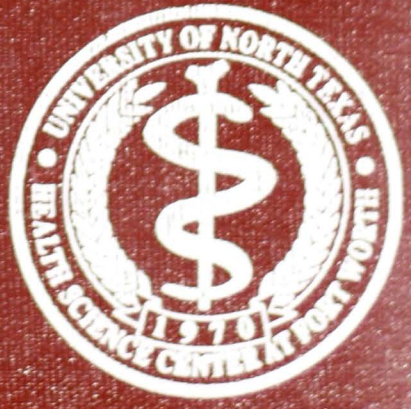
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SIGMA-1 RECEPTOR SIGNALING IN THE EYE. Kissaou T. Tchedre, Department of Biomedical Sciences, University of North Texas Health Sciences Center Fort Worth, TX 76107.

SUMMARY

The sigma-1 receptor is a discovered transmembrane protein that mediates the regulation of ion channels. Sigma-1 receptor ligands have exhibited a wide variety of actions in the central nervous system including attenuation of the neuronal death associated with glutamate excitotoxicity both *in vitro* and *in vivo*. Although the sigma-1 receptor was cloned almost a decade ago, the molecular mechanism of the neuroprotective effect remains to be elucidated. In the current proposal it was hypothesized that ***activation of sigma-1 receptors promotes retinal ganglion cells survival by decreasing calcium signaling pathways and factors linked to cell death.***

We showed by the ratiometric calcium imaging and patch clamp techniques that sigma-1 receptor activation could inhibit both calcium influx and intracellular calcium mobilization. The results showed that sigma-1 receptor overexpressing RGC-5 cells also had a lower glutamate-induced intracellular calcium mobilization compared to non-overexpressing RGC-5 cells. The survival assay data showed that the sigma-1 receptor agonist, (+)-SKF10047 protected RGC-5 cells from glutamate-induced cell death. Moreover, sigma-1 receptor overexpressing RGC-5 cells showed a significant resistance to glutamate-induced apoptosis compared to the control RGC-5 cells. The sigma-1 receptor neuroprotective mechanism also included the down regulation of Bax, and caveolin-1 protein expression levels and inhibited caspase-3 activation. We also demonstrated for the first time using a co-immunoprecipitation technique, the association

between L-type calcium channels and sigma-1 receptors. Thus sigma-1 receptor ligands may indirectly influence the voltage-gated calcium channels by interacting with the sigma-1 receptor associated voltage-gated calcium channel complex.

In conclusion, activation of sigma-1 receptors can regulate calcium homeostasis and signaling in retinal ganglion cells. Activation of sigma-1 receptors regulate intracellular calcium levels and pro-apoptotic gene expression to promote retinal ganglion cell survival. Sigma-1 receptor ligands may be neuroprotective and targets for potential glaucoma therapeutics.

SIGMA-1 RECEPTORS SIGNALING IN THE EYE.

Kissaou T. Tchedre, B.S., M.S.

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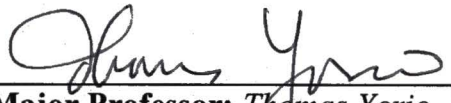
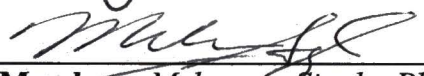
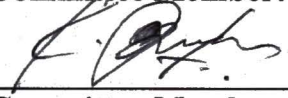


Major Professor: *Thomas Yorio, Ph.D.*

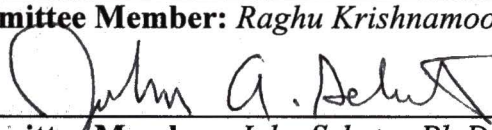


Committee Member: *Meharvan Singh , Ph.D.*

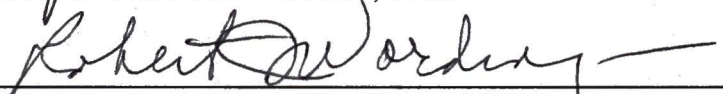
Committee Member: *Tina Machu , Ph.D.*



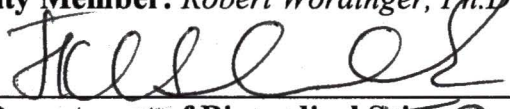
Committee Member: *Raghu Krishnamoorthy , Ph.D.*



Committee Member: *John Schetz , Ph.D.*



University Member: *Robert Wordinger, Ph.D.*



Chair, Department of Biomedical Sciences: *Jamboor K. Vishwanatha, Ph.D.*

Dean, Graduate School of Biomedical Sciences: *Jamboor K. Vishwanatha, Ph.D.*

SIGMA-1 RECEPTOR SIGNALING IN THE EYE.

Ph.D. DISSERTATION

Presented to the graduate council of the University of North Texas Health Sciences Center at Fort Worth.

In partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

Kissaou T. Tchedre, B.S. M.S.

Department of Biomedical Sciences
UNTHSC, Fort Worth, Texas 76107.

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"We do not know what are the right questions to ask, and we often do not find out until we are close to the answer."
Steven Weinberg

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CHAPTER 1

INTRODUCTION

The introduction is divided in three sections – the first part provides an extensive overview on sigma receptor biology, the second section provides a broad overview on sigma receptor expression in the eye and other organs, and the third section describes the biological functions of sigma-1 receptors and retinal ganglion cells and the clinical relevance associated with such interactions.

I - Sigma receptor biology

Sigma receptors (accession number NP 005857) were first described as one of the subtypes of opioid receptors. However, further studies have led to the complete distinction between sigma receptors from opioid receptors [1, 2]. Sigma receptors interact with phencyclidine (PCP), and the antipsychotic haloperidol. However, neither phencyclidine nor haloperidol have any interaction with opioid receptors. The sigma-1 receptor was first cloned from guinea pig liver by Hanner et al., [3]. Subsequently, using sequence homology from guinea pig sigma-1 receptors cDNA, human, mouse, and rat sigma-1 receptors were cloned [4-7]. Throughout the species, sigma-1 receptors show about 93% homology [6]. There is no sequence homology between sigma receptors and other known mammalian receptors or opioid receptors. However, the sigma-1 receptor closely resembles a C-8, 7 sterol isomerase found in fungi [3]. The putative structure of the sigma-1 receptor is a single polypeptide comprising 223 amino acids with the hydrophobicity plot predicting two membrane-spanning regions. Both the N and C terminals face the cytoplasm [1]. The longer C-terminal end has almost complete homology with the sterol-binding fungal isomerase. There is also a typical arginine-

arginine endoplasmic reticulum (ER)-locating signal near the N terminal end [1, 8]. The sigma-1 receptor gene, which is ~ 7 kilo base pairs long (kbp), contains four exons, which are interrupted by three introns. Exon 3, the shortest, is 93 base pairs (bp) long, while exon 4, the longest, is 1,132 bp long [9]. Using 5' rapid amplification of cDNA end reaction with mRNA from the human trophoblast cell line, it was possible to show that the initiation site for transcription is located at a site 56 bp upstream of the translation start codon [9]. The sigma 1 receptor gene promoter is TATA less. However, it has a CCAATC box and several GC boxes that are recognition sites for SP1, and many other transcription factors [9]. Alternative splicing has been observed for sigma receptors and five transcript variants, each encoding a distinct protein, have been identified [10]. However, only two sigma receptor subtypes have been characterized so far by ligand binding assay and photo affinity labeling. The sigma-1 receptor has a molecular weight of approximately 25 kDa and the sigma -2 receptor has a molecular weight of (~ 18-21 kDa) [3, 11, 12]. Sigma-2 receptors were first characterized in pheochromocytoma PC12 cells [11, 13]. In addition to these two sigma receptors subtypes, a sigma-3 receptor subtype was also proposed some years ago [14, 15]. There are less data available about sigma-2 receptors and sigma-3 receptors. Recently, sigma-2 receptors have been shown to be a biomarker for solid tumor proliferation [16]. In addition, sigma-2 receptors have been shown to be associated with cancer cell proliferation [16, 17]. The sigma-1 receptor subtype, however, is the most studied and increasing evidence suggests that it doesn't possess the characteristics of a true G protein coupled receptor, which has seven transmembrane domains. However, sigma-1 receptors show a well-characterized profile with high affinity ligands, obtained through several related pharmacological tests that

include selective agonists such as pentazocine, (+)-SKF10047 and antagonists such BD1047, and N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy) phenyl]-ethylamine monohydrochloride) [NE-100]. Sigma-1 receptors have been postulated to be associated with cellular functions of various tissues associated with the endocrine, immune, and nervous systems.

