In all the representative panels arrows indicate colocalization and arrowheads represent D1R-D5R positive neurons and DARPP-32 positive neurons are identified by asterisk (*). Panels A-C, E-G, I-K, M-O and Q-S scale bar = 100µm and for panel D, H, L, P and T scale bar = 20µm.

As illustrated in **Figure 2.7 I-L**, in case of colocalization studies for D3R two different populations of neurons in striatum were detected. First population displayed colocalization between D3R and DARPP-32 in projection neurons whereas the second neuronal population showed only D3R like immunoreactivity. Further analysis of immunoreactivity revealed that D3R like immunoreactivity was comparatively less than any other DR subtypes and also displayed weak colocalization except few neurons displaying very strong colocalization.

Figure 2.7 M-P showed the distributional pattern of D4R and DARPP-32 like immunoreactivity and colocalization in the striatum. The pattern of colocalization of D4R and DARPP-32 was comparable to D1R and D2R. Punctated DARPP-32 positive immunoreactivity was seen in nerve fibers all over the striatal region without any colocalization with D4R. Importantly, like any other DR subtypes distributional pattern of colocalization exhibited three distinct population in striatum either showing colocalization or independent staining for D4R and DARPP-32.

As shown in **Figure 2.7 Q-T** the pattern of colocalization of D5R with DARPP-32 in the striatum was greatly different than cortex. Some of the neurons positive to D5R and DARPP-32 displayed very strong colocalization. Interestingly, within the same region strongly positive D5R neurons with distinct morphological appearance were devoid of DARPP-32 positive immunoreactivity. Such neuronal populations were not seen positive for any other DR subtypes in cortical brain regions.

2.3.7 Quantitative analysis of DARPP-32 positive neurons demonstrating colocalization with DR subtypes in rat brain striatum

As shown in **Figure 2.8**, quantitative analysis revealed striking differences in colocalization of DARPP-32 with DRs. D1R was the most prominent receptor subtype which displayed highest degree of colocalization and constitutes 60% where as D2R-D5R represents 49%, 37%, 40% and 25 % colocalization respectively.

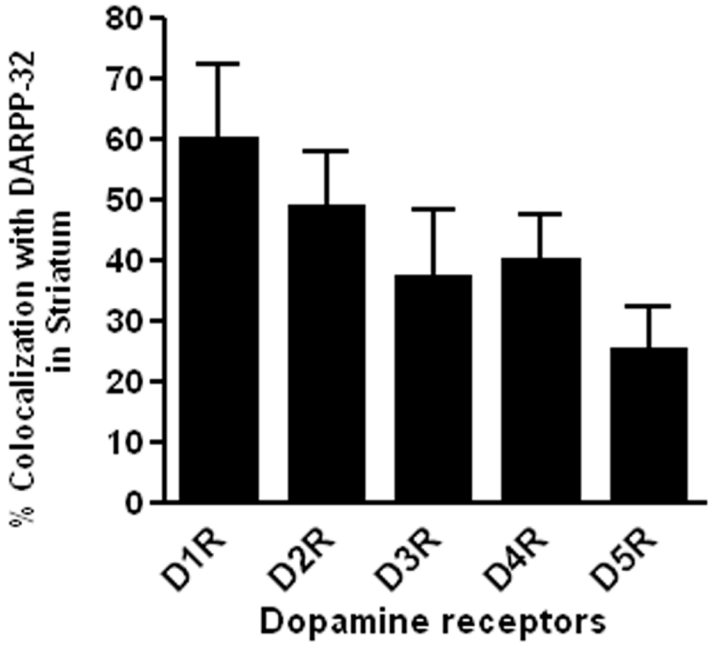

Figure 2.8 Quantitative analysis of D1R-D5R positive neurons showing colocalization with DARPP-32 in rat brain striatum.

Neurons positive for D1R-D5R were counted from striatum and analyzed for coexpression with DARPP-32. A total of 500-800 neurons were counted for each receptor subtype. The data are presented as percent of D1R-D5R neurons showing colocalization with DARPP-32 positive neurons. These data were obtained from three to four striatal sections from four different rats. Neurons positive for DARPP-32 and DR subtypes present on the edge of holes were not taken into consideration for quantitative analysis. Bars represent the mean ± SE (n= 4).

2.3.8 Western blot analysis of D1R and D2R subtype and DARPP-32 in cortical and striatal brain tissue

To validate our data for DRs and DARPP-32 like immunoreactivity we next determined the expression of DR subtypes and DARPP-32 in tissue lysate prepared from rat brain cortex and striatum using western blot analysis. As shown in **Figure 2.9**, D1R is well expressed in cortex and striatum at 65 kDa and 49 kDa.

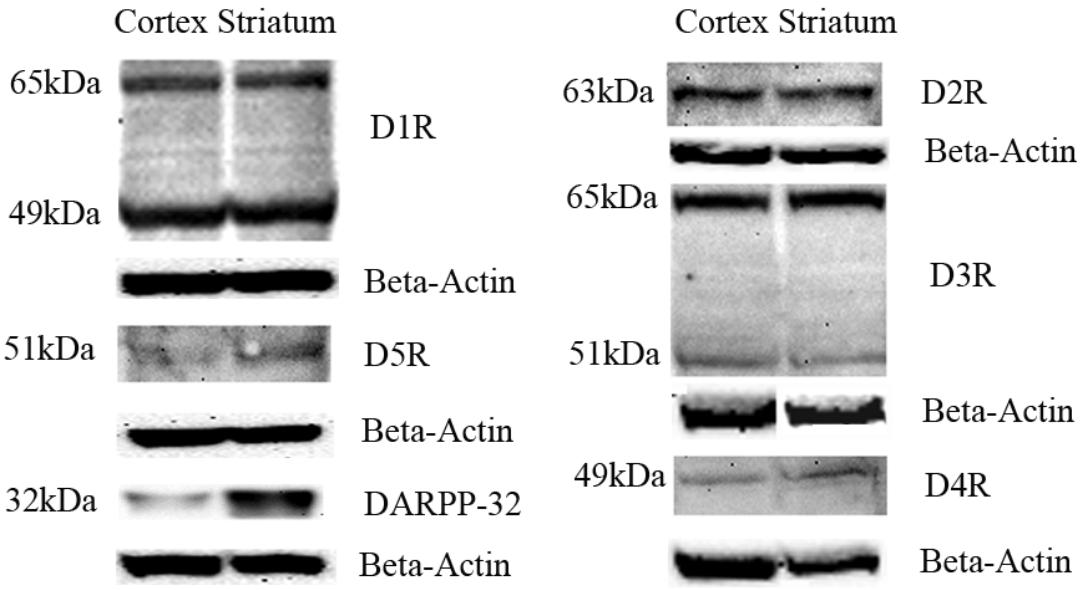

Figure 2.9 Western blot analysis showing the expression of D1R and D2R subtypes and DARPP-32 in tissue lysate prepared from rat brain cortex and striatum.

Tissue lysate was subjected to western blotting and immunoblotted for DR subtypes and DARPP-32 specific antibodies. Beta-actin was used as control for loading protein.

Expression levels were comparable in cortex and striatum. In comparison, D5R was weakly expressed in cortex and striatum at the expected size of 51 kDa. D2R (63 kDa) and D3R (65 kDa and 51 kDa) are well expressed at comparable levels in cortex and striatal tissue lysates. As shown, D4R at 49 kDa displayed significant differences between cortex

and striatum (**Figure 2.9**). When adjusted to beta-actin we observed high expression in striatum in comparison to cortex. DARPP-32 is also highly expressed in striatal tissue lysate in comparison to cortex and expressed at the expected size of 32 kDa.

2.3.9 Expression of DARPP-32 in DR subtype immunoprecipitate

To further validate our colocalization data we next determined the expression of DARPP-32 protein in DRs immunoprecipitate. Tissue lysate prepared from rat brain cortex and striatum was immunoprecipitated with DR subtypes antibodies and immunoprecipitate was fractionated on SDS gel, transferred to nitrocellulose membrane and blotted with DARPP-32 antibody.

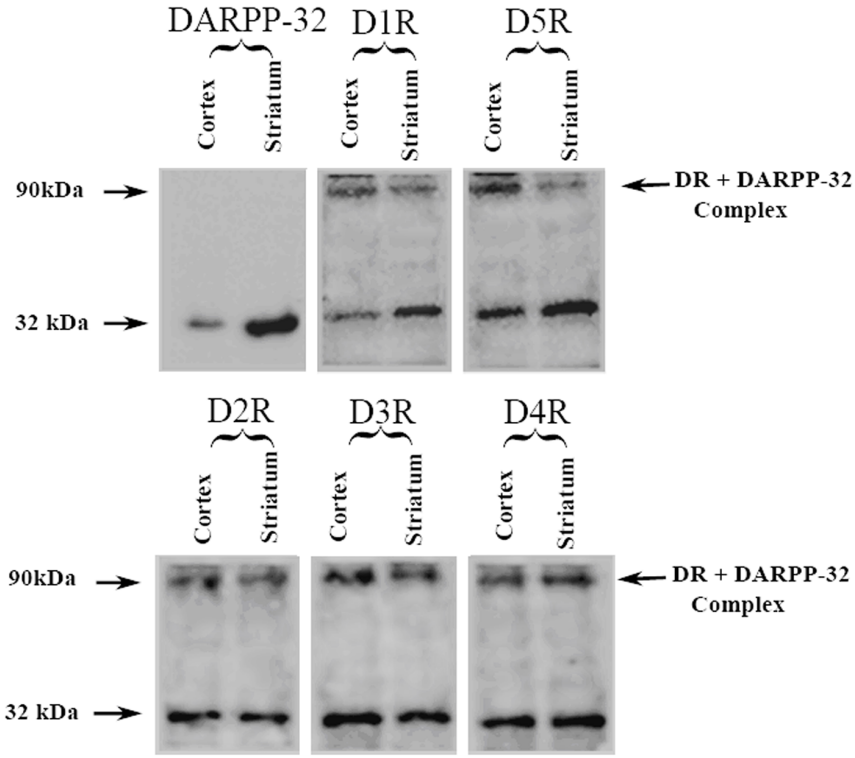

Figure 2.10 DARPP-32 is expressed in DR subtypes immunoprecipitate.

Tissue lysate prepared from cortex and striatum was immunoprecipitated with D1R–D5R antibodies and subjected to western blot analysis with DARPP-32 specific antibodies. Two specific bands at approximately

100 kDa and 32 kDa were seen representing a complex of DARPP-32 and DR subtype and DARPP-32 respectively. We also observed some additional nonspecific bands including IgG with or without primary antibody.

As shown in **Figure 2.10**, DARPP-32 immunoreactivity was detected in DRs immunoprecipitate from cortex as well as striatum in receptor specific manner. We observed the expression of DARPP-32 at 32 kDa and in a complex of DRs and DARPP-32 at approximate size of 100 kDa.

2.4 Discussion

Previous studies by using DARPP-32 or DR knockout mice have identified intimate association and functional dependence between dopaminergic neurons and DARPP-32 in CNS. DARPP-32 has drawn great attention for its critical role in several neurological diseases and most importantly in drugs of abuse as well as in schizophrenia. Such potential and critical role of DARPP-32 is modulated by DR subtypes in receptor specific manners as well as in the presence of other members of GPCR family in brain (Lindskog et al., 1999a). Present study is the first comprehensive description showing the comparative colocalization of all five DR subtypes with DARPP-32 in rat brain cortex and striatum. The distributional pattern of DARPP-32 and DR subtypes in single staining or in colocalization was comparable. This is possible that single neurons may also express multiple DR subtypes. The expression of DARPP-32 in DRs immunoprecipitate strongly supports our colocalization at cellular levels.

Several previous studies by using autoradiography or *insitu* hybridization have shown wide spread distribution of DR subtypes in CNS (Ariano et al., 1997; Bouthenet et al., 1991; Meador-Woodruff et al., 1992; Sunahara et al., 1991; Tiberi et al., 1991; Van Tol et al.,

1991). Although discrepancies exist, however distinct and region specific distributional pattern of mRNA for DR subtypes revealed intimate association of DA in cognitive function, movement disorder as well as regulation of endocrine functions. Our data showing the immunohistochemical localization of D2R subtypes in cortex and striatum are consistent with the distributional pattern at mRNA as described earlier (Bouthenet et al., 1991; Van Tol et al., 1991). D5R is least studied receptor from DRs family, however it has been shown that D5R has higher affinity for DA than D1R and these data suggest that D5R might exert prominent role in CNS. mRNA for D5R has previously been shown in motor and limbic regions of the rat brain significantly comparable to D1R (Sunahara et al., 1991). In contrast, D5R is distributed selectively in human brain (Tiberi et al., 1991). The distributional pattern of D5R described in the present study is comparable to the expression of D5R as described in human and rat brain recently (Khan et al., 2000). The discrepancies at the level of mRNA or proteins expression for DR subtypes might account for the type of probes and the antibodies used and cannot be eliminated from the discussion.

The distributional pattern of colocalization described herein is consistent with previous studies showing colocalization of DARPP-32 with D1R and D2R (Aizman et al., 2000; Langley et al., 1997). Several previous studies have shown the distribution of DARPP-32 in different parts of the brain in various species including human brain (Durstewitz et al., 1998; Schnabel et al., 1997). DARPP-32 positive neurons are widely distributed throughout the striatum and immunoreactivity mainly confined to the medium sized spiny projection neurons. In cortex DARPP-32 positive neurons were seen in deep layer of cortex and sparsely distributed in upper cortical layer. In cingulated and enterohinal cortex, DARPP-32 positive neurons displayed strong immunoreactivity in neuronal processes along with

neuronal cell bodies as described earlier (Ouimet et al., 1984). Our colocalization studies dealing with D1R and DARPP-32 are consistent with previous studies (Langley et al., 1997). In striatum the distribution of DARPP-32 is unique and expressed in majority of medium sized spiny projection neurons, which are susceptible in excitotoxicity. DARPP-32 positive medium sized spiny projection neurons in striatum are selectively susceptible to quinolinic acid or NMDA induced neurotoxicity as well as in HD and several other neuropathological states (Torres-Peraza et al., 2007). Consistent with previous studies, sodium channels are under the influence of D1R and D2R subtypes dopamine receptors to modulate Na^+ influx and efflux support the concept of colocalization (Aizman et al., 2000). Our data reveals receptors specific colocalization with DARPP-32 in cortex and striatum and suggests that DR positive neurons, which are lacking colocalization with DARPP-32, might be resistant in quinolinic acid or NMDA induced neurotoxicity as well as in pathophysiology of HD.

The role of DR subtypes to modulate the status of DARPP-32 phosphorylation is undisputed. However, whether this effect of DR is mediated directly on DARPP-32 or through the modulation of other signaling pathways is still uncertain. Most importantly, in this direction the role of protein kinase A (PKA), upon activation of D1R and stimulation of Ca^{2+} and calcineurin through D2R is well established (Andersson et al., 2005; Jay, 2003; Zhang et al., 2005). The phosphorylation of DARPP-32 in bidirectional manner is regulated by DA in a receptor specific manner either through activation of PKA via D1R the effect which is encountered by D2R (Lindskog et al., 1999b; Nishi et al., 1997). In CNS, DRs are not the only neurotransmitter that regulates DARPP-32 phosphorylation; other neurotransmitters such as glutamate, serotonin and adenosine also participate equally (Blank et al., 1997; Greengard et al., 1999; Nishi et al., 1997). Recent electron microscopic studies

have provided detail distributional pattern of DARPP-32 in association with glutamate in basal ganglia of domestic chick (Csillag et al., 2008). Activation of D2R decreases the phosphorylation of DARPP-32 (Nishi et al., 1997). Quinpirole, D2R agonist decreased D1 agonist mediated DARPP-32 phosphorylation (Lindskog et al., 1999b). These data suggest that this effect can be mediated by D1R and D2R, which are present in MSNs of striatal nigral projection. Indirectly, the distributional pattern of colocalization of DARPP-32 with DR subtypes also reveals that same neurons in striatum or cortex might contain more than one or two DR subtypes which also might express DARPP-32. D1 and D2 receptor exert opposite effect on DARPP-32 phosphorylation suggesting the presence of DR and DARPP-32 on the same neurons and indicate that DR might functionally interact with DARPP-32. The presence of D1R and D2R subtype has already shown on same neurons in colocalization studies (Aizman et al., 2000; Langley et al., 1997). Further in extension to these studies we found that DARPP-32 colocalized with D3R, D4R and D5R as well. At least in part our data are consistent with previous studies (Aizman et al., 2000; Langley et al., 1997). Furthermore, significantly, it will be interesting to see whether D1R positive neurons expressing DARPP-32 are devoid of D2R like immunoreactivity.

The complexity is driven due to the presence of other GPCR as well as other neurotransmitter receptors on striatal neurons such as cannabinoid receptors and adenosine A_2 receptor. Cannabinoid receptor -1 (CB1) is present in basal ganglia and exert pivotal role in regulation of neurotransmission, significantly on glutaminergic and GABAergic pathways (Andersson et al., 2005). Furthermore, CB1 receptors also modulate DR and adenosine A2 transmission which are intimately associated with PKA dependent DARPP-32 phosphorylation (Andersson et al., 2005). Recent studies have also shown that cannabinoid

receptor agonists (-)-cis-3-[2-hydroxy-4- (1,1-dimethylheptyl) phenyl] trans-4- (3-hydroxypropyl) cyclohexanol (CP55,940) decrease the state of DARPP-32 phosphorylation (Andersson et al., 2005). On the other hand tetrahydrocannabinol increases DARPP-32 phosphorylation via decreasing cAMP through D1R and anticipate possible interaction between two receptor subtypes (Borgkvist et al., 2008). In this direction the role of opioid receptors that are also present on MSNs in striatum cannot be avoided from discussion. Previously it has been shown that in the presence of μ opioid receptor agonist, D1 mediated increased DARPP-32 phosphorylation is blocked (Lindskog et al., 1999a) which further supported by the observation that D1 antagonist reverse the effect of D1 mediated DARPP-32 phosphorylation (Borgkvist et al., 2007) .

In striatum, like DARPP-32, majority of projection neurons also express N-methyl D-aspartate receptor (NMDAR) and upon activation exert determinant role in excitotoxicity and further reduce phosphorylation of DARPP-32. Most significantly, using organotypic culture from striatum activation of NMDAR reduces the diffusion coefficient and increases the recruitment of D1Rs but not D2Rs in the plasma membrane. Most importantly, NMDAR and DRs create a heteromeric complex and modulate the role of DR subtypes in receptor specific manner (Lee et al., 2002; Scott et al., 2006). The formation of heteromeric complex has also been shown to slow down diffusion (Scott et al., 2006). Previous studies have also shown opposing effect of D1R and D2R on phosphorylation of NMDAR1 in nucleus accumbens has been shown and D2R activation block D1R mediated NMDAR1 phosphorylation and such effect is attributed to cyclosporine A which inhibits calcineurin (Blank et al., 1997). In contrast D2R activate calcineurin and decreases phosphorylation of NMDAR1. D2R mediated decrease in NMDAR1 phosphorylation is blocked by neuroleptic drug D2R

antagonist raclopride. Phosphorylated DARPP-32 also modulate NMDAR functions through the inhibition of protein phosphatase-1 (Blank et al., 1997). These are the compelling evidence to believe that NMDAR might exert important effect on the role of DR subtypes in the status of DARPP-32 phosphorylation and its functional consequences. Recently, administration of drugs of abuse and psycho-stimulants also modulate extracellular signal-regulated kinases (ERKs) in striatum which is associated with the activation of D1R and NMDAR and blocked by D1R and NMDAR antagonists as well as in D1R or DARPP-32 knockout mice where gene for D1R or DARPP-32 is ablated (Bertran-Gonzalez et al., 2008; Gerfen et al., 2008; Valjent et al., 2000). Whether this interaction between NMDAR and DR subtypes functionally interferes with DARPP-32 is not known and need to be elucidated. The potential outcome of these data will elucidate the detailed molecular mechanism for the status of DARPP-32 phosphorylation and signaling cascade involved. In conclusion taken together our data provides first comprehensive and comparative colocalization of all five DR subtypes with DARPP-32. Data presented here give new insight for the role of DRs and DARPP-32 that might play important role in neuropathological conditions as well as in drugs of abuse and schizophrenia. Our colocalization data provide evidence for the first time that DARPP-32 might constitute functional complex with DR subtypes. However further studies like confocal FRET are required to elucidate such possibilities in single neurons expressing DARPP-32 and DR subtypes and further studies are in progress in this direction.

Chapter 3: [2]Colocalization of somatostatin receptors with DARPP-32 in cortex and striatum of rat brain

DRs and SSTRs share 30% structural similarities and functionally interact with each other in a heteromeric complex (Baragli et al., 2007; Rocheville et al., 2000a). Differential distribution of SSTR subtypes in striatal projection and interneurons is still not known. Accordingly, to determine the percentage distribution of SSTR subtypes inprojection neurons is essential to determine potential role of SSTR subtypes in excitotoxicity. Furthermore, SSTR positive neurons with lack of DARPP-32 colocalization anticipate the possibility that these neurons are interneurons in striatum and might escape excitotoxicity. Having seen the selective and preferential colocalization of DR subtypes with DARPP-32 we next determined the colocalization of SSTRs with DARPP-32 in cortical and striatal brain regions.

3.1 Background

Somatostatin (SST), was first isolated from sheep hypothalamus as growth hormone inhibiting peptide (Brazeau et al., 1973). At present, there are several evidences that SST is not only synthesized in hypothalamus but also present in other brain regions and major peripheral organs (Hokfelt et al., 1975; Patel and Reichlin, 1978; Reichlin, 1983a). SST exists in two bioactive isoforms, SST-14 and SST-28 derived from a common precursor and synthesized variably in SST-producing cells (Hobart et al., 1980; Montminy et al., 1984; Patel, 1999; Shen and Rutter, 1984). In the central nervous system (CNS), SST like

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immunoreactivity is seen in most of the brain regions and plays a critical role in neurotransmission and neuromodulation (Epelbaum et al., 1994; Oliva et al., 2000). Previous studies have demonstrated close association between SST and several neurological diseases such as Alzheimer's, Parkinson's and Huntington's disease (HD). Furthermore, impaired cognitive function, changes in locomotor behavior and other sensory and autonomic functions are linked with the loss of SST (Epelbaum et al., 1994; Reichlin, 1983a; Schindler et al., 1996).

In striatum, medium sized projection neurons (MSNs) expressing γ-Aminobutyric acid (GABA), N-methyl D-aspartate receptor (NMDAR) and dopamine-and cAMP-regulated phosphoprotein (DARPP-32) constitute large neuronal populations that are selectively vulnerable in excitotoxicity and neurological diseases. Conversely, the second population, which is selectively preserved in excitotoxicity and many pathological conditions, includes medium sized aspiny interneurons expressing SST/Neuropeptide Y (NPY)/brain nitric oxide synthase (bNOS). Previous studies have also shown increased SST expression in autopsy brain tissues from HD patients (Beal et al., 1984; Beal et al., 1991). Furthermore, cultured cortical neurons upon treatment with quinolinic acid (QUIN) and N-methyl-D-aspartic acid (NMDA) exhibited increased release of SST and mRNA expression (Patel et al., 1991; Patel et al., 1995b). Most significantly, knocking down SST by antisense oligonucleotide and immunoadsorption of released SST with SST antibodies potentiate the neuronal loss and exogenous addition of SST to cultured striatal neurons provides protection in QUIN and NMDA induced excitotoxicity (Kumar, 2008). This study provides the first evidence that presence of SST might be responsible for neuronal survival of interneurons and serve as neuroprotective peptide in excitotoxicity. In addition, SST via neprisilyn blocks the

aggregation of β-amyloid (Saito et al., 2005). These observations convincingly suggest a generalized neuroprotective role of SST in different models of excitotoxicity. The biological effect of SST is mediated by five different receptor subtypes commonly known as somatostatin receptors (SSTR1-5). SSTR subtypes belong to G protein-coupled receptors (GPCRs) family and are known to inhibit adenylyl cyclase thereby inhibiting intracellular cyclic adenosine monophosphate (cAMP) formation (Patel, 1999; Reisine, 1985; Reisine et al., 1985; Reisine et al., 1983). SST inhibits Ca^{2+} through modulation of cGMP-dependent protein kinase, the events which play crucial role in neurotoxicity (Meriney et al., 1994).

Unlike SST, DARPP-32 is expressed in almost >80% of medium-sized spiny neurons (projection neurons) in striatum (Ouimet et al., 1998; Walaas and Greengard, 1984). The loss of DARPP-32 immunoreactivity with progression of HD is well established (Torres-Peraza et al., 2007). DARPP-32 has been studied extensively and predominantly involved in regulation of signaling transduction pathway mediated by dopamine receptors (DRs) (Greengard, 2001; Greengard et al., 1999; Svenningsson et al., 2004; Walaas et al., 1983). We have recently shown that DRs colocalize with DARPP-32 (Rajput et al., 2009b). SSTR2 and SSTR5 functionally interact with dopamine receptor 2 in a heteromeric complex with enhanced pharmacological and signaling properties than the native receptors (Baragli et al., 2007; Rocheville et al., 2000a).

Although, several previous studies have shown differential distribution of SSTR subtypes in different parts of rat brain, the distribution of SSTR subtypes in projection and interneurons in cortex and striatum remains unknown. Such studies are critically important to understand the role of SSTRs in excitotoxicity and pathogenesis of neurodegenerative diseases. Accordingly, in the present study we used DARPP-32 as a marker of projection

neurons and applied to determine the colocalization with SSTR subtypes in cortex and striatum of rat brain using indirect immunofluorescence immunohistochemistry. We also determined the expression of DARPP-32 in SSTR1-5 immunoprecipitate prepared from rat brain cortex and striatum. Our results described here present the comprehensive distributional pattern of colocalization of SST and SSTRs with DARPP-32 in region and receptor specific manner in rat brain.

3.2 Materials and methods

3.2.1 Animals

Sprague-Dawley male rats weighing 150-200 g were obtained from UBC animal care unit and used for indirect immunofluorescence and co-immunoprecipitation studies. All experiments and procedures involving animals were performed in accordance to the guidelines of the Canadian Council on Animal Care and were reviewed and approved by the University of British Columbia committee on Animal Care (Protocol #A06-0419).

3.2.2 Materials

Rabbit polyclonal antibodies directed against SSTR1-5 were generated in our laboratory as described previously (Kumar, 2007; Kumar et al., 1999). Mouse anti-somatostatin monoclonal antibody was purchased from Santa Cruz Biotechnology (CA, USA). Rabbit polyclonal anti-DARPP-32 antibody and mouse monoclonal anti-DARPP-32 antibody was obtained from Cell signaling (MA, USA) and BD Biosciences (Pharmingen, San Diego, CA, USA) respectively. Alexa-488 and Alexa-594 secondary antibodies were purchased from Invitrogen (Burlington, ON, Canada). Protein A/G agarose beads were

obtained from Calbiochem, (Mississauga, ON, Canada). Nitrocellulose Hy-Bond membrane and enhanced chemiluminescence (ECL) obtained from Amersham Ltd. (Oakdale, ON, Canada). All other chemicals of analytical grade were purchased from various commercial sources.

3.2.3 Indirect immunofluorescence immunohistochemistry

Indirect immunofluorescence immunohistochemistry was performed as described previously (Kumar, 2007; Rajput et al., 2009b). Briefly, rats were anesthetized with halothane and perfused with heparinized cold saline and 4% paraformaldehyde with an infusion pump (KD Scientific Model 200S, Holliston, MA, USA). 40µm thick brain sections were cut using Leica 1200s vibratome and free floating sections were collected in phosphate buffer saline (PBS). Brain sections were incubated in 0.2% Triton X-100 in PBS for 15 min and followed by three subsequent washes with PBS. Sections were then incubated in 5% normal goat serum for 1h at room temperature (RT) and followed by overnight incubation with mouse anti-SST (1:500) in combination with rabbit anti-DARPP-32 (1:300) and rabbit anti- SSTR1-5 (1:400) with mouse DARPP-32 (1:250) antibodies. Following three subsequent washes in PBS brain sections were incubated in a cocktail of Alexa 594 (red) and Alexa 488 (green) conjugated secondary antibodies for 1h at RT. The sections were washed, mounted on slides and viewed under Leica DMLB microscope attached with Retiga 2000R camera. All merged images showing colocalization were generated using NIH, ImageJ software and Adobe Photoshop (San Jose, CA, USA) was used to make the composites.

3.2.4 Quantitative analysis of SSTR1-5 and DARPP-32 colocalization

Neuronal population displaying colocalization between SSTR1-5 and DARPP-32 was quantified in cortex and striatum as described previously (Kumar, 2007; Rajput et al., 2009b). Briefly, three to four brain sections from individual rats were taken for each receptor subtypes from five different rats. 12-15 randomly selected areas were selected from cortex and striatum according to rat brain atlas Paxinos and Watson (Paxinos and Watson, 2007). Neuronal population expressing receptor like immunoreactivity higher then background and intact morphology were included for quantification. Colocalization was determined using ImageJ software (National Institute of Health) and the Colocalization Highlighter plugin (P. Bourdoncle, Institute Jacques Monod, Service Imagerie, Paris, France). Neurons showing colocalization or individual staining for SSTRs or DARPP-32 were pooled and calculated for percentage distribution. Data for neuronal quantification are presented as mean ± SEM. Background staining is referred to the staining in absence of primary antibodies. Tissues stained for single staining were also photographed in green and red channels in order to verify specificity of immunofluorescence.

3.2.5 Co-immunoprecipitation

Co-immunoprecipitation (CO-IP) was performed on tissue lysate prepared from cortex and striatum of rat brain as described previously (Rajput et al., 2009b). Briefly, cortical and striatal tissue was homogenized in homogenization buffer containing 62.5 mM Tris-HCl, 50mM dithiothretiol [DTT], 2% SDS, 10% glycerol. Total protein in tissue extract was determined by Bradford protein assay. 200 µg of tissue protein was solubilized in 1 ml of radio-immunoprecipitation assay (RIPA) buffer for 1h at 4°C. The lysates were incubated

with polyclonal anti-SSTR1-5 antibodies (1:500) overnight at 4°C. The antibodies were further immunoprecipitated using protein A/G agarose beads for 2h at 4°C. The agarose beads were subsequently washed three times with RIPA buffer. The purified samples were fractionated by electrophoresis on 10% SDS-Page gel and transferred onto nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk for 1h at RT and subsequently incubated overnight at 4°C with primary antibody for DARPP-32 (1:250) diluted in 5% bovine serum albumin. The membrane was incubated with goat anti-mouse secondary antibodies for 1h at RT. The bands were detected with chemiluminescence reagent and images were taken using an Alpha Innotech Fluorchem 800 (Alpha Innotech Co., San Leandro, CA) gel box imager as described earlier (Rajput et al., 2009b).

3.3 Results

3.3.1 Specificity of somatostatin receptors (SSTR1-5) antibodies

The antibodies used for the expression of SSTR subtypes have been well characterized in our previous studies by using western blot analysis and immunohistochemistry in rat pancreas, brain and HEK-293 cells transfected with individual receptors (Kumar, 2007; Kumar et al., 1999; Rajput et al., 2009a). In the absence of primary antibodies, preabsorbed antibodies or in presence of preimmune serum only background staining was observed (Kumar, 2007; Kumar et al., 1999). Accordingly, receptor like immunoreactivity seen in the presence of receptor specific antibodies was considered specific.

3.3.2 Somatostatin positive neurons are devoid of DARPP-32 expression in rat brain cortex and striatum

As shown in **Figure 3.1 A, E** and **I**, SST positive neurons were identified in all three cortical regions including cingulate, temporal and enterohinal cortex. In cingulate and temporal cortex, SST like immunoreactivity was confined in cortical layers II, III and deep layer (**Figure 3.1 A** and **E**). The neuronal population displaying DARPP-32 like immunoreactivity in cingulate and temporal cortex confined densely in deep layer with scattered neuronal population in upper layer and a cluster of neurons in enterohinal cortex (**Figure 3.1 B, F** and **J**). As shown in merged images two distinct populations either positive for SST or DARPP-32 were seen without any colocalization (**Figure 3.1 C, D, G, H, K** and **L**). Like cortical brain regions, similar distributional pattern of colocalization was observed in striatum. As shown in **Figure 3.1 M-P**, medium sized aspiny interneurons positive for SST can be seen in striatum without any colocalization with DARPP-32 positive MSNs. The distributional pattern of SST and DARPP-32 positive neurons in cortex and striatum is in accordance to the concept that SST is present in interneurons whereas DARPP-32 is an integrated part of the projection neurons.

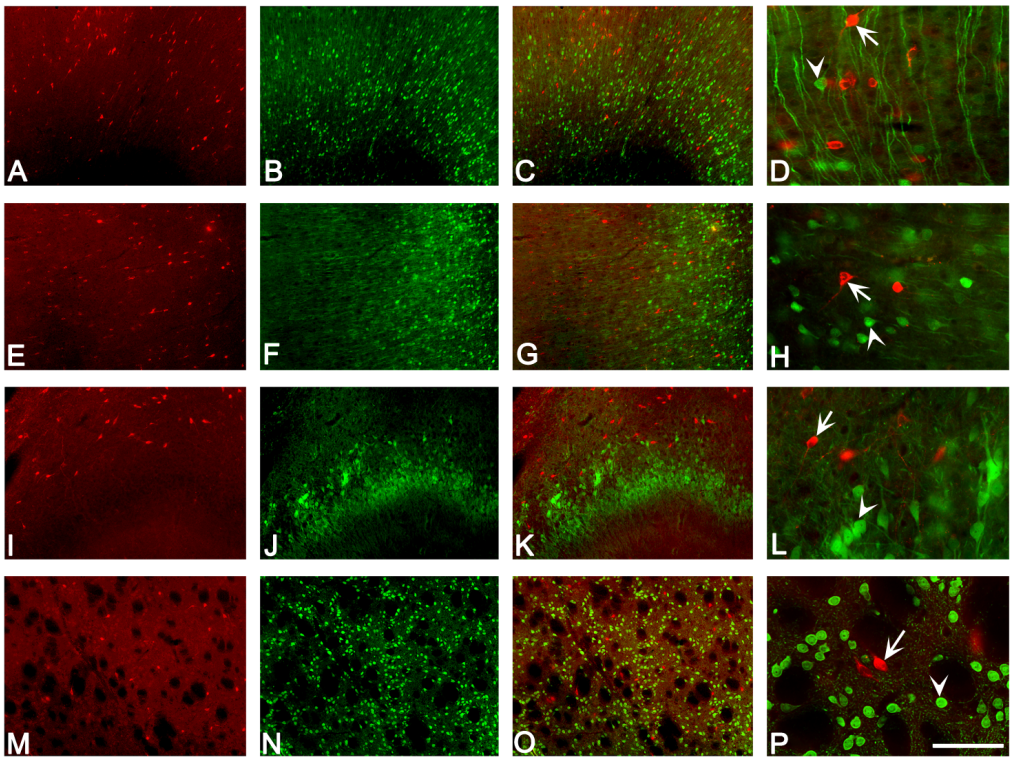

Figure 3.1 Somatostatin positive neurons are devoid of colocalization with DARPP-32 in rat brain cortex and striatum.

To visualize SST and DARPP-32 expression, 40 µm thick free floating brain sections were incubated with anti-SST monoclonal and anti-DARPP-32 polyclonal antibodies for 24h and followed by incubation with Alexa 594 (red) and Alexa 488 (green) to identify SST and DARPP-32 (please see methods section for details). In cingulate (panels A-D), temporal (panels E-H), enterohinal cortex (panels I-L) and striatum (panels M-P) the SST and DARPP-32 immunoreactivity was seen in neurons whereas DARPP-32 immunoreactivity was also seen in dendrites and innervated nerve fibers. Note medium sized aspiny interneurons positive to SST were devoid of colocalization with DARPP-32. Arrows indicate neurons expressing SST, whereas neurons positive for DARPP-32 like immunoreactivity are indicated by arrowhead. Scale bar = 100 µm for panels A-C, E–G, I–K and M-O and 20 µm for D, H, L and P.

3.3.3 Receptor and region specific colocalization of somatostatin receptor 1-5 with DARPP-32 in rat brain cortex

In cingulate, temporal and enterohinal cortex DARPP-32 like immunoreactivity (green) was confined in neuronal soma and dendrites as well as in nerve fibers. As shown in

Figure 3.2 A-C, SSTR1 displayed a differential distributional pattern of colocalization with DARPP-32 in all three cortical regions studied. In cingulate cortex, we identified three different neuronal populations displaying colocalization between SSTR1 and DARPP-32 and neurons with either DARPP-32 or SSTR1 immunoreactivity (**Figure 3.2 A**). In temporal cortex, SSTR1 strongly colocalized with DARPP-32 whereas majority of receptor positive neurons in deep layer were devoid of DARPP-32 immunoreactivity (**Figure 3.2 B**). As shown in **Figure 3.2 C**, in enterohinal cortex SSTR1 strongly colocalized with selective DARPP-32 positive neurons whereas rest of the neurons were either positive to SSTR1 or DARPP-32. In addition, neuronal processes and axonal extension seen positive for DARPP-32 were devoid of colocalization (**Figure 3.2**).

The pattern of colocalization between SSTR2 and DARPP-32 was comparable in cingulate, temporal and enterohinal cortex. As illustrated in **Figure 3.2 D-F**, only few DARPP-32 positive neurons exhibit colocalization with SSTR2. Three different populations of neurons were identified in the cortical regions first expressing only DARPP-32 (green), second expressing SSTR2 (red) like immunoreactivity and third population coexpressing SSTR2 and DARPP-32. However, intensity of colocalization between SSTR2 and DARPP-32 in cingulate and temporal cortex was relatively weak than enterohinal cortex. The colocalization between DARPP-32 and SSTR3 revealed a region specific distinction in all three cortical regions. As shown in **Figure 3.2 G-I**, in cingulate cortex SSTR3 and DARPP-32 displayed strong colocalization within neuronal cell bodies while dendrites and innervated nerve fibers were only positive for DARPP-32. Conversely few neurons in temporal and enterohinal cortical region showed moderate degree of colocalization between DARPP-32 and SSTR3.

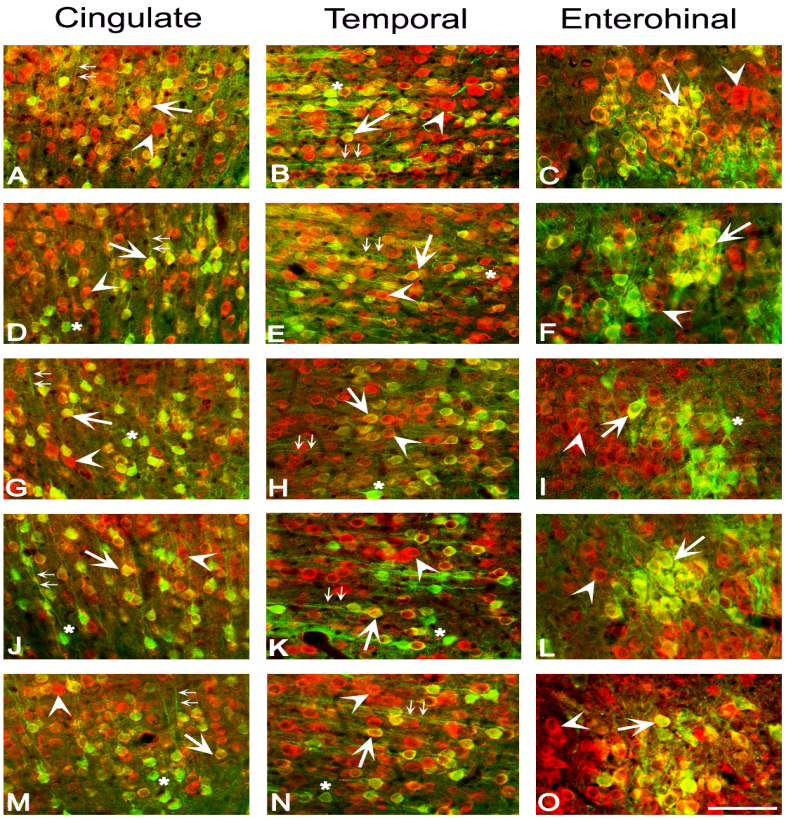

Figure 3.2 Representative photomicrographs showing the colocalization of SSTR1-5 and DARPP-32 in rat brain cortex.

Coronal brain sections of 40 µm thickness were used for indirect immunofluorescence. Briefly, sections were incubated overnight with polyclonal SSTR1-5 and monoclonal DARPP-32 antibodies and followed by incubation with Alexa 594 (red) and Alexa 488 (green) to visualize SSTRs and DARPP-32 (please see methods section for details). Panels A-C show colocalization of SSTR1 with DARPP-32 in cingulate, temporal and enterohinal cortex in a region specific manner. Similarly, SSTR2 (D-F), SSTR3 (G-I), SSTR4 (J-L) and SSTR5 (M-O) panels represent colocalization with DARPP-32 in region and receptor specific manner. All the three cortical regions showed neurons expressing either only SSTR1-5 or DARPP-32 as well as neurons showing colocalization. Arrows indicate neurons showing colocalization; SSTR1-5 positive neurons are indicated by arrowhead, DARPP-32 positive neurons by asterisk and paired thin arrows indicate DARPP-32 positive dendrites/nerve fibers. Scale bar = 20µm.

As depicted in **Figure 3.2 J-L**, in cortical brain regions we observed significant variation in colocalization between DARPP-32 and SSTR4. The number of neurons expressing SSTR4 positive immunoreactivity colocalized with DARRP-32 was less in the

cingulate and temporal regions of cortex whereas enterohinal cortex exhibited moderate colocalization. The innervated nerve fibers in all three cortical regions were strongly positive for DARPP-32 and were lacking colocalization with SSTR4 (**Figure 3.2 J-L**). The colocalization of DARPP-32 with SSTR5 in cingulate cortex was comparable to SSTR1. SSTR5 is well-expressed receptor subtype in temporal cortex and displayed strong colocalization in selective neurons (**Figure 3.2 M-O**). In enterohinal cortex SSTR5 showed strong colocalization with DARPP-32. Three different neuronal populations either showing SSTR5 or DARPP-32 and colocalization were identified in all cortical brain regions.

3.3.4 Quantitative analysis of DARPP-32 positive neurons demonstrating colocalization with SSTR subtypes in rat brain cortex

To determine the percentage distribution of DARPP-32 positive neurons colocalizing with SSTRs in rat brain cortex quantitative analysis was performed. There were no significant changes in SSTRs and DARPP-32 colocalization in frontal and temporal cortex hence quantitative analysis was performed in the aforementioned regions in five different rats and neurons were counted from 10-15 fields in frontal/temporal cortex. As shown in **Figure 3.3**, SSTR1 was the most prominent receptor subtype colocalized with DARPP-32 (38 ± 4%) followed by SSTR5 (32 ± 2%), SSTR4 (27 ± 2%) whereas SSTR2 and SSTR3 constituted 24 ± 0.9% and 18 ± 0.39% respectively.

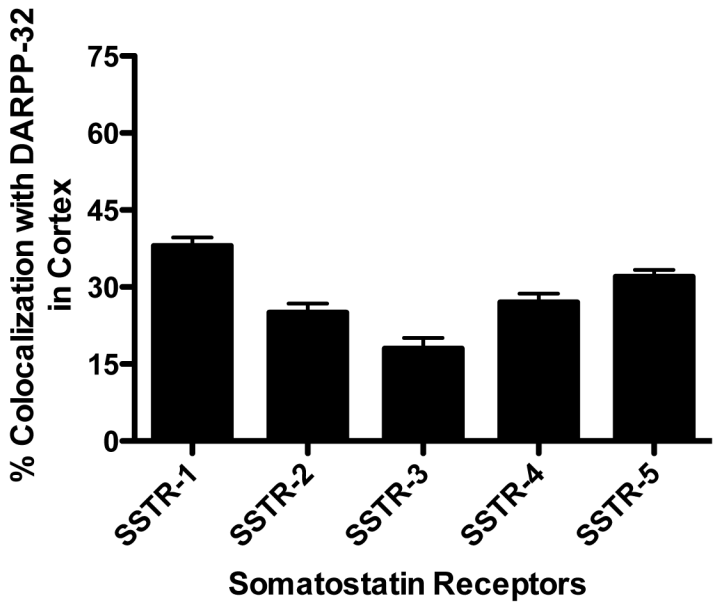

Figure 3.3 Quantitative analysis of SSTR subtypes showing colocalization with DARPP-32 in rat brain cortex.

SSTR1-5 and DARPP-32 positive neurons were counted from frontal/temporal cortex and neuronal population showing colocalization was calculated as percentage as described in Material and Methods section. Briefly, 12-15 randomly selected areas; from three to four sections for each receptor subtypes were used for the quantification. Percentage colocalization was quantified using ImageJ software and Colocalization highlighter plugin. SSTR1 and SSTR5 colocalized with DARPP-32 in a comparable manner and constitute 38 ± 4% and 32 ± 2% respectively. SSTR4 and SSTR2 exhibit comparable degree of colocalization i.e., 27 ± 2% and 24 ± 0.9% respectively. SSTR3 is the receptor subtype colocalized the least with DARPP-32 and constitutes 18 ± 0.39% of colocalization. Each bar graph represents mean ± SEM (n=5)

3.3.5 Colocalization of SSTR subtypes and DARPP-32 in rat brain striatum

As described earlier >80% projection neurons in striatum are positive for DARRP-32 (Ouimet et al., 1998; Walaas and Greengard, 1984). In comparison, SSTRs are expressed in medium sized aspiny interneurons as well as projection neurons in a receptor specific manner. In striatum, most of the interneurons displaying SSTR subtypes like immunoreactivity were lacking DARPP-32 expression. Conversely, projection neurons displayed receptor specific colocalization. As shown in **Figure 3.4 A-D**, SSTR1 strongly

colocalized with DARPP-32 in MSNs whereas large interneurons positive for SSTR1 were devoid of DARPP-32 like immunoreactivity. As illustrated in **Figure 3.4 E-H**, colocalization of SSTR2 with DARPP-32 was relatively weak and large populations of neurons were devoid of colocalization. As shown in **Figure 3.4 I-L**, the pattern of colocalization of SSTR3 with DARPP-32 in the striatum was greatly different than SSTR1 and SSTR2. Some of the neurons positive to SSTR3 and DARPP-32 displayed strong colocalization. Interestingly, within the same region strongly positive SSTR3 neurons with distinct morphological appearance probably interneurons were devoid of DARPP-32 like immunoreactivity.

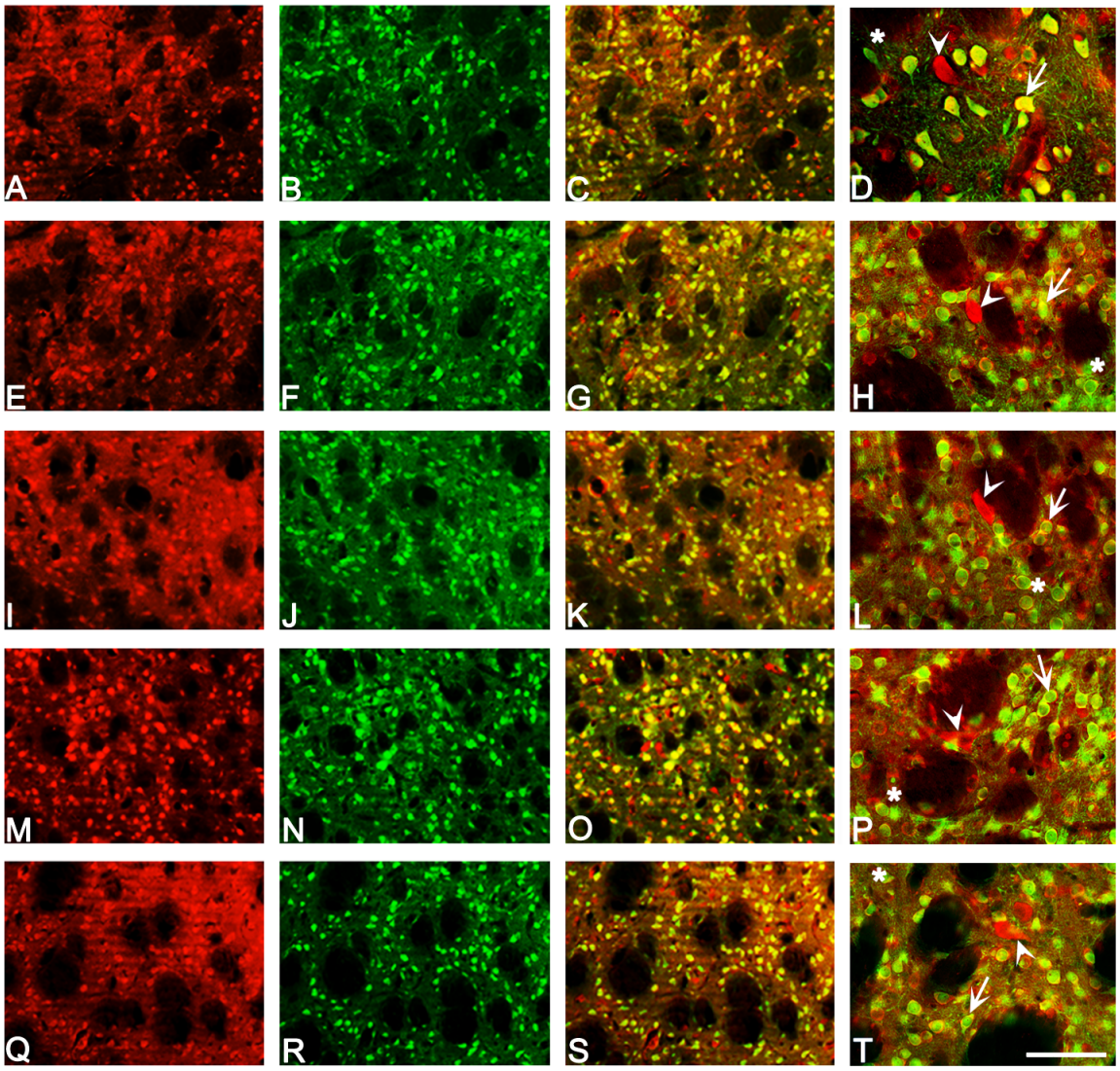

Figure 3.4 Photomicrographs illustrating colocalization of SSTR1-5 and DARPP-32 in rat brain striatum. Immunofluorescence detection of SSTR1-5 and DARPP-32 in the rat brain striatum was performed using anti-SSTR1-5 polyclonal and anti-DARPP-32 monoclonal antibodies as described in figure 2 legend. In striatum three different neuronal populations expressing SSTR1-5 or DARPP-32 and neurons showing colocalization between SSTR subtypes and DARPP-32 were observed. Note, the majority of interneurons positive for SSTR subtypes were devoid of DARPP-32 immunoreactivity (arrowheads). In contrast large number of projection neurons positive for DARPP-32 selectively display colocalization with SSTR subtypes in receptor specific manner. In all the representative panels arrows indicate colocalization and arrowheads represent SSTR1-5 positive neurons and DARPP-32 positive neurons are identified by asterisk (*). Scale bar = 100 μm for panels A-C, E–G, I–K, M-O and Q-S and 20 μm for panels D, H, L, P and T.

As illustrated in **Figure 3.4 M-P**, SSTR4 positive neurons exhibited strong to weak colocalization with DARPP-32 and large neuronal population was positive for either SSTR4 or DARPP-32 in striatum. SSTR5 colocalization was comparable to SSTR1 showing strong colocalization in **Figure 3.4 Q-T**. These results revealed three different populations of projection neurons either expressing SSTR subtypes or DARPP-32 alone or colocalization. Majority of innervated nerve fibers positive to DARPP-32 were devoid of SSTR subtypes like immunoreactivity.

3.3.6 Quantitative analysis of SSTR subtypes colocalization with DARPP-32 in striatum

Quantitative analysis revealed a distinctive pattern of colocalization of SSTR subtypes with DARPP-32. The neurons positive for SSTRs also showed positive immunoreactivity for DARPP-32. Like cortical region, highest degree of colocalization was displayed by SSTR1 constituting 69 ± 0.98% followed by SSTR5 with 63 ± 0.71%, whereas, SSTR2, 3 and SSTR4 positive neurons showed less colocalization when compared with SSTR1 and SSTR5 representing 52 ± 0.17%, 45 ± 0.41% and 48 ± 0.37% colocalization respectively (**Figure 3.5**).

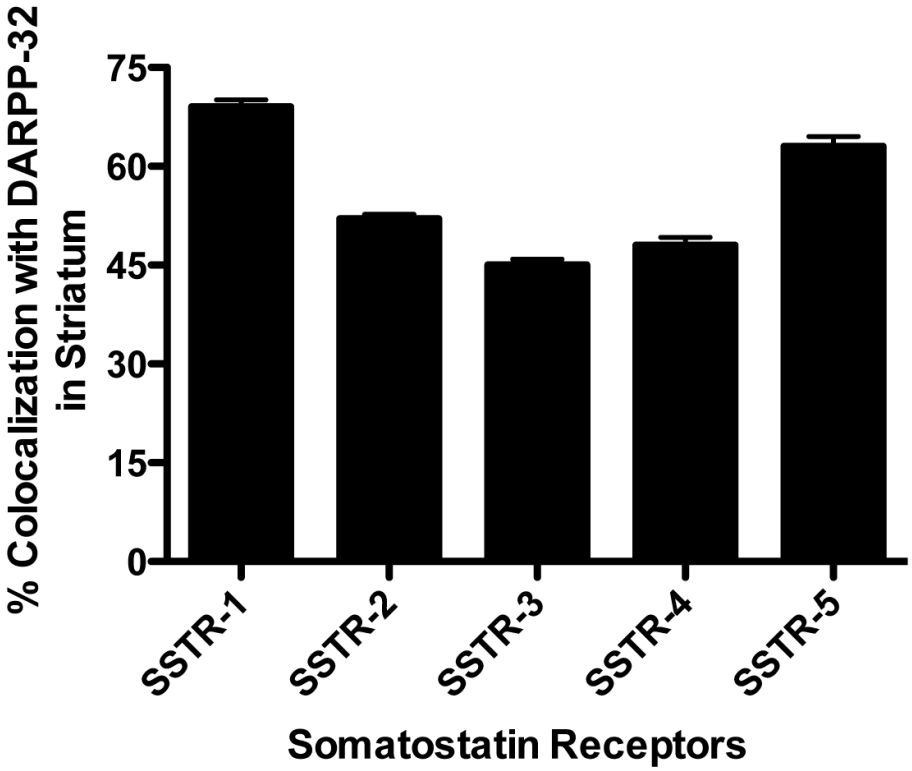

Figure 3.5 Quantitative analysis of SSTR1-5 positive neurons showing colocalization with DARPP-32 in rat brain striatum.

Neurons positive for SSTR1-5 were counted from striatum and analyzed for colocalization with DARPP-32. A total of 1200-1400 neurons were counted from each receptor subtype (as described in methods section). SSTR1 and SSTR5 showed more colocalization (69 ± 0.98% and 63 ± 0.71% respectively) compared to SSTR2 (52 ± 0.17%), SSTR3 (45 ± 0.41%) and SSTR4 (48 ± 0.37%). Colocalization highlighter plugin for ImageJ software was used to quantify colocalization. The data are presented as percent of SSTR1-5 neurons showing colocalization with DARPP-32 positive neurons. Bars represent the mean ± SEM (n = 5)

3.3.7 Expression of DARPP-32 in SSTR1-5 subtypes immunoprecipitate

The colocalization of SSTR subtypes with DARPP-32 in cortex and striatum is the indication that SSTR may interact with DARPP-32 and possibly, like DRs might involve in DARPP-32 phosphorylation. Accordingly, we next determined the expression of DARPP-32 in SSTRs immunoprecipitate prepared from cortex and striatum. Briefly, tissue lysate

prepared from rat brain cortex and striatum was immunoprecipitated with SSTR subtypes antibodies, fractionated on SDS-PAGE and blotted for DARPP-32 antibody.

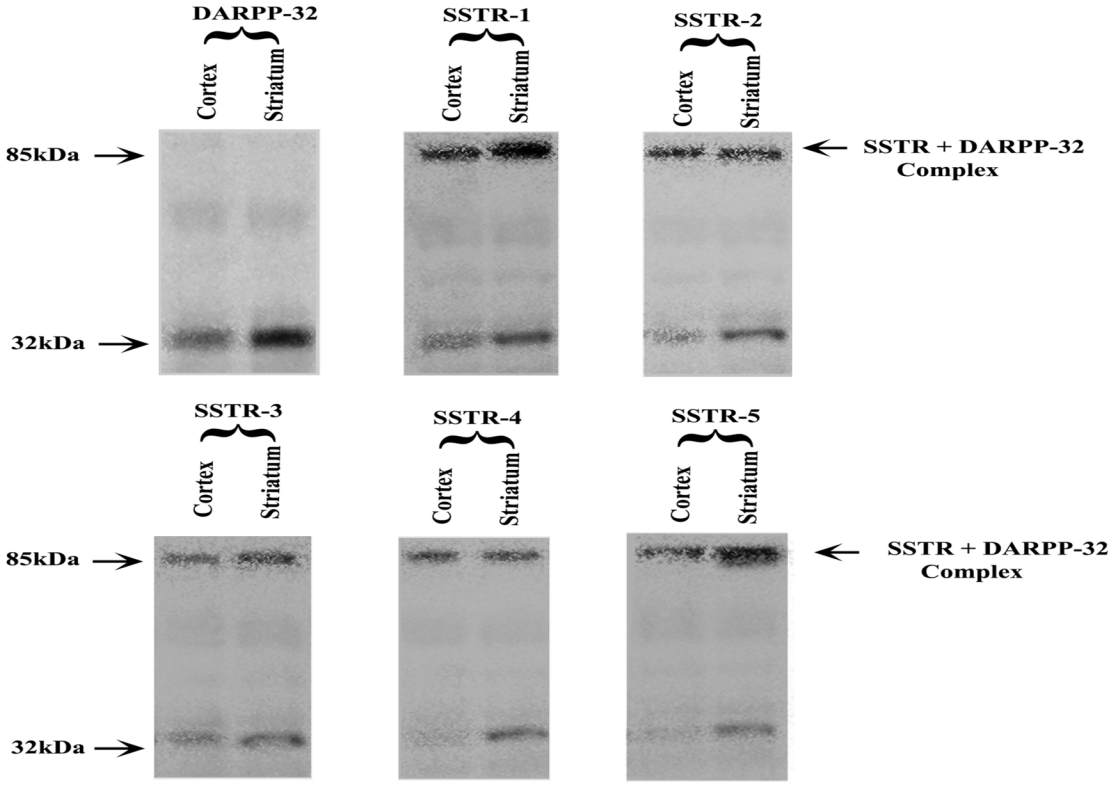

Figure 3.6 DARPP-32 is expressed in SSTR subtypes immunoprecipitate.

Tissue lysate prepared from cortex and striatum was immunoprecipitated overnight for SSTR1-5 and fractionated on SDS page and immunoblotted for DARPP-32 (please see materials and methods section). Two specific bands at approximately 80-90 kDa and 32 kDa where; 80-90 kDa represents a complex of DARPP-32 and SSTR subtype and 32 kDa represent DARPP-32.

As illustrated in **Figure 3.6**, SSTR subtypes immunoprecipitate from cortex and striatum exhibited DARPP-32 immunoreactivity. We observed the complex of SSTRs and DARPP-32 at approximate size of 80-90 kDa and additionally we also detected DARPP-32 at 32kDa. Taken together, these results suggest that SSTR might interact with DARPP-32 in CNS.

3.4 Discussion

We recently described that knocking down of SST using antisense oligonucleotide and immunoadsorption of released SST with SST antibodies accelerates QUIN/NMDA induced neuronal cell death in cultured striatal neurons including the neuronal population expressing enzyme nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) (Kumar, 2008). These results provide first evidence that the presence of SST in medium sized aspiny interneurons and released SST is necessary for the survival of projection neurons. However which receptor might be involved in SST mediated neuroprotection is not known. In the present study, we characterize the distributional pattern of SSTRs in projection neurons using DARPP-32 as an index of projection neurons. In cortex and striatum SST positive neurons are devoid of DARPP-32 immunoreactivity. Receptor specific colocalization with DARPP-32 in cortex and striatum revealed that SSTR 1 and SSTR 5 are the prominent receptor subtype colocalized with DARPP-32. Furthermore, the direct evidence in support of our colocalization is the expression of DARPP-32 in SSTRs immunoprecipitate prepared from cortex and striatum. Such preferential and selective colocalization of SSTRs with DARPP-32 indicates the specific role of SSTR subtypes in excitotoxicity and specifically in pathophysiology of HD. This is the first comprehensive description elucidating the comparative colocalization of SSTRs with DARPP-32 in receptor and region specific manner and suggests that SSTRs positive neurons lacking colocalization with DARPP-32 might be preserved in pathophysiology of HD and QUIN/NMDA induced neurotoxicity.

Role of SST as multifunctional peptide and its widespread distribution in different parts of CNS is linked to the pathophysiology of several diseases (Cervia and Bagnoli, 2007;

Panteris and Karamanolis, 2005). SSTRs are expressed in CNS and are involved in various neurological diseases like HD (Aronin et al., 1983; Menkes, 1988), Alzheimer's disease (Doggrell, 2004; Kumar, 2005), Parkinson's Disease (Agnati et al., 2003), epilepsy (Binaschi et al., 2003), HIV encephalitis (Fox et al., 1997), dementia (Doggrell and Evans, 2003) and psychiatric disorder like schizophrenia (De Wied and Sigling, 2002). Consistent with these studies, pattern of colocalization described here provides new insight for the role of SSTR subtypes in association with DARPP-32 in neurodegenerative diseases and neuropsychological disorders.

Selective preservation of interneurons expressing SST in excitotoxicity was proposed due to the lack of NMDARs (Chen et al., 1999; Koh et al., 1986). However, recent studies have shown the presence of NMDARs in medium sized aspiny interneurons (Augood et al., 1994; Kumar et al., 1997a). Consistent with these observations, preferential low preponderance of NMDAR and lack of DARPP-32 in SST positive neurons strengthen the concept of interneurons survival in excitotoxicity. The distributional pattern of SST and DARPP-32 in MSNs is consistent with previous studies showing single staining in different neuronal populations (Yoshioka et al., 2011). SSTR subtypes function as autoreceptors in region and receptor specific manner and subtype devoid of DARPP-32 like immunoreactivity might be resistant in excitotoxicity as well as in HD. Conversely, SSTRs subtypes displaying colocalization with DARPP-32 might be susceptible in excitotoxicity as well as in HD. Consistent with our speculation in our recent studies we have shown in SSTR1 and SSTR5 double knockout mice a marked decrease in DARPP-32 like immunoreactivity as seen in R6/2 HD transgenic mice (Rajput et al., 2011b). This study further validates our colocalization findings, which shows an extensive colocalization of DARPP-32 with SSTR1

and SSTR5 in comparison to other subtypes In HD pathophysiology and excitotoxicity NMDARs that are expressed in majority of MSNs play key role upon activation (Cepeda et al., 2001; Fan and Raymond, 2007). Studies have shown that phosphorylation of DARPP-32 modulates NMDAR functions via inhibition of Protein Phosphatase-1 (PPtase1) which play a key role in modulating cAMP Response Element-Binding Protein (CREB). Recent studies have shown the role of DARPP-32 phosphorylation and modulation of signaling via PPtase1 and Protein Phosphatase-2 (PPtase2) in various HD models (Metzler et al., 2010). We have previously reported that NMDARs colocalize with SST positive neurons (Kumar et al., 1997a). The data from our study indicates a possible interaction between SSTRs and NMDARs that could potentially modulate the signaling in HD.

The role of DRs in DARPP-32 phosphorylation has been studied in detail and DRs mediated DARPP-32 phosphorylation modulates the downstream signaling linked to many neurodegenerative disorders. Previous studies have shown that SSTRs share a structural homology with DRs and function as heterodimers with enhanced pharmacological activity (Baragli et al., 2007; Rocheville et al., 2000a). In agreement with these observations, colocalization of DARPP-32 with SSTRs and its expression in SSTR immunoprecipitate suggest that SSTRs might be involved in DARPP-32 phosphorylation and might modulate downstream signaling pathways associated with neurodegeneration and excitotoxicity in HD. In conclusion our, data provides first comprehensive distributional pattern of colocalization of SSTRs with DARPP-32 and evidence that SSTRs and DARPP-32 might constitute functional complex that might play an important role in neurological diseases. The potential outcome of our study provides the new dimension in understanding the molecular mechanism

for DARPP-32 phosphorylation and receptor specific role of SSTR subtypes in brain specifically during progression of neurodegenerative diseases.

Chapter 4: [3]Somatostatin receptor 1 and 5 double knockout mice mimic neurochemical changes of huntington's disease transgenic mice

Results described in previous chapter suggested that SSTR 1 and SSTR5 receptor subtype colocalize predominantly with DARPP-32 in striatum. These observations are the first indication that SSTR1 and SSTR5 receptor subtype might play a crucial role in neuroprotection. To study in depth and ascertain the physiological implications of SSTR1 and SSTR5 we next determined the changes in key receptors and signaling pathways involved in excitotoxicity in SSTR1/5 double *ko* mice and compared with HD transgenic mice (R6/2).

4.1 Background

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder caused by mutation in the *huntingtin (Htt)* gene and characterized by progressive chorea and impaired cognitive function (de la Monte et al., 1988; Giampa et al., 2006). Genetic abnormality of expanded polyglutamine repeat sequence is confined in the coding region of a gene IT15 located on chromosome 4 encoding the Htt protein (HDCRgroup, 1993; Kumar et al., 1997a). The length of CAG repeat is one of the factors that plays an important role in the onset of HD symptoms (Brinkman et al., 1997; Gusella and MacDonald, 2000). Pathological characteristics of the disease are the intranuclear inclusion of mutated Htt and neostriatum atrophy and gliosis.

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In addition to genetic mutation and histopathological hallmarks, the critical determinant of HD is the degeneration of medium size spiny neurons (MSNs) expressing γ-aminobutyric acid (γ-GABA), N-methyl-D-aspartic acid receptors (NMDARs) and dopamine and cAMP regulated phosphoprotein of 32 kDa (DARPP-32). In contrast, in striatum, a subset of neuronal population consisting of medium sized aspiny interneurons positive to somatostatin (SST), neuropeptide Y (NPY) and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)/brain nitric oxide synthase (bNOS) are selectively spared (Cicchetti and Parent, 1996; Ferrante et al., 1985). In addition, the expression of calbindin D-28K is increased in HD patients, transgenic mouse models and quinolinic acid (QUIN)-induced excitotoxicity (Huang et al., 1995; Kiyama et al., 1990; Sun et al., 2005). Activation of NMDARs in striatum mimics the pathological, neurochemical and behavioral changes of HD (Li and Li, 2004). Furthermore, the analysis of HD patient's postmortem brain reveals the degeneration of NMDAR-positive neurons and association with the pathogenesis in HD (Albin et al., 1990; Young et al., 1988). NMDARs are composed of two subunits of NR1 and two subunits of NR2A, NR2B or NR2C (Monyer et al., 1994; Ozawa et al., 1998). Previous studies have shown enhanced NMDAR-mediated toxicity in cells expressing mutated Htt as well as in HD mouse models (Chen et al., 1999; Fan and Raymond, 2007). Recently, the functional importance of NMDARs emerged from a study describing the role of NMDAR antagonist memantine to block the nuclear inclusion of Htt in yeast artificial chromosome (YAC) mice (Okamoto et al., 2009). These data suggest that NMDARs play an important role in HD and may contribute to neuronal loss.

NMDA replicate the neuropathological features of HD and have been used as models of the disease (Beal et al., 1991; Beal et al., 1986). In the striatum of experimental mice,

medium-sized aspiny interneurons expressing SST, NPY and NADPH-d/bNOS are selectively resistant to QUIN-induced excitotoxicity. Similarly, such interneurons are relatively well spared observations in the brains of HD patients (Aronin et al., 1983; Beal et al., 1984; Beal et al., 1986; Dawbarn et al., 1985; Nemeroff et al., 1983; Okamoto et al., 2009; Vincent and Johansson, 1983). Previous studies have also shown increased SST secretion and gene expression in HD brain and NMDA/QUIN-induced excitotoxicity (Beal et al., 1984; Beal et al., 1986; Patel et al., 1991). In support of the selective preservation of interneurons, it was argued that these neurons lack NMDARs (Koh et al., 1986). In contrast, several recent studies have shown the presence of NMDARs in SST/NPY/NOS positive neurons in striatum of rat brain and cultured striatal neurons (Augood et al., 1994; Chen et al., 1996; Kumar et al., 1997a). Most importantly, we have recently shown that immunoblockade of SST by using antisense oligonucleotides and immunoneutralization of released SST by using SST specific antibodies potentiate neuronal loss in QUIN/NMDA-induced excitotoxicity in cultured striatal neurons, including NPY, NADPH-d and bNOS positive neurons (Kumar, 2008). Furthermore, selective sparing of SST positive neurons in bNOS knockout mice suggests that the presence of SST is essential for the survival of interneurons (Dawson et al., 1996).

The presence of SST in the central and peripheral nervous system is associated with several physiological functions, which are attributed to different receptor subtypes, namely somatostatin receptor 1-5 (SSTR1-5), which are members of G-protein coupled receptor (GPCR) family. All five SSTR subtypes display-overlapping distribution in different parts of brain and importantly couple to G_i protein and inhibit cAMP in a pertussis toxin sensitive manner. SSTRs are involved in the regulation of ion channels; inhibition of Ca^{2+} and

activation of K^+ channels involved in the release of several neurotransmitters and modulation of neurotransmission (Patel, 1999). These functional properties of SSTR subtypes can be further enhanced by interaction with members of their own family as well as other GPCRs, including dopamine and opioid receptors via heterodimerization (Grant et al., 2004a; Grant et al., 2004b; Pfeiffer et al., 2002; Rocheville et al., 2000a). Widespread distribution of SSTRs in CNS is involved in various neurological diseases such as Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), epilepsy, HIV encephalitis, dementia and psychiatric disorders, including schizophrenia (Aronin et al., 1983; Binaschi et al., 2003; De Wied and Sigling, 2002; Doggrell, 2004; Fox et al., 1997; Kumar, 2005). These studies cumulatively suggest the critical and pivotal role of SSTR subtypes in neurodegenerative diseases.

We recently observed that the knock-down of SSTR 1 and SSTR 5 using antisense oligonucleotides accelerated neuronal death upon NMDA treatment in cultured striatal neurons (unpublished observations). Accordingly, in an attempt to elucidate the possible functions of SSTR1 and 5, the present study was undertaken to determine the expression of NMDARs, DARPP-32, calbindin, bNOS/SST and SSTRs in striatum of R6/2 and SSTR1/5$^{-/-}$ mice. In addition, we also studied the downstream signaling cascades including calcineurin, calpain, PKC-α, ERK1/2, synapsin-IIa and enkephalin associated in the process of neurodegeneration in HD pathology as well as in experimental models of the disease. In the present study, for the first time, we describe that the SSTR1/5 complex is a critical regulator of NMDARs, DARPP-32 and downstream signaling cascades normally seen in R6/2 transgenic mice. Importantly, this study revealed that SSTR1/5$^{-/-}$ mice mimic neuro-and biochemical changes of presymptomatic HD transgenic mice.