The endogenous ligand of sigma-1 receptors is not known. However, it has been suggested that neurosteroids might be sigma receptor endogenous ligands [18]. The first evidence for an effective interaction between steroids and sigma-1 receptors was provided by *in vitro* binding studies. Among the steroids studied, progesterone was shown to be the most potent competitor of [^{3}H]-(+)-SKF10047, a prototypical sigma-1 receptor agonist, binding in the guinea pig brain and [^{3}H]-haloperidol binding in the spleen, with K_i values in the 300 nM range [19]. Progesterone also inhibited [^{3}H]-(+)-SKF10047 binding in the rat brain membrane preparations [19]. Progesterone also binds with the highest affinity to human sigma-1 receptors, with a reported affinity (K_i) as high as 30 nM while the other steroids exhibit lower affinity [20]. For this reasons, sigma-1 receptors have been proposed as a link between the central nervous system and the endocrine and reproductive systems [18].

Hayashi and collaborators have shown that sigma-1 receptors are localized on both the endoplasmic reticulum (ER) and the plasma membrane [21]. In addition, Hayashi and collaborators have shown that sigma receptors translocate to the endoplasmic reticulum-associated reticular network and subsequently to the cell periphery upon stimulation [21]. Recently, a complex formation has been shown between sigma-1 receptors at the mitochondria-associated ER membrane (MAM) with a

chaperone, BiP. Sigma-1 receptors dissociate from BiP when ER Ca^{2+} depletion occurs or via ligand stimulation. This dissociation leads to a prolonged Ca^{2+} signaling into mitochondria via IP3 receptors, which can lead to the ER stress [22]. Under ER stress or calcium overload, sigma-1 receptors translocate to the cell periphery. The translocation of sigma-1 receptors in cells counteracts the ER stress response to promote cell survival [22]. Sigma-1 receptors are also involved in lipid raft modeling by altering cholesterol distribution in lipid rafts in breast cancer cell lines [23]. Cholesterol recognition domains in the COOH terminus of the sigma-1 receptor have been identified and characterized. The cholesterol-binding domains are part of the sigma-1 receptor drug-binding site since, cholesterol binding to sigma-1 receptors can be blocked by sigma-1 receptor ligands or by mutations of the sigma-1 receptor binding site [23]. Recently, regions of the sigma-1 receptor ligand binding site(s) were identified using a novel photoprobe. The sigma-1 receptor ligand binding domains were shown to be located in a peptide containing steroid binding domain-like I (SBDLI) (amino acids 91–109) and in a peptide containing steroid binding domain-like II (SBDLII) (amino acids 176–194) [24]. The anticonvulsant phenytoin has also been shown to bind to an associated allosteric site on the sigma site complex [25, 26].

Sigma-1 receptor knockout mice were generated by Montoliu et *al.* [27]. Although sigma-1 receptors are broadly expressed in many tissues and organs, homozygous mutant mice are viable, fertile and do not display any overt phenotype, compared with their wild-type littermates, in mixed genetic backgrounds. However, upon challenge with N-allylnormetazocine ((+)-SKF-10047), a significant decrease in the hypermotility response was observed in knockout mice. That observation is in agreement

with the involvement of sigma 1-receptors in the induction of the psychostimulant actions. The activity of sigma-2 receptors seems to be unaffected in sigma-1 mutant mice. These knockout mice could contribute to a better understanding of the *in vivo* role of sigma-receptors [27].

II - Sigma receptor expression in the eye and in other systems.

Sigma-1 receptors have been shown to be expressed in lachrymal gland, retina [28], iris-ciliary body, cornea and lens in the eye [8] similar to that demonstrated in other tissues [18, 29]. Retinal ganglion cells also express sigma-1 receptors [30]. Recently, the expression pattern of sigma-1 receptors was characterized in brain. *In situ* hybridization studies demonstrated that sigma-1 receptor mRNA is detectable primarily in the cerebral cortex, hippocampus, and Purkinje cells of the cerebellum [7]. It was suggested that the localization of sigma-1 receptors in these cells might be useful in the regulation of neuronal activity. Immunohistochemical studies of sigma receptors in rat brain demonstrated high levels of immunostaining associated with neurons located in the granular layer of the olfactory bulb, various hypothalamic nuclei, the septum, the central gray matter, motor nuclei of the hindbrain, and the dorsal horn of the spinal cord [31, 32]. The respective distribution of the sigma-1 receptor and sigma-2 receptor binding sites in the brain of various species including mouse, rat, guinea pig, cat, monkey and human have been demonstrated using receptor binding studies of membrane homogenates and quantitative autoradiography [33-43]. However, the anatomical distribution of the sigma-1 receptor mRNA in the mammalian brain and the specific role of the sigma-1 receptor subtypes in the central nervous system remains to be fully characterized.

III - Sigma-1 receptor signaling

The functions of sigma receptors are poorly understood mainly because an endogenous ligand has yet to be identified. The sigma receptor gene encodes a receptor protein that interacts with a variety of psychotomimetic drugs, including cocaine and amphetamines [12, 31]. Activation of sigma receptors by an agonist ligand may induce hallucinogenic effects and also may be responsible for the paradoxical convulsions sometimes seen in opiate overdose. Drugs known to be sigma receptor agonists in addition to their major mechanisms of action, include cocaine, heroin, PCP, fluvoxamine methamphetamine and dextromethorphan [12, 19, 44]. Sigma-1 receptors exhibit high affinity and stereoselectivity for ligands such as pentazocine, N-allylnormetazocine ((+)-SKF-10 047), and dextromethorphan, while sigma-2 receptor have lower affinity and are not selective for these compounds [19]. Haloperidol, DTG, and (+)-3-PPP are non-discriminating ligands with high affinity for both sigma-1 and 2 receptors. Currently, there are selective and high affinity sigma-2 receptor ligands such as LU 28-179 [45], and BD1008 [46]. **Table 1** summarizes the nomenclature of some of the sigma receptor ligands with their respective affinities values generated in whole tissue and from cloned human sigma-1 receptor expressed in MCF-7 cells [44]. **Table 2** summarizes the affinities of two sigma receptors antagonists [47].

Table 1. Affinities values of sigma receptors ligands from whole tissue and cloned human sigma-1 receptor [44].

Compound names	K_i values for sigma-1 receptor	K_i values measured in whole tissue	Average K_i values from whole tissue
(+)-SKF10047	250 ± 79.1	9.15	250 ± 339
BD1063	3.11 ± 2.40		9

Table 2. Affinities of two sigma receptors antagonists [47].

Compounds names	Sigma-1 receptor	Sigma-2 receptor	Sigma-1 receptor / Sigma-2 receptor
BD1063	9.15 ± 1.28	449 ± 11	0.020
BD1047	0.93 ± 0.14	47 ± 0.60	0.019

The transport pathway for exogenous sigma-1 receptor ligands throughout the cell membrane is still unclear [24]. Currently, two uptake pathways for sigma receptor ligands have been suggested in cultured neuronal cells [48]. The uptake of sigma-1 receptors ligands was shown to be temperature and energy dependant [48]. Sigma-1 receptors have multiple binding/storage sites inside the cells. These multiple binding/storage sites are involved in different cellular signaling caused by various sigma-1 receptor ligands [48, 49]. Sigma-1 receptors are allosterically modulated by phenytoin, insensitive to the modulatory effect of guanosine triphosphate (GTP) [50] and are down regulated by sub chronic treatment with haloperidol [51, 52]. There is less information available on sigma-2 receptors, which exhibit low affinity for (+)-benzomorphans [46]. Sigma-2 receptors also mediate several functions such as regulation of motor functions, induction of dystonia [53, 54], and blockade of tonic potassium channels [55].