4.2 Materials and methods

4.2.1 Materials

Rabbit polyclonal antibodies for SSTR subtypes have been previously characterized (Geci et al., 2007; Kumar, 2005; Rajput et al., 2009a). Mouse monoclonal antibodies for DARPP-32 and calcineurin were purchased from BD Biosciences (Mississauga, ON, Canada). Antibodies against NMDAR-1, NMDAR-2A, and NMDAR-2B were obtained from Millipore (CA, USA) and anti-calbindin D-28K from Sigma-Aldrich (MO, USA). PKC-α, ERK1/2, synapsin-IIa and Met/Liu enkephalin were obtained from Cell Signaling Technology (MA, USA). The peroxidase vectastain ABC kit was purchased from Vector Laboratories (CA, USA). Nitrocellulose Hy-bond membrane and enhanced chemiluminescence (ECL) detection kit was obtained from Amersham Ltd. (Oakdale, ON, Canada). All other chemicals of analytical grade were obtained from various commercial sources.

4.2.2 Animals

Perfused and frozen brains from 8-week old HD (C57BL/6 X CBA)F1(B6CBA)-TgN (HDexon1)62 (R6/2) carrying 120 +/- 5 CAG repeat expansions and [wild-type(*WT*)]B6CBAF_1/J mice were obtained from Jackson Laboratory (Sacramento, CA, USA). Similarly, frozen and perfused brains from 8 weeks old SSTR1/5$^{-/-}$ mice and *wt* were kindly provided by Dr. F. C. Brunicardi (Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, Texas, USA). Further details for the creation and characterization of SSTR1/5$^{-/-}$ mice have been described in detail previously (Wang et al., 2004). The protocols regarding animal care were followed in compliance with the Institute of

Laboratory Animal Resources, Commission on Life Sciences, National Research Council. The *wt* and SSTR1/5$^{-/-}$ mice were housed at Baylor college of Medicine, Houston, Texas, USA with 12-hour light/dark cycle.

4.2.3 Immunohistochemistry studies

In the present study, three male adult mice from each group, *wt* and R6/2 and five male adult mice from *wt* and SSTR1/5$^{-/-}$ mice were used. 30μm thick coronal brain sections were cut on Leica vibratome. Immunostaining was performed on free-floating sections using the avidin–biotin peroxidase Vectastain ABC kit as described previously (Billova et al., 2007; Geci et al., 2007; Rajput et al., 2009a). Briefly, brain sections were incubated with 0.3% hydrogen peroxide followed by incubation with 0.02% Triton X-100. Following three washes, sections were incubated with 5% NGS for 1 h at room temperature (RT) and followed by overnight incubation with specific primary antibodies against rabbit-anti-SSTR1-5 (1:500), rabbit-anti-SST (1:600), mouse anti-DARPP-32 (1:250), mouse anti-NMDAR-1, NMDAR-2A, NMDAR-2B and calbindin D-28K (1:500) and rabbit anti-bNOS (1:400) in 1 % NGS at 4°C in humid atmosphere. The sections were incubated with the biotinylated secondary antibodies; goat-anti-rabbit to detect SSTRs, SST, and bNOS and goat anti-mouse to detect DARPP-32, calbindin and NMDARs and followed by incubation in ABC complex for 30 min. The final color was developed by adding a mixture containing 0.001% hydrogen peroxide and 0.2mg/ml of 3,3-diaminobenzidine (DAB) for 3-4 min in 50mM Tris buffer. Sections were washed in PBS, mounted onto slides and viewed and photographed under Leica microscope attached with the Retiga 2000R camera.

4.2.4 Western blot analysis

Striatal tissues isolated from R6/2 or SSTR1/5$^{-/-}$ mice and respective *wt* brains were homogenized in homogenizing buffer containing (62.5mM Tris-HCl, 50mM dithiothretiol [DTT], 10% glycerol, 2% SDS). 15µg of total protein was fractioned by electrophoresis on 10% SDS polyacrylamide gel, transferred onto 0.2µm-nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk at RT for 1 h and incubated overnight at 4°C with specific primary antibodies against– SSTRs1-5, NMDARs, bNOS (1:600), SST, enkephalin, synapsin-IIa, calpain, calcineurin, calbindin, phospho and total ERK1/2, PI3K (1:1000), DARPP-32 (1:250) and PKC-α (1:500). Following three subsequent washes, the membrane was incubated with peroxidase conjugated goat anti-rabbit or a goat anti-mouse secondary antibody at RT for 1 h. The bands were detected using chemiluminescence reagent and images were taken using an Alpha Innotech FluorChem 8800 (Alpha Innotech Co., San Leandro, CA) gel box imager. β-actin was used as the housekeeping protein for loading control. The bands intensity was quantified using densitometric analysis and the changes in protein expression were calculated as the ratio of band of interest and the density of β-actin.

4.2.5 Quantitative analysis

Quantitative analysis on brain sections was performed using NIH Image J software as described earlier (Rajput et al., 2009a). Neurons were considered immunoreactive if the labeling of their cell bodies was distinctly higher than the background staining obtained in the presence of pre-absorbed or in the absence of primary antibodies. For quantitative analysis five mice were taken from *wt*, SSTR1/5$^{-/-}$ mice and three for *wt* and R6/2 mice. 12-15 randomly selected areas from 6-7 sections from individual mouse brain were used for neuronal counting. Since our immunohistochemical studies provide semiquantitative analysis

of immunoreactivity in R6/2 or SSTR1/5$^{-/-}$ and respective *wt* mice we took all the possible precaution to keep our experimental conditions consistent in all aspects, i.e., incubation timings with antibodies (18 h) and 3, 3-diaminobenzidine for the final color development (4 mins).

4.2.6 Statistical analysis

Data are presented as percentage changes in neuronal population for positive immunoreactivity from 6-7 brain sections per mice. Total of (6000-6500) neurons were counted in each condition. Bars represent the mean ± SEM (n= 3 for R6/2 and n=5 for SSTR1/5$^{-/-}$). Statistical comparison between *wt* and R62 (n=3) and *wt* and SSTR1/5$^{-/-}$ (n=5) mice were analyzed using student t-test (*$p< 0.05$). GraphPad Prism 5.0 (San Diego, CA) was used to perform all the statistical analysis.

4.3 Results

4.3.1 Decreased DARPP-32 expression and increased calbindin D-28K expression in striatum of SSTR1/5$^{-/-}$ mice and R6/2 mice brain

Neuronal population in striatum is largely composed of medium sized projection neurons, which are positive for DARPP-32 and constitute 80% of total neuronal population. Several previous studies have shown significant loss of DARPP-32 like immunoreactivity in HD patients and/or in experimental models of disease (Bibb et al., 2000; Torres-Peraza et al., 2007). Accordingly, here we examined the DARPP-32 like immunoreactivity using immunohistochemical and Western blot analysis in R6/2 and SSTR1/5$^{-/-}$ as well as respective *wt* mice brains. As illustrated in **Figure 4.1 a**, consistent with previous studies, a marked

decrease in signal intensity of DARPP-32 was observed in R6/2 when compared to *wt* mice. Like R6/2 mice, a similar decrease in DARPP-32 like immunoreactivity was observed in SSTR1/5$^{-/-}$ when compared to *wt* mice. DARPP-32 like immunoreactivity is mostly expressed as cytoplasmic protein. Consistent with our immunocytochemistry data, Western blot analysis showed a significant decrease in DARPP-32 expression in comparison to *wt* in the brains of both R6/2 and SSTR1/5$^{-/-}$ mice (**Figure 4.1 b**).

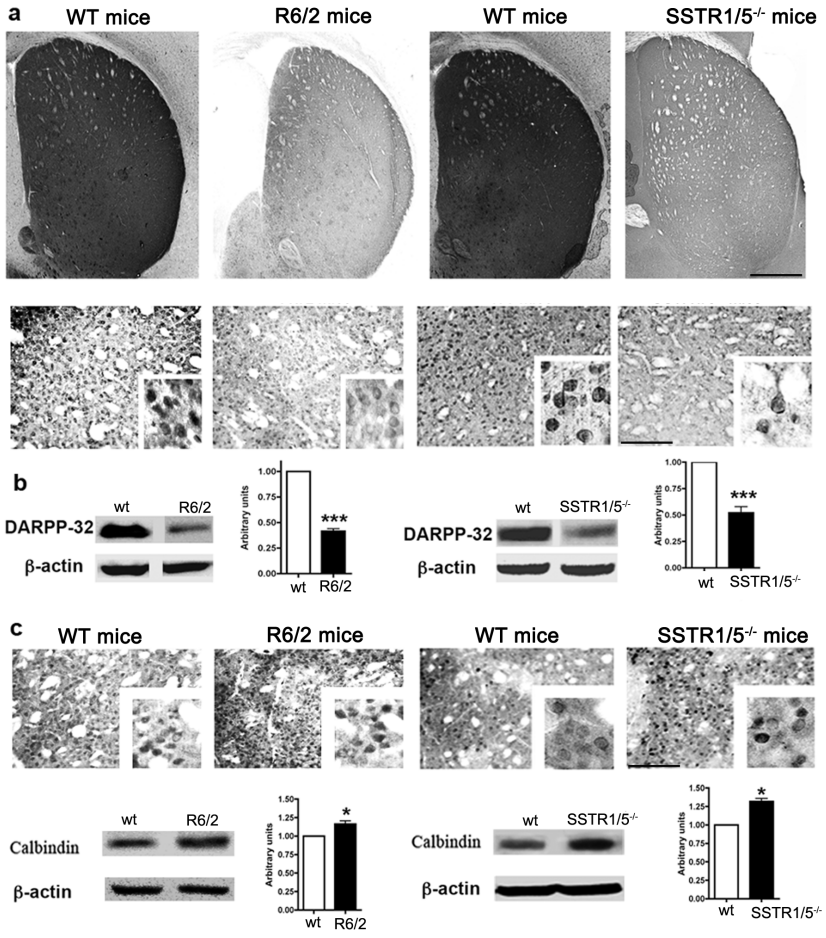

Figure 4.1 Representative low- and high-magnification photomicrogrpahs depicting comparative distribution of DARPP-32 immunoreactivity in striatum of R6/2 and SSTR1/5$^{-/-}$ mice.

DARPP-32 like immunoreactivity was lost in striatum of R6/2 and SSTR1/5$^{-/-}$ mice, respectively (a). Comparable decreased expression of DARPP-32 (32 kDa) in R6/2 and SSTR1/5$^{-/-}$ mice was also seen in

Western blot analysis (b). Calbindin like immunoreactivity was found to be increased in both R6/2 and SSTR1/5$^{-/-}$ mice compared to respective *wt* mice (c upper panel). Increased calbindin (28 kDa) expression was further confirmed with Western blot analysis (c bottom panel). Data presented as mean ± SD for Western blot analysis (SSTR1/5$^{-/-}$, n=5 and R6/2 mice, n=3) in comparison to *wt* mice brain,***P<0.001 and *P<0.05. Scale bar = 150µm upper panel a; 20µm for lower panel and upper panel c and 5µm for inset.

Similar to DARPP-32, calbindin D-28K immunoreactivity was detected in the striatum and has been used as an index of MSN. As illustrated in Figure 5.1c, increased expression of calbindin-like immunoreactivity was observed in R6/2 and SSTR1/5$^{-/-}$ when compared to *wt* mice. Western blot analysis showed consistent results as seen in immunohistochemistry with increased expression of calbindin in striatum of R6/2 and SSTR1/5$^{-/-}$ mice (**Figure 4.1 c**).

4.3.2 SST and bNOS positive neurons are selectively spared in striatum of R6/2 and SSTR1/5$^{-/-}$ mice

Previous studies have shown that medium-sized aspiny interneurons positive for bNOS/ NADPH-d also co-express SST and NPY and are selectively preserved in HD patients as well as experimental models of disease (Cicchetti and Parent, 1996; Ferrante et al., 1985). Accordingly, we determined the expression of bNOS and SST-positive neurons in striatum of R6/2 and SSTR1/5$^{-/-}$ mice brain. As shown in **Figure 4.2 a**, bNOS immunoreactivity was observed in sparsely distributed neurons all over the striatum with arborizing neuronal processes without discernable changes in R6/2 and SSTR1/5$^{-/-}$ mice when compared to *wt* mice. In addition, Western blot analysis also revealed no significant changes in bNOS expression levels in R6/2 and SSTR1/5$^{-/-}$ mice in comparison to their counterpart *wt*.

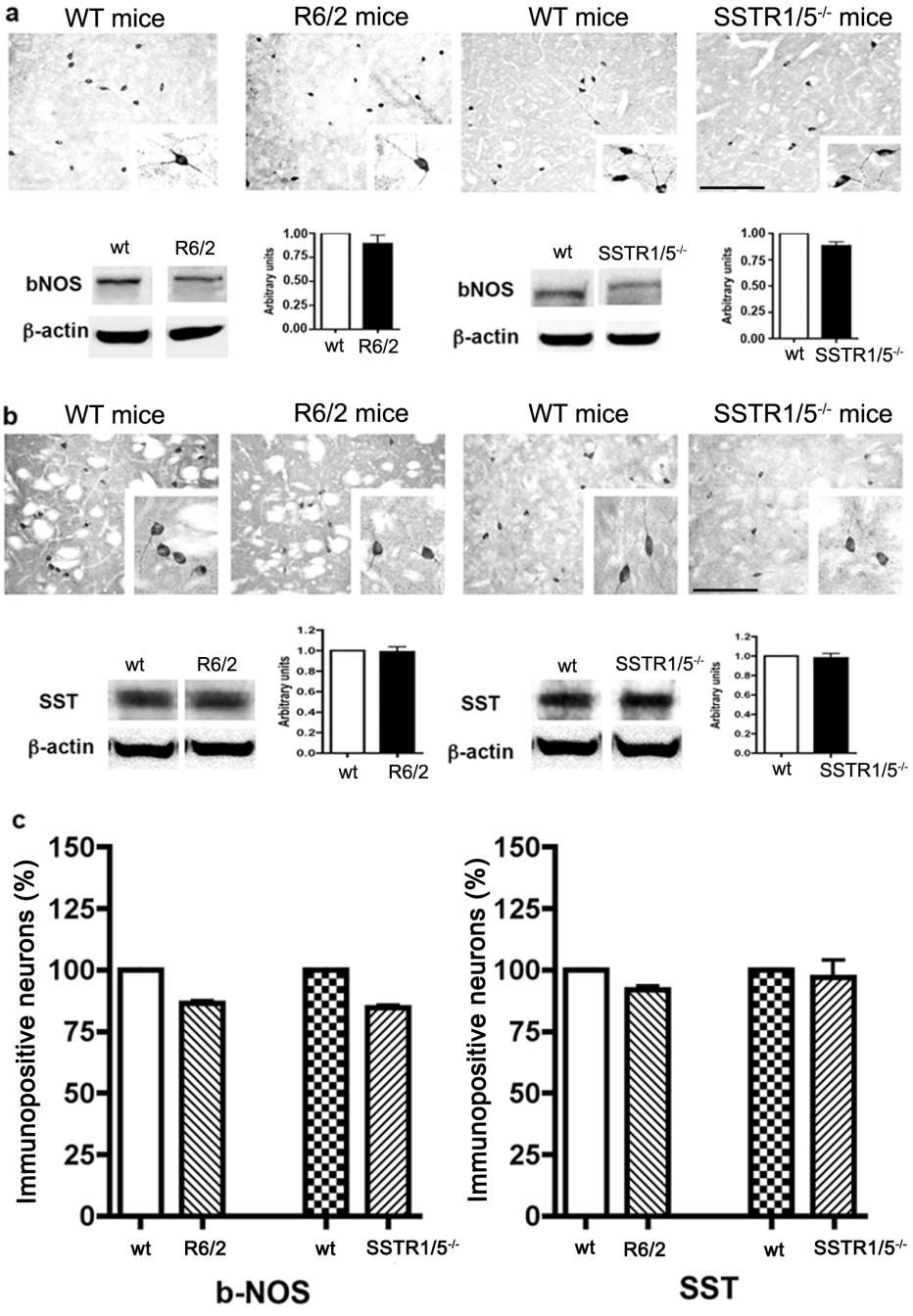

Figure 4.2 Comparative immunohistochemical localization of bNOS and SST in striatum of R6/2 and SSTR1/5$^{-/-}$ mice.

The expression level of bNOS (a upper panel) was comparable in both strains. Furthermore, Western blot analysis revealed no significant changes in bNOS (160 kDa) expression (a bottom panel) . As shown in upper panel b expression of SST in both the strains was comparable. Western blot analysis reveals no change in

expression of SST (28 kDa) in both the strains (b bottom panel) when compared to respective *wt*. Histogram represent quantitative analysis of bNOS and SST positive neurons (c). Data presented as mean ± SD (SSTR1/5$^{-/-}$, n=5 and R6/2 mice, n=3) in comparison to *wt* mice brain. Scale bar = 20 µm panel a and b and 5 µm for inset.

As shown in **Figure 4.2 b**, the neuronal population exhibiting SST like immunoreactivity was comparable in R6/2 and SSTR1/5$^{-/-}$ mice using immunohistochemistry and Western blot analysis. As shown in **Figure 4.2 c**, further quantitative analysis revealed comparable changes in bNOS and SST positive neurons in R6/2 and SSTR1/5$^{-/-}$ mice as well as in comparison to *wt*. R6/2 transgenic mice exhibited 14 and 8% loss of bNOS and SST positive neurons, respectively. Conversely, SSTR1/5$^{-/-}$ mice showed 15 and 3% loss of bNOS and SST positive neurons respectively, not significantly different from *wt* mice.

4.3.3 Expression of NMDA receptor subtypes in the striatum of SSTR1/5$^{-/-}$ and R6/2 mice

The role of activated NMDARs in degeneration of medium spiny neurons in HD as well as in excitotoxicity is indisputable. Several previous studies have shown the activation of NMDAR subtypes in the brain of R6/2 transgenic mice (Fan and Raymond, 2007). We sought to determine whether R6/2 and SSTR1/5$^{-/-}$ mice exhibit comparable cellular distribution pattern in NMDAR subunits and determined the expression levels of NR1, NR2A and NR2B using immunohistochemistry and Western blot analysis. As shown in **Figure 4.3 a**, in *wt* mice brain NR1-like immunoreactivity in striatal neurons was confined to the cell membranes, as well as, intracellularly. Additionally, NR1-like immunoreactivity was also seen in nerve fibers. In comparison to *wt*, R6/2 mice brain displayed strong NR1 immunoreactivity that was restricted to cell membrane. Consistent with immunohistochemistry, increased receptor expression was observed in striatal tissue lysate

prepared from R6/2 mice in comparison to *wt*. Similar pattern of NR-1 immunoreactivity was observed in SSTR1/5$^{-/-}$ mice in comparison to *wt* using immunohistochemistry and Western blot analysis (**Figure 4.3 a**). Similar to the NR1 expression, NR2A subunit expression was increased in R6/2 and SSTR1/5$^{-/-}$ mice when compared to respective *wt* (**Figure 4.3 b**). Interestingly, NR2A immunoreactivity expressed at cell surface in R6/2 mice in comparison to SSTR1/5$^{-/-}$ mice where receptor immunoreactivity was markedly intracellular. Western blot analysis revealed enhanced NR2A expression in SSTR1/5$^{-/-}$ mice in comparison to R6/2 mice as well as respective *wt*. In comparison to NR1 or NR2A expression, R6/2 and SSTR1/5$^{-/-}$ mice exhibited the loss of NR2B immunoreactivity. The loss of NR2B was to a greater extent in R6/2 brain than SSTR1/5$^{-/-}$ mice (**Figure 4.3 c**). The distributional pattern of NR2B-like immunoreactivity was significantly different in R6/2 and SSTR1/5$^{-/-}$ mouse striatum. As illustrated in **Figure 4.3 c**, in R6/2 mice, NR2B in striatal neurons was expressed at cell surface, whereas in SSTR1/5$^{-/-}$ mice, receptor-like immunoreactivity was present intracellularly. These data collectively provide comparable changes in NMDAR subunits in R6/2 and SSTR1/5$^{-/-}$ mice with a distinct distribution pattern. As illustrated in **Figure 4.3 d**, quantitative analysis revealed that R6/2 and SSTR1/5$^{-/-}$ mice displayed 10 and 16% increase in neuronal population positive to NR1 whereas 15 and 12% increase was observed in NR2A. In contrast, neuronal population expressing NR2B declined by 21% in R6/2 mice without any significant changes in SSTR1/5$^{-/-}$ mice brain when compared to *wt* mice.

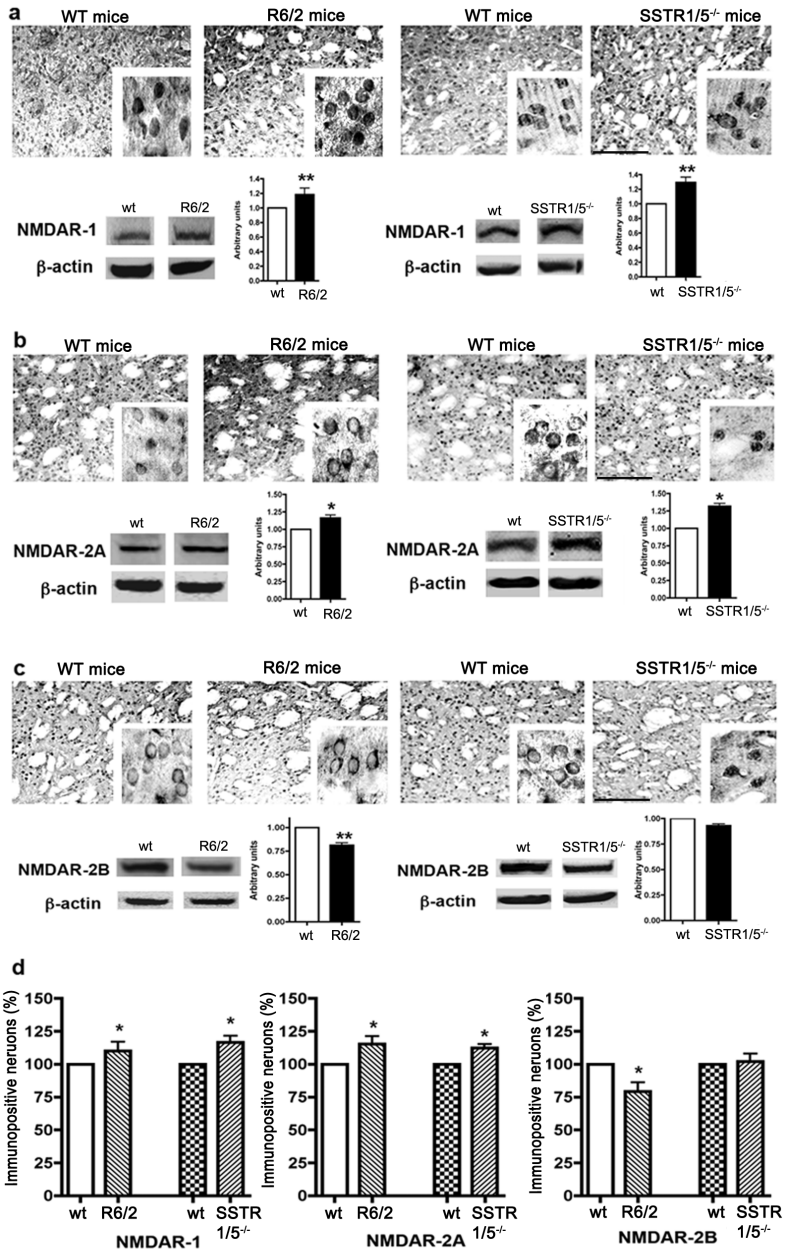

Figure 4.3 Representative photomicrographs illustrating distribution of NMDAR subtypes expression in striatum of R6/2 and SSTR1/5$^{-/-}$ mice.

Comprarable distribution pattern of NR1 (a), NR2A (b) and NR2B (c) was seen between R6/2 mice and SSTR1/5$^{-/-}$ mice. Quantitaive analysis of receptor immunoreactivity was accomplished using Western blot analysis. Note the increased expression of NR1 (120 kDa) immunoreactivity (a upper panel) and expression by Western blot (a lower panel) in R6/2 and SSTR1/5$^{-/-}$ mice. NR2A (180 kDa) expression was increased in R6/2 and SSTR1/5$^{-/-}$ mice (b). Decreased expression of NR2B (190 kDa) was seen in R6/2 mice whereas no significant changes in SSTR1/5$^{-/-}$ mice when compared to *wt* mice (c). Note cytosolic accumulation of NR2A

and NR2B in SSTR1/5$^{-/-}$ mice and membrane expression in R6/2 mice. Percentage changes in NMDARs positive neurons in R6/2, SSTR1/5$^{-/-}$ mice and *wt* mice are shown in panel (d). Data presented as mean ± SD for Western blot analysis (SSTR1/5$^{-/-}$, n=5 and R6/2 mice, n=3) in comparison to *wt* mice brain, *P<0.05, ** P<0.01. Scale bar = 20 μm panel a-c and 5 μm for inset.

4.3.4 Receptor-specific changes in SSTR subtype in striatum of SSTR1/5$^{-/-}$ and R6/2 mice

In our recent study, we have shown the expression of SSTRs in both MSNs and interneurons (Rajput et al., 2009a). We have recently shown the receptor specific changes in cortical brain region of SSTR5$^{-/-}$mice (Ramirez et al., 2004). Here we describe the comparative distribution pattern of SSTR subtypes in striatal brain regions of R6/2 and SSTR1/5$^{-/-}$ mice. In both cases, SSTRs like immunoreactivity was observed in medium-sized projection neurons, as well as in medium-sized aspiny interneurons in a receptor-specific manner. As shown in **Figure 4.4**, SSTR1-like immunoreactivity was increased in R6/2 mice in comparison to *wt,* as expected in SSTR1/5$^{-/-}$ mice, no SSTR1 expression was detected, while *wt* mice exhibited receptor-like immunoreactivity (**Figure 4.4 a**). Like SSTR1, in R6/2 and SSTR1/5$^{-/-}$ mice striatum, SSTR2 like immunoreactivity was increased as determined by immunohistochemistry and Western blot analysis (**Figure 4.4 b**). Interestingly, SSTR3 like immunoreactivity in R6/2 and SSTR1/5$^{-/-}$ mice exhibited distinct distribution pattern.

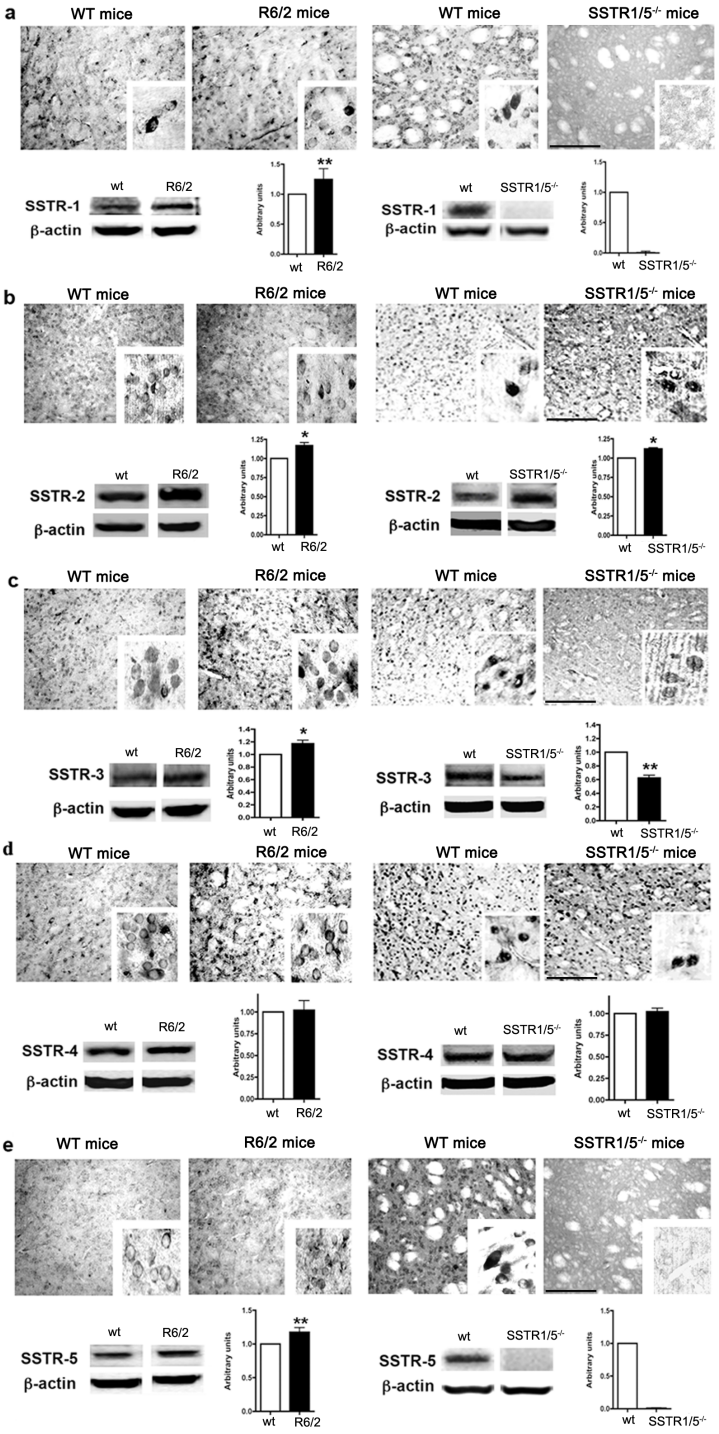

Figure 4.4 Peroxidase immunohistochemistry illustrating the changes in distribution pattern of SSTR1-5 in striatum of R6/2 and SSTR1/5$^{-/-}$ mice.

SSTR1(53 kDa) like immunoreactivity increased in striatum of R6/2 mice and abolished completely in SSTR1/5$^{-/-}$ mice (a). SSTR2 (57 kDa) like immunoreactivity was increased in R6/2 and SSTR1/5$^{-/-}$ mice (b). SSTR3 (60 kDa) expression was increased in R6/2 while it decreased in SSTR1/5$^{-/-}$ mice striatum (c).

Comparable expression of SSTR4 (44 kDa) was observed in both R6/2 and SSTR1/5$^{-/-}$ mice brain (d). SSTR5 (58 kDa) expression was selectivley higher in R6/2 mice brain than *wt* and receptor like immunoreactivity was not detected in SSTR1/5$^{-/-}$ mice (e). Data presented as mean ± SD (SSTR1/5$^{-/-}$, n=5 and R6/2 mice, n=3) in comparison to *wt* mice brain, *P<0.05, ** P<0.01. Scale bar = 20 µm panel a-e and 5 µm for inset

As illustrated in **Figure 5-4 c**, increased expression of SSTR3 was seen in R6/2 mice in comparison to *wt*, whereas SSTR1/5$^{-/-}$ displayed significant loss in SSTR3 likes immunoreactivity when compared with *wt*. Conversely, SSTR4-like immunoreactivity was comparable in both R6/2 and SSTR1/5$^{-/-}$ mice without any discernable changes (**Figure 5-4 d**). R6/2 transgenic mice exhibited increased expression of SSTR5 like immunoreactivity when compared with *wt* (**Figure 5-4 e**). Although no SSTR5 expression was seen in SSTR1/5$^{-/-}$ mice, SSTR5 like receptor expression was well expressed in *wt*.

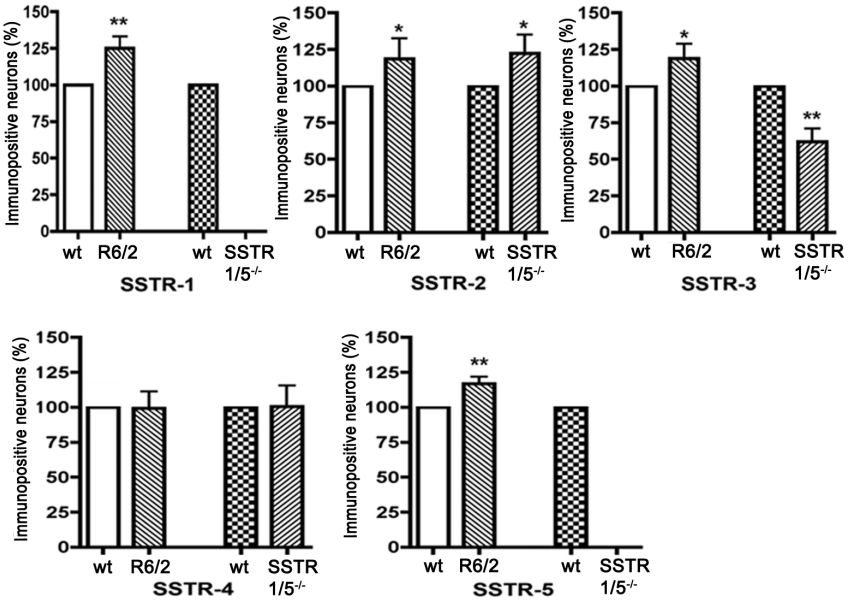

Figure 4.5 Quantitative analysis of SSTR postive neurons for receptor specific changes in SSTR1/5$^{-/-}$ and R6/2 mice strains.

Note receptor specific changes in the numbers of SSTR1–5 positive neurons in SSTR1/5$^{-/-}$and R6/2 mice compared to the respective *wt*. Data presented as mean ± SD for neuronal quantification (SSTR1/5$^{-/-}$, n=5 and R6/2 mice, n=3) in comparison to *wt* mice brain, *P<0.05, ** P<0.01.

As illustrated in **Figure 4.5**, quantitative analysis of SSTR subtypes indicates receptor specific changes in R6/2 and SSTR1/5$^{-/-}$. In R6/2 transgenic mice SSTR1, SSTR2, SSTR3 and SSTR5 positive immunoreactive neurons were increased by 25, 18, 19 and 17%, respectively, in comparison to *wt*. Conversely, SSTR4 positive neurons were not changed significantly in R6/2 and SSTR1/5$^{-/-}$ in comparison to *wt.* However, in SSTR1/5$^{-/-}$ mice SSTR2-positive neurons increased by 25%, whereas SSTR3 positive neurons declined by 38% when compared to *wt*.

4.3.5 Comparable changes in signaling cascades in striatum of SSTR1/5$^{-/-}$ and R6/2 mice

The physiological response of neurons upon activation or inhibition of GPCR or ionotropic receptors is intimately associated with the modulation of signaling pathways. Whether the ablation of SSTR modulates signaling cascades similar to the model of neurodegenerative disease has not been studied yet. Several previous studies have shown significant changes in mitogen activated protein kinases (MAPK); extracellular regulated kinase (ERK1/2) and several other key regulators associated with neuronal degeneration and pathogenesis of neurodegenerative diseases. To ascertain the underlying mechanism and functional consequences of the changes seen in SSTR and NMDAR subtypes here we determined the expression levels of pERK1/2, PKC-α, PI3K, calcineurin, calpain, synapsin-IIa, and enkephalin in striatum of R6/2 and SSTR1/5$^{-/-}$ mice brain by Western blot analysis. As shown in **Figure 4.6 a**, the expression levels of pERK1/2 were not altered in R6/2 mice whereas the status of pERK1/2 was increased in SSTR1/5$^{-/-}$ mice but not significantly when

compared to *wt*. The expression level of cell survival pathway PI3K was increased in SSTR1/5$^{-/-}$ mice, whereas, it decreased significantly in R6/2 transgenic mice (**Figure 4.6 b**). In contrast, decreased expression of PKC-α (**Figure 4.6 c**) was observed in SSTR1/5$^{-/-}$ mice and R6/2 mice when compared to *wt*.

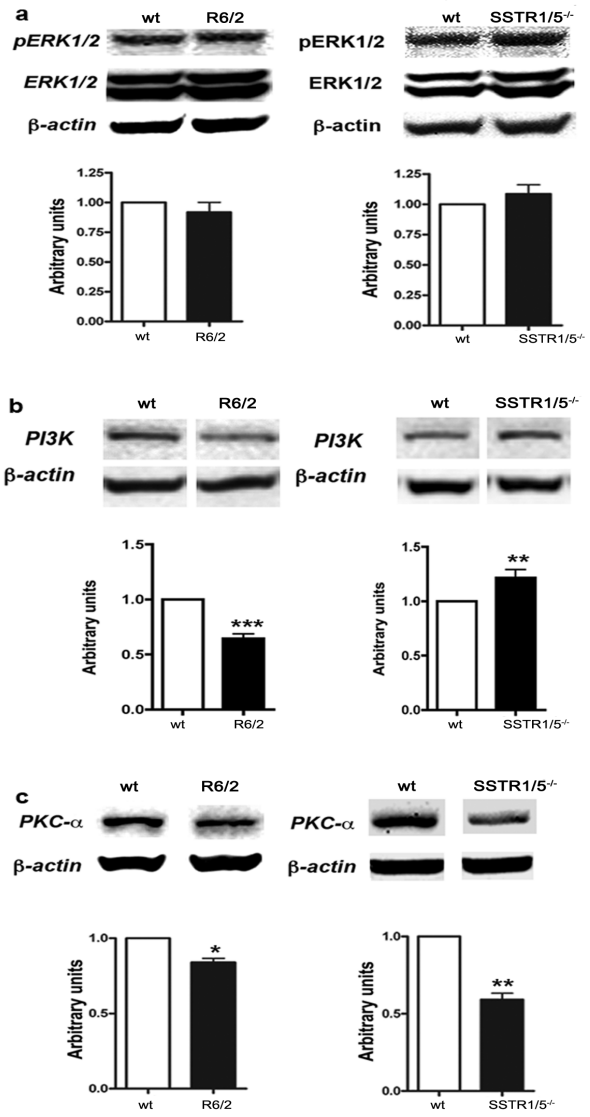

Figure 4.6 Downstream signaling pathways are differentialy regulated in R6/2 and SSTR1/5$^{-/-}$ mice.

Tissue lysate prepared from striatum of R6/2 and SSTR1/5$^{-/-}$ mice brain was fractionated on 10% SDS PAGE and membrane was blotted for total and phosphorylated ERK1/2 and PI3K and PKC-α. The status of p-ERK1/2

is not changed in R6/2 and SSTR1/5$^{-/-}$ mice striatum (a). Note the decreased expression level of PI3K (110 kDa) in R6/2 mice whereas SSTR1/5$^{-/-}$ mice brain exhibited increased PI3K expression (b). In contrast PKC-α (80 kDa) decreased in R6/2 and SSTR1/5$^{-/-}$ mice brain (c). Data presented as mean ± SD (SSTR1/5$^{-/-}$, n=5 and R6/2 mice, n=3) in comparison to *wt* mice brain, *P<0.05, ** P<0.01, ***P<0.001.

Previous studies have shown that the inhibitors of calcineurin accelerate HD neurological phenotype in R6/2 mice (Hernandez-Espinosa and Morton, 2006). Accordingly, we determined the expression levels of calcineurin in R6/2 and SSTR1/5$^{-/-}$ mouse brains. As illustrated in **Figure 4.7 a**, calcineurin expression decreased in R6/2 and SSTR1/5$^{-/-}$ mice, when compared to *wt* mice. Studies using YAC transgenic mice models have shown increased calpain expression, which is involved in neuronal apoptosis (Cowan et al., 2008). However, no data is presently available for calpain expression in R6/2 mice. As shown in **Figure 4.7 b**, increased expression of calpain in R6/2 mice as well as in SSTR1/5$^{-/-}$ mice was observed. Consistent with previous studies, these data strongly suggest the possible interaction between activation of NMDARs and increased expression of calpain in YAC and R6/2 transgenic mice.

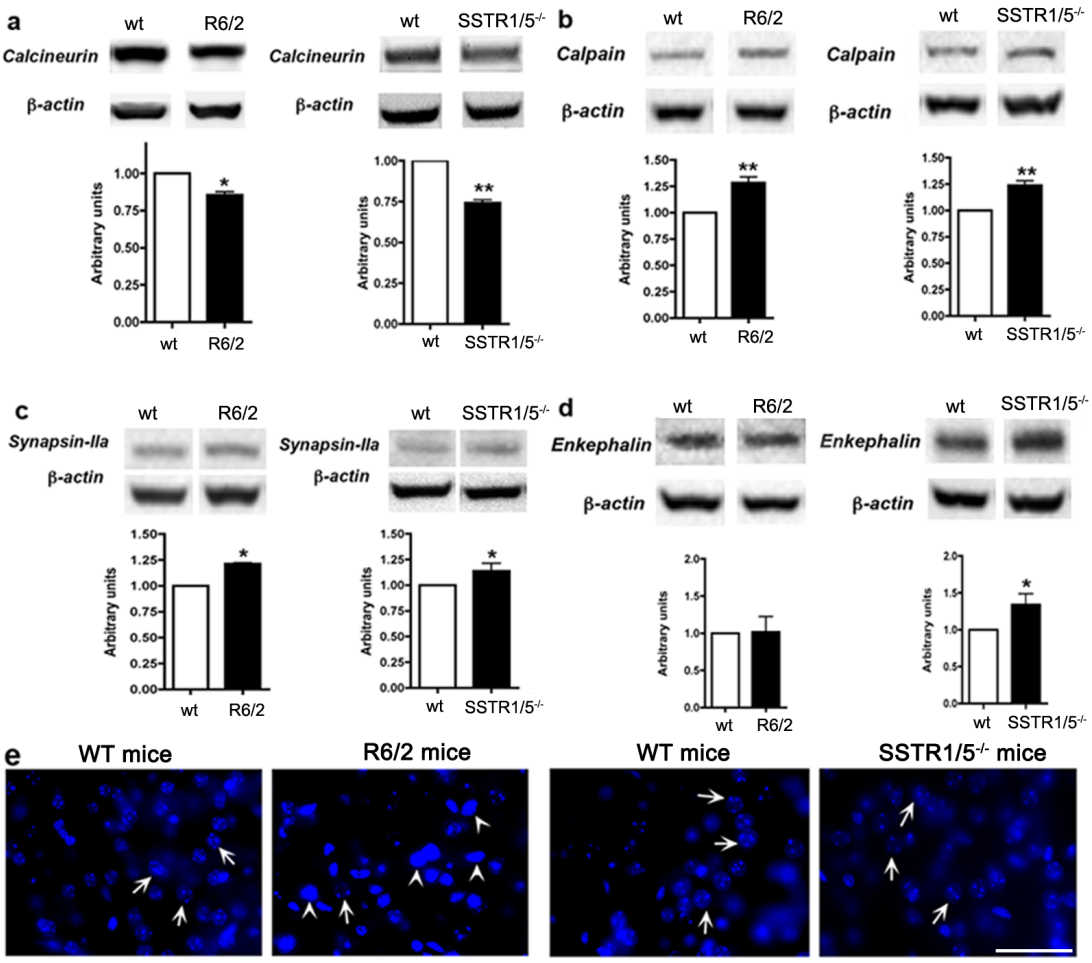

Figure 4.7 Western blot analysis demonstrating the changes in expression of downstreeam signaling protiens.

Western blot analysis demonstrating the changes in expression of calcineurin (61kDa), calpain (90 kDa), synapsin-IIa (74 kDa) and enkephalin (55 kDa) in tissue lysate prepared from striatum R6/2 and SSTR1/5$^{-/-}$ mice. Note the loss of calcineurin in SSTR1/5$^{-/-}$ and R6/2 mice brain striatum (a) and increased expression of calpain and synapsin-IIa (b,c). In contrast enkephalin was not changed in R6/2 mice whereas increased in SSTR1/5$^{-/-}$ mice brain (d). Data presented as mean ± SD (SSTR1/5$^{-/-}$, n=5 and R6/2 mice, n=3) in comparison to *wt* mice brain. *P<0.05, ** P<0.01. e) Photomicrographs showing apoptosis in R6/2 and SSTR1/5$^{-/-}$ mice striatum. R6/2 transgenic mice displayed significantly increased number of apoptotic neuronal cells in striatum when compared to *wt* (e panel left). Unlike R6/2 mice, only few apoptotic neuronal cells were observed in SSTR1/5$^{-/-}$ mice (e panel right). Arrows represent surviving cells and arrowheads represent apoptotic cells. Scale bar = 20 µm panel e.

Decreased expression of synapsin-IIa mRNA in striatum of R6/2 mice without significant changes in protein expression has been described earlier (Bibb et al., 2000). In further extension to our study, we next determined the expression level of synapsin-IIa and enkephalin in striatum of R6/2 and SSTR1/5$^{-/-}$ mice and compared with *wt* using Western blot analysis. As shown in **Figure 4.7 c**, synapsin-IIa expression was increased in both R6/2 and SSTR1/5$^{-/-}$ mice, whereas, no significant change in enkephalin protein expression was observed in R6/2. However, increased expression was seen in SSTR1/5$^{-/-}$ mice striatum in comparison to *wt* (**Figure 4.7 d**).

In HD transgenic mice, namely YAC and R6/2, increased apoptosis has been described in brain due to the toxic effect of mutated *Htt* (Fernandes et al., 2007). Here we determined whether occurrence of apoptosis is comparable in R6/2 and SSTR1/5$^{-/-}$ mice using Hoechst 33258 staining. As shown in **Figure 4.7 e**, in the absence of SSTR1/5 no apoptosis was observed. In comparison, R6/2 mice exhibited increased number of apoptotic cells when compared to their respective *wt*. These data strongly suggest that lack of SSTR1/5 does not induce excitotoxicity and the changes seen in several markers might be associated with the physiological and pharmacological effects of SSTR subtypes.

4.4 Discussion

Receptor specific ablation of SSTR subtypes have shown behavioral changes, impaired cognitive function, loss of analgesic effect and sustained pain in a receptor-specific manner (Helyes et al., 2009; Viollet et al., 2000). Whether SSTR *ko* or any other GPCRs *ko* mice mimic the neurochemical changes similar to the neurodegenerative disorders has not been studied yet. In the present study, we compared the expression levels of key molecular

markers that exert a determining role in the pathogenesis of HD in SSTR1/5$^{-/-}$ and R6/2 mice striatum. We demonstrate that, SSTR1/5$^{-/-}$ and R6/2 mice display comparable changes in distribution pattern of SST, NMDA receptor (NR1, NR2A and NR2B), DARPP-32, calbindin and bNOS, as well as the downstream signaling pathways including pERK1/2, calpain, synapsin IIa, PKC-α, and calcineurin in striatum. In contrast, SSTR subtypes, enkephalin, and PI3K are modulated in strain specific manner. This is the first comprehensive description showing comparable neurochemical and biochemical changes in a transgenic model of HD and SSTR1/5$^{-/-}$ mice brains.

The most coherent finding in HD pathology as well as in transgenic mice is the loss of DARPP-32 (Bibb et al., 2000). Consistent with this study, we describe comparable changes in DARPP-32 like immunoreactivity in R6/2 and SSTR1/5$^{-/-}$ mice, exhibiting >60% loss of DARPP-32. For the first time, we have demonstrated a similar pattern of DARPP-32 loss in the striatum of SSTR1/5 ablated mice and HD transgenic mice R6/2 indicating that SSTRs might be involved in regulation of DARPP-32. The DARPP-32 mediated downstream signaling pathway is modulated by dopamine receptors (DRs), another prominent member of GPCR family (Greengard et al., 1999; Svenningsson et al., 2000). SSTRs share ~30 % structural homology with DRs and constitute functional heterodimers with distinct pharmacological properties and enhanced signaling (Baragli et al., 2007; Rocheville et al., 2000a). In agreement with this information, SSTRs, directly or indirectly, via interaction with DR, may modulate DARPP-32. This concept is further strengthened by our recent studies showing receptor- and region-specific colocalization of DARPP-32 and DRs in rat brain (Rajput et al., 2009b). DARPP-32 becomes phosphorylated in response to the activation of D1R (Bibb et al., 2000). Conversely, D2R activation inhibits DARPP-32 phosphorylation.

Whether SSTR subtype regulates DARPP-32 phosphorylation is not known. Here we provide the evidence that in the absence of SSTR subtypes, DARPP-32 expression is decreased in striatum in a similar manner as seen in HD brain and/or HD transgenic mice. The loss of DARPP-32 like immunoreactivity in SSTR1/5$^{-/-}$ mice is not surprising; as previous studies have also shown that DARPP-32$^{-/-}$ mice resemble HD mice in dopamine signaling (Bibb et al., 2000).

Consistent with the existing notion that bNOS positive neurons are preserved in HD, the expression pattern and quantification of bNOS positive neurons in R6/2 mice as well as SSTR1/5$^{-/-}$ mice is comparable. Similar is the pattern of SST expression in both strains. In line with previous observations, our results further strengthen the concept of selective preservation of medium-sized aspiny interneurons expressing SST/b-NOS (Ferrante et al., 1985). Furthermore, our previous studies have shown that blockade of SST using antisense oligonucleotide leads to the loss of bNOS/NADPH-d positive neurons upon QUIN/NMDA treatment (Kumar, 2008). Taken together, these results support the notion that the presence of SST is likely responsible for the survival of aspiny interneurons in excitotoxicity.

In HD, the activation of NMDAR is one of the leading causes of neuronal loss, in addition to the mutation in *Htt*. Functional and physiological significance of NMDARs has recently been described in pathophysiology of HD and reported that the NMDAR antagonist, memantine, blocks the nuclear inclusion of mutated *Htt* seen in HD (Okamoto et al., 2009). Furthermore, recent studies have shown the distinct role of synaptic and extrasynaptic NMDARs in early and late onset of HD (Gladding and Raymond, 2011; Hardingham, 2009; Hardingham and Bading, 2010). NMDAR positive neurons are most vulnerable in HD as well as in various mouse models of excitotoxicity (Ali and Levine, 2006; Cepeda et al., 2001;

Fan and Raymond, 2007; Milnerwood and Raymond, 2010). Cumulatively, these studies indicate an increase NR1 and the loss of NR2B expression. However, studies for NR2A are controversial and such discrepancies may be due to the mouse strain used as a model. In the current study, an increased NR1 and NR2A expression, with the loss of NR2B immunoreactivity in both R6/2 and SSTR1/5$^{-/-}$ mice may be linked to the neurodegeneration of MSNs, which is attributed to an increased Ca^{2+} influx. Moreover, in SSTR1/5$^{-/-}$ mice, NR2A and NR2B immunoreactivity accumulates intracellularly while in R6/2 both receptors are well expressed at cell surface. In light of these results, we propose two different mechanisms for the role in NMDAR-mediated neurotoxicity. First, the membrane expression of NR2A and NR2B in HD transgenic mice allows receptor interaction at cell surface, which results in excitotoxicity. Consistent with the existing concept that NMDARs are functionally active in heteromeric complex, the increased cell surface expression of NR1 and NR2A in R6/2 might be involved in excitotoxicity through heterodimerization. In the absence of SSTR subtypes, NMDAR trafficking might be impaired, leading to receptor accumulation intracellularly. Whether SSTR and NMDAR functionally interact with each other is not known and further studies are in progress to determine this. Studies are warranted to delineate the molecular mechanism for the intracellular accumulation of NMDAR in SSTR1/5$^{-/-}$ mice. Furthermore, the possibility of impaired mitochondrial function in these processes cannot be ruled out.

The physiological response of cells upon the activation of SSTRs is receptor specific and can display multiple effects. SSTR2 is known to inhibit Ca^{2+} activated channels and increased neuronal Ca^{2+} is detrimental in excitotoxicity *in vitro* as well as in HD. Our results show increased expression of SSTR2 in the absence of SSTR1/5 and concomitantly in HD

transgenic mice, suggesting a compensatory mechanism to inhibit Ca^{2+} due to enhanced excitatory input via the activation of NMDAR. In support, we have recently shown that SSTR2 and SSTR5 heterodimerize with significant changes in receptor pharmacological properties as well as enhanced signaling (Grant and Kumar, 2010). Furthermore, SSTR1/5$^{-/-}$ mice exhibit increased expression of D2R in comparison to *wt* (unpublished observations). Since SSTR5 and D2R functionally interact and exist in a heteromeric complex, the increased expression of D2R in SSTR1/5$^{-/-}$ mice supports the compensatory role in the absence of SSTR5 *in vivo* (Rocheville et al., 2000a).

Increased expression of NR1 and NR2A might be linked with the decreased expression of calcineurin and increased expression of calpain in R6/2 and SSTR1/5$^{-/-}$ mice. Calcineurin is involved in the phosphorylation of DARPP-32, which further regulates the cell survival pathways. Calcineurin knockout mice show the inhibition of motor functions, loss of synaptic plasticity, learning and memory (Miyakawa et al., 2003). The loss of calcineurin expression in R6/2 and SSTR1/5$^{-/-}$ mice might correlate with the symptoms of HD. Furthermore, the loss of calcineurin and DARPP-32 expression can be correlated with the decreased expression of PKC-α. PKC-α plays a role in regulation of membrane associated signal transduction pathways mediated by Ca^{2+} homeostasis (Blank et al., 1997). DARPP-32 phosphorylated at Thr34 (threonine) via PKC-α converts into protein phosphatase-1 (PPtase-1), which in turn inhibits the phosphorylated forms of calcium response element binding protein (CREB) and leads to cell survival (Bastia and Schwarzschild, 2003). Alternatively, the loss of PKC-α expression can also be correlated with increased apoptosis detected in R6/2 mice. However, decreased expression of PKC-α may also be associated with decreased expression of DARPP-32 in R6/2 and SSTR1/5$^{-/-}$ mice. Changes in pERK1/2 can be

correlated with the variable expression of SSTRs. Increased pERK1/2 in SSTR1/5$^{-/-}$ mice may be due to the loss of SSTR5, which is known to inhibit pERK1/2 and increased expression of SSTR5 in R6/2 might exert inhibitory role on pERK1/2.

Previous studies have shown increased calpain activity in HD human brain tissue and YAC transgenic mice (Cowan et al., 2008; Gafni and Ellerby, 2002; Gafni et al., 2004). Increased expressions of calpain in R6/2 and SSTR1/5$^{-/-}$ mice further suggest a role of NMDAR-mediated cell death (Cowan et al., 2008). Enkephalin primarily functions as an anti-nociceptive but is also disrupted in early-onset of HD (Menalled et al., 2000). In agreement with previous studies, we detected no changes in enkephalin expression in R6/2 mice (Menalled et al., 2000). However, these results contradict other findings, which report a decrease in enkephalin expression (Sun et al., 2002). Such discrepancies may be due to the isoform of enkephalin targeted. In SSTR1/5$^{-/-}$ mice, increased expression of enkephalin, in part, may be indirectly due to changes in opioid receptors, since SSTRs share 40% structural homology and exhibit heterodimerization (Pfeiffer et al., 2002).

Post-mortem brains from HD patients and transgenic mice have shown augmented apoptosis in striatum (Fan and Raymond, 2007). Amongst all SSTRs, SSTR3 is the only known receptor subtype that can induce apoptosis (Patel, 1999). Whether increased expression of SSTR3 as seen in R6/2 mice is associated with apoptosis in HD needs further investigation. However, decreased SSTR3 expression in SSTR1/5$^{-/-}$ mice may account, albeit to a lesser degree, for apoptotic cell death in comparison to R6/2. SST via activation of SSTR subtypes blocks Ca^{2+} influx through the interference with NMDA function. This suggests that mice lacking SSTR1/5 may cause pronounced NMDA-induced toxicity due to the activation of NMDA receptors in SSTR1/5$^{-/-}$ mice. This speculation is consistent with

previous studies from HD transgenic mice (Fan and Raymond, 2007). Furthermore, the inhibitory effect of SST on excitatory synaptic transmission is also well established in rodent brain (Patel, 1999).

Previous studies have shown that presymptomatic HD mice (6-8 weeks old R6/2 mice) not only display physiological characteristics but also behavioral and neurochemical changes similar to those observed in HD (Lione et al., 1999). These observations indicate that SSTR1/5$^{-/-}$ might serve as a model to elucidate the role of SSTR subtypes to characterize the neurochemical changes in different proteins and pathways involved in HD. SSTR5 blocks the L-type Ca^{2+} channels and may correlate with the increased expression of SSTR5 in R6/2 mice (Tallent et al., 1996). As SSTR5 is not predominantly expressed in mouse striatum and SSTR1 and SSTR5 are known to form heterodimers, we speculate that the increased expression of SSTR1 in R6/2 mice may be due to a compensatory mechanism of SSTR5. Our observations raise a potential question about the role of SSTR1 or SSTR5 independently. We did not eliminate this question from our discussion and propose that knocking out SSTR1 or SSTR5 might have same effect as seen in double *ko* mice, albeit to the lesser degree. We have recently characterized the SSTR subtypes for homo-and heterodimerization in detail with distinct pharmacological and enhanced functional properties (Grant et al., 2004a; Grant et al., 2004b; Somvanshi et al., 2009).

Taken together, our results indicate that SST and SSTRs might play an important neuroprotective role in neurodegenerative diseases. Targeting this pathway can provide novel insights in understanding the pathophysiology of neurodegenerative diseases. This is the first study showing similar disease characteristics related to neurochemical changes upon ablation of the GPCR gene (i.e., SSTRs). We here reinforced the concept that mice in the absence of

SSTR subtype might have greater susceptibility to neurodegenerative diseases as well as excitotoxicity. We propose that in HD transgenic mice as well as in HD patient's pharmacological restoration and maintenance of somatostatinergic system might serve as a beneficial therapeutic approach in treatment of HD.

Chapter 5: [4]Somatostatin receptors-mediated inhibition of NMDA currents and dissociation of NMDARs complex in striatum: implication in excitotoxicity

SSTR1 and SSTR5 double *ko* mice resulted in similar neurochemical changes as seen in HD transgenic models leading to the hypothesis for the role of SSTR1 and SSTR5 specific agonist and their role in excitotoxicity. The speculation is based on critical findings including activation of NMDARs and signaling pathways associated with neuronal cell death. We next determine the effects of SSTR1 and SSTR5-specific agonist on NMDARs heterodimerization and NMDARs mediated signaling pathway in striatal brain slices using morphological, biological and electrophysiological approaches.

5.1 Background

In the striatum, N-Methyl-D-aspartic acid (NMDA) induced excitotoxicity represents a model of neurodegeneration attributed to several neurological diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), human immunodeficiency virus (HIV)-associated dementia, multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (Lau and Zukin, 2007; Lipton, 2006). NMDA receptor (NMDARs) and dopamine and cAMP-regulated phosphoprotein (DARPP-32) positive neurons are highly susceptible in excitotoxicity (Albin et al., 1990; Sun et al., 2005; Torres-

[4] A version of this chapter is will be submitted as: Somatostatin Receptor Mediated Inhibition of NMDA Currents and Dissociation of NMDARs Complex in Striatum: Implication in Excitotoxicity.
Rajput PS, Yang, B, Sastry BR, Kumar U.

Peraza et al., 2007; Young et al., 1988). In contrast, medium sized aspiny interneurons positive to somatostatin (SST) and coexpressing neuropeptide Y (NPY)/brain nitric oxide synthase (bNOS) are selectively spared in excitotoxicity as well as in several neurological diseases such as AD, HD as well as PD (Beal et al., 1984; Beal et al., 1988; Beal et al., 1985; Kumar, 2004; Kumar, 2005; Kumar, 2008; Patel et al., 1991; Patel et al., 1995b; Saito et al., 2005). Previous studies demonstrate increased release of SST and mRNA expression upon treatment with Quinolinic acid (QUIN)/NMDA in cultured cortical neurons without any changes in NPY expression (Patel et al., 1991; Patel et al., 1995b). Furthermore, immunoneutralization of released SST using SST antibodies and knocking down of SST using SST antisense oligonucleotides resulted in extensive neuronal loss in response to QUIN/NMDA in cultured striatal neurons (Kumar, 2008). We recently described that SSTR1 and 5 double knockout mice show similar neurochemical changes as seen in HD transgenic R6/2 mice (Rajput et al., 2011b). These changes include increased expression of NMDARs, key signaling molecules which are involved in neuronal degeneration like DARPP-32, calpain, calbindin and calcineurin, suggesting a potential role of SSTR1 and 5 in NMDA/QUIN induced excitotoxicity as well as HD (Rajput et al., 2011b).

NMDARs at synaptic and extrasynaptic location play a detrimental role, whether NMDARs activation is neuroprotective or triggers neuronal loss (Hardingham, 2009; Hardingham and Bading, 2003; Hardingham and Bading, 2010). Activation of synaptic NMDARs leads to an activation of cAMP response element binding (CREB)/calmodulin kinases (CaMK) pathway and affords neuroprotection (Papadia et al., 2005). Whereas, activation of extrasynaptic NMDARs leads to an increase in Ca^{2+} influx, oxidative stress, mitochondrial damage and consequent neuronal cell death (Milnerwood and Raymond, 2010;

Raymond et al., 2011). Furthermore, NMDA mediated neurodegeneration is determined with the formation of NMDARs specific complex formation. NMDARs comprise NR1 as the main receptor subunit and NR2 (A-D) or NR3 (A and B) (Monyer et al., 1994; Ozawa et al., 1998; Roy et al., 1994). In this direction, partial inhibition of NMDARs can be used for potential therapeutic intervention to protect neurons in excitotoxicity and neurodegenerative diseases (Okamoto et al., 2009).

There is growing evidence that neuronal cell death in excitotoxicity is not due to a single cause but rather associated with well-integrated events responsible for impaired cell response. Whether or not SSTRs interfere in NMDA induced currents and NMDARs heteromeric complex, this could represent a novel therapeutic application of SSTRs in regulation of neurodegeneration in excitotoxicity. Previous studies support the concept of receptor heterodimerization in improving receptor functional activity and modulation of effective signaling compared to native receptors (Kumar and Grant, 2010; Rocheville et al., 2000a). However, in contrast, dissociation of preformed receptor complex might also exert a beneficial role specifically in excitotoxicity. Accordingly, in the present study, we explored the role of SSTR1 and SSTR5 in modulation of NMDARs mediated signaling in slices prepared from rat brain striatum. We applied morphological, biochemical and electrophysiological approaches to determine the interaction between NMDARs and SSTRs in the striatum of rat brain. In addition, we also explored the role of key proteins and signaling pathways upon treatment with NMDA and SSTR1 or 5 agonist alone or in combination. Our results suggest that SSTR1/5 abrogate the signaling cascade associated with neuronal cell death upon activation or over expression of NMDARs.

5.2 Materials and methods

5.2.1 Materials

Rabbit polyclonal antibodies for SSTR1 and 5 subtypes have been characterized previously (Kumar, 2005; Kumar, 2007; Rajput et al., 2009a). Mouse monoclonal antibodies for DARPP-32 and PSD-95 were purchased from BD Biosciences (Mississauga, ON, Canada). Mouse monoclonal antibodies against NR-1, NR-2A, and NR-2B were obtained from Millipore (CA, USA) and NR1 rabbit polyclonal from Sigma (KY, USA). Phosphoinositide 3-kinase (PI3K), protein kinase C-alpha (PKC-α), extracellular signal-regulated kinases (ERK1/2), Calpain, CREB, p-DARPP-32 Thr34 and Thr75 were obtained from Cell Signaling Technology (MA, USA). The non-peptide agonists L-797591 (hSSTR1) and L-817818 (hSSTR5) were provided by Dr. S.P. Rohrer from Merck & Co. Nitrocellulose Hy-bond membrane and enhanced chemiluminescence (ECL) detection kit were obtained from Amarsham Ltd. (Oakdale, ON, Canada). All other chemicals of analytical grade were obtained from various commercial sources.

5.2.2 Indirect immunofluorescence immunohistochemistry

Indirect immunofluorescence immunohistochemistry was performed as described previously (Rajput et al., 2011a; Rajput et al., 2009b). Briefly, rats were anesthetized with halothane and perfused with heparinized cold saline and 4% paraformaldehyde with an infusion pump (KD Scientific Model 200S, Holliston, MA, USA). 40µm thick brain sections were cut using Leica 1200s vibratome and free floating sections were collected in phosphate buffer saline (PBS). Brain sections were incubated in 0.02% Triton X-100 in PBS for 15 min

and followed by three subsequent washes with PBS. Sections were then incubated in 5% normal goat serum for 1h at room temperature (RT) and followed by overnight incubation with rabbit anti-SSTR1 and SSTR5 (1:400) and mouse NR1, NR2A and NR2B (1:1000) antibodies. Following three subsequent washes in PBS brain sections were incubated in a cocktail of Alexa 594 (red) and Alexa 488 (green) conjugated secondary antibodies for 1 h at RT. The sections were washed, mounted on slides and viewed under Leica DMLB microscope attached with Retiga 2000R camera. All merged images showing colocalization were generated using NIH, ImageJ software and Adobe Photoshop (San Jose, CA, USA) was used to make the composites.

5.2.3 Western blot analysis

Brain was removed from anesthetized rats and kept in artificial cerebrospinal fluid equilibrated with 95% O_2 -5% CO_2 carbogen (pH 7.4). The striatal slices were cut using a Lecia vibratome and slices were treated with NMDA (300µm), SSTR1 agonist (L-797,591, 20 nM), SSTR5 agonist (L-817,818, 20 nM) and in combination with SSTR1 or 5 agonist with NMDA for 30 min. Post treatment striatal slices were homogenized in homogenizing buffer. Total protein in tissue extract was determined by Bradford protein assay and 15µg of total protein was fractionated by electrophoresis on 10% SDS-PAGE, and transferred onto 0.2µm-nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk at RT for 1 h and incubated overnight at 4°C with specific primary antibodies against– SSTR1 and 5 (1:500), NR1, NR2A, NR2B, calpain, PSD-95, p- DARPP-32^{Thr75}, p-DARPP-32^{Thr34}, phospho and total PI3K, CREB and ERK1/2 (1:1000) and PKC-α (1:500). Following three subsequent washes, the membrane was incubated with peroxidase conjugated goat anti-rabbit

or goat anti-mouse secondary antibody at RT for 1 h. The bands were detected using chemiluminescence reagent and images were taken using an Alpha Innotech FluorChem 8800 (Alpha Innotech Co., San Leandro, CA) gel box imager. β-actin was used as the housekeeping protein for loading control. The bands intensity was quantified using densitometric analysis and the changes in protein expression were calculated as the ratio of band of interest and the density of β-actin.

5.2.4 Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed on lysate prepared from striatal slices following different treatment conditions as described previously (Rajput et al., 2011a; Rajput et al., 2009b). 200 µg of tissue protein was solubilized in 1 ml of radio-immunoprecipitation assay (RIPA) buffer for 1 h at 4°C. The lysates were incubated with polyclonal anti-SSTR1 and SSTR5 antibodies (1:500) overnight at 4°C. The antibodies were further immunoprecipitated using protein A/G agarose beads for 2 h at 4°C. The agarose beads were subsequently washed three times with RIPA buffer. The purified samples were fractionated by electrophoresis on 10% SDS-Page gel and transferred onto nitrocellulose membrane. The membranes was blocked with 5% non-fat dried milk for 1h at RT and subsequently incubated overnight at 4°C with primary monoclonal antibodies for NR1, NR2A and NR2B diluted in 5% bovine serum albumin. The membrane was incubated with goat anti-mouse secondary antibodies for 1 h at RT. For co-immunoprecipitation of NR1 with NR2A and NR2B the membranes were incubated with rabbit polyclonal NR1 antibody overnight and followed by similar procedure described earlier and immunobloted for NR2A and NR2B. The bands were detected with chemiluminescence reagent and images were taken

using an Alpha Innotech Fluorchem 800 (Alpha Innotech Co., San Leandro, CA) gel box imager as described earlier.

5.2.5 Electrophysiology

Rat striatal slices were prepared as described previously (Venance et al., 2004) and superfused with Mg^{2+} -free artificial cerebrospinal fluid (ACSF) containing (in mM): 120 NaCl, 3 KCl, 26 $NaHCO_3$, 4 $CaCl_2$ and 10 dextrose, equilibrated with 95% O_2–5% CO_2 carbogen (pH 7.35–7.4) at 1.5~2 ml/min. Recordings were made with a patch pipette while voltage-clamping the neurons at around -60 mV. Excitatory postsynaptic currents (EPSCs) were recorded in response to stimulations (at 0.05 Hz) in presence of 50 μM DNQX (6,7-dinitroquinoxaline-2,3-dione). In preliminary experiments, concentrations of L-797,591 and L-817,818 that produced effects on the EPSC were determined and for each drug, three concentrations were chosen for testing dose-response relationships. To examine the effects of combined agonists (L-797,591 and L-817,818), a concentration of 20 nM was chosen for each agonist. Six consecutive EPSCs were averaged and used for quantitative analysis. Control recordings were made for 10 min and cells that showed stable control responses were chosen for further studies. All drugs were applied to the superfusing medium and their effects expressed as percentage of control.

5.2.6 Data Aquisation and statistical analysis

For each cell, amplitudes of evoked currents (EPSCs) were normalized relative to responses prior to drug application. EPSCs were recorded in the whole-cell voltage clamp mode using an Axopatch 200A (Molecular Devices, CA) amplifier. Records were digitized using Digidata 1322A interface and Clampex Ver. 9.0 software (Molecular Devices,

Sunnyvale, CA). Low pass filtering was set at 2 kHz. Data were analyzed using Clampfit Ver. 9.2 software (Molecular Devices, CA) and expressed, as mean ± SEM. Statistical significance of changes in amplitude of EPSCs was determined using one-way ANOVA and the post hoc Dunnett's. Differences were considered significant if $P < 0.05$. Since one cell per striatal slice was used in this study, n refers to number of cells (n = 7). The data presented in the study were analyzed using GraphPad Prism 4.0. The data for western blot analysis were analyzed using one-way ANOVA and the post hoc Dunnett's was applied according to the experimental conditions Significant statistical differences were taken at $*P < 0.05$. Representative data is presented from studies in five different animals (n = 5).

5.3 Results

5.3.1 Expression of SSTR1, SSTR5 and NMDA receptor subtypes in rat brain striatum

We first examined the distributional pattern of NMDARs, SSTR1 and 5 in striatum. As shown in **Figure 5.1 A**, NR1, NR2A and NR2B like immunoreactivity was expressed in majority of the medium sized projection neurons with strong expression on neuronal cell membrane. As shown, SSTR1 was expressed in majority of neuronal population including dendrites. In comparison to SSTR1 and NMDARs, only a few neurons showed SSTR5 positive immunoreactivity in the striatum. These results indicate that NMDARs and SSTR1 and 5 could be present in the same neuronal population therefore, as a next step we wanted to determine whether these receptors colocalize with each other in the striatum.