Sigma-1 receptors are involved in a wide range of physiological processes such as cognition, locomotion, cellular differentiation, and neuroprotection [56, 57]. Sigma receptors are believed to play an important role in the cellular functions of various tissues associated with the endocrine, immune, and nervous systems. One of the major roles of sigma receptors is to exert a potent modulation on different neurotransmitter systems, including modulation of NMDA receptor mediated responses [58], cholinergic responses [59], regulation of IP3 receptors, potassium channels [60], voltage-gated calcium channels [61], calcium signaling at the endoplasmic reticulum, mobilization of cytoskeleton adaptor proteins, potentiation of nerve growth factor-induced neurite sprouting, decrease of neurotransmitter release and neuronal firing [62-64]. Several sigma receptor selective ligands possess important neuroprotective ability. For example, in vitro

exposure of cultured rat brain neurons to selective sigma receptor agonists protects cells against glutamate or NMDA exposure [65]. Similarly, sigma receptor activation protect retinal ganglion cells from glutamate and homocysteine excitotoxicity [66]. Sigma-1 receptor agonists also inhibit agonist-stimulated phospholipase C activity in rat brain tissue [67], and in adrenal medullar cells sigma receptors are activated upon the stimulation of the glutamatergic, and dopaminergic systems [68, 69]. Since sigma-1 receptor are activated upon the stimulation of the glutamatergic, dopaminergic, IP3-related metabotropic, or nerve growth factor-related systems. Hayashi et al. hypothesized that sigma-1 receptors create a supersensitized state for signal transduction in the biological system in response to calcium overload. This supersensitized state may be part of the protective mechanism of sigma-1 receptor [70]. Nicotine-stimulated catecholamine release and increased the intracellular calcium (Ca^{2+}) levels were also inhibited by sigma-1 receptor agonists. Sigma receptors can modulate signal transduction pathways involving calcium [71]. **Diagram 1** depicts the cellular signaling pathways involved in sigma-1 receptor neuroprotection.

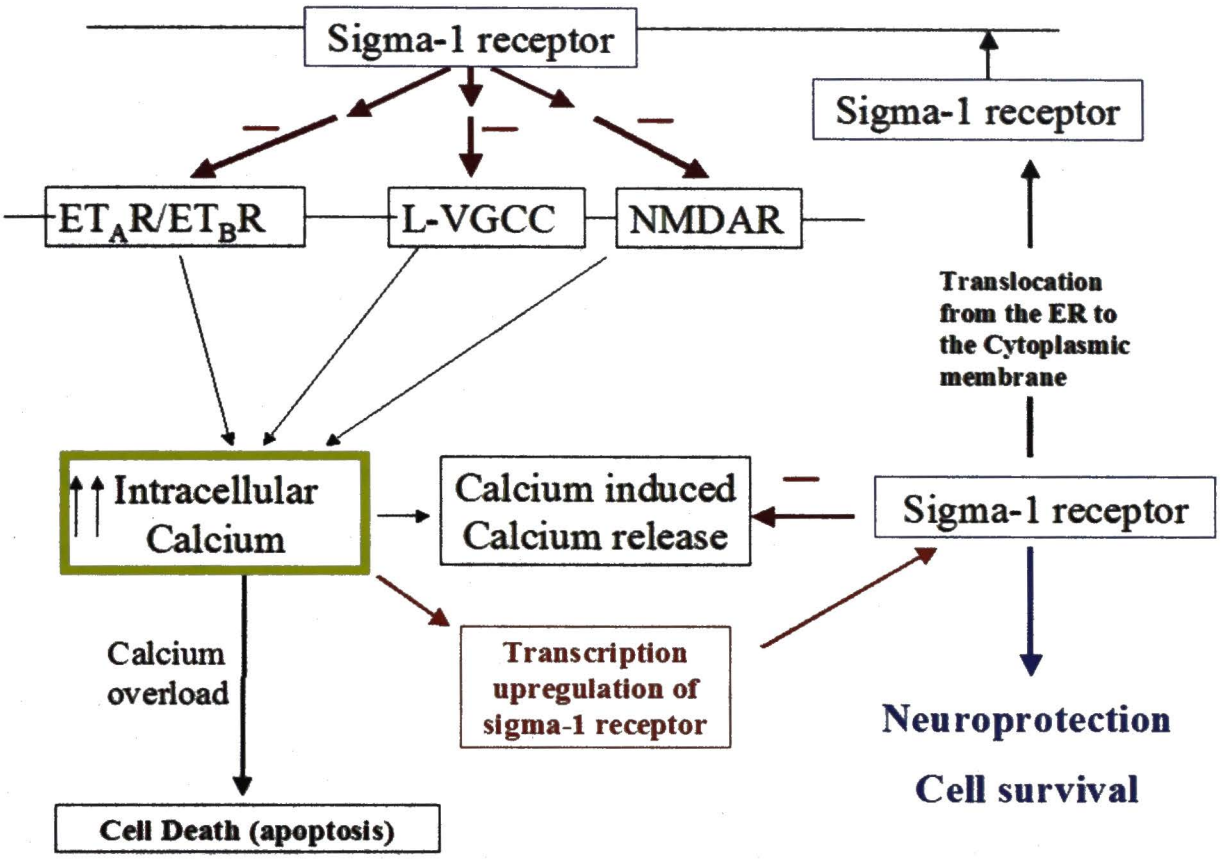

Diagram 1. The proposed mechanism of cellular signaling pathways involved in sigma-1 receptor neuroprotection action.

Multiple stimuli lead to the expression and activation of sigma-1 receptors (S1R). Activation of N-methyl-D-aspartate receptors (NMDAR), or L-type voltage-gated calcium channels (L-VGCCs) allows the influx of calcium. Endothelin-1 through ET_AR/ET_BR activates phospholipase C (PLC), which cleaves phosphatidylinositol- 4,5-biphosphate to form diacylglycerol (DAG and inositol-1, 4,5-triphosphate (IP_3). IP_3 through IP_3 receptors (IP_3R), located on the endoplasmic reticulum (ER), stimulates calcium release from ER stores. Increased intracellular calcium levels affect the transcription of calcium responsive elements containing genes by activating transcription factors. Increased intracellular calcium levels may lead to the transcription and/or translocation of sigma-1 receptors from the ER to the plasma membrane where it decreases the excitatory neurotransmitter-induced Ca^{2+} influx. By controlling calcium levels, sigma-1 receptors regulate the transcription of many genes to promote cell survival.

Sigma receptors show an unusual promiscuous ability not only to bind a variety of drugs, but also to associate with a surprisingly large number of critical cellular components. These include the endoplasmic reticulum IP3 receptor calcium channel, voltage sensitive potassium channels [68], and the cytoskeletal element ankyrin [1, 21]. In addition to calcium homeostasis regulation, the neuroprotective mechanism of sigma-1 receptors *in vivo* involves the attenuation of Neuronal Nitric Oxide Synthase (nNOS) activity and ischemia-evoked NO production [72, 73]. Moreover, sigma-1 receptors reduce cell death *in vitro* by a mechanism involving receptor-dependent preservation of protective genes such as bcl-2 [74]. Furthermore, sigma receptors have also been shown to modulate the activity of the eye, by lowering the IOP and protecting RGCs from apoptosis and in the lens, by modulating cell growth and pigmentation [75].