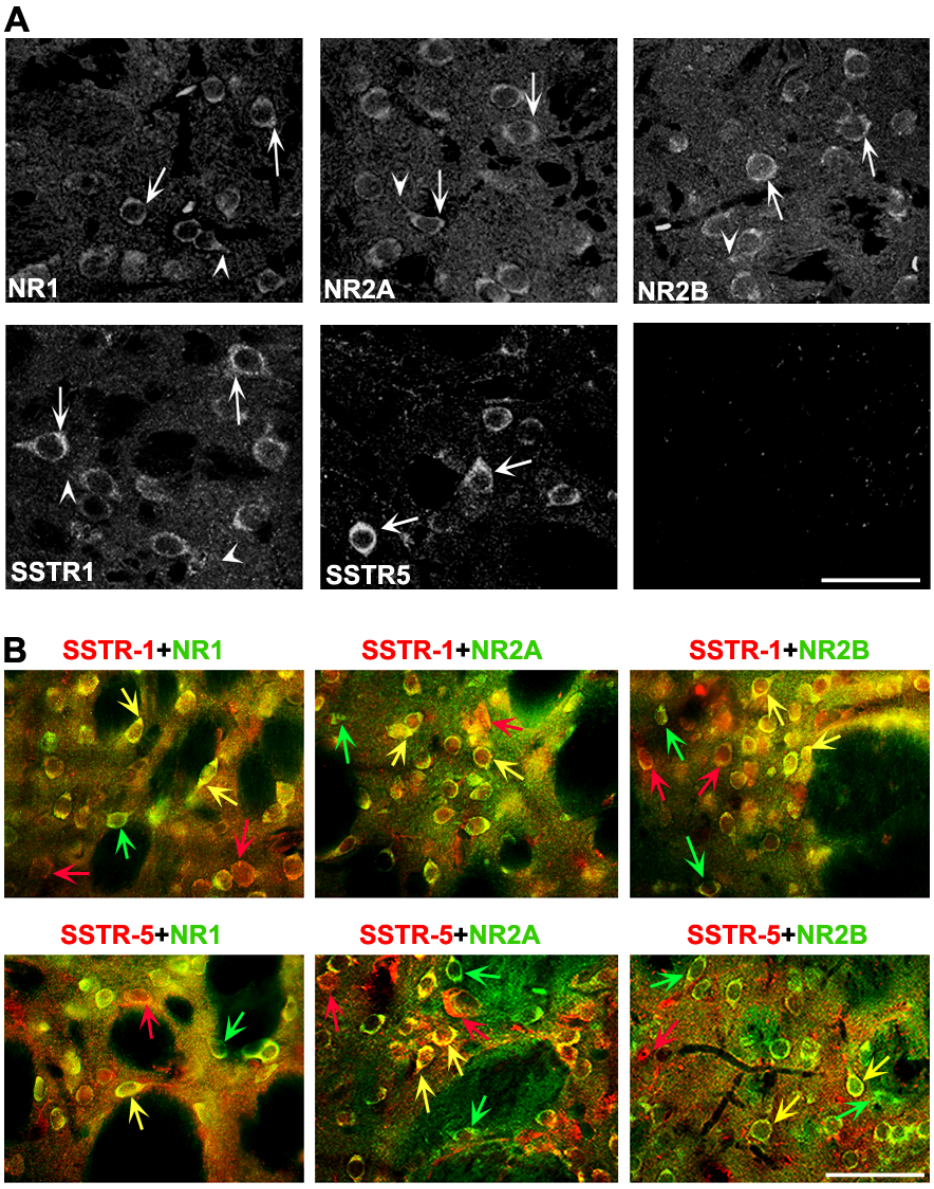

Figure 5.1 Expression and colocalization of SSTR1, SSTR5 and NMDARs (NR1, NR2A and NR2B) in striatum.

A) NR1-like immunoreactivity was highly expressed in majority of striatal neurons followed by NR2B and NR2A (upper panel). SSTR1 positive neurons display strong immunoreactivity in neuronal cell body. SSTR5-like immunoreactivity was strongly expressed relatively in less number of neurons in comparison to SSTR1 (bottom panel). Specificity of immunoreactivity was confirmed in absence of primary antibodies. In the absence of primary antibody no receptor-like immunoreactivity were observed. Arrows indicate receptor positive neurons and arrowhead shows nerve fibers. **B)** Representative photomicrograph illustrating colocalization of

SSTR1 and SSTR5 with NR1, NR2A and NR2B in striatum of rat brain. As described in materials and methods section brain sections were incubated overnight with polyclonal SSTR1 or SSTR5 and monoclonal NMDARs antibodies and followed by incubation with Alexa 594 (red) and Alexa 488 (green) to visualize SSTRs and NMDARs. SSTR1 and SSTR5 coexpressed with NR1, NR2A and NR2B in a receptor specific manner (upper and lower panel respectively). Three different populations of neurons expressing SSTRs or NMDARs and colocalizing neurons were observed. SSTRs positive neurons are indicated by red arrow, NMDARs positive neurons by green arrow and colocalization is indicated by yellow arrow. Scale bar = 5µm

5.3.2 SSTR1 and SSTR5 colocalizes with NMDA receptor subtypes in striatum

In excitotoxicity, the concept of selective preservation of aspiny interneurons was proposed due to the lack of NR1 immunoreactivity (Koh et al., 1986). However, later studies using cultured striatal neurons and brain slices revealed that SST positive neurons coexpressed NMDARs like immunoreactivity, but relatively less than the projections neurons (Augood et al., 1994; Kumar et al., 1997a). Whether, SSTR subtypes positive neurons colocalized with NMDARs is not known. Therefore, to confirm any possible interaction between SSTRs and NMDARs colocalization studies were performed. **Figure 5.1 B**, illustrates comparative colocalization of SSTR1 and 5 with NR1, NR2A and NR2B in striatum. We found that, SSTR1 positive neurons show a selective colocalization with NR1 and few neurons were devoid of NR1-like immunoreactivity while strongly positive for SSTR1. However, a large number of neurons show selective colocalization of SSTR1 with NR2A and neurons with distinct morphology (interneurons) were devoid of colocalization with NR2A. In case of NR2B only a selective population of neurons displayed mild to strong colocalization whereas number of neurons show only SSTR1 or NR2B-like immunoreactivity.

As illustrated in **Figure 5.1 B**, SSTR5 positive neurons exhibited lesser degree of colocalization in comparison to SSTR1 with NMDARs. Sparsely distributed SSTR5-positive neurons exhibit colocalization with NMDARs. Colocalization analysis exhibited three distinct neuronal population either showing colocalization or independent staining for SSTR5 or NR1. However more SSTR5-positive neurons show colocalization with NR2A then NR1. Like colocalization with NR1, NR2B and SSTR5 colocalization data shows three different neuronal populations with selective neuronal population expressing colocalization and neuronal population expressing SSTR5 or NR2B only (**Figure 5.1 B**). These results strongly suggest that same neuronal population could be positive to NMDARs as well as SSTRs.

5.3.3 SSTR1 and 5 agonist decreases expression of NMDA receptor subtypes in striatum

We recently described increased expression of NMDARs in SSTR1/5 double knock out mice, which were comparable to HD transgenic mice (Rajput et al., 2011b). These results suggest inhibitory influence of SSTR1/5 on NMDARs expression and association with HD pathology. To ascertain, direct activation of SSTR1 and 5 on NMDARs expression striatal slices were treated with NMDA (300μM), SSTR1 (L-797,591, 20nM) and SSTR5 (L-817,818, 20nM) agonist alone or in combination.

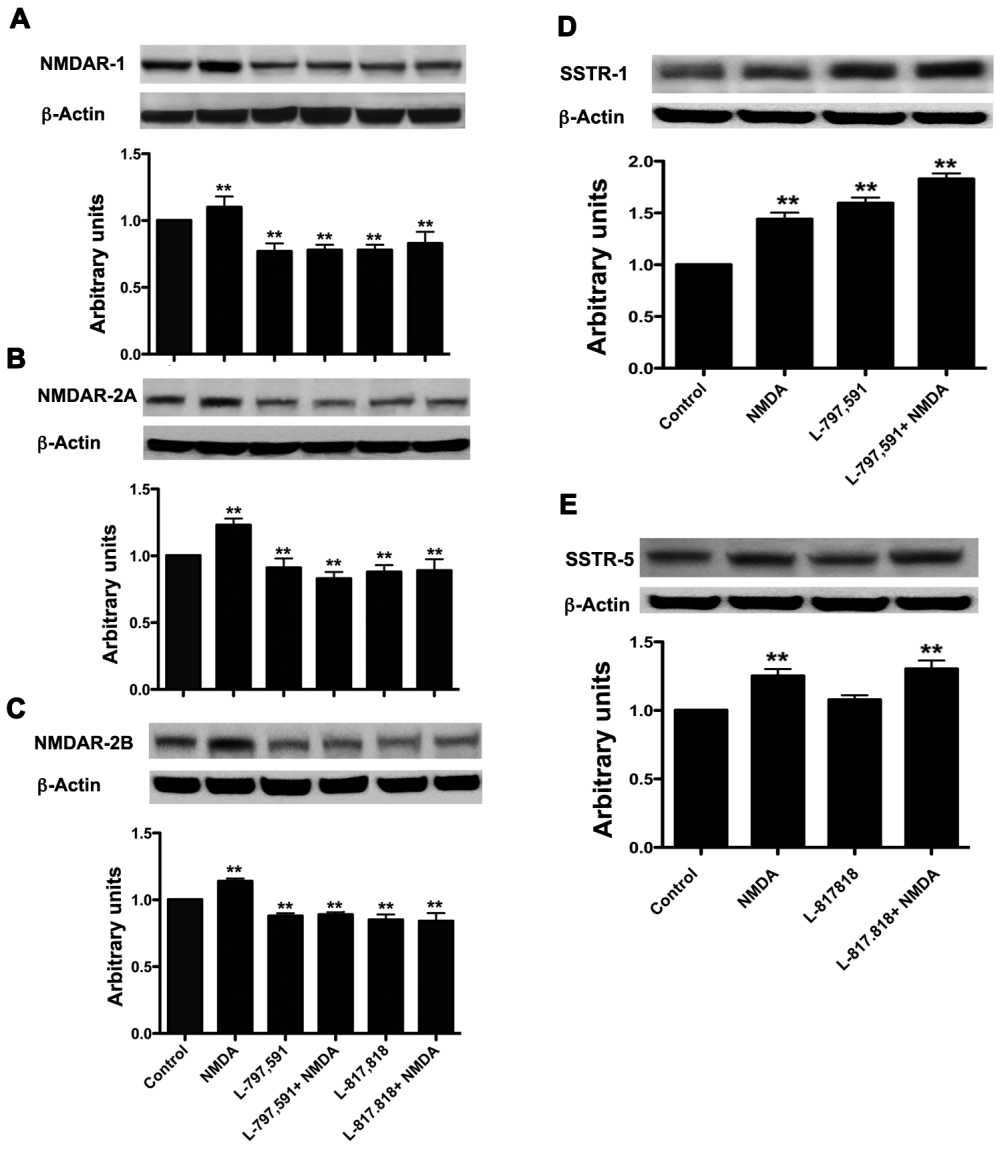

Figure 5.2 Western blot analysis depicting expression of NMDARs, SSTR1 and SSTR5 in tissue lysate prepared from striatal brain slices.

The slices were treated with NMDA (300μM), SSTR1 agonist (L-797,591, 20nM), SSTR5 agonist (L-817,818, 20nM) alone or in combination with NMDA for 30 min at room temperature. Post treatment slices were homogenized in homogenizing buffer and tissue lysate was used for Western blot analysis as described in methods section. NR1, NR2A and NR2B expression was significantly elevated in response to NMDA treatment. Note significant decrease in NMDARs expression upon SSTR1 and SSTR5 agonist treatments **(panels A-C)**. Striatal tissue lysate exhibit increased expression of SSTR1 and SSTR5 upon NMDA and SSTRs

specific agonist treatments panel **D and E**. Increased SSTR1 and SSTR5 expression might support the inhibitory role of SSTRs on NMDARs expression. * P< 0.05.

Tissue lysate prepared from striatal slices were processed for western blot analysis. As shown in **Figure 5.2**, NR1 (**A**) NR2A (**B**) and NR2B (**C**) are well expressed in striatal lysate at expected size of >120 kDa, >180 kDa and >190 kDa and receptor expression increased upon treatment with NMDA. Interestingly, striatal slices treated with SSTR1 or 5 specific agonist displayed decreased NMDARs expression and NMDA effect was attenuated in the presence of SSTR1 and 5 agonist. Conversely, SSTR1 (>53 kDa) and SSTR5 (>58kDa) expression increased upon treatment in comparison to control as indicated (**Figure 5.2D** and **E**).

5.3.4 NMDARs are expressed in SSTR1 or SSTR5 immunoprecipitate and changed upon receptor specific activation.

Receptor specific colocalization and changes in receptor immunoreactivity following treatment with respective agonists suggest that SSTRs might constitute a functional receptor complex with NMDARs. Next we investigated the expression of NMDARs in SSTR1 and SSTR5 immunoprecipitate prepared from striatal tissue lysate upon treatment as indicated. As shown, NR1 was expressed in SSTR1 and SSTR5 immunoprecipitate at the expected molecular size of >180 kDa and >190 kDa in basal as well as in the presence of NMDA treatment (**Figure 5.3A**). Expression levels of NR1 significantly decreased upon treatment with SSTR1 or SSTR5 specific agonist with or without NMDA. Interestingly, as illustrated in **Figure 3 A** (middle panel), NR2A was expressed in SSTR1 immunoprecipitate at >240kDa without any discernable changes upon treatment as indicated. In contrast, SSTR5

and NR2A complex formation at >250 kDa was relatively strong in presence of SSTR5 agonist alone and with NMDA in comparison to control and NMDA treatment.

We next determined the effect of SSTR1 and 5-specific agonist on association of NR2B with SSTR1 and SSTR5 respectively. As shown in **Figure 5.3 A** (bottom panel), in basal condition no expression of NR2B was observed in SSTR1 immunoprecipitate whereas, NR2B was expressed in SSTR5 immunoprecipitate. Conversely, NMDA, L-797,591 and L-817,818 treatment showed enhanced expression of NR2B in SSTR1 immunoprecipitate attesting the complex formation between two receptors (**Figure 5.3 A**). In contrast, expression of NR2B in SSTR5 immunoprecipitate was comparable to control upon NMDA treatment, but enhanced significantly with SSTR5 agonist alone or in combination. Co-IP results strongly suggest that treatment with SSTR1 and 5 agonist can modulate the heteromeric complex formation of SSTRs and NMDARs, indicating dissociation of NMDARs complex and can be correlated to decrease in NMDARs expression.

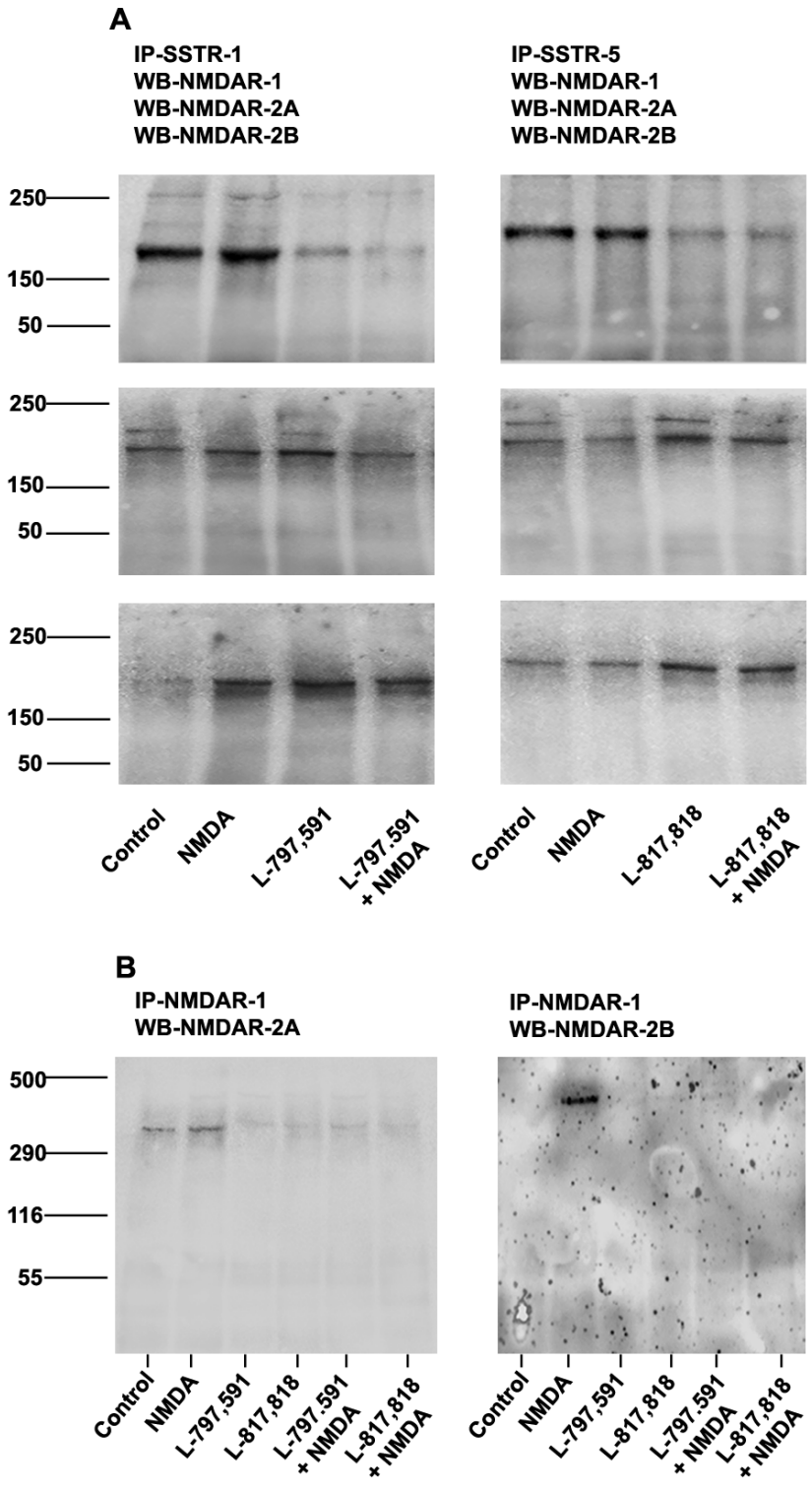

Figure 5.3 SSTR1 and SSTR5 immunoprecipitate expresses NMDARs in striatal tissue lysate.

A) Western blot analysis in SSTR-1 and SSTR-5 immunoprecipitate illustrating the expression of NR1, NR2A and NR2B in tissue lysate prepared from striatal slices treated with SSTR1 and SSTR5-specific agonist alone or

in combinations with NMDA. Western blot analysis indicates the formation of heteromeric complex between SSTR1 and NMDARs in control or NMDA treatment in striatal tissue lysate immunoprecipitated with SSTR1 antibodies. Note the dissociation of heteromeric complex between NMDARs and SSTR1 upon treatment with SSTR1 agonist alone or in combination with NMDA (upper left hand panel). SSTR1 and NR2A display stable rather weak complex with or without treatment as indicated (middle left hand panel). NR2B is not expressed in SSTR1 immunoprecipitate in basal condition but induced upon agonist treatment (bottom panel). SSTR5 immunoprecipitate displayed comparable expression of NR1 (upper right hand panel). NR2A is well expressed in SSTR5 immunoprecipitate with slight increase upon SSTR5 agonist treatment alone or in combination with NMDA (middle right hand panel) NR2B is also well expressed in control or NMDA treated SSTR5 immunoprecipitate and increased in presence of SSTR5 agonist in absence or presence of NMDA (bottom right hand panel). **B)** SSTRs activation impedes NMDARs heteromeric complex. Post treatment with NMDA or SSTR1 or SSTR5 agonist alone or in combinations NR1 immunoprecipitate prepared from tissue lysate of striatal slices was immunobloted for NR2A and NR2B expression. Note the expression of NR2A in control or NMDA treated tissue lysate was completely dimisnished upon SSTR agonist alone or in combination with NMDA (left hand panel) Conversely, NR2B expression in NR1 immunoprecipitate was observed only when striatal slices were treated with NMDA whereas, no complex formation was observed in basal as well as agonist treatment as indicated. Note the data indicate that treatment with SSTR1 and 5-specific agonist dissociates the NMDARs preformed heteromeric complex in striatum.

5.3.5 SSTR1 and SSTR5 activation impedes the formation of NR1 heteromeric complex with NR2A and NR2B.

In order to determine whether the activation of SSTR1 and 5 impedes NMDARs heterodimerization, NR1 immunoprecipitate prepared from striatal tissue lysate following indicated treatment was probed for NR2A and NR2B expression. NR2A was expressed in NR1 immunoprecipitate (>300 kDa) in basal condition with relatively higher expression upon treatment with NMDA (**Figure 5.3 B**). Interestingly, upon treatment with L-797,591 and L-817,818 alone or in combination with NMDA no expression of NR2A was detected in

NR1 immunoprecipitate suggesting the disruption of NR1 and NR2A preformed complex. NR2B was expressed strongly in NR1 immunoprecipitate upon treatment with NMDA at the expected molecular size of >320 kDa suggesting agonist induced complex formation between NR1 and NR2B. In rest of the experimental conditions as indicated NR2B expression in NR1 immunoprecipitate was not detectable including control. These results support that activation of SSTR1 and 5 lead to the dissociation of herterodimerization between NR1/NR2A and NR1/NR2B. Furthermore, diminished heterodimerization between NMDARs upon SSTR1 and 5 agonist treatment suggests the role of SSTR1 and SSTR5 in NMDA induced excitotoxicity.

5.3.6 NMDA current in striatal slices is inhibited by activation of SSTR1 and SSTR5

To determine direct functional consequence of NR1 dissociation from NR2A or NR2B and impact of SSTR activation, we processed striatal slices for NMDA currents. To record NMDA currents, striatal slices were voltage-clamped at -60mV in the presence of DNQX in Mg^{2+} free medium. As illustrated in **Figure 5.4**, addition of SSTR1 and SSTR5 specific agonist to bath medium resulted in inhibition of NMDA current by 25 and 28 %, respectively.

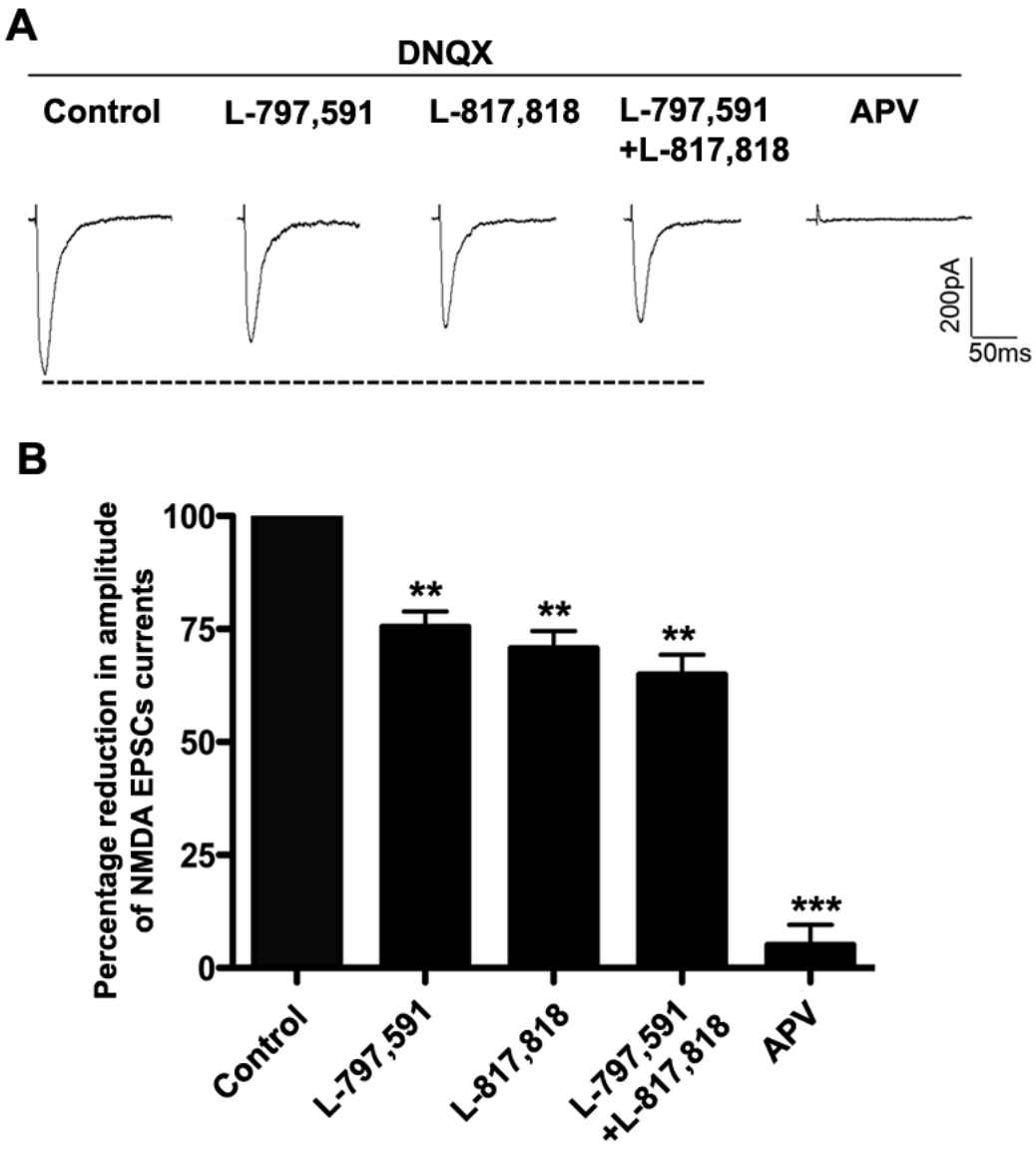

Figure 5.4 Activation of SSTR1 and 5 suppressed NMDA-current in brain slices.

To monitor NMDA currents, slices were superfused continuously with DNQX (50μM) that blocks α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor-mediated excitatory post-synaptic currents, EPSCs and Mg^{2+}-free medium to unblock the NMDA channels. In A, both SSTR1 agonist (20 nM) and SSTR5 agonist (20 nM) decreased the NMDA EPSCs whereas, inhibition of EPSCs was further enhanced upon combined application of SSTR agonists. APV (50μM), the NMDA receptor antagonist, completely blocked the EPSC. In B, histograms show that both SSTR1 and SSTR5 agonists markedly suppressed the amplitudes of EPSCs (n=6, **P<0.001).

Interestingly, inhibitory effect by combined activation of SSTR1 and 5 was relatively higher, albeit not significantly different than the activation of SSTR1 or 5 alone. Furthermore, NMDA current, in striatal slices was completely abolished in presence of 2-amino-5-phosphonovaleric acid (APV), a selective antagonist of NMDA receptor. These results from electrophysiological data lend support to inhibition of NMDA receptor immunoreactivity in the presence of SSTR1 or SSTR5 agonist.

5.3.7 Activation of SSTR 1 and SSTR 5 modulates DARPP-32 phosphorylation

Majority of striatal projection neurons are positive for DARPP-32 and are susceptible in excitotoxicity (Sun et al., 2005; Torres-Peraza et al., 2007). The phosphorylation of DARPP-32 at threonine 34 and 75 positions is critical regulator of DARPP-32 activity (Nishi et al., 2000). To ascertain whether SSTRs mediated neuroprotection is associated with the regulation of DARPP-32 site-specific phosphorylation, we determined the effect of NMDA and SSTR1 or 5 specific agonist on phosphorylation of DARPP-32 at Thr34 and Thr75 in striatal tissue lysate prepared from control and treated conditions as indicated. As shown in **Figure 5.5 A**, p-DARPP-32^{Thr34} was increased significantly upon NMDA treatment in comparison to control whereas, upon treatment with SSTR1 or 5 agonist the status of p-DARPP-32^{Thr34} was decreased in comparison to NMDA treatment and remained comparable to control. Interestingly, NMDA induced DARPP-32^{Thr34} phosphorylation was completely abolished in the presence of SSTR1 or 5 agonist. Conversely, p-DARPP-32^{Thr75} was decreased upon NMDA treatment whereas SSTR1 or 5 agonist enhanced DARPP-32^{Thr75} phosphorylation. Interestingly, SSTR1 and 5 agonist in combination with NMDA augmented p-DARPP-32^{Thr75} in comparison to NMDA treatment alone and expression level remains comparable to control. As illustrated in **Figure 5.5 A**, expression of DARPP-32 was not

changed upon NMDA treatment when compared to control; however, SSTR1 specific agonist L-797,591 treatment increased the expression of DARPP-32. On the other hand SSTR5 specific agonist L-817,818 alone exhibit increased expression of DARPP-32 and blocked NMDA induced inhibitory effect in combination.

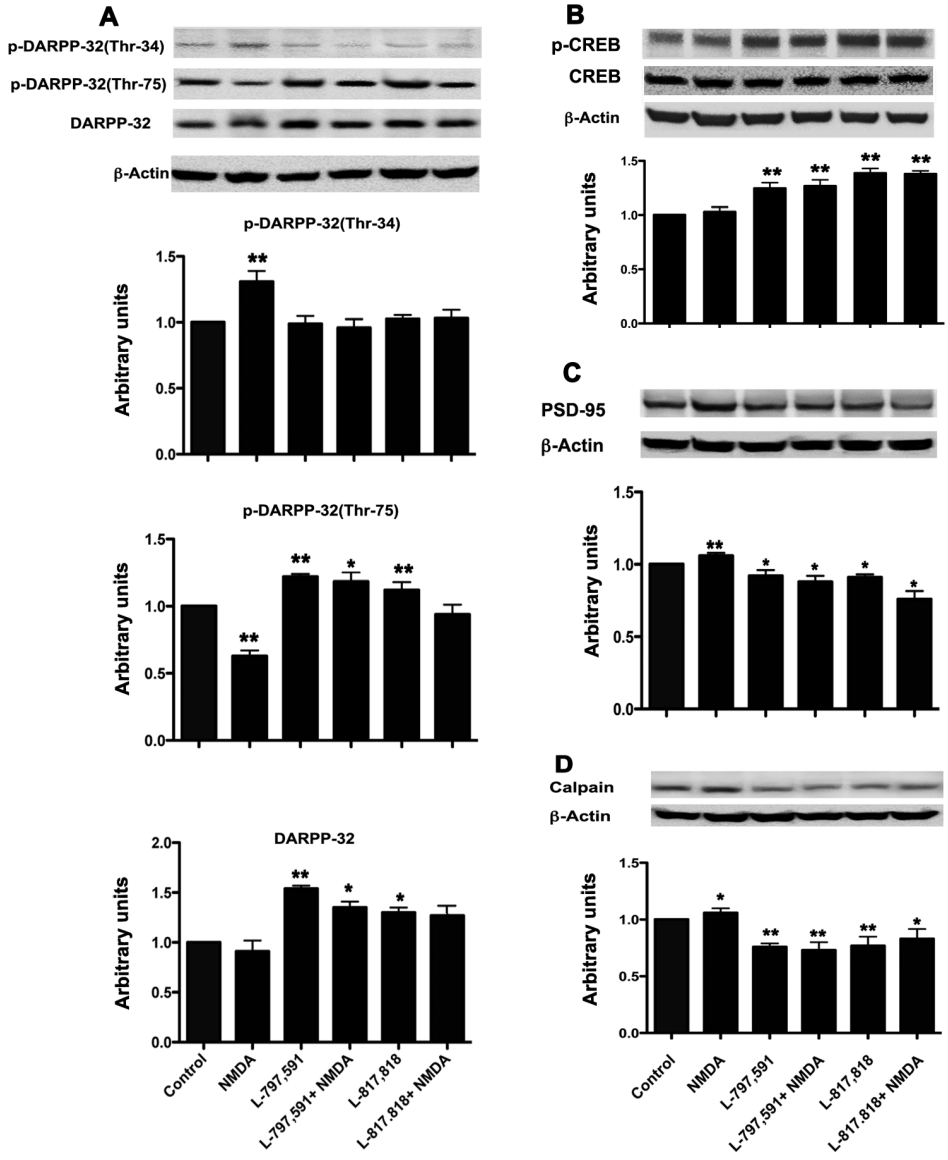

Figure 5.5 Western blot analysis showing changes in key proteins associated with NMDARs activity.

Tissue lysate prepared from striatal slices following treatment with NMDA or SSTR1 agonist or SSTR5 agonist alone or in combination were processed for Western blot analysis for phospho and total DARPP-32, CREB,

PSD-95 and calpain. The phosphorylation of DARPP-32^{Thr34} increased upon NMDA treatment and decreased upon SSTR1 and 5-specific agonist treatments. However, p-DARPP-32^{Thr75} decreased upon NMDA treatment and increased following SSTRs agonist-specific treatment. DARPP-32 expression was significantly increased upon SSTR1 and 5-specific treatment (**panel A**). Note enhanced phosphorylation of CREB upon SSTR1 and SSTR5 agonist treatments in combination or alone indicating neuronal survival (**panel B**). Decrease in Calpain **(panel C)** activity was also observed after SSTR1 and SSTR5 agonist treatments indicating decrease in NMDARs activity. PSD-95 **(panel D)** expression was decreased upon SSTR1 and SSTR5 agonist treatment indicating dissociation between NMDARs and PSD-95 complex. * < P 0.05.

5.3.8 Enhanced expression of CREB in the presence of SSTR1 and SSTR5 is an indication of synaptic NMDA receptor activation

Increased phosphorylation of DARPP-32 at Thr75 position leads to the phosphorylation of CREB, which is further involved in neuronal survival. Additionally, the status of p-CREB also distinguishes activation of synaptic or extrasynaptic NMDARs and decides the fate of neurons. Therefore, we next determined the effect of SSTR1 and 5 specific agonist on phosphorylation of CREB which is a key transcription factor involved in neuronal cell survival and regulating SST gene (Montminy and Bilezikjian, 1987). As shown in **Figure 5.5 B**, the status of phosphorylated CREB was comparable to control in presence of NMDA however significantly enhanced upon treatment with SSTR1 and 5-specific agonist alone or in combination with NMDA. These results indicate that SSTR1 and 5 may play a key role in neuronal cell survival.

5.3.9 Postsynaptic density protein PSD 95 is inhibited upon activation of somatostatin receptors

Studies have shown that PSD-95 binds with NMDARs and is involved in assembly of NMDARs at cell membrane and opening of NMDARs mediated calcium channels causing

Ca^{2+} overload and consequent neuronal death. To ascertain whether the changes in NMDARs complex formation and NMDA current are under the influence of PSD-95, we next determined the expression of PSD-95 in striatal slices. As shown in **Figure 5.5 C**, NMDA treatment shows significant increase in PSD-95 activity, however, L-797,591 and L-817,818 treatment results in decreased expression of PSD-95 when compared to NMDA treatment correlating to the expression of NMDARs as demonstrated in **Figure 5.2 A-C**. Importantly, PSD-95 expression levels were not changed when NMDA was used in combination with SSTRs agonist. These results suggest that SSTR1 and SSTR5 activation blocked NMDA induced PSD-95 expression in the striatum.

5.3.10 Inhibition of calpain is the indication of reduced Ca^{2+} influx upon SSTR activation

Having seen the significant changes in DARPP-32, PSD-95 and CREB, we next determined the expression of calpain, a protein linked to increased Ca^{2+} influx in response to NMDA induced neurotoxicity. As shown in **Figure 5.5 D**, calpain expression was increased in response to NMDA treatment in comparison to the control. SSTR1 and 5 specific agonist not only inhibit calpain expression but also abrogated NMDA mediated increased calpain expression in striatal tissue lysate.

5.3.11 SSTR 1 and SSTR 5 induced dissociation of NMDA receptor complex modulates NMDA induced signaling pathways

Direct implication and physiological significance of receptor heterodimerization although well established *in vitro* still awaits *in vivo* confirmation. To ascertain the underlying mechanism and functional consequences of the dissociation of NMDARs complex and the complex formation between NMDARs with SSTR1 or SSTR5 upon agonist

treatment we next determined the changes in downstream signaling pathways including PKC-α, PI3K and ERK1/2 by western blot analysis.

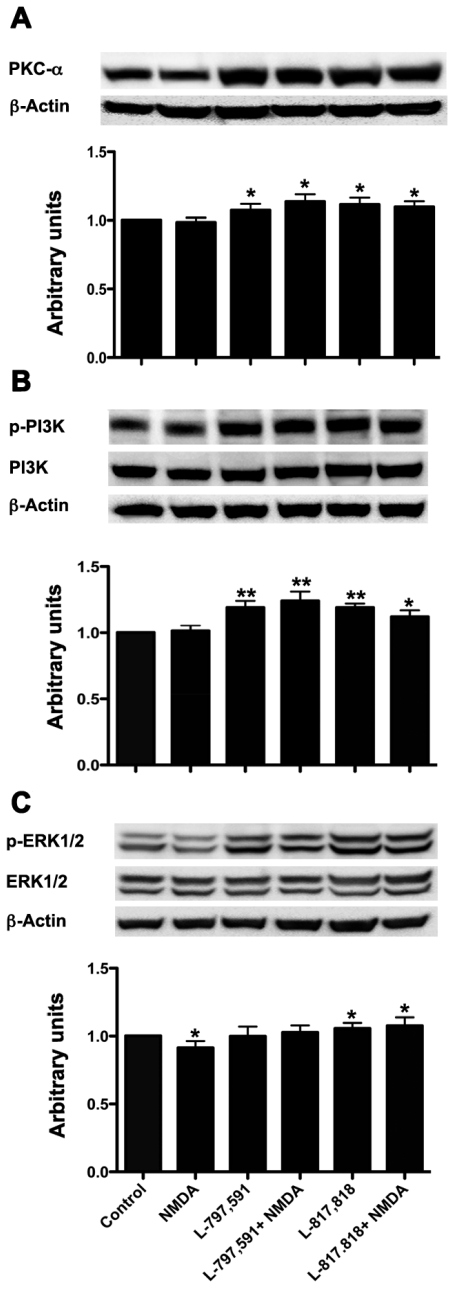

Figure 5.6 Representative immunoblots showing changes in PKC-α, PI3K and ERK1/2 in striatal slices. Striatal slices were treated with NMDA and SSTRs agonist alone or in combination as indicated for 30 min at 37°C. Tissue lysate prepared were processed for downstream signaling pathways including PKC-α, PI3K and

ERK1/2. SSTR1 and SSTR5 agonist treatment shows significant increase in PKC-α, PI3K cell survival pathway. The status of phosphorylated ERK1/2 signaling pathways was with out any discernable changes. * P< 0.05, ** P< 0.01.

As shown in **Figure 5.6 A**, insignificant decrease in expression of PKC-α was observed upon treatment with NMDA however, SSTR1 and 5 agonist in absence or presence of NMDA significantly increased PKC-α expression in comparison to basal and NMDA treatment. The phosphorylation of PI3K was not changed upon NMDA treatment significantly, but increased upon treatment with SSTR1 and 5 specific agonist alone and in combination with NMDA (**Figure 5.6 B**). ERK1/2 phosphorylation is associated with neuronal degeneration and NMDA mediated excitotoxicity. NMDA treatment decreased the p-ERK1/2 levels whereas, SSTR1 agonist in the presence or absence of NMDA resulted in enhanced ERK1/2 phosphorylation without any significant changes in comparison to control. Interestingly, significant increase in p-ERK1/2 was observed upon treatment with SSTR5 agonist alone or in combination with NMDA (**Figure 5.6 C**). These data strongly suggest that activation of SSTR1 and SSTR5 upon agonist treatment modulate NMDARs mediated signaling pathways.

5.4 Discussion

We recently described that mice lacking SSTR1/5 receptors exhibit neurochemical changes comparable to HD transgenic R6/2 mice with increased expression of NMDARs (Rajput et al., 2011b). Because, inhibition of NMDA receptors is a prerequisite in neuroprotection, these observations indicate that SSTR might exert a neuroprotective role in excitotoxicity. This speculation is further supported by our previous *in vitro* study showing that SST provides neuroprotection against QUIN/ NMDA induced neurotoxicity (Kumar, 2008). In excitotoxicity, over activation and increased NMDARs complex formation and

increased Ca^{2+} influx play determinant role in neuronal cell death and correlate with pathology of HD as well as many other neurological diseases (Chen and Lipton, 2006; Lipton, 2006). In the present study, we used striatal brain slices to delineate the effect of SSTR1 and 5 specific agonists with or without NMDA on NMDARs heterodimerization and signaling pathways involved in NMDA-induced excitotoxicity. We established that activation of SSTR1 or 5 diminished heteromeric complex between SSTR1 or SSTR5 with NR1 seen in control and NMDA treated striatal tissue lysate, decreases the expression of NMDARs and increases the expression of SSTR1 and SSTR5. Interestingly, agonist treatment not only triggers receptor or treatment specific in complex formation of NMDARs with SSTR1 or SSTR5 but also prompts the dissociation of NR1 with NR2A and NR2B in immunoprecipitation assay. Furthermore, significant decrease in NMDA currents in presence of SSTR agonist supports the role of SSTRs in NMDARs heterodimerization, dissociation of NMDARs complex and its implication in signaling associated with NMDA induced excitotoxicity. This is the first comprehensive description providing a mechanistic explanation in support of neuroprotective role of SST via its receptor through modulation of NMDARs functions.

Receptor specific colocalization in striatum and inhibition of NMDARs immunoreactivity in striatal tissue lysate tempted us to determine the complex formation between SSTR and NMDARs in striatal tissue lysate. The expression of NMDARs in SSTR1 and SSTR5 immunoprecipitate upon agonist treatment indicates dissociation of preformed NMDARs complex in basal or NMDA induced treatment. Selective and preferential functional complex formation between NMDARs is essential in excitotoxicity (Fan and Raymond, 2007; Lipton, 2006). Consistent with these observations, the lack of NR2A and

NR2B expression in NR1 immunoprecipitate upon treatment with SSTR1 and 5-specific agonist alone or with NMDA provides first evidence in support of SSTRs mediated neuroprotective role in excitotoxicity.

DARPP-32 phosphorylation at Thr34 and Thr75 decreases upon activation of NMDARs and AMPA receptors in Ca^{2+} dependent manner involving protein phosphatase-2B (PP2B) and protein phosphatase-2A (PP2A) respectively (Nishi et al., 2005). Our observations while consistent with Thr75 phosphorylation, contradict Thr34 phosphorylation upon NMDA treatment. This discrepancy may arise due to the concentration of NMDA and time of treatment. In previous studies, striatal slices were treated with 100μM NMDA for 5 min. whereas in present study striatal slices were treated for 30 min. with 300μM NMDA. Nishi et al., have also shown increased Thr34 phosphorylation at very early time point in response to NMDA treatment. In contrast mGluR enhances Thr34 phosphorylation in a cAMP dependent manner whereas, glutamate exerts rapid and transient Thr34 phosphorylation which was abolished by inhibitors of bNOS and guanylyl cyclase which support the concept that glutamate activates bNOS in SST and GABAergic positive neurons in striatum (Nishi et al., 2005). In contrast, we speculate that glutamate activated NMDARs also increase SST release that activates SSTRs and inhibits cAMP and PKA formation and consequently modulates DARPP-32^{Thr34} phosphorylation. Previously, it has been shown that activation of NMDARs attenuates the PKA inhibition via dephosphorylation of DARPP-32^{Thr75}(Ferraro et al., 1995; Nishi et al., 2000). DARPP-32^{Thr75} phosphorylation is regulated through the increased NMDARs mediated Ca^{2+} influx. In agreement with these, we show decrease in DARPP-32^{Thr75} phosphorylation upon NMDA treatment. Furthermore, the presence of SSTR1 and 5-specific agonist not only enhanced phosphorylation of DARPP-

32^{Thr75} but also diminished NMDA induced inhibition of DARPP-32. Previous studies have reported decrease in DARPP-32 expression in striatum of chemically induced excitotoxicity models as well as HD transgenic mouse models (Bibb et al., 2000; Rajput et al., 2011b; Torres-Peraza et al., 2007). Meanwhile increased DARPP-32 expression in presence of SSTR1 and 5-specific agonist without any significant difference in presence or absence of NMDA indicates major contribution of SSTRs in DARPP-32 signaling. It has been previously proposed that activation of D1R, which activates cAMP, enhanced NMDA function involving cAMP, PKA and DARPP-32. Accordingly, the inhibition of cAMP upon SSTR activation a well-established role of SSTR subtypes, can consequently inhibit NMDARs function.

The neuroprotective role of increased p-CREB is well established in NMDA induced excitotoxicity as well as in HD or other neurological disorders (Lonze and Ginty, 2002; Lonze et al., 2002). Interestingly, SST positive interneurons display increased CREB levels in QUIN induced lesioned animals in comparison to parvalbumin and calretinin positive interneurons as well as projection neurons (Giampa et al., 2006). As discussed previously, NMDA-mediated toxicity results in increased SST mRNA expression, interestingly; studies have also shown that increased CREB activity results in upregulation of SST synthesis via modulation of transcription factors (Montminy and Bilezikjian, 1987). In agreement with the information, we provide evidence that upon NMDA treatment no significant changes were observed in phosphorylated CREB however, SSTR1 and 5-specific agonist treatment resulted in elevated phosphorylated CREB levels.

Previous studies have shown that PSD-95 enhances NMDARs clustering at synapses and inhibits NR2-mediated internalization and reduced PSD-95 that inhibits NMDA

receptor-mediated excitotoxicity (Kim et al., 1996; Lavezzari et al., 2004; Roche et al., 2001; Sattler et al., 1999). Suppressed PSD-95 expression upon treatment with SSTR1 and 5-specific agonist also prompt the dissociation NMDARs receptor heteromeric complex as PSD-95 play crucial role in anchoring NMDARs at cell surface. Taken together, our results anticipate that activated SSTR1 and 5 modulate PSD-95 mediated changes in NMDA receptors functions. Increased Ca^{2+} influx upon stimulation of NMDARs leads to the activation of calcium-dependent calpain and influence diverse cellular function including excitotoxic neuronal death and neurodegeneration (Abe and Takeichi, 2007; Bano et al., 2005; Gafni and Ellerby, 2002; Saito et al., 1993; Xu et al., 2007). In contrast, inhibition of calpain activity in NMDA mediated excitotoxicity leads to neuronal survival (Nimmrich et al., 2008). Consistent with these observations, we show an increased expression of calpain in presence of NMDA, which was abrogated upon SSTR1 and 5-specific treatment. PKC-α plays a key role in cell swelling and neuronal death due to activation of Ca^{2+} influx and also enhances NMDARs mediated NMDA currents (Bickler et al., 2004; Liao et al., 2001; Wagey et al., 2001). Moreover, studies have also shown decreased expression of PKC in NMDA induced toxicity and HD with its implication in long-term potentiation and effects on synaptic plasticity and learning (Harris et al., 2001). SSTR1 and 5-specific agonist treatment reveals increased expression of PKC-α indicating survival of neurons and inhibition Ca^{2+} influx. The presence of NMDARs at different locations has been associated with neuroprotection or neurodegeneration upon activation of NMDARs. Supporting the neuroprotective role of synaptic NMDARs is the elevated status of phosphorylated PI3K and ERK1/2 in association of enhanced CREB phosphorylation. Consistent with these observation, we show that SSTR1 and 5-specific agonist treatment enhanced CREB, PI3K

and ERK1/2 phosphorylation.

NMDA receptor are expressed in large neuronal population of striatum and over activation of NMDARs is involved in pathogenesis of several neurodegenerative disorders. Colocalization of NMDARs with SSTRs, dissociation of NMDA receptor complex upon SSTR activation and modulation of associated proteins and signaling pathways opens a new avenue in the possible intervention of NMDA-mediated excitotoxicity without changing physiological expression of NMDARs. NMDARs activation, enhanced Ca^{2+} influx, PSD-95 and Calpain along with decreased CREB and DARPP-32 phosphorylation at Thr 34 or 75 are associated with NMDA induced excitotoxicity and our data shows SSTR subtype activating avert these changes. Although, results of our study indicate inhibition of extrasynaptic NMDA receptors and activation at synaptic NMDA receptors however, further studies are warranted to draw any conclusive decision in this direction.

Chapter 6: Overall general discussion and significance

Neurodegeneration, in NMDARs agonist induced excitotoxicity in striatum is exactly similar to HD that is associated with severe atrophy of the neostriatum with marked neuronal loss and gliosis (Coyle and Puttfarcken, 1993; Kumar, 2004). In striatum, large neuronal population is composed of medium sized projection neurons which make up >80% of all striatal neurons and are selectively vulnerable in excitotoxicity (Torres-Peraza et al., 2007). A subclass of medium sized aspiny neurons also known as interneurons that coproduce SST/NPY/bNOS are selectively spared in excitotoxicity as well as in HD (Beal, 1990; Kumar, 2004; Patel et al., 1991). Interestingly, in addition to selective sparing of SST positive neurons, striatal concentrations of SST and NPY are not simply maintained but actually increased for SST in HD brain as well as in a model of excitotoxicity induced by NMDARs agonist QUIN (Beal et al., 1984; Patel et al., 1991; Patel et al., 1995b). Multifunctional nature and widespread distribution of SST in different regions of the CNS has led to many studies correlating SST to the pathophysiology of several neurodegenerative diseases (Beal, 1990; Patel, 1999). However, there are many other questions to answer regarding the physiological function of SST in the brain. SST and its analogs, octreotide and newly identified cortistatin have been shown to be neuroprotective in ischemic brain damage, NMDA/kainate-induced excitotoxicity in cortical neurons, as well as during seizures and epileptogenesis (Braun et al., 1998; Rauca et al., 1999; Tallent and Qiu, 2008). NMDA/QUIN induced excitotoxicity is due to the activation of NMDARs associated Ca^{2+} channels leading to overload cytosolic Ca^{2+} and resulting in severe neuronal death (McBain and Mayer, 1994; Waxman and Lynch, 2005).

The finding that all striatal and a major subset of cortical SST neurons possess a specialized phenotype with co-expression of NADPH-d and NPY those are selectively resistant in clinical and experimental models of neurodegeneration. In support, it was suggested that these neurons survive due to the lack of NMDA receptors and presence of NADPH-d (Koh et al., 1986). Soon after this theory was challenged and it was noticed that SST/NPY/NOS positive neurons are not lacking NMDA receptors but rather had low expression in comparison to surrounding projection neurons (Augood et al., 1994; Chen et al., 1996; Kumar et al., 1997a). SST-14 added to the incubation medium with QUIN or NMDA rescued the neurons virtually completely from excitotoxic death. These results clearly indicate a potent neuroprotective role of SST on all striatal neurons including SST producing NADPH-d positive neurons. The preferential localization of Mn-superoxide dismutase (Mn-SOD) in SST positive striatal neurons indicates another mechanism for protecting these cells against toxic free radicals (Kumar, 2004). Furthermore, direct evidence in support of neuroprotective role for SST emerged from recent studies showing that immunoneutralization of released SST by using SST antibodies and knocking down SST using antisense oligonucleotides lead to 95% of neuronal death including NADPH-d neurons which are selectively spared in QUIN and NMDA induced neurotoxicity (Kumar, 2008).

SST via binding to five different SSTR subtypes executes its biological functions. SSTR subtypes are well expressed in various regions of CNS and are involved in various neurological diseases. However, which SSTR subtype is associated with SST mediated neuroprotective role in excitotoxicity is largely elusive and poorly understood. Accordingly, the characterization of SSTR subtype distribution in MSNs and interneurons was critical to determine the population of neurons, which could be vulnerable to neurodegeneration in

excitotoxicity and pathophysiology of several neurological disorders, specifically in HD. One of the major proteins associated with MSNs in striatum is DARPP-32, which is expressed in >80% of MSNs. Because these neurons are most vulnerable in QUIN/NMDA induced excitotoxicity, accordingly, DARPP-32 has been used as a marker of MSNs. However, a review of the literature showed no functional and physiological correlation between DARP-32 and SSTRs in CNS. In contrast, DARPP-32 has been extensively characterized with DRs and studies have shown that DRs modulate the phosphorylation of DARPP-32 at various threonine sites (Greengard et al., 1999; Matsuyama et al., 2003; Nishi et al., 2000). Interestingly, previous studies have shown that SSTRs and DRs share 30% sequence homology and are known to form active functional heterodimers (Baragli et al., 2007; Rocheville et al., 2000a). Taking these facts in consideration, the first colocalization study was accomplished between DRs and DARPP-32 in different brain regions including cortex and striatum to confirm if DARPP-32 can be used as marker for differentiating projection and interneurons with respect to characterizing DRs member of GPCR family **(Chapter 2)**.

The distributional pattern of colocalization between DARPP-32 with DR subtypes reveals that all five DR subtypes and DARPP-32 were expressed in rat brain cortex and striatum. DARPP-32 positive neurons displayed comparative colocalization with DR1-5. D1R is one of the most predominant subtypes that colocalized with DARPP-32 in cortex as well as striatum and followed by D2R, D3R, D4R and D5R. Amongst all DR subtypes D5R, was the least coexpressed with DARPP-32 positive neurons. Importantly, these data also reveal that the same neurons in striatum or cortex might contain more than one or two DR subtypes which also might express DARPP-32. This study further confirmed the approach to

use DARPP-32 as a marker for MSNs and validated the use of technique for characterization of other GPCRs in different neuronal sub population.

To ascertain differential pattern of expression of SSTR in projection neurons we further determined the colocalization of SST and SSTRs with DARPP-32 in rat brain cortical and striatal regions using immunofluorescence immunohistochemistry **(Chapter 3)**. SST positive neurons in cortex and striatum are devoid of colocalization with DARPP-32. These data suggest that SST is not synthesized in projection neurons; rather this peptide is an integral part of interneurons. However, in cortical and striatal brain regions three different neuronal populations either expressing SSTRs and DARPP-32 alone or displaying colocalization were identified. Quantitative analysis revealed that in cortex and striatum, SSTR1 and 5 are the most predominant receptor subtypes colocalized with DARPP-32 followed by SSTR4, 2 and 3 in cortex whereas, SSTR2, 4 and 3 in striatum. SSTRs positive neurons lacking colocalization with DARPP-32 might be preserved in the pathophysiology of HD and QUIN/NMDA induced neurotoxicity. Conversely, SSTRs displaying colocalization with DARPP-32 might be susceptible in excitotoxicity as well as in HD. These results demonstrate that SSTR1 and SSTR5 receptor subtypes colocalize predominantly with DARPP-32 in striatum suggesting these receptor subtypes might play a crucial role in phosphorylation of DARPP-32 and modulate the DARPP-32 mediated downstream pathways involved in excitotoxicity.

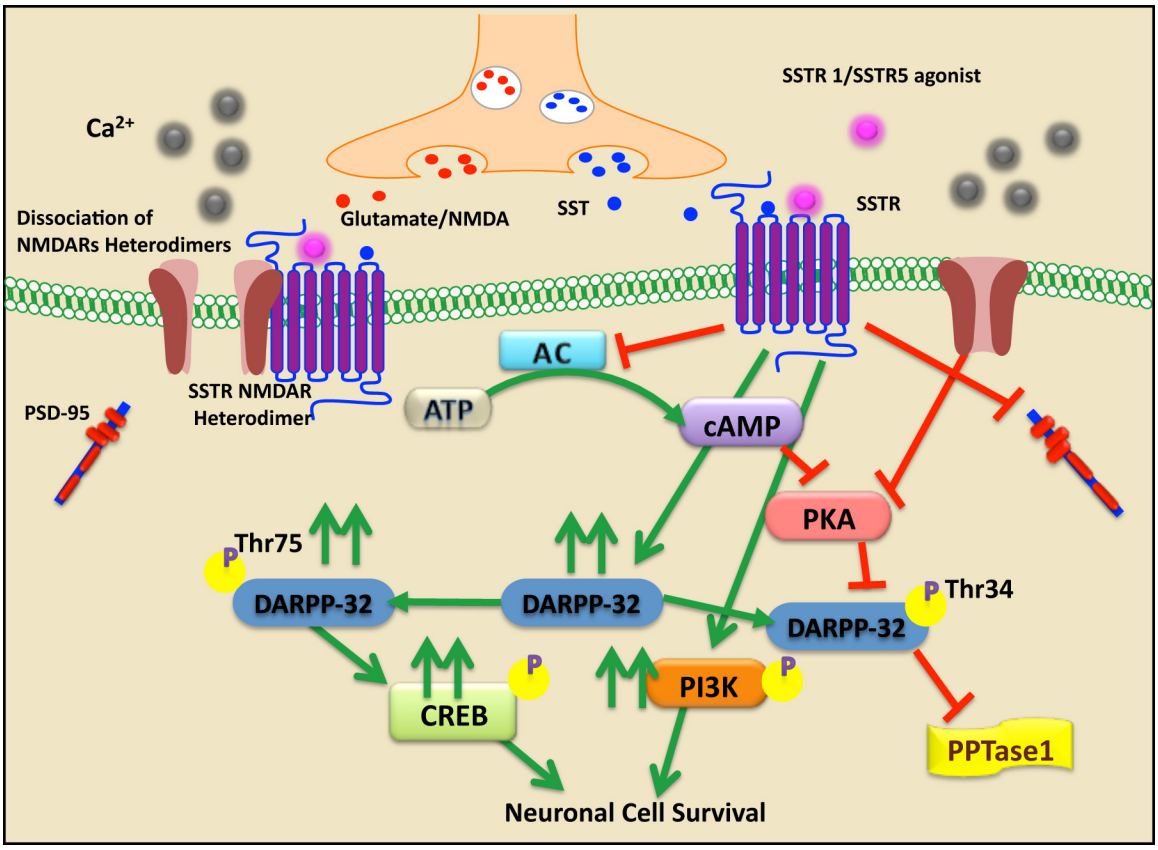

Figure 6.1 Schematic illustration showing SSTR mediated signaling involved in neuronal cell survival.

To provide direct evidence for the physiological role of SSTR1 and SSTR5 we next delineated receptor subtype selective neurochemical changes in SSTR1/5$^{-/-}$ mice and compared with HD transgenic R6/2 mice in striatum **(Chapter 4)**. Our study revealed for the first time significant loss of DARPP-32 and comparable changes in SST, calbindin and bNOS expression. A similar pattern of changes in the expression of NMDARs was observed in both R6/2 and SSTR1/5$^{-/-}$. Increased expression of both SSTR1 and SSTR5 in R6/2 mice indicates that these receptors upregulate in HD. Interestingly, increased SSTR2 like immunoreactivity in both R6/2 and SSTR1/5$^{-/-}$ mice indicate possible mechanism to decrease the Ca^{2+} overload caused due to activated NMDARs. Changes in key signaling proteins including calpain, phospho-ERK1/2, synapsin-IIa, protein kinase C-α and calcineurin in

SSTR1/5$^{-/-}$ and R6/2 mice strongly indicate the key neuroprotective role played by SSTR1 and SSTR5 in pathophysiology of HD. SSTR1/5 appears to be important in regulating NMDARs, DARPP-32 and signaling molecules in similar fashion as seen in HD transgenic mice.

Ablation of SSTR1 and SSTR5 resulted in similar neurochemical changes as seen in HD transgenic models suggesting that SSTR1 and SSTR5 govern crucial role in HD as well as in excitotoxicity. Based on our previous observations from SSTR1/5$^{-/-}$ mice we next extended our study to determine the role of SSTR1 and 5 on NMDARs complex formation and its consequences on downstream signaling pathways in striatal brain slices using morphological, electrophysiological and biochemical analysis **(Chapter 5)**. This study using striatal brain slices, revealed selective and preferential colocalization between SSTR1 and SSTR5 with NR1, NR2A and 2B. These results indicate a receptors specific colocalization pattern and possibility of functional interaction. Interestingly, while NR1 exists in a complex with NR2A and 2B in striatal tissue lysate, treatment with a SSTR specific agonist leads to the dissociation of this complex and consequently leads to the complex formation between SSTR and NMDARs. Treatment with SSTR agonists further decreases the NMDA currents in striatal brain slices indicating that SSTRs might be interrupting the NMDARs heteromeric complex and thereby inhibiting the excitotoxic effects of NMDARs. Furthermore, striatal brain sections treated with NMDA, L-797,591 and L-817,818 were used to determine the changes in receptor expression. Significant decrease in NMDARs receptor expression upon SSTR agonist treatment was in correlation with the changes in calpain and PSD-95, which further enhances the NMDARs, mediated excitotoxicity in neurons. Taken together the data

reveals that SSTR1 and SSTR 5 could play an important neuroprotective role in NMDA-induced excitotoxicity.

Chapter 7: Conclusion

In this thesis, I have characterized the possible association of SSTR subtypes with different proteins that are known to exert crucial and determinant role in excitotoxicity and further correlated closely with neurological disease such as HD. The specific emphasis is on HD, because NMDARs activation mimics the neurochemical phenotype of HD and SST via different SSTRs prevents neuronal loss in NMDA induced neurotoxicity. Furthermore, observations described in second and third chapter elucidate the characterization of SSTR and DRs with DARPP-32. Importantly, characterization of SSTR subtypes revealed that SSTR positive neurons devoid of DARPP-32 might escape in excitotoxicity as well as in pathology of HD. In support of this, results presented in chapter four provide further direct evidence that SSTR1/5$^{-/-}$ displayed exactly similar neurochemical phenotype and changes in signaling pathways linked to neuronal loss in HD transgenic mice R6/2. The last chapter of this thesis described direct evidence for the role of SSTR1 and 5 in modulation of NMDARs function. Most importantly, dissociation of NMDARs heteromeric complex formation upon activation SSTR1 and 5 in rat brain slices provide first evidence that SSTR might serves as potential neuroprotective receptor target in excitotoxicity and pathophysiology of HD. Although, the mechanistic profile for SSTR subtypes need to be addressed the activation of SSTR with partial inhibition of NMDARs may open an avenue for the development of new drugs for the treatment of neurological diseases which are partly attributed to over-activation of NMDA receptor induced Ca^{2+} influx and oxidative stress.

In many of the neurological diseases, excitotoxicity is one of the major contributing factors in neurodegeneration and involve activation of NMDARs. Neuroprotective approaches are largely elusive, poorly understood and utmost need in neurological disorder.

Although, the clinical implication of SSTRs is highly possible numerous difficulties need to overcome to develop effective drugs. The observations described in this thesis speculate a chimeric molecule as novel drugs in combination of SSTR activation and subsequent blockade of NMDARs. Such new drugs if developed will help not only a potential treatment of HD but also in many neurological disorders associated with abnormal function of NMDARs.

References

Abe, K., and Takeichi, M. (2007). NMDA-receptor activation induces calpain-mediated beta-catenin cleavages for triggering gene expression. Neuron *53*, 387-397.

Agnati, L. F., Ferre, S., Lluis, C., Franco, R., and Fuxe, K. (2003). Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with examples from the striatopallidal GABA neurons. Pharmacol Rev *55*, 509-550.

Aguado-Llera, D., Puebla-Jimenez, L., Yebenes-Gregorio, L., and Arilla-Ferreiro, E. (2007). Alteration of the somatostatinergic system in the striatum of rats with acute experimental autoimmune encephalomyelitis. Neuroscience *148*, 238-249.

Aguila, M. C. (1994). Growth hormone-releasing factor increases somatostatin release and mRNA levels in the rat periventricular nucleus via nitric oxide by activation of guanylate cyclase. Proc Natl Acad Sci U S A *91*, 782-786.

Aizman, O., Brismar, H., Uhlen, P., Zettergren, E., Levey, A. I., Forssberg, H., Greengard, P., and Aperia, A. (2000). Anatomical and physiological evidence for D1 and D2 dopamine receptor colocalization in neostriatal neurons. Nat Neurosci *3*, 226-230.

Albin, R. L., Young, A. B., Penney, J. B., Handelin, B., Balfour, R., Anderson, K. D., Markel, D. S., Tourtellotte, W. W., and Reiner, A. (1990). Abnormalities of striatal projection neurons and N-methyl-D-aspartate receptors in presymptomatic Huntington's disease. N Engl J Med *322*, 1293-1298.

Ali, N. J., and Levine, M. S. (2006). Changes in expression of N-methyl-D-aspartate receptor subunits occur early in the R6/2 mouse model of Huntington's disease. Dev Neurosci *28*, 230-238.

Allen, J. P., Hathway, G. J., Clarke, N. J., Jowett, M. I., Topps, S., Kendrick, K. M., Humphrey, P. P., Wilkinson, L. S., and Emson, P. C. (2003). Somatostatin receptor 2 knockout/lacZ knockin mice show impaired motor coordination and reveal sites of somatostatin action within the striatum. Eur J Neurosci *17*, 1881-1895.

Amara, S. G., Sonders, M. S., Zahniser, N. R., Povlock, S. L., and Daniels, G. M. (1998). Molecular physiology and regulation of catecholamine transporters. Adv Pharmacol *42*, 164-168.

Andersson, M., Usiello, A., Borgkvist, A., Pozzi, L., Dominguez, C., Fienberg, A. A., Svenningsson, P., Fredholm, B. B., Borrelli, E., Greengard, P., and Fisone, G. (2005). Cannabinoid action depends on phosphorylation of dopamine- and cAMP-regulated phosphoprotein of 32 kDa at the protein kinase A site in striatal projection neurons. J Neurosci *25*, 8432-8438.

Arancibia, S., Epelbaum, J., Boyer, R., and Assenmacher, I. (1984). In vivo release of somatostatin from rat median eminence after local K+ infusion or delivery of nociceptive stress. Neurosci Lett *50*, 97-102.

Ariano, M. A., Wang, J., Noblett, K. L., Larson, E. R., and Sibley, D. R. (1997). Cellular distribution of the rat D1B receptor in central nervous system using anti-receptor antisera. Brain Res *746*, 141-150.

Aronin, N., Cooper, P. E., Lorenz, L. J., Bird, E. D., Sagar, S. M., Leeman, S. E., and Martin, J. B. (1983). Somatostatin is increased in the basal ganglia in Huntington disease. Ann Neurol *13*, 519-526.

Augood, S. J., McGowan, E. M., and Emson, P. C. (1994). Expression of N-methyl-D-aspartate receptor subunit NR1 messenger RNA by identified striatal somatostatin cells. Neuroscience *59*, 7-12.

Ballian, N., Brunicardi, F. C., and Wang, X. P. (2006). Somatostatin and its receptors in the development of the endocrine pancreas. Pancreas *33*, 1-12.

Banki, C. M., Karmacsi, L., Bissette, G., and Nemeroff, C. B. (1992a). CSF corticotropin releasing hormone, somatostatin, and thyrotropin releasing hormone in schizophrenia. Psychiatry Res *43*, 13-21.

Banki, C. M., Karmacsi, L., Bissette, G., and Nemeroff, C. B. (1992b). CSF corticotropin-releasing hormone and somatostatin in major depression: response to antidepressant treatment and relapse. Eur Neuropsychopharmacol *2*, 107-113.

Bano, D., Young, K. W., Guerin, C. J., Lefeuvre, R., Rothwell, N. J., Naldini, L., Rizzuto, R., Carafoli, E., and Nicotera, P. (2005). Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. Cell *120*, 275-285.

Baragli, A., Alturaihi, H., Watt, H. L., Abdallah, A., and Kumar, U. (2007). Heterooligomerization of human dopamine receptor 2 and somatostatin receptor 2 Co-immunoprecipitation and fluorescence resonance energy transfer analysis. Cell Signal *19*, 2304-2316.

Barinaga, M., Bilezikjian, L. M., Vale, W. W., Rosenfeld, M. G., and Evans, R. M. (1985). Independent effects of growth hormone releasing factor on growth hormone release and gene transcription. Nature *314*, 279-281.

Barnea, A., Roberts, J., and Ho, R. H. (1999). Evidence for a synergistic effect of the HIV-1 envelope protein gp120 and brain-derived neurotrophic factor (BDNF) leading to enhanced expression of somatostatin neurons in aggregate cultures derived from the human fetal cortex. Brain Res *815*, 349-357.

Bastia, E., and Schwarzschild, M. A. (2003). DARPP chocolate: a caffeinated morsel of striatal signaling. Sci STKE *2003*, PE2.

Beal, M. F. (1990). Somatostatin in neurodegenerative illnesses. Metabolism *39*, 116-119.

Beal, M. F., Bird, E. D., Langlais, P. J., and Martin, J. B. (1984). Somatostatin is increased in the nucleus accumbens in Huntington's disease. Neurology *34*, 663-666.

Beal, M. F., Brouillet, E., Jenkins, B. G., Ferrante, R. J., Kowall, N. W., Miller, J. M., Storey, E., Srivastava, R., Rosen, B. R., and Hyman, B. T. (1993). Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. J Neurosci *13*, 4181-4192.

Beal, M. F., Clevens, R. A., and Mazurek, M. F. (1988). Somatostatin and neuropeptide Y immunoreactivity in Parkinson's disease dementia with Alzheimer's changes. Synapse *2*, 463-467.

Beal, M. F., Ferrante, R. J., Swartz, K. J., and Kowall, N. W. (1991). Chronic quinolinic acid lesions in rats closely resemble Huntington's disease. J Neurosci *11*, 1649-1659.

Beal, M. F., Kowall, N. W., Ellison, D. W., Mazurek, M. F., Swartz, K. J., and Martin, J. B. (1986). Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. Nature *321*, 168-171.

Beal, M. F., Mazurek, M. F., Tran, V. T., Chattha, G., Bird, E. D., and Martin, J. B. (1985). Reduced numbers of somatostatin receptors in the cerebral cortex in Alzheimer's disease. Science *229*, 289-291.

Beaudet, A., Greenspun, D., Raelson, J., and Tannenbaum, G. S. (1995). Patterns of expression of SSTR1 and SSTR2 somatostatin receptor subtypes in the hypothalamus of the adult rat: relationship to neuroendocrine function. Neuroscience *65*, 551-561.

Berelowitz, M., Kronheim, S., Pimstone, B., and Sheppard, M. (1978). Potassium stimulated calcium dependent release of immunoreactive somatostatin from incubated rat hypothalamus. J Neurochem *31*, 1537-1539.

Bertran-Gonzalez, J., Bosch, C., Maroteaux, M., Matamales, M., Herve, D., Valjent, E., and Girault, J. A. (2008). Opposing patterns of signaling activation in dopamine D1 and D2 receptor-expressing striatal neurons in response to cocaine and haloperidol. J Neurosci *28*, 5671-5685.

Bibb, J. A., Yan, Z., Svenningsson, P., Snyder, G. L., Pieribone, V. A., Horiuchi, A., Nairn, A. C., Messer, A., and Greengard, P. (2000). Severe deficiencies in dopamine signaling in presymptomatic Huntington's disease mice. Proc Natl Acad Sci U S A *97*, 6809-6814.

Bickler, P. E., Fahlman, C. S., and Ferriero, D. M. (2004). Hypoxia increases calcium flux through cortical neuron glutamate receptors via protein kinase C. J Neurochem *88*, 878-884.

Billova, S., Galanopoulou, A. S., Seidah, N. G., Qiu, X., and Kumar, U. (2007). Immunohistochemical expression and colocalization of somatostatin, carboxypeptidase-E and prohormone convertases 1 and 2 in rat brain. Neuroscience *147*, 403-418.

Binaschi, A., Bregola, G., and Simonato, M. (2003). On the role of somatostatin in seizure control: clues from the hippocampus. Rev Neurosci *14*, 285-301.

Bissette, G., Widerlov, E., Walleus, H., Karlsson, I., Eklund, K., Forsman, A., and Nemeroff, C. B. (1986). Alterations in cerebrospinal fluid concentrations of somatostatinlike immunoreactivity in neuropsychiatric disorders. Arch Gen Psychiatry *43*, 1148-1151.

Blank, T., Nijholt, I., Teichert, U., Kugler, H., Behrsing, H., Fienberg, A., Greengard, P., and Spiess, J. (1997). The phosphoprotein DARPP-32 mediates cAMP-dependent potentiation of striatal N-methyl-D-aspartate responses. Proc Natl Acad Sci U S A *94*, 14859-14864.

Borgkvist, A., Marcellino, D., Fuxe, K., Greengard, P., and Fisone, G. (2008). Regulation of DARPP-32 phosphorylation by Delta9-tetrahydrocannabinol. Neuropharmacology *54*, 31-35.

Borgkvist, A., Usiello, A., Greengard, P., and Fisone, G. (2007). Activation of the cAMP/PKA/DARPP-32 signaling pathway is required for morphine psychomotor stimulation but not for morphine reward. Neuropsychopharmacology *32*, 1995-2003.