Flunarizine, a selective calcium entry blocker, has been shown to lower the intraocular pressure (IOP) through sigma-1 receptor when applied topically. Flunarizine, through sigma-1 receptor lower the intraocular pressure by inhibiting first norepinephrine release through ocular sigma receptors, and second by inhibiting cAMP accumulation. These effects of sigma ligands were sensitive to *N, N-dipropyl-2- [4-methoxy-3-2phenylethoxy) phenyl]-ethylamine* (NE-100), a sigma 1 receptor antagonist [76, 77]. The lowering of intraocular pressure by the Sigma-1 agonist (+)-pentazocine was effective in both normotensive rabbits and in the α-chymotrypsin model of hypertension [76]. Moreover, the IOP lowering effect was dose-dependent and occurred in the absence of ocular inflammation or change in pupil diameter. It also appeared that sigma receptors may be responsible for the intraocular pressure lowering ability of a number of other

agents [77]. Due to their large spectrum of action, sigma receptors have recently been a target of drug development related to psychiatric and neurological disorders.

Sigma receptors interaction with other receptors

Sigma receptors have the ability to interact with a huge range of different classes of drugs. There are also reports on the effect of sigma receptor ligands on other neurotransmission systems such as GABAergic neurotransmission. It has been reported that (+)-SKF10047 and opioid receptor agonists inhibited potassium-induced [^{3}H]-GABA efflux in rat substantia nigra slices [78]. In addition it has been shown that the sigma receptor ligand DTG inhibited the firing rate of GABA-containing midbrain inter-neuron in anesthetized rats [79]. These few reports suggest that sigma receptors may act as negative regulators of the GABA receptors or that the ligands may have direct action on GABA transmission.

In several models sigma receptors have been shown to potentiate differentially the cholinergic systems according to the brain structure [80-82]. Behavioral and neurochemical data have shown that sigma receptors ligands regulate dopaminergic system [44, 83].

It is possible that sigma-1 receptor ligands used in our research project interact with others receptors or ion channel complexes. But the micromolar concentrations used are in the affinity range of sigma-1 receptors, and therefore, should untoward actions on other receptors.

Sigma Receptors and Cancer

Sigma receptor antagonists have been shown to inhibit proliferation in mammary and colon carcinoma cell lines, and this has led to the development and use of sigma ligands

for diagnostic tumor imaging [84]. The potential effectiveness of sigma receptor drugs on proliferation of tumor cells *in vitro* has been studied using different techniques. The effects of various sigma receptors drugs (haloperidol, DTG, SKF10047, pentazocine, and rimcazole) on the *in vitro* growth of human mammary adenocarcinoma, colon carcinomas, and melanomas have been studied in detail by Brent and Pang [85]. They found using light microscopy, inhibition of cellular proliferation, induction of cell detachment and rounding, characteristic of cell death. Of the drugs tested, the sigma-1 and sigma-2 nonspecific agent rimcazole [86] and the reduced haloperidol, which is the main metabolite of haloperidol in humans, were the most potent inhibitors of cell proliferation [85, 87]. Sigma drugs such as (*N*- [2-(piperidino) ethyl]-2-iodobenzamide (2-IBP), haloperidol, and 2-piperidinyl- (aminoethyl)-4-iodobenzamide (IPAB)] also had similar inhibitory effects on small-cell lung cancer (NCI-H209 and NCI-N417) cells [88]. In addition, IPAB or 2-IBP also inhibited the *in vivo* xenograft proliferation of NCI-N417 cells [88]. Sigma receptors inhibit cell proliferation by inhibiting potassium (K+) channels and promoting p27kip1 accumulation [89].

Sigma-1 receptors are also involved in the regulation of endothelial cell proliferation and angiogenesis. Because of their potent effect on cancer cell proliferation, sigma-1 receptor ligands are a promising target for oncology applications [1, 84, 86]. Other areas currently being investigated include treatment of gastrointestinal, cardiovascular, endocrine and immune system disorders.

Taken together, the above information highlights an important yet largely unexplored but promising area of research to examine the biological function and therapeutic potential of sigma receptors [19, 90].

Disease involvement

Sigma-1 receptors, either through the use of agonists, antagonists or expression markers are the subject of clinical trials in fields as diverse as drug addiction [90, 91] and cancer treatment [1, 84]. Sigma receptor polymorphisms have been shown to be associated with alcoholism [92]. There are at present no such trials underway in the ophthalmology field, but there are promising *in vitro* studies indicating that sigma agonists may alleviate some of the problems associated with glaucoma [30]. Sigma-1 receptor agonist may also prove to be useful in preventing cataracts [75].

The sigma receptor gene is located on chromosome: 9, band p13.3, a region known to be associated with different types of psychiatric disorders [9]. Some preclinical evidence suggests that sigma-1 receptors, which play several roles in learning and memory, may also be involved in the pathogenesis of Alzheimer's disease (AD) [93]. In addition, binding studies in the CA1 stratum pyramidal region between hippocampi affected by Alzheimer's and normal hippocampi have shown a significant reduction in the densities of [3H]-1,3-di-o-tolylguanidine ([3H]-DTG) in hippocampi affected by Alzheimer's disease [94]. It was also shown that donepezil, a high affinity sigma-1 receptor agonist, can alleviate the memory deficits induced by $A\beta_{25-35}$ peptide injection in mice [95]. Therefore, the development of sigma-1 receptor acting selective drugs may lead to original neuroprotective strategies for the treatment of β-amyloid-induced toxicity [95]. However, since donepezil is a cholinesterase inhibitor, it is likely that part of donepezil's actions is mediated through cholinesterase inhibition. Sigma-1 receptors are also potential

targets in the treatment of cocaine addiction and the decline in cognitive functions associated with old age [90, 96]. A recent report has shown an increase in pre-synaptic function during NMDAR-independent LTP, via a sigma receptor-dependent mechanism in brain slices from early-adolescent chronic intermittent ethanol exposed animals. Sigma receptors and neuroactive steroids have been demonstrated to mediate hippocampal synaptic plasticity, and memory pathway [97]. Sigma-1 receptor agonists have been shown to have a neuroprotective effect, however, sigma-1 receptor antagonists such as haloperidol has been shown to protect the brain against ischemia [98]. Although sigma-1 receptors are well characterized in the CNS, little is known of the role in the eye.

CHAPTER 2

SPECIFIC AIMS, SIGNIFICANCE, EXPERIMENTAL DESIGN

A). SPECIFIC AIMS.

The sigma receptor is a transmembrane protein that has been shown to be involved in a variety of physiological processes such as cognition, locomotion, and neuroprotection. It has been reported that the effect of sigma receptor activation is mediated via modulation of the activity of glutamatergic transmission at the (*N*-methyl-D-aspartate) NMDA receptor complex and voltage gated calcium channels [49, 61]. Calcium influx in neurons can serve as both a signal for survival as well as, in some cases, cell death [99]. It has been shown that differential regulation of calcium channels and NMDA receptor activities can control neuronal survival by regulating transcription factors such as CAAT/enhancer-binding protein (C/EBPs), calcium- dependent kinases such as CaMKIV, and calcium-dependent phosphatases such as calcineurin. In addition, it has been shown that glutamate excitotoxicity is mediated by a rapid increase of C/EBPβ, and the increase activity of calcineurin [100]. Using transformed retinal ganglion cells (RGC-5) and overexpressing sigma-1 receptor retinal ganglion (RGC-5) cells our laboratory was able to show that activation of sigma-1 receptor can inhibit glutamate or potassium chloride (KCl) induced calcium influx. However, the exact function of sigma receptors in the eye remains to be elucidated. Sigma-1 receptors are expressed extensively in retinal ganglion cells, lachrymal gland, iris-ciliary body, cornea and lens in the eye[24, 28]. This suggests that sigma-1 receptors may be playing an important role in the eye and particularly in retinal ganglion cells. We have recently identified sigma-1 receptors in

transformed retinal ganglion cells (RGC-5) that function as a negative regulator of NMDA receptors and the voltage gated L-type calcium channels. Based on the above-mentioned information, ***we hypothesized that Activation of sigma-1 receptors promotes retinal ganglion cells survival by decreasing calcium signaling pathways and factors linked to cell death.***

The overall objective of this proposal was to identify and characterize retinal ganglion cell sigma-1 receptors so that we can understand the functional responses linked to sigma-1 receptors at this site and perhaps in the central nervous system.

To address the objectives of this research project, we pursued the following two specific aims:

***B).* EXPERIMENTAL DESIGN**

Different experiments were designed to investigate specific aims II & I. These experiments are discussed in detail in chapter 3, and 4. However, a brief description of the experiment rational and methodology is provided below.

Study # 1. (*Kissaou T. Tchedre, Thomas Yorio· Sigma-1 receptors protect RGC-5 cells from apoptosis by regulating intracellular calcium, Bax levels, and caspase-3 activation.* ***In press, IOVS.***)

Aim 1: *Determine neuroprotection capabilities of the sigma-1 receptor ligand (+)-SKF10047 in primary retinal ganglion, normal retinal ganglion cells line (RGC-5), and sigma-1 receptor overexpressing RGC-5 cells.*

1a). *Measure the survival of retinal ganglion cells after treating retinal ganglion cells with glutamate in the presence or absence of sigma-1 agonist (+)-SKF10047. A sigma-1*

receptor antagonist (BD1047) was used to confirm that the neuroprotection is mediated by sigma-1 receptors.

1b). *Measure caspase-3 activation after glutamate treatment of retinal ganglion cells. A sigma-1 receptor antagonist (BD1047) was used to confirm that the neuroprotection is mediated by sigma-1 receptor.*

1c). *Measure changes in intracellular calcium concentrations to sigma-1 receptor ligands (agonist and antagonist) in the presence of glutamate.*

Hypothesis:

The hypothesis in this part of the project was that sigma-1 receptor expression and activation occurs following an increase in calcium levels that subsequently leads to retinal ganglion cells survival and/or decreases factors involved in calcium signaling pathways linked to cell death.

Rationale:

Sigma-1 receptor ligands have been shown to exhibit a wide variety of actions in the central nervous system and to prevent neuronal death associated with glutamate excitotoxicity both *in vitro* and *in vivo* [101, 102]. There is not much known about the mechanism of sigma-1 receptor mediated retinal ganglion cell survival. Therefore, the control of the intracellular calcium levels, and factors linked to cell death could represent the signaling involved in sigma-1 receptor-mediated cell survival and neuroprotection.

Results:

These studies allowed us to determine that sigma-1 receptors regulate intracellular calcium levels, and prevent the activation of pro-apoptotic genes to promote retinal

ganglion cell survival. Sigma-1 ligands appear to be neuroprotective and a potential target for neuroprotective therapeutics.

Study # 2: *(Kissaou T. Tchedre, Renqi Huang, Raghu R. Krishnamoorthy, Adnan Dibas, Glenn H. Dillon, and Thomas Yorio. Sigma-1 receptor regulation of voltage-gated calcium channels involves a direct interaction between sigma-1 receptor and voltage-gated L-type calcium channel).* ***Submitted to IOVS.***

Aim 2: *Determine the effect of sigma-1 receptor activation on calcium influx in retinal ganglion cells.*

2a. *Measure the changes in calcium influx to sigma-1 receptor ligands (agonist and antagonist) in the presence of potassium chloride (KCl).*

2b. *Measure the calcium currents in primary retinal ganglion cells in the presence and absence of sigma-1 receptors ligands ((+)-SKF10047, BD1047), and calcium channel blocker nifedipine.*

2c. *Determine if there is any physical interaction between the sigma-1 receptors and voltage gated calcium channels by utilizing the co-immunoprecipitation technique.*

Hypothesis:

The hypothesis in this part of the study was that sigma-1 receptors regulate differentially NMDA and voltage gated calcium channels to promote cell survival in retinal ganglion cells. Regulation of voltage gated calcium channels by sigma-1 receptors might require a

direct coupling between sigma-1 receptors and voltage gated calcium channels as already shown between Kv1.4 potassium channels and sigma-1 receptors [103].

Rationale:

Sigma receptors have been suggested to be involved in the regulation of intracellular calcium. For example, the contractility of cardiac myocytes was affected by sigma receptor ligands [104]. Sigma ligands have also been shown to regulate N-methyl-D-aspartate receptors (NMDAR) in rat primary neurons [49, 58] Sigma receptor ligands also inhibit high voltage-gated calcium channels in sympathetic neurons [59]. However, none of these studies has clearly defined the mechanism by which sigma ligands or sigma-1 receptors control the intracellular calcium levels. It is known that sigma-1 receptors modulate voltage-gated K^+ channels (Kv1.4 or Kv1.5) in different ways in the presence and absence of ligands by serving as auxiliary subunits to voltage-gated K^+ channels [68]. Therefore, regulation of intracellular calcium in RGC by sigma-1 receptors could be mediated via a coupling between calcium channels and sigma-1 receptors or via second messenger signaling.

Results: This study allowed us to determine that sigma-1 receptor activation can regulate calcium influx in retinal ganglion cells. This study also allowed us to report for the first time the association between L-type calcium channels and sigma-1 receptors. Regulation of calcium influx in retinal ganglion cells by sigma-1 receptor ligands may represent in part the neuroprotective effect of sigma-1 receptors.

C). METHODS:

The methodology used in each investigation is described in detail in each of the chapters 3, and 4. However, it is appropriate to discuss these techniques briefly.

Measurement of calcium influx in retinal ganglion cells

In order to determine whether sigma-1 receptor ligand (+)-SKF10047 has any effect on calcium influx in retinal ganglion cells, potassium chloride (KCl) induced calcium influx was measured in control and sigma-1 receptor over-expressing retinal ganglion (RGC-5) cells. The calcium influx in single cells was measured with a Nikon fluorescent microscope using Imaging Metafluor software (Universal Imaging Co., West Chester, PA). KCl (20 mM) was added and changes in fluorescence ratios were recorded. BD1047 (3μM) was used to determine whether the observed calcium influx inhibition was mediated through sigma-1 receptors. Nifedipine (10μM) was used to determine whether the calcium influx was mainly mediated through L-type voltage-gated calcium channels.

Calcium Currents Measurement by Whole Cell Patch Clamp.

Macroscopic calcium currents were measured in rat primary retina ganglion using whole-cell patch clamp [105]. Patch pipettes of borosilicate glass (1B150F, World Precision Instrument, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 12.5 MΩ. The pipette solution contained (mM): 140 CsCl, 10 EGTA, 4 Mg-ATP, and 0.2 Na_3-GTP, pH 7.2. The external bath solution contained (in mM) 125 NaCl, 1 MgCl, 10 HEPES, 5 $CaCl_2$, 10 glucose, 0.5 TTX (μM), pH 7.3. Coverslips containing cells were placed in a small

chamber (~ 1.5 ml) and perfused continuously (7-10 ml/min). The whole-cell Ca^{2+} currents were obtained using a patch clamp amplifier (PC-505B, Warner Instruments, Hamden, CT) equipped with 201B headstage. Currents were evoked with voltage step from -90 to 0 mV for 55 or 200 ms. All recordings were carried out at room temperature (20-21°C). All drugs, including sigma-1 receptor ligands ((+)-SKF10047, BD1047), and L-type voltage-gated calcium channels were applied at room temperature.

Primary rat retinal ganglion cell isolation.

Rat primary retinal ganglion cells were isolated and used as the experimental model in the patch clamp experiment and caspase-3 activation assay. See chapter 3 and chapter 4 for the methodology detail. Primary retinal ganglion cells were characterized by immunocytochemistry for a normally expressed retinal ganglion cell marker, thy-1, and neurofilament-200.