Bouthenet, M. L., Souil, E., Martres, M. P., Sokoloff, P., Giros, B., and Schwartz, J. C. (1991). Localization of dopamine D3 receptor mRNA in the rat brain using in situ hybridization histochemistry: comparison with dopamine D2 receptor mRNA. Brain Res *564*, 203-219.

Brakch, N., Galanopoulou, A. S., Patel, Y. C., Boileau, G., and Seidah, N. G. (1995). Comparative proteolytic processing of rat prosomatostatin by the convertases PC1, PC2, furin, PACE4 and PC5 in constitutive and regulated secretory pathways. FEBS Lett *362*, 143-146.

Braun, H., Schulz, S., Becker, A., Schroder, H., and Hollt, V. (1998). Protective effects of cortistatin (CST-14) against kainate-induced neurotoxicity in rat brain. Brain Res *803*, 54-60.

Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., and Guillemin, R. (1973). Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science *179*, 77-79.

Bresson, J. L., Clavequin, M. C., Fellmann, D., and Bugnon, C. (1984). Ontogeny of the neuroglandular system revealed with HPGRF 44 antibodies in human hypothalamus. Neuroendocrinology *39*, 68-73.

Brinkman, R. R., Mezei, M. M., Theilmann, J., Almqvist, E., and Hayden, M. R. (1997). The likelihood of being affected with Huntington disease by a particular age, for a specific CAG size. Am J Hum Genet *60*, 1202-1210.

Brouillet, E., Guyot, M. C., Mittoux, V., Altairac, S., Conde, F., Palfi, S., and Hantraye, P. (1998). Partial inhibition of brain succinate dehydrogenase by 3-nitropropionic acid is sufficient to initiate striatal degeneration in rat. J Neurochem *70*, 794-805.

Brownstein, M., Arimura, A., Sato, H., Schally, A. V., and Kizer, J. S. (1975). The regional distribution of somatostatin in the rat brain. Endocrinology *96*, 1456-1461.

Bugnon, C., Fellmann, D., and Bloch, B. (1978). Immunocytochemical study of the ontogenesis of the hypothalamic somatostatin-containing neurons in the human fetus. Metabolism *27*, 1161-1165.

Bunzow, J. R., Van Tol, H. H., Grandy, D. K., Albert, P., Salon, J., Christie, M., Machida, C. A., Neve, K. A., and Civelli, O. (1988). Cloning and expression of a rat D2 dopamine receptor cDNA. Nature *336*, 783-787.

Buscail, L., Vernejoul, F., Faure, P., Torrisani, J., and Susini, C. (2002). Regulation of cell proliferation by somatostatin. Ann Endocrinol (Paris) *63*, 2S13 - 12S18.

Cepeda, C., Ariano, M. A., Calvert, C. R., Flores-Hernandez, J., Chandler, S. H., Leavitt, B. R., Hayden, M. R., and Levine, M. S. (2001). NMDA receptor function in mouse models of Huntington disease. J Neurosci Res *66*, 525-539.

Cervera, P., Videau, C., Viollet, C., Petrucci, C., Lacombe, J., Winsky-Sommerer, R., Csaba, Z., Helboe, L., Daumas-Duport, C., Reubi, J. C., and Epelbaum, J. (2002). Comparison of somatostatin receptor expression in human gliomas and medulloblastomas. J Neuroendocrinol *14*, 458-471.

Cervia, D., and Bagnoli, P. (2007). An update on somatostatin receptor signaling in native systems and new insights on their pathophysiology. Pharmacol Ther *116*, 322-341.

Chen, H. S., and Lipton, S. A. (2006). The chemical biology of clinically tolerated NMDA receptor antagonists. J Neurochem *97*, 1611-1626.

Chen, N., Luo, T., Wellington, C., Metzler, M., McCutcheon, K., Hayden, M. R., and Raymond, L. A. (1999). Subtype-specific enhancement of NMDA receptor currents by mutant huntingtin. J Neurochem *72*, 1890-1898.

Chen, Q., Veenman, C. L., and Reiner, A. (1996). Cellular expression of ionotropic glutamate receptor subunits on specific striatal neuron types and its implication for striatal vulnerability in glutamate receptor-mediated excitotoxicity. Neuroscience *73*, 715-731.

Chihara, K., Arimura, A., Kubli-Garfias, C., and Schally, A. V. (1979a). Enhancement of immunoreactive somatostatin release into hypophysial portal blood by electrical stimulation of the preoptic area in the rat. Endocrinology *105*, 1416-1418.

Chihara, K., Arimura, A., and Schally, A. V. (1979b). Effect of intraventricular injection of dopamine, noreprinephrine, acetylcholine, and 5-hydroxytryptamine on immunoreactive somatostatin release into rat hypophyseal portal blood. Endocrinology *104*, 1656-1662.

Choi, D. W., and Rothman, S. M. (1990). The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu Rev Neurosci *13*, 171-182.

Christie, J. M., Jane, D. E., and Monaghan, D. T. (2000). Native N-methyl-D-aspartate receptors containing NR2A and NR2B subunits have pharmacologically distinct competitive antagonist binding sites. J Pharmacol Exp Ther *292*, 1169-1174.

Cicchetti, F., Gould, P. V., and Parent, A. (1996). Sparing of striatal neurons coexpressing calretinin and substance P (NK1) receptor in Huntington's disease. Brain Res *730*, 232-237.

Cicchetti, F., and Parent, A. (1996). Striatal interneurons in Huntington's disease: selective increase in the density of calretinin-immunoreactive medium-sized neurons. Mov Disord *11*, 619-626.

Compston, A., and Coles, A. (2002). Multiple sclerosis. Lancet *359*, 1221-1231.

Cowan, C. M., Fan, M. M., Fan, J., Shehadeh, J., Zhang, L. Y., Graham, R. K., Hayden, M. R., and Raymond, L. A. (2008). Polyglutamine-modulated striatal calpain activity in YAC transgenic huntington disease mouse model: impact on NMDA receptor function and toxicity. J Neurosci *28*, 12725-12735.

Coyle, J. T., and Puttfarcken, P. (1993). Oxidative stress, glutamate, and neurodegenerative disorders. Science *262*, 689-695.

Coyle, J. T., and Schwarcz, R. (1976). Lesion of striatal neurones with kainic acid provides a model for Huntington's chorea. Nature *263*, 244-246.

Csaba, Z., and Dournaud, P. (2001). Cellular biology of somatostatin receptors. Neuropeptides *35*, 1-23.

Csaba, Z., Pirker, S., Lelouvier, B., Simon, A., Videau, C., Epelbaum, J., Czech, T., Baumgartner, C., Sperk, G., and Dournaud, P. (2005). Somatostatin receptor type 2 undergoes plastic changes in the human epileptic dentate gyrus. J Neuropathol Exp Neurol *64*, 956-969.

Csillag, A., Balint, E., Adam, A., and Zachar, G. (2008). The organisation of the basal ganglia in the domestic chick (Gallus domesticus): anatomical localisation of DARPP-32 in relation to glutamate. Brain Res Bull *76*, 183-191.

Cull-Candy, S., Brickley, S., and Farrant, M. (2001). NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol *11*, 327-335.

Da Cunha, A., Rausch, D. M., and Eiden, L. E. (1995). An early increase in somatostatin mRNA expression in the frontal cortex of rhesus monkeys infected with simian immunodeficiency virus. Proc Natl Acad Sci U S A *92*, 1371-1375.

Dahms, P., and Mentlein, R. (1992). Purification of the main somatostatin-degrading proteases from rat and pig brains, their action on other neuropeptides, and their identification as endopeptidases 24.15 and 24.16. Eur J Biochem *208*, 145-154.

Davies, P., Katzman, R., and Terry, R. D. (1980). Reduced somatostatin-like immunoreactivity in cerebral cortex from cases of Alzheimer disease and Alzheimer senile dementa. Nature *288*, 279-280.

Dawbarn, D., De Quidt, M. E., and Emson, P. C. (1985). Survival of basal ganglia neuropeptide Y-somatostatin neurones in Huntington's disease. Brain Res *340*, 251-260.

Dawson, V. L., Kizushi, V. M., Huang, P. L., Snyder, S. H., and Dawson, T. M. (1996). Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. J Neurosci *16*, 2479-2487.

de la Monte, S. M., Vonsattel, J. P., and Richardson, E. P., Jr. (1988). Morphometric demonstration of atrophic changes in the cerebral cortex, white matter, and neostriatum in Huntington's disease. J Neuropathol Exp Neurol *47*, 516-525.

De Wied, D., and Sigling, H. O. (2002). Neuropeptides involved in the pathophysiology of schizophrenia and major depression. Neurotox Res *4*, 453-468.

Doggrell, S. A. (2004). The potential of activation of somatostatinergic neurotransmission with FK960 in Alzheimer's disease. Expert Opin Investig Drugs *13*, 69-72.

Doggrell, S. A., and Evans, S. (2003). Treatment of dementia with neurotransmission modulation. Expert Opin Investig Drugs *12*, 1633-1654.

Dournaud, P., Boudin, H., Schonbrunn, A., Tannenbaum, G. S., and Beaudet, A. (1998). Interrelationships between somatostatin sst2A receptors and somatostatin-containing axons in rat brain: evidence for regulation of cell surface receptors by endogenous somatostatin. J Neurosci *18*, 1056-1071.

Dournaud, P., Gu, Y. Z., Schonbrunn, A., Mazella, J., Tannenbaum, G. S., and Beaudet, A. (1996). Localization of the somatostatin receptor SST2A in rat brain using a specific anti-peptide antibody. J Neurosci *16*, 4468-4478.

Durstewitz, D., Kroner, S., Hemmings, H. C., Jr., and Gunturkun, O. (1998). The dopaminergic innervation of the pigeon telencephalon: distribution of DARPP-32 and co-occurrence with glutamate decarboxylase and tyrosine hydroxylase. Neuroscience *83*, 763-779.

Dutour, A., Kumar, U., Panetta, R., Ouafik, L., Fina, F., Sasi, R., and Patel, Y. C. (1998). Expression of somatostatin receptor subtypes in human brain tumors. Int J Cancer *76*, 620-627.

Epelbaum, J. (1986). Somatostatin in the central nervous system: physiology and pathological modifications. Prog Neurobiol *27*, 63-100.

Epelbaum, J., Dournaud, P., Fodor, M., and Viollet, C. (1994). The neurobiology of somatostatin. Crit Rev Neurobiol *8*, 25-44.

Epelbaum, J., Ruberg, M., Moyse, E., Javoy-Agid, F., Dubois, B., and Agid, Y. (1983). Somatostatin and dementia in Parkinson's disease. Brain Res *278*, 376-379.

Esclapez, M., and Houser, C. R. (1995). Somatostatin neurons are a subpopulation of GABA neurons in the rat dentate gyrus: evidence from colocalization of pre-prosomatostatin and glutamate decarboxylase messenger RNAs. Neuroscience *64*, 339-355.

Fan, M. M., and Raymond, L. A. (2007). N-methyl-D-aspartate (NMDA) receptor function and excitotoxicity in Huntington's disease. Prog Neurobiol *81*, 272-293.

Ferland, L., Labrie, F., Jobin, M., Arimura, A., and Schally, A. V. (1976). Physiological role of somatostatin in the control of growth hormone and thyrotropin secretion. Biochem Biophys Res Commun *68*, 149-156.

Fernandes, H. B., Baimbridge, K. G., Church, J., Hayden, M. R., and Raymond, L. A. (2007). Mitochondrial sensitivity and altered calcium handling underlie enhanced NMDA-induced apoptosis in YAC128 model of Huntington's disease. J Neurosci *27*, 13614-13623.

Ferrante, R. J., Beal, M. F., Kowall, N. W., Richardson, E. P., Jr., and Martin, J. B. (1987). Sparing of acetylcholinesterase-containing striatal neurons in Huntington's disease. Brain Res *411*, 162-166.

Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Jr., Bird, E. D., and Martin, J. B. (1985). Selective sparing of a class of striatal neurons in Huntington's disease. Science *230*, 561-563.

Ferrante, R. J., Kowall, N. W., Cipolloni, P. B., Storey, E., and Beal, M. F. (1993). Excitotoxin lesions in primates as a model for Huntington's disease: histopathologic and neurochemical characterization. Exp Neurol *119*, 46-71.

Ferraro, L., Tanganelli, S., O'Connor, W. T., Bianchi, C., Ungerstedt, U., and Fuxe, K. (1995). Neurotensin increases endogenous glutamate release in the neostriatum of the awake rat. Synapse *20*, 362-364.

Ferrier, I. N., Roberts, G. W., Crow, T. J., Johnstone, E. C., Owens, D. G., Lee, Y. C., O'Shaughnessy, D., Adrian, T. E., Polak, J. M., and Bloom, S. R. (1983). Reduced cholecystokinin-like and somatostatin-like immunoreactivity in limbic lobe is associated with negative symptoms in schizophrenia. Life Sci *33*, 475-482.

Finley, J. C., Maderdrut, J. L., Roger, L. J., and Petrusz, P. (1981). The immunocytochemical localization of somatostatin-containing neurons in the rat central nervous system. Neuroscience *6*, 2173-2192.

Forloni, G., Lucca, E., Angeretti, N., Chiesa, R., and Vezzani, A. (1997). Neuroprotective effect of somatostatin on nonapoptotic NMDA-induced neuronal death: role of cyclic GMP. J Neurochem *68*, 319-327.

Fox, L., Alford, M., Achim, C., Mallory, M., and Masliah, E. (1997). Neurodegeneration of somatostatin-immunoreactive neurons in HIV encephalitis. J Neuropathol Exp Neurol *56*, 360-368.

Gabriel, S. M., Davidson, M., Haroutunian, V., Powchik, P., Bierer, L. M., Purohit, D. P., Perl, D. P., and Davis, K. L. (1996). Neuropeptide deficits in schizophrenia vs. Alzheimer's disease cerebral cortex. Biol Psychiatry *39*, 82-91.

Gafni, J., and Ellerby, L. M. (2002). Calpain activation in Huntington's disease. J Neurosci *22*, 4842-4849.

Gafni, J., Hermel, E., Young, J. E., Wellington, C. L., Hayden, M. R., and Ellerby, L. M. (2004). Inhibition of calpain cleavage of huntingtin reduces toxicity: accumulation of calpain/caspase fragments in the nucleus. J Biol Chem *279*, 20211-20220.

Gaspar, P., Duyckaerts C, Febvret A, Benoit R, Beck B, and Berger B. (1989). Subpopulations of somatostatin 28-immunoreactive neurons display different vulnerability in senile dementia of the Alzheimer type. . Brain Research, 1-13.

Geci, C., How, J., Alturaihi, H., and Kumar, U. (2007). Beta-amyloid increases somatostatin expression in cultured cortical neurons. J Neurochem *101*, 664-673.

Gemignani, A., Paudice, P., Pittaluga, A., and Raiteri, M. (2000). The HIV-1 coat protein gp120 and some of its fragments potently activate native cerebral NMDA receptors mediating neuropeptide release. Eur J Neurosci *12*, 2839-2846.

Gerfen, C. R., Paletzki, R., and Worley, P. (2008). Differences between dorsal and ventral striatum in Drd1a dopamine receptor coupling of dopamine- and cAMP-regulated phosphoprotein-32 to activation of extracellular signal-regulated kinase. J Neurosci *28*, 7113-7120.

Ghasemzadeh, M. B., Sharma, S., Surmeier, D. J., Eberwine, J. H., and Chesselet, M. F. (1996). Multiplicity of glutamate receptor subunits in single striatal neurons: an RNA amplification study. Mol Pharmacol *49*, 852-859.

Giampa, C., DeMarch, Z., D'Angelo, V., Morello, M., Martorana, A., Sancesario, G., Bernardi, G., and Fusco, F. R. (2006). Striatal modulation of cAMP-response-element-binding protein (CREB) after excitotoxic lesions: implications with neuronal vulnerability in Huntington's disease. Eur J Neurosci *23*, 11-20.

Gladding, C. M., and Raymond, L. A. (2011). Mechanisms underlying NMDA receptor synaptic/extrasynaptic distribution and function. Mol Cell Neurosci.

Goodyer, C. G., Grigorakis, S. I., Patel, Y. C., and Kumar, U. (2004). Developmental changes in the expression of somatostatin receptors (1-5) in the brain, hypothalamus, pituitary and spinal cord of the human fetus. Neuroscience *125*, 441-448.

Grant, M., Collier, B., and Kumar, U. (2004a). Agonist-dependent dissociation of human somatostatin receptor 2 dimers: a role in receptor trafficking. J Biol Chem *279*, 36179-36183.

Grant, M., and Kumar, U. (2010). The role of G-proteins in the dimerisation of human somatostatin receptor types 2 and 5. Regul Pept *159*, 3-8.

Grant, M., Patel, R. C., and Kumar, U. (2004b). The role of subtype-specific ligand binding and the C-tail domain in dimer formation of human somatostatin receptors. J Biol Chem *279*, 38636-38643.

Greenamyre, J. T. (1986). The role of glutamate in neurotransmission and in neurologic disease. Arch Neurol *43*, 1058-1063.

Greengard, P. (2001). The neurobiology of slow synaptic transmission. Science *294*, 1024-1030.

Greengard, P., Allen, P. B., and Nairn, A. C. (1999). Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. Neuron *23*, 435-447.

Gulyas, A. I., Hajos, N., Katona, I., and Freund, T. F. (2003). Interneurons are the local targets of hippocampal inhibitory cells which project to the medial septum. Eur J Neurosci *17*, 1861-1872.

Gurevich, E. V., Bordelon, Y., Shapiro, R. M., Arnold, S. E., Gur, R. E., and Joyce, J. N. (1997). Mesolimbic dopamine D3 receptors and use of antipsychotics in patients with schizophrenia. A postmortem study. Arch Gen Psychiatry *54*, 225-232.

Gusella, J. F., and MacDonald, M. E. (2000). Molecular genetics: unmasking polyglutamine triggers in neurodegenerative disease. Nat Rev Neurosci *1*, 109-115.

Handel, M., Schulz, S., Stanarius, A., Schreff, M., Erdtmann-Vourliotis, M., Schmidt, H., Wolf, G., and Hollt, V. (1999). Selective targeting of somatostatin receptor 3 to neuronal cilia. Neuroscience *89*, 909-926.

Hardingham, G. E. (2009). Coupling of the NMDA receptor to neuroprotective and neurodestructive events. Biochem Soc Trans *37*, 1147-1160.

Hardingham, G. E., and Bading, H. (2003). The Yin and Yang of NMDA receptor signalling. Trends Neurosci *26*, 81-89.

Hardingham, G. E., and Bading, H. (2010). Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. Nat Rev Neurosci *11*, 682-696.

Hardingham, G. E., Fukunaga, Y., and Bading, H. (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci *5*, 405-414.

Harris, A. S., Denovan-Wright, E. M., Hamilton, L. C., and Robertson, H. A. (2001). Protein kinase C beta II mRNA levels decrease in the striatum and cortex of transgenic Huntington's disease mice. J Psychiatry Neurosci *26*, 117-122.

HDCRgroup (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. Cell *72*, 971-983.

Heikkila, L. (1993). Somatostatin in the cerebrospinal fluid of schizophrenic patients before and after neuroleptic drug treatment. Schizophr Res *8*, 273-277.

Helboe, L., Stidsen, C. E., and Moller, M. (1998). Immunohistochemical and cytochemical localization of the somatostatin receptor subtype sst1 in the somatostatinergic parvocellular neuronal system of the rat hypothalamus. J Neurosci *18*, 4938-4945.

Helyes, Z., Pinter, E., Sandor, K., Elekes, K., Banvolgyi, A., Keszthelyi, D., Szoke, E., Toth, D. M., Sandor, Z., Kereskai, L.*, et al.* (2009). Impaired defense mechanism against inflammation, hyperalgesia, and airway hyperreactivity in somatostatin 4 receptor gene-deleted mice. Proc Natl Acad Sci U S A *106*, 13088-13093.

Hendry, S. H., Jones, E. G., and Emson, P. C. (1984). Morphology, distribution, and synaptic relations of somatostatin- and neuropeptide Y-immunoreactive neurons in rat and monkey neocortex. J Neurosci *4*, 2497-2517.

Hernandez-Espinosa, D., and Morton, A. J. (2006). Calcineurin inhibitors cause an acceleration of the neurological phenotype in a mouse transgenic for the human Huntington's disease mutation. Brain Res Bull *69*, 669-679.

Hervieu, G., and Emson, P. C. (1998a). The localization of somatostatin receptor 1 (sst1) immunoreactivity in the rat brain using an N-terminal specific antibody. Neuroscience *85*, 1263-1284.

Hervieu, G., and Emson, P. C. (1998b). Visualisation of non-glycosylated somatostatin receptor two (ngsst2) immunoreactivity in the rat central nervous system. Brain Res Mol Brain Res *58*, 138-155.

Hobart, P., Crawford, R., Shen, L., Pictet, R., and Rutter, W. J. (1980). Cloning and sequence analysis of cDNAs encoding two distinct somatostatin precursors found in the endocrine pancreas of anglerfish. Nature *288*, 137-141.

Hofland, L. J., van der Hoek, J., Feelders, R., van der Lely, A. J., de Herder, W., and Lamberts, S. W. (2005). Pre-clinical and clinical experiences with novel somatostatin ligands: advantages, disadvantages and new prospects. J Endocrinol Invest *28*, 36-42.

Hokfelt, T., Efendic, S., Hellerstrom, C., Johansson, O., Luft, R., and Arimura, A. (1975). Cellular localization of somatostatin in endocrine-like cells and neurons of the rat with special references to the A1-cells of the pancreatic islets and to the hypothalamus. Acta Endocrinol Suppl (Copenh) *200*, 5-41.

Hornung, J. P., De Tribolet, N., and Tork, I. (1992). Morphology and distribution of neuropeptide-containing neurons in human cerebral cortex. Neuroscience *51*, 363-375.

Huang, Q., Zhou, D., Sapp, E., Aizawa, H., Ge, P., Bird, E. D., Vonsattel, J. P., and DiFiglia, M. (1995). Quinolinic acid-induced increases in calbindin D28k immunoreactivity in rat striatal neurons in vivo and in vitro mimic the pattern seen in Huntington's disease. Neuroscience *65*, 397-407.

Hyman, B. T., Marzloff, K., Wenniger, J. J., Dawson, T. M., Bredt, D. S., and Snyder, S. H. (1992). Relative sparing of nitric oxide synthase-containing neurons in the hippocampal formation in Alzheimer's disease. Ann Neurol *32*, 818-820.

Hyman, S. E., and Malenka, R. C. (2001). Addiction and the brain: the neurobiology of compulsion and its persistence. Nat Rev Neurosci *2*, 695-703.

Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M., and et al. (1993). Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. J Biol Chem *268*, 2836-2843.

Iversen, L. L., Iversen, S. D., Bloom, F., Douglas, C., Brown, M., and Vale, W. (1978). Calcium-dependent release of somatostatin and neurotensin from rat brain in vitro. Nature *273*, 161-163.

Jaquet, P., Ouafik, L., Saveanu, A., Gunz, G., Fina, F., Dufour, H., Culler, M. D., Moreau, J. P., and Enjalbert, A. (1999). Quantitative and functional expression of somatostatin receptor subtypes in human prolactinomas. J Clin Endocrinol Metab *84*, 3268-3276.

Jay, T. M. (2003). Dopamine: a potential substrate for synaptic plasticity and memory mechanisms. Prog Neurobiol *69*, 375-390.

Johansson, O., Hokfelt, T., and Elde, R. P. (1984). Immunohistochemical distribution of somatostatin-like immunoreactivity in the central nervous system of the adult rat. Neuroscience *13*, 265-339.

Joyce, J. N. (2001). D2 but not D3 receptors are elevated after 9 or 11 months chronic haloperidol treatment: influence of withdrawal period. Synapse *40*, 137-144.

Kendall, D. M., Poitout, V., Olson, L. K., Sorenson, R. L., and Robertson, R. P. (1995). Somatostatin coordinately regulates glucagon gene expression and exocytosis in HIT-T15 cells. J Clin Invest *96*, 2496-2502.

Kew, J. N., and Kemp, J. A. (2005). Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology (Berl) *179*, 4-29.

Khan, Z. U., Gutierrez, A., Martin, R., Penafiel, A., Rivera, A., and de la Calle, A. (2000). Dopamine D5 receptors of rat and human brain. Neuroscience *100*, 689-699.

Kharmate, G., Rajput, P. S., Watt, H. L., Somvanshi, R. K., Chaudhari, N., Qiu, X., and Kumar, U. (2011a). Dissociation of epidermal growth factor receptor and ErbB2 heterodimers in the presence of somatostatin receptor 5 modulate signaling pathways. Endocrinology *152*, 931-945.

Kharmate, G., Rajput, P. S., Watt, H. L., Somvanshi, R. K., Chaudhari, N., Qiu, X., and Kumar, U. (2011b). Role of somatostatin receptor 1 and 5 on epidermal growth factor receptor mediated signaling. Biochim Biophys Acta *1813*, 1172-1189.

Kim, E., Cho, K. O., Rothschild, A., and Sheng, M. (1996). Heteromultimerization and NMDA receptor-clustering activity of Chapsyn-110, a member of the PSD-95 family of proteins. Neuron *17*, 103-113.

Kiyama, H., Seto-Ohshima, A., and Emson, P. C. (1990). Calbindin D28K as a marker for the degeneration of the striatonigral pathway in Huntington's disease. Brain Res *525*, 209-214.

Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G., and Wittig, B. (1991). Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. Nature *353*, 43-48.

Koh, J. Y., Peters, S., and Choi, D. W. (1986). Neurons containing NADPH-diaphorase are selectively resistant to quinolinate toxicity. Science *234*, 73-76.

Kohler, C., Eriksson, L. G., Davies, S., and Chan-Palay, V. (1987). Co-localization of neuropeptide tyrosine and somatostatin immunoreactivity in neurons of individual subfields of the rat hippocampal region. Neurosci Lett *78*, 1-6.

Koob, G. F., and Le Moal, M. (1997). Drug abuse: hedonic homeostatic dysregulation. Science *278*, 52-58.

Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science *269*, 1737-1740.

Kowall, N. W., and Beal, M. F. (1988). Cortical somatostatin, neuropeptide Y, and NADPH diaphorase neurons: normal anatomy and alterations in Alzheimer's disease. Ann Neurol *23*, 105-114.

Kowall, N. W., Ferrante, R. J., Beal, M. F., Richardson, E. P., Jr., Sofroniew, M. V., Cuello, A. C., and Martin, J. B. (1987). Neuropeptide Y, somatostatin, and reduced nicotinamide adenine dinucleotide phosphate diaphorase in the human striatum: a combined immunocytochemical and enzyme histochemical study. Neuroscience *20*, 817-828.

Krantic, S., Robitaille, Y., and Quirion, R. (1992). Deficits in the somatostatin SS1 receptor sub-type in frontal and temporal cortices in Alzheimer's disease. Brain Res *573*, 299-304.

Krulich, L., Dhariwal, A. P., and McCann, S. M. (1968). Stimulatory and inhibitory effects of purified hypothalamic extracts on growth hormone release from rat pituitary in vitro. Endocrinology *83*, 783-790.

Kumar, U. (2004). Characterization of striatal cultures with the effect of QUIN and NMDA. Neurosci Res *49*, 29-38.

Kumar, U. (2005). Expression of somatostatin receptor subtypes (SSTR1-5) in Alzheimer's disease brain: An immunohistochemical analysis. Neuroscience *134*, 525-538.

Kumar, U. (2007). Colocalization of somatostatin receptor subtypes (SSTR1-5) with somatostatin, NADPH-diaphorase (NADPH-d), and tyrosine hydroxylase in the rat hypothalamus. J Comp Neurol *504*, 185-205.

Kumar, U. (2008). Somatostatin in medium-sized aspiny interneurons of striatum is responsible for their preservation in quinolinic acid and N-methyl-D-asparate-induced neurotoxicity. J Mol Neurosci *35*, 345-354.

Kumar, U., Asotra, K., Patel, S. C., and Patel, Y. C. (1997a). Expression of NMDA Receptor-1 (NR1) and Huntingtin in Striatal Neurons Which Colocalize Somatostatin, Neuropeptide Y, and NADPH Diaphorase: A Double-Label Histochemical and Immunohistochemical Study. Experimental Neurology *145*, 412-424.

Kumar, U., and Grant, M. (2010). Somatostatin and somatostatin receptors. In Results Probl Cell Differ, J. Rehfeld, and J. Bundgaard, eds., pp. 137-184.

Kumar, U., Laird, D., Srikant, C. B., Escher, E., and Patel, Y. C. (1997b). Expression of the five somatostatin receptor (SSTR1-5) subtypes in rat pituitary somatotrophes: quantitative analysis by double-layer immunofluorescence confocal microscopy. Endocrinology *138*, 4473-4476.

Kumar, U., and Patel, S. C. (2007). Immunohistochemical localization of dopamine receptor subtypes (D1R-D5R) in Alzheimer's disease brain. Brain Res *1131*, 187-196.

Kumar, U., Sasi, R., Suresh, S., Patel, A., Thangaraju, M., Metrakos, P., Patel, S. C., and Patel, Y. C. (1999). Subtype-selective expression of the five somatostatin receptors (hSSTR1-5) in human pancreatic islet cells: a quantitative double-label immunohistochemical analysis. Diabetes *48*, 77-85.

Kuppenbender, K. D., Standaert, D. G., Feuerstein, T. J., Penney, J. B., Jr., Young, A. B., and Landwehrmeyer, G. B. (2000). Expression of NMDA receptor subunit mRNAs in neurochemically identified projection and interneurons in the human striatum. J Comp Neurol *419*, 407-421.

Lamberts, S. W., Krenning, E. P., and Reubi, J. C. (1991). The role of somatostatin and its analogs in the diagnosis and treatment of tumors. Endocr Rev *12*, 450-482.

Landwehrmeyer, G. B., Standaert, D. G., Testa, C. M., Penney, J. B., Jr., and Young, A. B. (1995). NMDA receptor subunit mRNA expression by projection neurons and interneurons in rat striatum. J Neurosci *15*, 5297-5307.

Langley, K. C., Bergson, C., Greengard, P., and Ouimet, C. C. (1997). Co-localization of the D1 dopamine receptor in a subset of DARPP-32-containing neurons in rat caudate-putamen. Neuroscience *78*, 977-983.

Lau, C. G., and Zukin, R. S. (2007). NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat Rev Neurosci *8*, 413-426.

Lavezzari, G., McCallum, J., Dewey, C. M., and Roche, K. W. (2004). Subunit-specific regulation of NMDA receptor endocytosis. J Neurosci *24*, 6383-6391.

Le Moine, C., and Bloch, B. (1995). D1 and D2 dopamine receptor gene expression in the rat striatum: sensitive cRNA probes demonstrate prominent segregation of D1 and D2 mRNAs in distinct neuronal populations of the dorsal and ventral striatum. J Comp Neurol *355*, 418-426.

Lee, F. J., Xue, S., Pei, L., Vukusic, B., Chery, N., Wang, Y., Wang, Y. T., Niznik, H. B., Yu, X. M., and Liu, F. (2002). Dual regulation of NMDA receptor functions by direct protein-protein interactions with the dopamine D1 receptor. Cell *111*, 219-230.

Lenard, J. (1992). Mammalian hormones in microbial cells. Trends Biochem Sci *17*, 147-150.

Li, L., Fan, M., Icton, C. D., Chen, N., Leavitt, B. R., Hayden, M. R., Murphy, T. H., and Raymond, L. A. (2003). Role of NR2B-type NMDA receptors in selective neurodegeneration in Huntington disease. Neurobiol Aging *24*, 1113-1121.

Li, S. H., and Li, X. J. (2004). Huntingtin-protein interactions and the pathogenesis of Huntington's disease. Trends Genet *20*, 146-154.

Liao, G. Y., Wagner, D. A., Hsu, M. H., and Leonard, J. P. (2001). Evidence for direct protein kinase-C mediated modulation of N-methyl-D-aspartate receptor current. Mol Pharmacol *59*, 960-964.

Lindskog, M., Svenningsson, P., Fredholm, B., Greengard, P., and Fisone, G. (1999a). Mu- and delta-opioid receptor agonists inhibit DARPP-32 phosphorylation in distinct populations of striatal projection neurons. Eur J Neurosci *11*, 2182-2186.

Lindskog, M., Svenningsson, P., Fredholm, B. B., Greengard, P., and Fisone, G. (1999b). Activation of dopamine D2 receptors decreases DARPP-32 phosphorylation in striatonigral and striatopallidal projection neurons via different mechanisms. Neuroscience *88*, 1005-1008.

Lione, L. A., Carter, R. J., Hunt, M. J., Bates, G. P., Morton, A. J., and Dunnett, S. B. (1999). Selective discrimination learning impairments in mice expressing the human Huntington's disease mutation. J Neurosci *19*, 10428-10437.

Lipton, S. A. (2006). Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond. Nat Rev Drug Discov *5*, 160-170.

Liu, L., Wong, T. P., Pozza, M. F., Lingenhoehl, K., Wang, Y., Sheng, M., Auberson, Y. P., and Wang, Y. T. (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science *304*, 1021-1024.

Lloyd, K., and Hornykiewicz, O. (1970). Parkinson's disease: activity of L-dopa decarboxylase in discrete brain regions. Science *170*, 1212-1213.

Loftis, J. M., and Janowsky, A. (2003). The N-methyl-D-aspartate receptor subunit NR2B: localization, functional properties, regulation, and clinical implications. Pharmacol Ther *97*, 55-85.

Lonze, B. E., and Ginty, D. D. (2002). Function and regulation of CREB family transcription factors in the nervous system. Neuron *35*, 605-623.

Lonze, B. E., Riccio, A., Cohen, S., and Ginty, D. D. (2002). Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. Neuron *34*, 371-385.

Loudes, C., Petit, F., Kordon, C., and Faivre-Bauman, A. (2000). Brain-derived neurotrophic factor but not neurotrophin-3 enhances differentiation of somatostatin neurons in hypothalamic cultures. Neuroendocrinology *72*, 144-153.

Low, M. J., Otero-Corchon, V., Parlow, A. F., Ramirez, J. L., Kumar, U., Patel, Y. C., and Rubinstein, M. (2001). Somatostatin is required for masculinization of growth hormone-regulated hepatic gene expression but not of somatic growth. J Clin Invest *107*, 1571-1580.

Lu, J. Q., and Stoessl, A. J. (2002). Somatostatin modulates the behavioral effects of dopamine receptor activation in parkinsonian rats. Neuroscience *112*, 261-266.

Mantamadiotis, T., Lemberger, T., Bleckmann, S. C., Kern, H., Kretz, O., Martin Villalba, A., Tronche, F., Kellendonk, C., Gau, D., Kapfhammer, J.*, et al.* (2002). Disruption of CREB function in brain leads to neurodegeneration. Nat Genet *31*, 47-54.

Martin, J. B., and Gusella, J. F. (1986). Huntington's disease. Pathogenesis and management. N Engl J Med *315*, 1267-1276.

Matsuyama, S., Fukui, R., Higashi, H., and Nishi, A. (2003). Regulation of DARPP-32 Thr75 phosphorylation by neurotensin in neostriatal neurons: involvement of glutamate signalling. Eur J Neurosci *18*, 1247-1253.

McBain, C. J., and Mayer, M. L. (1994). N-methyl-D-aspartic acid receptor structure and function. Physiol Rev *74*, 723-760.

Meador-Woodruff, J. H., Mansour, A., Grandy, D. K., Damask, S. P., Civelli, O., and Watson, S. J., Jr. (1992). Distribution of D5 dopamine receptor mRNA in rat brain. Neurosci Lett *145*, 209-212.

Menalled, L., Zanjani, H., MacKenzie, L., Koppel, A., Carpenter, E., Zeitlin, S., and Chesselet, M. F. (2000). Decrease in striatal enkephalin mRNA in mouse models of Huntington's disease. Exp Neurol *162*, 328-342.

Menkes, J. H. (1988). Huntington disease: finding the gene and after. Pediatr Neurol *4*, 73-78.