Co-immunoprecipitation of Sigma-1 Receptor and Voltage gated calcium channels:

In order to determine whether the regulation of L-type calcium channels involves a physical interaction between the two receptors, we performed the co-immunoprecipitation. Non-overexpressing and sigma-1 receptor overexpressing retinal ganglion (RGC-5) cells were collected and lysed with ice-cold non-denaturing lysis buffer. The co-immunoprecipitation was achieved using The Exacta Cruz™ (F: sc-45043) immunoprecipitation kit method (as described in Exacta Cruz™ datasheet: http://www.scbt.com/datasheets_list/sc-45043.pdf). After the co-immunoprecipitation, the samples were resolved on a SDS-PAGE gel and immunoblotted via standard methods using anti-sigma-1 receptor antibody (1/500) or anti-human α1C antibody (1/500) (L-type

channel α1C subunit). The blots will be developed using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Retinal ganglion (RGC-5) cells culture and differentiation

RGC-5 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 100 U/ml of penicillin and 100 μg/ml of streptomycin. RGC-5 cells were grown in culture media and incubated at 37°C in 5% CO_2 and air. For all the experiments, cells were differentiated using human non-pigmented ciliary epithelial (HNPE) cells conditioned media collected from 100% confluent T-150 cm^2 flask (Rachel Dauphin et al., 2007). Briefly, 24 hours after seeding the RGC-5 cells the DMEM was removed from the culture dishes and replaced by the HNPE conditioned media (50ml of HNPE conditioned media and 1 ml complete DMEM (10% heat inactivated fetal bovine serum and 100 U/ml of penicillin and 100 μg/ml of streptomycin)). The cultures dishes were then incubated at 37°C in 5% CO_2 and air for 48 hours to allow the cells to differentiate.

Preparation of stably transfected sigma-1 receptor RGC-5 Cells

We prepared sigma-1 receptor overexpressing RGC-5 cells in order to assess the effect of up regulation of sigma-1 receptors in retinal ganglion cells. The lipofectamine ™ 2000 (Invitrogen) method was used for transfection of retinal ganglion (RGC-5) cells as instructed by the manufacturer's guide (http://www.adobe.com/products/acrobat/readstep2.html). Western blot was used to detect the sigma-1 receptor expression using polyclonal anti-sigma-1 receptor antibody.

Calcein-AM/propidium iodide cell survival assay

In order to determine the neuroprotective capabilities of sigma-1 receptor ligands in retinal ganglion cells, we used a cell viability kit containing calcein/propidium iodide (Molecular Probes, Eugene, OR). RGC-5 cells were cultured in complete Dulbecco's Modified Eagle Medium, and differentiated as previously described above. Then 2µl of 2µM calcein and 2µg/ml of propidium iodide in 1X PBS were added to the each culture well. The culture dishes with the cells were incubated at 37°C for 30 minutes and fluorescence was measured using Nikon Microphot FXA digital fluorescent microscope. The following concentrations of glutamate (50µM, 100µM, 250µM, 500µM, and 1mM) were used to determine the maximum number of RGC-5 cells death. The experiment was performed in the presence and absence of sigma-1 receptor ligands ((+)-SKF10047, BD1047), and NMDA receptor antagonist, MK801.

Caspase-3 activation by glutamate treatment.

In order to determine whether glutamate-induced retinal ganglion cells death was mediated through the activation of caspase-3, we performed the caspase-3 assay in both primary retinal ganglion cells and RGC-5 cells line. The APOLOGIX carboxyfluorescein caspase detection kit (cat. # FAM200-1, Cell Technology, Inc.) was used in this part of the study as instructed by the manufacturer guide: (http://www.celltechnology.com/documents/protocols/Apo%20Logix%20FAM%20Instructions.pdf). Cells were analyzed using confocal laser scanning microscope (Zeiss LSM 410).

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CHAPTER 3

Title: Sigma-1 receptors protect RGC-5 cells from apoptosis by regulating intracellular calcium, Bax levels, and caspase-3 activation.

Abbreviated title: Sigma-1 receptor neuroprotection in RGC-5 cells

Authors:

Kissaou T. Tchedre

University of North Texas Health Science Center

Department of Biomedical Sciences

3500 Camps Bowie Blvd

Fort Worth, TX 76107

Phone: 817 735 2490

Email: ktchedre@hsc.unt.edu

Corresponding author:

Thomas Yorio

University of North Texas Health Science Center

Department of Pharmacology& Neurosciences

3500 Camps Bowie Blvd

Fort Worth, TX 76107

Phone: 817 735 -0258

Email: yoriot@hsc.unt.edu

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ABSTRACT

PURPOSE: Sigma-1 receptor ligands prevent neuronal death associated with glutamate excitotoxicity both *in vitro* and *in vivo*. However, the molecular mechanism of the neuroprotective effect remains to be elucidated. Presently, we determined that sigma-1 receptor agonists provide neuroprotection by decreasing glutamate-induced calcium mobilization and prevent apoptotic gene expression. **METHODS:** Cell death was measured using the calcein-AM/propidium iodide cell survival assay. Western blot analysis determined the expression levels of Bax in normal RGC-5 cells. Caspase-3 activation after glutamate treatment was determined using the carboxyfluorescein caspase-3 detection kit. Glutamate-induced intracellular calcium mobilization was measured using ratiometric calcium imaging. **RESULTS:** Sigma-1 receptor overexpressing RGC-5 (RGC-5-S1R) cells have lower glutamate-induced intracellular calcium mobilization compared to normal RGC-5 cells and the sigma-1 receptor ligand (+)-SKF10047 reduced the glutamate calcium response in normal and RGC-5-S1R cells. (+)-SKF10047 protected RGC-5 cells from glutamate-induced cell death and RGC-5-S1R cells showed a significant resistance to glutamate-induced apoptosis compared to the control RGC-5 cells. BD1047, a sigma-1 receptor antagonist, blocked the protective effect of (+)-SKF10047. Western blot showed that (+)-SKF10047 inhibited the increase in Bax after glutamate treatments. Glutamate-mediated cell death involved activation of caspase-3, and sigma-1 receptor activation prevented an increase in caspase-3 expression. **CONCLUSIONS:** These results suggest that sigma-1 receptors regulate intracellular calcium levels and prevent activation of pro-apoptotic genes promoting retinal ganglion

cell survival. Sigma-1 ligands appear to be neuroprotective and a potential target for neuroprotective therapeutics.

INTRODUCTION

The Sigma-1 receptor was first cloned from guinea pig liver by Hanner et al[1]. Although first described as one of the subtypes of opioid receptors, further studies have led to the distinction between sigma receptors from opioid receptors [2]. The biology of sigma-1 receptors is poorly understood. The sigma-1 receptor has been studied extensively in the central nervous system[3], but it is also recognized to be overabundant in the eye[4-6]. Two types of sigma receptors have been described. The Sigma-1 receptors with a molecular weight of approximately 25 kDa and the sigma-2 receptors with a molecular weight of ~ 18-21 kDa[1, 7]. Hayashi et al.[8] have shown that sigma-1 receptors are localized on both the endoplasmic reticulum (ER) and the plasma membrane. In addition, Hayashi and collaborators have shown that sigma-1 receptors translocate from the endoplasmic reticulum-associated reticular network to the cell periphery upon stimulation. Sigma-1 receptors are also associated with lipid raft microdomains and control the endoplamsmic reticulum lipid compartmentalization and export[8], as well as oligodendrocyte differentiation[9].

In vitro exposure of cultured rat brain neurons to selective sigma receptor ligands protects cells against glutamate or NMDA exposure[10]. Sigma-2 receptors have been shown to be a biomarker for solid tumors proliferation [11]. In addition, Sigma-2 receptor have been shown to be associated with cancer cell proliferation and survival[12, 13]. Glutamate, an NMDA receptor agonist, when present in excess, can lead to accumulation of intracellular calcium via NMDA receptor activation, which is a well documented

process leading to neuronal death or injury[14, 15]. Recently, sigma-1 receptors have also been shown to lower the intraocular pressure (IOP)[16, 17] and protect retinal ganglion cells from stress[18] and, in the lens, modulating cell growth and pigmentation[6]. Sigma-1 receptor knock down in human lens cells induces apoptosis[19]. Caspases, a unique class of aspartate-specific proteases, are the central components of the apoptotic response. The release of cytochrome c from mitochondria into the cytosol leads to the activation of the initiator caspase-9, which in turn activates the effector caspase-3. Once activated, caspase-3 is responsible for the proteolytic cleavage of a broad spectrum of cellular targets, which ultimately leads to cell death[20, 21]. It has recently been shown that glutamate-induced excitotoxicity is mediated through the activiation of caspase-3,[22] and the upregulation of Bax [22].