Meriney, S. D., Gray, D. B., and Pilar, G. R. (1994). Somatostatin-induced inhibition of neuronal Ca2+ current modulated by cGMP-dependent protein kinase. Nature *369*, 336-339.

Metzler, M., Gan, L., Mazarei, G., Graham, R. K., Liu, L., Bissada, N., Lu, G., Leavitt, B. R., and Hayden, M. R. (2010). Phosphorylation of huntingtin at Ser421 in YAC128 neurons is associated with protection of YAC128 neurons from NMDA-mediated excitotoxicity and is modulated by PP1 and PP2A. J Neurosci *30*, 14318-14329.

Millar, R. P., Sheward, W. J., Wegener, I., and Fink, G. (1983). Somatostatin-28 is an hormonally active peptide secreted into hypophysial portal vessel blood. Brain Res *260*, 334-337.

Milnerwood, A. J., and Raymond, L. A. (2010). Early synaptic pathophysiology in neurodegeneration: insights from Huntington's disease. Trends Neurosci *33*, 513-523.

Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., and Caron, M. G. (1998). Dopamine receptors: from structure to function. Physiol Rev *78*, 189-225.

Miyakawa, T., Leiter, L. M., Gerber, D. J., Gainetdinov, R. R., Sotnikova, T. D., Zeng, H., Caron, M. G., and Tonegawa, S. (2003). Conditional calcineurin knockout mice exhibit multiple abnormal behaviors related to schizophrenia. Proc Natl Acad Sci U S A *100*, 8987-8992.

Monaghan, D. T., Bridges, R. J., and Cotman, C. W. (1989). The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. Annu Rev Pharmacol Toxicol *29*, 365-402.

Montminy, M. R., and Bilezikjian, L. M. (1987). Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature *328*, 175-178.

Montminy, M. R., Goodman, R. H., Horovitch, S. J., and Habener, J. F. (1984). Primary structure of the gene encoding rat preprosomatostatin. Proc Natl Acad Sci U S A *81*, 3337-3340.

Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B., and Seeburg, P. H. (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron *12*, 529-540.

Morishita, W., Lu, W., Smith, G. B., Nicoll, R. A., Bear, M. F., and Malenka, R. C. (2007). Activation of NR2B-containing NMDA receptors is not required for NMDA receptor-dependent long-term depression. Neuropharmacology *52*, 71-76.

Morrison, J. H., Benoit, R., Magistretti, P. J., and Bloom, F. E. (1983). Immunohistochemical distribution of pro-somatostatin-related peptides in cerebral cortex. Brain Res *262*, 344-351.

Morrison, J. H., Benoit, R., Magistretti, P. J., Ling, N., and Bloom, F. E. (1982). Immunohistochemical distribution of pro-somatostatin-related peptides in hippocampus. Neurosci Lett *34*, 137-142.

Nairn, A. C., Svenningsson, P., Nishi, A., Fisone, G., Girault, J. A., and Greengard, P. (2004). The role of DARPP-32 in the actions of drugs of abuse. Neuropharmacology *47 Suppl 1*, 14-23.

Nakamura, Y., Tsuji, K., Shuto, M., Ogita, K., Yoneda, Y., Shimamoto, K., Shibata, T., and Kataoka, K. (1997). Protection by diphenyliodonium against glutamate neurotoxicity due to blocking of N-methyl-D-aspartate receptors. Neuroscience *76*, 459-466.

Nemeroff, C. B., Youngblood, W. W., Manberg, P. J., Prange, A. J., Jr., and Kizer, J. S. (1983). Regional brain concentrations of neuropeptides in Huntington's chorea and schizophrenia. Science *221*, 972-975.

Nestler, E. J. (2001). Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci *2*, 119-128.

Newman, C. B., Melmed, S., George, A., Torigian, D., Duhaney, M., Snyder, P., Young, W., Klibanski, A., Molitch, M. E., Gagel, R.*, et al.* (1998). Octreotide as primary therapy for acromegaly. J Clin Endocrinol Metab *83*, 3034-3040.

Nimmrich, V., Szabo, R., Nyakas, C., Granic, I., Reymann, K. G., Schroder, U. H., Gross, G., Schoemaker, H., Wicke, K., Moller, A., and Luiten, P. (2008). Inhibition of Calpain Prevents N-Methyl-D-aspartate-Induced Degeneration of the Nucleus Basalis and Associated Behavioral Dysfunction. J Pharmacol Exp Ther *327*, 343-352.

Nishi, A., Bibb, J. A., Snyder, G. L., Higashi, H., Nairn, A. C., and Greengard, P. (2000). Amplification of dopaminergic signaling by a positive feedback loop. Proc Natl Acad Sci U S A *97*, 12840-12845.

Nishi, A., Snyder, G. L., and Greengard, P. (1997). Bidirectional regulation of DARPP-32 phosphorylation by dopamine. J Neurosci *17*, 8147-8155.

Nishi, A., Watanabe, Y., Higashi, H., Tanaka, M., Nairn, A. C., and Greengard, P. (2005). Glutamate regulation of DARPP-32 phosphorylation in neostriatal neurons involves activation of multiple signaling cascades. Proc Natl Acad Sci U S A *102*, 1199-1204.

Noguchi, T., Ishii, K., Ohtubo, Y., Shuto, S., Ono, S., Matsuda, A., and Yoshii, K. (1999). Open channel block of NMDA receptors by conformationally restricted analogs of milnacipran and their protective effect against NMDA-induced neurotoxicity. Synapse *31*, 87-96.

Norris, P. J., Waldvogel, H. J., Faull, R. L., Love, D. R., and Emson, P. C. (1996). Decreased neuronal nitric oxide synthase messenger RNA and somatostatin messenger RNA in the striatum of Huntington's disease. Neuroscience *72*, 1037-1047.

Okamoto, S., Pouladi, M. A., Talantova, M., Yao, D., Xia, P., Ehrnhoefer, D. E., Zaidi, R., Clemente, A., Kaul, M., Graham, R. K.*, et al.* (2009). Balance between synaptic versus extrasynaptic NMDA receptor activity influences inclusions and neurotoxicity of mutant huntingtin. Nat Med *15*, 1407-1413.

Oliva, A. A., Jr., Jiang, M., Lam, T., Smith, K. L., and Swann, J. W. (2000). Novel hippocampal interneuronal subtypes identified using transgenic mice that express green fluorescent protein in GABAergic interneurons. J Neurosci *20*, 3354-3368.

Ouimet, C. C., LaMantia, A. S., Goldman-Rakic, P., Rakic, P., and Greengard, P. (1992). Immunocytochemical localization of DARPP-32, a dopamine and cyclic-AMP-regulated phosphoprotein, in the primate brain. J Comp Neurol *323*, 209-218.

Ouimet, C. C., Langley-Gullion, K. C., and Greengard, P. (1998). Quantitative immunocytochemistry of DARPP-32-expressing neurons in the rat caudatoputamen. Brain Res *808*, 8-12.

Ouimet, C. C., Miller, P. E., Hemmings, H. C., Jr., Walaas, S. I., and Greengard, P. (1984). DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. III. Immunocytochemical localization. J Neurosci *4*, 111-124.

Ozawa, S., Kamiya, H., and Tsuzuki, K. (1998). Glutamate receptors in the mammalian central nervous system. Prog Neurobiol *54*, 581-618.

Palacios, J. M., Rigo, M., Chinaglia, G., and Probst, A. (1990). Reduced density of striatal somatostatin receptors in Huntington's chorea. Brain Res *522*, 342-346.

Palkovits, M., Brownstein, M. J., Arimura, A., Sato, H., Schally, V., and Kizer, J. S. (1976). Somatostatin content of the hypothalamic ventromedial and arcuate nuclei and the circumventricular organs in the rat. Brain Res *109*, 430-434.

Panteris, V., and Karamanolis, D. G. (2005). The puzzle of somatostatin: action, receptors, analogues and therapy. Hepatogastroenterology *52*, 1771-1781.

Papadia, S., Stevenson, P., Hardingham, N. R., Bading, H., and Hardingham, G. E. (2005). Nuclear Ca2+ and the cAMP response element-binding protein family mediate a late phase of activity-dependent neuroprotection. J Neurosci *25*, 4279-4287.

Patel, S., Asotra, K, and Patel, YC. (1996). Huntington's Disease. In Neurotherapeutics: Emerging Strategies, , (Totowa, NJ, : Humana Press,).

Patel, S. C., Papachristou, D. N., and Patel, Y. C. (1991). Quinolinic acid stimulates somatostatin gene expression in cultured rat cortical neurons. J Neurochem *56*, 1286-1291.

Patel, Y. C. (1997). Molecular pharmacology of somatostatin receptor subtypes. J Endocrinol Invest *20*, 348-367.

Patel, Y. C. (1999). Somatostatin and Its Receptor Family. Frontiers in Neuroendocrinology *20*, 157-198.

Patel, Y. C., Greenwood, M. T., Panetta, R., Demchyshyn, L., Niznik, H., and Srikant, C. B. (1995a). The somatostatin receptor family. Life Sci *57*, 1249-1265.

Patel, Y. C., Liu, J. L., Warszynska, A., Kent, G., Papachristou, D. N., and Patel, S. C. (1995b). Differential stimulation of somatostatin but not neuropeptide Y gene expression by quinolinic acid in cultured cortical neurons. J Neurochem *65*, 998-1006.

Patel, Y. C., Murthy, K. K., Escher, E. E., Banville, D., Spiess, J., and Srikant, C. B. (1990). Mechanism of action of somatostatin: An overview of receptor function and studies of the molecular characterization and purification of somatostatin receptor proteins. Metabolism *39*, 63-69.

Patel, Y. C., and Reichlin, S. (1978). Somatostatin in hypothalamus, extrahypothalamic brain, and peripheral tissues of the rat. Endocrinology *102*, 523-530.

Patel, Y. C., and Srikant, C. B. (1994). Subtype selectivity of peptide analogs for all five cloned human somatostatin receptors (hsstr 1-5). Endocrinology *135*, 2814-2817.

Patel, Y. C., and Srikant, C. B. (1997). Somatostatin Receptors. Trends in Endocrinology and Metabolism *8*, 398-405.

Paxinos, G., and Watson, C. (2007). The Rat Brain in Stereotaxic Coordinates, 6th edn: Elsevier Academic Press, San Diego).

Perez, J., Rigo, M., Kaupmann, K., Bruns, C., Yasuda, K., Bell, G. I., Lubbert, H., and Hoyer, D. (1994). Localization of somatostatin (SRIF) SSTR-1, SSTR-2 and SSTR-3 receptor mRNA in rat brain by in situ hybridization. Naunyn Schmiedebergs Arch Pharmacol *349*, 145-160.

Perez, J., Vezzani, A., Civenni, G., Tutka, P., Rizzi, M., Schupbach, E., and Hoyer, D. (1995). Functional effects of D-Phe-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Trp-NH2 and differential changes in somatostatin receptor messenger RNAs, binding sites and somatostatin release in kainic acid-treated rats. Neuroscience *65*, 1087-1097.

Pfeiffer, M., Koch, T., Schroder, H., Laugsch, M., Hollt, V., and Schulz, S. (2002). Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. J Biol Chem *277*, 19762-19772.

Piwko, C., Thoss, V. S., Samanin, R., Hoyer, D., and Vezzani, A. (1996). Status of somatostatin receptor messenger RNAs and binding sites in rat brain during kindling epileptogenesis. Neuroscience *75*, 857-868.

Qiu, C., Zeyda, T., Johnson, B., Hochgeschwender, U., de Lecea, L., and Tallent, M. K. (2008). Somatostatin receptor subtype 4 couples to the M-current to regulate seizures. J Neurosci *28*, 3567-3576.

Rajput, P. S., Billova, S., Patel, S. C., Kharmate, G., Somvanshi, R. K., and Kumar, U. (2009a). Expression of somatostatin and somatostatin receptor subtypes in Apolipoprotein D (ApoD) knockout mouse brain: An immunohistochemical analysis. J Chem Neuroanat *38*, 20-33.

Rajput, P. S., Kharmate, G., and Kumar, U. (2011a). Colocalization of Somatostatin Receptors with DARPP-32 in Cortex and Striatum of Rat Brain. J Mol Neurosci.

Rajput, P. S., Kharmate, G., Norman, M. A., Liu, S.-H., Bhagavatula, S. R., Brunicardi, F. C., and Kumar, U. (2011b). Somatostatin Receptor 1 and 5 Double knockout mice Mimic Neurochemical changes of Huntington's disease transgenic mice. PLoS One.

Rajput, P. S., Kharmate, G., Somvanshi, R. K., and Kumar, U. (2009b). Colocalization of dopamine receptor subtypes with dopamine and cAMP-regulated phosphoprotein (DARPP-32) in rat brain. Neurosci Res *65*, 53-63.

Ramirez, J. L., Grant, M., Norman, M., Wang, X. P., Moldovan, S., de Mayo, F. J., Brunicardi, C., and Kumar, U. (2004). Deficiency of somatostatin (SST) receptor type 5 (SSTR5) is associated with sexually dimorphic changes in the expression of SST and SST receptors in brain and pancreas. Mol Cell Endocrinol *221*, 105-119.

Ramirez, J. L., Mouchantaf, R., Kumar, U., Otero Corchon, V., Rubinstein, M., Low, M. J., and Patel, Y. C. (2002). Brain somatostatin receptors are up-regulated in somatostatin-deficient mice. Mol Endocrinol *16*, 1951-1963.

Rauca, C., Schafer, K., and Hollt, V. (1999). Effects of somatostatin, octreotide and cortistatin on ischaemic neuronal damage following permanent middle cerebral artery occlusion in the rat. Naunyn Schmiedebergs Arch Pharmacol *360*, 633-638.

Raymond, L. A., Andre, V. M., Cepeda, C., Gladding, C. M., Milnerwood, A. J., and Levine, M. S. (2011). Pathophysiology of Huntington's disease: time-dependent alterations in synaptic and receptor function. Neuroscience *198*, 252-273.

Redmon, J. B., Towle, H. C., and Robertson, R. P. (1994). Regulation of human insulin gene transcription by glucose, epinephrine, and somatostatin. Diabetes *43*, 546-551.

Reichlin, S. (1983a). Somatostatin. N Engl J Med *309*, 1495-1501.

Reichlin, S. (1983b). Somatostatin (second of two parts). N Engl J Med *309*, 1556-1563.

Reisine, T. (1985). Multiple mechanisms of somatostatin inhibition of adrenocorticotropin release from mouse anterior pituitary tumor cells. Endocrinology *116*, 2259-2266.

Reisine, T., and Bell, G. I. (1995). Molecular biology of somatostatin receptors. Endocr Rev *16*, 427-442.

Reisine, T., Zhang, Y. L., and Sekura, R. (1985). Pertussis toxin treatment blocks the inhibition of somatostatin and increases the stimulation by forskolin of cyclic AMP accumulation and adrenocorticotropin secretion from mouse anterior pituitary tumor cells. J Pharmacol Exp Ther *232*, 275-282.

Reisine, T. D., Zhang, Y., and Sekura, R. D. (1983). Pertussis toxin blocks somatostatin's inhibition of stimulated cyclic AMP accumulation in anterior pituitary tumor cells. Biochem Biophys Res Commun *115*, 794-799.

Riekkinen, P. J., and Pitkanen, A. (1990). Somatostatin and epilepsy. Metabolism *39*, 112-115.

Rigby, M., Le Bourdelles, B., Heavens, R. P., Kelly, S., Smith, D., Butler, A., Hammans, R., Hills, R., Xuereb, J. H., Hill, R. G.*, et al.* (1996). The messenger RNAs for the N-methyl-D-aspartate receptor subunits show region-specific expression of different subunit composition in the human brain. Neuroscience *73*, 429-447.

Roberts, G. W., Ferrier, I. N., Lee, Y., Crow, T. J., Johnstone, E. C., Owens, D. G., Bacarese-Hamilton, A. J., McGregor, G., O'Shaughnessey, D., Polak, J. M., and et al. (1983). Peptides, the limbic lobe and schizophrenia. Brain Res *288*, 199-211.

Roche, K. W., Standley, S., McCallum, J., Dune Ly, C., Ehlers, M. D., and Wenthold, R. J. (2001). Molecular determinants of NMDA receptor internalization. Nat Neurosci *4*, 794-802.

Rocheville, M., Lange, D. C., Kumar, U., Patel, S. C., Patel, R. C., and Patel, Y. C. (2000a). Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. Science *288*, 154-157.

Rocheville, M., Lange, D. C., Kumar, U., Sasi, R., Patel, R. C., and Patel, Y. C. (2000b). Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. J Biol Chem *275*, 7862-7869.

Rogaeva, E. A., Chumakova, M. M., Dergunova, N. N., Pozharitskaia, D. A., Kopeiko, G. I., Tsutsul'kovskaia, M., and Tsibezov, V. V. (1990). [Anti-somatostatin autoantibodies in the blood serum of patients with schizophrenia]. Zh Nevropatol Psikhiatr Im S S Korsakova *90*, 82-84.

Rossor, M. N., Emson, P. C., Mountjoy, C. Q., Roth, M., and Iversen, L. L. (1980). Reduced amounts of immunoreactive somatostatin in the temporal cortex in senile dementia of Alzheimer type. Neurosci Lett *20*, 373-377.

Roy, B. F., Benkelfat, C., Hill, J. L., Pierce, P. F., Dauphin, M. M., Kelly, T. M., Sunderland, T., Weinberger, D. R., and Breslin, N. (1994). Serum antibody for somatostatin-14 and prodynorphin 209-240 in patients with obsessive-compulsive disorder, schizophrenia, Alzheimer's disease, multiple sclerosis, and advanced HIV infection. Biol Psychiatry *35*, 335-344.

Rubinow, D. R., Davis, C. L., and Post, R. M. (1995). Somatostatin in the central nervous system. In In: Psychopharmacologyy: the foruth generation of progress, F.E. Bloom, and D.J. Kupfer, eds. (New York: Raven), pp. 553-562.

Rushlow, W., Flumerfelt, B. A., and Naus, C. C. (1995). Colocalization of somatostatin, neuropeptide Y, and NADPH-diaphorase in the caudate-putamen of the rat. J Comp Neurol *351*, 499-508.

Saito, K., Elce, J. S., Hamos, J. E., and Nixon, R. A. (1993). Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. Proc Natl Acad Sci U S A *90*, 2628-2632.

Saito, T., Iwata, N., Tsubuki, S., Takaki, Y., Takano, J., Huang, S. M., Suemoto, T., Higuchi, M., and Saido, T. C. (2005). Somatostatin regulates brain amyloid beta peptide Abeta42 through modulation of proteolytic degradation. Nat Med *11*, 434-439.

Saiz-Ruiz, J., Carrasco, J. L., Martin, M., Manzanares, J., and Hernanz, A. (1992). Plasmatic somatostatin as a marker of positive symptoms of schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry *16*, 203-210.

Sattler, R., Xiong, Z., Lu, W. Y., Hafner, M., MacDonald, J. F., and Tymianski, M. (1999). Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science *284*, 1845-1848.

Schally, A. V. (1988). Oncological applications of somatostatin analogues. Cancer Res *48*, 6977-6985.

Schindler, M., Holloway, S., Humphrey, P. P., Waldvogel, H., Faull, R. L., Berger, W., and Emson, P. C. (1998). Localization of the somatostatin sst2(a) receptor in human cerebral cortex, hippocampus and cerebellum. Neuroreport *9*, 521-525.

Schindler, M., Humphrey, P. P., and Emson, P. C. (1996). Somatostatin receptors in the central nervous system. Prog Neurobiol *50*, 9-47.

Schindler, M., Sellers, L. A., Humphrey, P. P., and Emson, P. C. (1997). Immunohistochemical localization of the somatostatin SST2(A) receptor in the rat brain and spinal cord. Neuroscience *76*, 225-240.

Schnabel, R., Metzger, M., Jiang, S., Hemmings, H. C., Jr., Greengard, P., and Braun, K. (1997). Localization of dopamine D1 receptors and dopaminoceptive neurons in the chick forebrain. J Comp Neurol *388*, 146-168.

Schonbrunn, A., and Tashjian, H., Jr. (1978). Characterization of functional receptors for somatostatin in rat pituitary cells in culture. J Biol Chem *253*, 6473-6483.

Schreff, M., Schulz, S., Handel, M., Keilhoff, G., Braun, H., Pereira, G., Klutzny, M., Schmidt, H., Wolf, G., and Hollt, V. (2000). Distribution, targeting, and internalization of the sst4 somatostatin receptor in rat brain. J Neurosci *20*, 3785-3797.

Schulz, S., Schreff, M., Schmidt, H., Handel, M., Przewlocki, R., and Hollt, V. (1998). Immunocytochemical localization of somatostatin receptor sst2A in the rat spinal cord and dorsal root ganglia. Eur J Neurosci *10*, 3700-3708.

Schwarcz, R., Foster, A. C., French, E. D., Whetsell, W. O., Jr., and Kohler, C. (1984). Excitotoxic models for neurodegenerative disorders. Life Sci *35*, 19-32.

Scott, L., Zelenin, S., Malmersjo, S., Kowalewski, J. M., Markus, E. Z., Nairn, A. C., Greengard, P., Brismar, H., and Aperia, A. (2006). Allosteric changes of the NMDA receptor trap diffusible dopamine 1 receptors in spines. Proc Natl Acad Sci U S A *103*, 762-767.

Seeman, P., Bzowej, N. H., Guan, H. C., Bergeron, C., Reynolds, G. P., Bird, E. D., Riederer, P., Jellinger, K., and Tourtellotte, W. W. (1987). Human brain D1 and D2 dopamine receptors in schizophrenia, Alzheimer's, Parkinson's, and Huntington's diseases. Neuropsychopharmacology *1*, 5-15.

Seeman, P., and Van Tol, H. H. (1993). Dopamine receptor pharmacology. Curr Opin Neurol Neurosurg *6*, 602-608.

Self, D. W., Barnhart, W. J., Lehman, D. A., and Nestler, E. J. (1996). Opposite modulation of cocaine-seeking behavior by D1- and D2-like dopamine receptor agonists. Science *271*, 1586-1589.

Selmer, I. S., Schindler, M., Humphrey, P. P., and Emson, P. C. (2000a). Immunohistochemical localization of the somatostatin sst(4) receptor in rat brain. Neuroscience *98*, 523-533.

Selmer, I. S., Schindler, M., Humphrey, P. P., Waldvogel, H. J., Faull, R. L., and Emson, P. C. (2000b). First localisation of somatostatin sst(4) receptor protein in selected human brain areas: an immunohistochemical study. Brain Res Mol Brain Res *82*, 114-125.

Shen, L. P., and Rutter, W. J. (1984). Sequence of the human somatostatin I gene. Science *224*, 168-171.

Shin, Y., Kumar, U., Patel, Y., Patel, S. C., and Sidhu, A. (2003). Differential expression of D2-like dopamine receptors in the kidney of the spontaneously hypertensive rat. J Hypertens *21*, 199-207.

Sidhu, A., Kumar, U., Uh, M., and Patel, S. (1998). Diminished expression of renal dopamine D1A receptors in the kidney inner medulla of the spontaneously hypertensive rat. J Hypertens *16*, 601-608.

Siler, T. M., Yen, S. C., Vale, W., and Guillemin, R. (1974). Inhibition by somatostatin on the release of TSH induced in man by thyrotropin-releasing factor. J Clin Endocrinol Metab *38*, 742-745.

Somvanshi, R. K., Billova, S., Kharmate, G., Rajput, P. S., and Kumar, U. (2009). C-tail mediated modulation of somatostatin receptor type-4 homo- and heterodimerizations and signaling. Cell Signal *21*, 1396-1414.

Somvanshi, R. K., Chaudhari, N., Qiu, X., and Kumar, U. (2011a). Heterodimerization of beta2 adrenergic receptor and somatostatin receptor 5: Implications in modulation of signaling pathway. J Mol Signal *6*, 9.

Somvanshi, R. K., War, S. A., Chaudhari, N., Qiu, X., and Kumar, U. (2011b). Receptor specific crosstalk and modulation of signaling upon heterodimerization between beta1-adrenergic receptor and somatostatin receptor-5. Cell Signal *23*, 794-811.

Soriano, F. X., Papadia, S., Hofmann, F., Hardingham, N. R., Bading, H., and Hardingham, G. E. (2006). Preconditioning doses of NMDA promote neuroprotection by enhancing neuronal excitability. J Neurosci *26*, 4509-4518.

Sotnikova, T. D., Beaulieu, J. M., Gainetdinov, R. R., and Caron, M. G. (2006). Molecular biology, pharmacology and functional role of the plasma membrane dopamine transporter. CNS Neurol Disord Drug Targets *5*, 45-56.

Sperk, G., Reynolds, G. P., and Riederer, P. (1987). HPLC analysis of somatostatin related peptides in putamen of Huntington's disease patients. J Neural Transm *69*, 153-160.

Srikant, C. B., and Patel, Y. C. (1981). Somatostatin receptors: identification and characterization in rat brain membranes. Proc Natl Acad Sci U S A *78*, 3930-3934.

Steinman, L. (1996). Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. Cell *85*, 299-302.

Sun, Y., Savanenin, A., Reddy, P. H., and Liu, Y. F. (2001). Polyglutamine-expanded huntingtin promotes sensitization of N-methyl-D-aspartate receptors via post-synaptic density 95. J Biol Chem *276*, 24713-24718.

Sun, Z., Del Mar, N., Meade, C., Goldowitz, D., and Reiner, A. (2002). Differential changes in striatal projection neurons in R6/2 transgenic mice for Huntington's disease. Neurobiol Dis *11*, 369-385.

Sun, Z., Wang, H. B., Deng, Y. P., Lei, W. L., Xie, J. P., Meade, C. A., Del Mar, N., Goldowitz, D., and Reiner, A. (2005). Increased calbindin-D28k immunoreactivity in striatal projection neurons of R6/2 Huntington's disease transgenic mice. Neurobiol Dis *20*, 907-917.

Sunahara, R. K., Guan, H. C., O'Dowd, B. F., Seeman, P., Laurier, L. G., Ng, G., George, S. R., Torchia, J., Van Tol, H. H., and Niznik, H. B. (1991). Cloning of the gene for a human dopamine D5 receptor with higher affinity for dopamine than D1. Nature *350*, 614-619.

Surmeier, D. J., Reiner, A., Levine, M. S., and Ariano, M. A. (1993). Are neostriatal dopamine receptors co-localized? Trends Neurosci *16*, 299-305.

Susini, C., and Buscail, L. (2006). Rationale for the use of somatostatin analogs as antitumor agents. Ann Oncol *17*, 1733-1742.

Svenningsson, P., Lindskog, M., Ledent, C., Parmentier, M., Greengard, P., Fredholm, B. B., and Fisone, G. (2000). Regulation of the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa in vivo by dopamine D1, dopamine D2, and adenosine A2A receptors. Proc Natl Acad Sci U S A *97*, 1856-1860.

Svenningsson, P., Nairn, A. C., and Greengard, P. (2005). DARPP-32 mediates the actions of multiple drugs of abuse. Aaps J *7*, E353-360.

Svenningsson, P., Nishi, A., Fisone, G., Girault, J. A., Nairn, A. C., and Greengard, P. (2004). DARPP-32: an integrator of neurotransmission. Annu Rev Pharmacol Toxicol *44*, 269-296.

Tallent, M., Liapakis, G., O'Carroll, A. M., Lolait, S. J., Dichter, M., and Reisine, T. (1996). Somatostatin receptor subtypes SSTR2 and SSTR5 couple negatively to an L-type Ca2+ current in the pituitary cell line AtT-20. Neuroscience *71*, 1073-1081.

Tallent, M. K., and Qiu, C. (2008). Somatostatin: an endogenous antiepileptic. Mol Cell Endocrinol *286*, 96-103.

Tannenbaum, G. S., Zhang, W. H., Lapointe, M., Zeitler, P., and Beaudet, A. (1998). Growth hormone-releasing hormone neurons in the arcuate nucleus express both Sst1 and Sst2 somatostatin receptor genes. Endocrinology *139*, 1450-1453.

Tiberi, M., Jarvie, K. R., Silvia, C., Falardeau, P., Gingrich, J. A., Godinot, N., Bertrand, L., Yang-Feng, T. L., Fremeau, R. T., Jr., and Caron, M. G. (1991). Cloning, molecular characterization, and chromosomal assignment of a gene encoding a second D1 dopamine receptor subtype: differential expression pattern in rat brain compared with the D1A receptor. Proc Natl Acad Sci U S A *88*, 7491-7495.

Tolis, G., Angelopoulos, N. G., Katounda, E., Rombopoulos, G., Kaltzidou, V., Kaltsas, D., Protonotariou, A., and Lytras, A. (2006). Medical treatment of acromegaly: comorbidities and their reversibility by somatostatin analogs. Neuroendocrinology *83*, 249-257.

Tomioka, R., Okamoto, K., Furuta, T., Fujiyama, F., Iwasato, T., Yanagawa, Y., Obata, K., Kaneko, T., and Tamamaki, N. (2005). Demonstration of long-range GABAergic connections distributed throughout the mouse neocortex. Eur J Neurosci *21*, 1587-1600.

Torres-Peraza, J., Pezzi, S., Canals, J. M., Gavalda, N., Garcia-Martinez, J. M., Perez-Navarro, E., and Alberch, J. (2007). Mice heterozygous for neurotrophin-3 display enhanced vulnerability to excitotoxicity in the striatum through increased expression of N-methyl-D-aspartate receptors. Neuroscience *144*, 462-471.

Valjent, E., Corvol, J. C., Pages, C., Besson, M. J., Maldonado, R., and Caboche, J. (2000). Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. J Neurosci *20*, 8701-8709.

Van Tol, H. H., Bunzow, J. R., Guan, H. C., Sunahara, R. K., Seeman, P., Niznik, H. B., and Civelli, O. (1991). Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. Nature *350*, 610-614.

Venance, L., Glowinski, J., and Giaume, C. (2004). Electrical and chemical transmission between striatal GABAergic output neurones in rat brain slices. J Physiol *559*, 215-230.

Vezzani, A., and Hoyer, D. (1999). Brain somatostatin: a candidate inhibitory role in seizures and epileptogenesis. Eur J Neurosci *11*, 3767-3776.

Videau, C., Hochgeschwender, U., Kreienkamp, H. J., Brennan, M. B., Viollet, C., Richter, D., and Epelbaum, J. (2003). Characterisation of [125I]-Tyr0DTrp8-somatostatin binding in sst1- to sst4- and SRIF-gene-invalidated mouse brain. Naunyn Schmiedebergs Arch Pharmacol *367*, 562-571.

Vincent, S. R., and Johansson, O. (1983). Striatal neurons containing both somatostatin- and avian pancreatic polypeptide (APP)-like immunoreactivities and NADPH-diaphorase activity: a light and electron microscopic study. J Comp Neurol *217*, 264-270.

Viollet, C., Vaillend, C., Videau, C., Bluet-Pajot, M. T., Ungerer, A., L'Heritier, A., Kopp, C., Potier, B., Billard, J., Schaeffer, J.*, et al.* (2000). Involvement of sst2 somatostatin receptor in locomotor, exploratory activity and emotional reactivity in mice. Eur J Neurosci *12*, 3761-3770.

Vonsattel, J. P., Myers, R. H., Stevens, T. J., Ferrante, R. J., Bird, E. D., and Richardson, E. P., Jr. (1985). Neuropathological classification of Huntington's disease. J Neuropathol Exp Neurol *44*, 559-577.

Wagey, R., Hu, J., Pelech, S. L., Raymond, L. A., and Krieger, C. (2001). Modulation of NMDA-mediated excitotoxicity by protein kinase C. J Neurochem *78*, 715-726.

Walaas, S. I., Aswad, D. W., and Greengard, P. (1983). A dopamine- and cyclic AMP-regulated phosphoprotein enriched in dopamine-innervated brain regions. Nature *301*, 69-71.

Walaas, S. I., and Greengard, P. (1984). DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. I. Regional and cellular distribution in the rat brain. J Neurosci *4*, 84-98.

Wang, X. P., Norman, M. A., Yang, J., Cheung, A., Moldovan, S., Demayo, F. J., and Brunicardi, F. C. (2004). Double-gene ablation of SSTR1 and SSTR5 results in hyperinsulinemia and improved glucose tolerance in mice. Surgery *136*, 585-592.

War, S. A., Somvanshi, R. K., and Kumar, U. (2011). Somatostatin receptor-3 mediated intracellular signaling and apoptosis is regulated by its cytoplasmic terminal. Biochim Biophys Acta *1813*, 390-402.

Watt, H. L., Kharmate, G., and Kumar, U. (2008). Biology of somatostatin in breast cancer. Mol Cell Endocrinol *286*, 251-261.

Waxman, E. A., and Lynch, D. R. (2005). N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. Neuroscientist *11*, 37-49.

Weckbecker, G., Lewis, I., Albert, R., Schmid, H. A., Hoyer, D., and Bruns, C. (2003). Opportunities in somatostatin research: biological, chemical and therapeutic aspects. Nat Rev Drug Discov *2*, 999-1017.

White, R. E., Schonbrunn, A., and Armstrong, D. L. (1991). Somatostatin stimulates Ca(2+)-activated K+ channels through protein dephosphorylation. Nature *351*, 570-573.

Wilson, R. S., Como, P. G., Garron, D. C., Klawans, H. L., Barr, A., and Klawans, D. (1987). Memory failure in Huntington's disease. J Clin Exp Neuropsychol *9*, 147-154.

Wulfsen, I., Meyerhof, W., Fehr, S., and Richter, D. (1993). Expression patterns of rat somatostatin receptor genes in pre- and postnatal brain and pituitary. J Neurochem *61*, 1549-1552.

Xu, J., Zhang, Q. G., Li, C., and Zhang, G. Y. (2007). Subtoxic N-methyl-D-aspartate delayed neuronal death in ischemic brain injury through TrkB receptor- and calmodulin-mediated PI-3K/Akt pathway activation. Hippocampus *17*, 525-537.

Yang, S.-K., Parkington, H. C., Epelbaum, J., Keating, D. J., and Chen, C. (2007). Somatostatin decreases voltage-gated Ca2+ currents in GH3 cells through activation of somatostatin receptor 2. Am J Physiol Endocrinol Metab *292*, E1863-1870.

Yankner, B. A. (1996). Mechanisms of neuronal degeneration in Alzheimer's disease. Neuron *16*, 921-932.

Yoshioka, H., Niizuma, K., Katsu, M., Sakata, H., Okami, N., and Chan, P. H. (2011). Consistent injury to medium spiny neurons and white matter in the mouse striatum after prolonged transient global cerebral ischemia. J Neurotrauma *28*, 649-660.

Young, A. B., Greenamyre, J. T., Hollingsworth, Z., Albin, R., D'Amato, C., Shoulson, I., and Penney, J. B. (1988). NMDA receptor losses in putamen from patients with Huntington's disease. Science *241*, 981-983.

Zappone, C. A., and Sloviter, R. S. (2001). Commissurally projecting inhibitory interneurons of the rat hippocampal dentate gyrus: a colocalization study of neuronal markers and the retrograde tracer Fluoro-gold. J Comp Neurol *441*, 324-344.

Zeron, M. M., Chen, N., Moshaver, A., Lee, A. T., Wellington, C. L., Hayden, M. R., and Raymond, L. A. (2001). Mutant huntingtin enhances excitotoxic cell death. Mol Cell Neurosci *17*, 41-53.

Zeron, M. M., Hansson, O., Chen, N., Wellington, C. L., Leavitt, B. R., Brundin, P., Hayden, M. R., and Raymond, L. A. (2002). Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron *33*, 849-860.

Zhang, T. A., Hendricson, A. W., and Morrisett, R. A. (2005). Dual synaptic sites of D(1)-dopaminergic regulation of ethanol sensitivity of NMDA receptors in nucleus accumbens. Synapse *58*, 30-44.