Presently, we report that the sigma-1 receptor agonist (+)-SKF10047 protects retinal ganglion cells from glutamate-induced apoptosis. The protective effect of the sigma-1 receptor agonist (+)-SKF10047 was prevented by the sigma-1 receptor selective antagonist BD1047. Glutamate-mediated retinal ganglion cell death appears to be signaled by calcium, followed by an upregulation of Bax, and caspase-3 activation, leading to cell death. Sigma-1 receptors are thus neuroprotective.

Materials and Methods

Glutamate was purchased from Sigma (St., Louis, MO). (+)-SKF10047, BD1047, and MK801 were purchased from Tocris bioscience (Ellisville, MO, USA). Rabbit polyclonal NMDAR1 were purchased from Cell signaling technology (Danvers, MA). Rabbit polyclonal Bax (N-20) (Catalog number: sc-493) antibody was purchased from Santa Cruz Biotechnology (Delaware Ave, CA). Rabbit anti-Sigma-1 receptor was a gift from

Dr. Hayashi, Teruo (NIH/NIDA/IRP, Baltimore, MD). Rabbit normal IgG was purchased from Sigma (St. Louis, MO). Secondary antibodies including donkey anti-rabbit IgG and donkey anti-mouse IgG conjugated to HRP were purchased from Amersham Biosciences, Piscataway, NJ. Fluorescent probes including goat anti-rabbit Alexa 488, goat anti-Rabbit Alexa 633 and fura-2-AM were purchased from Molecular Probes, Eugene, OR. The APOLOGIX^{TM} carboxyfluorescein caspase-3 detection kit (catalog number: FAM200-1) was purchased from Cell technology Inc. (Mountain view, CA).

Retinal ganglion (RGC-5) cells culture and differentiation

The RGC-5 cell line was developed specifically to establish a permanent rat retinal ganglion cell culture[23]. RGC-5 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco Cat #23700-040) supplemented with 10% fetal bovine serum (Gibco Cat #26140-079) and 100 U/ml of penicillin and 100 µg/ml of streptomycin. RGC-5 cells were grown in growth media and incubated at 37°C in 5% CO_2 and air.

For all the experiment cells were differentiated using human non-pigmented ciliary epithelial (HNPE) cells conditioned media collected from 100% confluent T-150 cm^2 flask (Rachel Dauphin et al., 2007). Briefly, 24 hours after seeding the RGC-5 cells the DMEM was removed from the culture dishes and replaced by the HNPE conditioned media (50ml of HNPE conditioned media and 1 ml complete DMEM (10% fetal bovine serum and 100 U/ml of penicillin and 100 µg/ml of streptomycin)). The cultures dishes were then incubated at 37°C in 5% CO_2 and air for 48 hours to allow the cells to differentiate (**Fig 1, panel I & II**).

Preparation of stably transfected sigma-1 receptor RGC-5 Cells

The plasmid pSPORT-Sigma-l-R cDNA [24] was a gift from Dr. Ganapathy (Medical College of Georgia, Augusta, GA,).

Large Scale plasmid preparation:

E. Coli competent strain SURE 2 cells were transformed with a plasmid pSPORT-Sigma-1-R. After transformation, cells were plated on ampicillin containing Luris-Bertani (LB) plates. Many colonies were obtained after overnight incubation. Colonies were picked up and grown in LB media for large-scale preparation of plasmid DNA. Plasmid DNA was purified through equilibrium cesium chloride gradient method. After plasmid preparation, the purity of the DNA was checked on agarose gel (data not shown).

Permanent transfection

The lipofectamine ™ 2000 (Invitrogen) method was used for transfection of retinal ganglion (RGC-5) cells as instructed by the manufacturer's guide (http://www.adobe.com/products/acrobat/readstep2.html). Since RGC-5 cells are neomycin (G418) resistant, we performed a co-transfection using pSPORT-Sigma-1R and psiRNA-hH1GFPzeo (Invivogen). The sigma-1 receptor empty vector transfection was performed with psiRNA-hH1GFPzeo alone. After transfection, zeocin resistant clones were selected and the expression of Sigma-1 receptors was assessed by western blot using anti-sigma-1 receptor rabbit polyclonal antibody (1/500 dilution) **(Fig. 2)**.

Preparation of Cells Lysates and western blotting

Normal RGC-5 cells lysates were prepared as described by Bu et al[25]. Protein concentrations of cell lysates were determined using a BioRad DC protein kit (Bio-Rad Laboratories, Hercules, CA, USA). 20μg of samples protein were run on SDS-PAGE gel

and immunoblotted via standard methods as described by Laemmli[26] and Towbin et al.[27] using rabbit polyclonal NMDAR1 antibody (1/500 dilution) (for NMDA receptors expression), and rabbit polyclonal Bax (N-20) antibody (1/500 dilution) (for Bax expression). The blots were developed using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Beta actin was used as control for equal loading.

Primary rat retinal ganglion cell isolation

All procedures were performed in accordance with the ARVO guidelines for care and use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee at the UNT Health Science Center. RGCs were purified by a modification[28] of immunopanning with antibodies against Thy 1.1 specific for RGCs. Briefly, Wistar Kyoto rats (3-4 weeks old, either sex) were purchased from Harlan Laboratory (Indianapolis, IN) and the rats were anesthetized with isoflurane and then decapitated. The eyes were enucleated and the retina were gently peeled off with fine forceps and placed in sterile phosphate buffer saline (PBS). Retina were collected in DMEM and incubated at 37°C in 3ml of dispase for 1 hour. After 1-hour incubation, the retina was gently washed once with complete neurobasal medium. Retinal ganglion cells were obtained by trituration of the retina in neurobasal medium (GTBCO, Grand Island, NY) with a fire-polished Pasteur pipette.

Panning dishes (Falcon, Becton & Dickinson, NY, USA) were incubated with goat anti-mouse IgG antibodies (2 μg/ml, Sigma, USA) in Tris-HCl buffer (pH 9.5) for 24 h at 4°C. Dishes were then washed three times with phosphate-buffered saline (PBS) prior to

each of the subsequent steps. Incubation with antibodies against Thy1.1 (monoclonal antibody 1.5 –2.5 μg/ml, Chemicon International, CA, Cat# MAB1406) was performed for at least 2 h at room temperature in PBS. To prevent nonspecific binding of cells, dishes were then incubated with 0.2% bovine serum albumin in PBS for 20 min at room temperature. Approximately 5 ml of cell suspension were added per dish and incubated for 20 min at 37°C. Dishes were gently swirled every 5 min to ensure access of all RGCs to the surface of the plate. To remove nonadherent cells, dishes were washed repeatedly with PBS and swirled moderately until only adherent cells remained. Washing was monitored under the microscope. RGCs were removed from the panning dish with trypsin (0.0625%) solution. After centrifugation at 200*g* for 5 min, purified RGCs were suspended in culture medium. The cell suspension collected was seeded (500 μl) on coverslips previously coated first with poly-D-lysine (overnight). The coverslips were covered with 2 ml of complete neurobasal medium supplemented with B27 and Pen-Strep (Gibco, Carlsbad, CA), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic growth factor (CTNF) (biosource PHC 7074), and forskolin (Sigma-Aldricht F6886). Cell culture dishes were subsequently incubated in a 5% CO2 incubator at 37°C for up to 7 days. Isolated retinal ganglion cells were characterized by immunocytochemistry for a normally expressed retinal ganglion cell marker, thy-1[49].

Immunofluorescence microscopy:

Primary retinal ganglion (7 DIV) cells were grown on coverslips and subsequently fixed with 4% paraformaldehyde and blocked with blocking solution (5% BSA + 5% normal goat serum) for 1 hour at room temperature. The blocking buffer was discarded and the

coverslips were washed three times with 1X PBS, before the addition of the Thy1.1 monoclonal antibody (1/500 dilution) with 1% normal goat serum and 5% bovine serum albumin (Sigma-Aldrich, St Louis, MN). The incubation was performed overnight at 4°C. Coverslips were washed three times and 1/500 secondary antibodies (Alexa Fluor 633) was added and the coverslips were incubated for 1 hour in the dark. After the incubation, the coverslips were washed again three times with 1X PBS. The mounting was done on glass slides using ProlongR Gold antifade reagent with DAPI (Invitrogen , CA) and allowed to dry for 20 minutes in the dark. Cells were viewed with confocal laser scanning microscope (Zeiss LSM 510).

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement

Glutamate-induced intracellular calcium mobilization in normal RGC-5, green fluorescence protein transfected RGC-5 (RGC-5-GFP) cells (sigma-1 receptor empty vector), and sigma-1 receptor overexpressing RGC-5 cells (RGC-5-S1R) was measured at 37 °C by the ratiometric technique using fura-2-AM (excitation at 340 nm and 380 nm, emission at 510 nm) according to Prasanna et al.[29] Nikon Diaphot microscope using Metafluor software (Universal Imaging, West Chester, PA). Intracellular calcium ($[Ca^{2+}]_I$) in nanomolar (nM) was calculated using the Grynkiewicz equation[30]. In calcium imaging studies, cells were pretreated with NMDA receptors antagonist, MK801, and sigma-1 receptor antagonist for 30 minutes before stimulating the cells with glutamate.

Calcein-AM/propidium iodide cell survival assay

Cell viability was determined using a calcein/propidium iodide (Catalog number: C3099, Molecular Probes, Eugene, OR) dual staining assay. The calcein/propidium iodide assay relies on the intracellular esterase activity within living cells, through which the calcein

AM, a cell-permeable fluorogenic esterase substrate, hydrolyzes to a green fluorescent product, calcein. Living cells will be showing green fluorescence while dead cells will be stained red by the cell impermeant propidium iodide. Propidium iodide is a nucleic acid stain usually used as a counter stain in multicolor fluorescent techniques. Propidium iodide is used to identify nuclei showing apoptotic changes. RGC-5 cells were seeded on coverslips. RGC-5 cells were treated with glutamate (1mM) with (10μM) glycine for 4 days with or without sigma-1 receptor ligands ((+)-SKF10047 (1μM), BD1047 (3μM), and MK801 (10μM). After treatment of the RGC-5 cells, the culture medium was removed and the coverslips were rinsed with 1X PBS. Then 2ml of 2μM calcein and 2μg/ml of propidium iodide in 1X PBS was added to the each culture well. The culture dishes with the cells were incubated at 37°C for 30 minutes and fluorescence was measured using Nikon Microphot FXA digital fluorescent microscope.

A 1mM concentration of Glutamate was chosen after a concentration dose-response killing study using calcein/propidium iodide dual staining survival assay. The following concentrations of glutamate (50μM, 100μM, 250μM, 500μM, and 1mM) were used to determine the maximum number of RGC-5 cells death. The 1mM glutamate presented the highest cell death **(Fig.3A, B)**. Three different types of RGC-5 cells (normal, GFP-transfected, and sigma-1 receptor overexpressing) were used in this part of the study.

Caspase-3 activation by glutamate treatment.

Conversion of procaspase-3 to active caspase-3 was assessed by the APOLOGIX carboxyfluorescein caspase detection kit (cat.# FAM200-1, Cell Technology, Inc.). Caspase-3 activation was assessed as instructed by the manufacturer guide

(http://www.celltechnology.com/documents/protocols/Apo%20Logix%20FAM%20Instructions.pdf). This kit is based on carboxyfluorescein labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and non-cytotoxic. Once inside the cell, the inhibitor binds covalently to the active caspase[31]. Cells that contain bound inhibitor can be analyzed by fluorescence microscopy. In this experiment both retinal ganglion cells (RGC-5) line and primary rat retinal ganglion cells were used. Cells were analyzed using confocal laser scanning microscope (Zeiss LSM 410).

RESULTS

Expression of sigma-1 receptors in RGC-5 cells.

Sigma-1 receptors are expressed in many organs including the eye[4]. In this study we examined the expression of sigma-1 receptors using western blot. The western blot results showed the expression of sigma-1 receptors in normal RGC-5 cells, RGC-5-GFP transfected (sigma-1 receptor empty vector) cells, and sigma-1 receptor over-expression RGC-5 cells (clone 4, see method section for the preparation of stably transfected RGC-5 cells) (**Fig.2, lane 3**). In addition, the western blot data showed that normal RGC-5 cells and RGC-5-GFP transfected cells expressed basal sigma-1 receptors (**Fig.2, lane 1&2**).

Characterization of the isolated primary retinal ganglion cells

Isolated retinal ganglion cells were characterized by immunocytochemistry for detection of a normally expressed retinal ganglion cell marker, thy-1 (**Fig. 10 A, B**).

Sigma-1 receptor overexpressing RGC-5 cells have low calcium response to glutamate compare to sigma-1 receptor empty vector RGC-5 cells.

Sigma-1 receptors have been shown to inhibit NMDA receptors in neurons[3, 32, 33]. We first examined the effect of glutamate and the sigma-1 receptor agonist (+)-SKF10047 on

intracellular calcium mobilization. Glutamate mediated increase in intracellular calcium was concentration dependent **(Table 1)**. The sigma-1 receptor agonist (+)-SKF10047, dose dependently significantly inhibited glutamate-induced intracellular calcium mobilization **(Table 2).** To investigate further the effect of sigma-1 receptors on NMDA receptors in the retinal ganglion cells, we stably overexpressed sigma-1 receptors in RGC-5 cells using the sigma-1 receptor expression construct pSPORT-Sigma-l-R plasmid, and compared the glutamate-induced intracellular calcium mobilization among transfected and untransfected RGC-5 cells. Intracellular calcium mobilization in retinal ganglion cells (RGC-5) was measured by a ratiometric technique using fura- 2-AM (Molecular probe, OR). The sigma-1 receptor agonist (+)-SKF10047 (1μM) was able to inhibit the glutamate-induced calcium mobilization peak **(Table 3)** from (1086 ± 102nM) to (502 ± 43 nM) *($p<0.05$)* in sigma-1 receptor empty vector RGC-5 cells and from (630 ±.40) to (199 ± 8) *($p<0.05$)* in sigma-1 receptor overexpressing RGC-5 cells **(Table 3)**. In sigma-1 receptor overexpressing RGC-5 cells the glutamate response was lower compared to the sigma-1 receptor empty vector RGC-5-GFP cells glutamate response **(Table 3).** The sigma-1 receptor antagonist BD1047 didn't have any inhibitory effect on its own with respect to glutamate-induced calcium mobilization, but was able to block the inhibitory effect of (+)-SKF10047 on glutamate-induced calcium mobilization **(Table 4)**. To test whether the lower glutamate response of sigma-1 receptor overexpressing RGC-5 cells was due to the down regulation of NMDA receptors, we performed a western blot analysis using rabbit polyclonal NMDAR1 antibody. The results of the western blot data showed that the NMDA receptors levels were not affected **(Fig. 4)**. This suggested that

sigma-1 receptor mediated NMDA receptor inhibition occured via other mechanisms but not by downregulation of the NMDA receptors expression level.

Sigma-1 receptor agonist (+)-SKF10047 inhibits glutamate-induced excitotoxicity in RGC-5 cells.

Sigma-1 receptor ligands have been shown to protect neurons by preventing the neurotoxicity associated with CNS injury and neurodegenerative disorders [35]. In addition, the neuroprotective effects of sigma-1 receptor ligands are thought to include modulation of NMDA receptors,[36, 37] attenuation of postsynaptic glutamate-evoked calcium influx [35, 38], depression of neuronal responsivity to NMDA receptor stimulation, and reduction of nitric oxide production[18]. We therefore studied the effect of high glutamate concentrations on RGC-5 cell death.

Calcein-AM/propidium iodide cell survival assay.

To assess the consequences of acute exposure to high levels of glutamate, normal RGC-5, RGC-5-GFP (sigma-1 receptor empty vector), and sigma-1 receptor overexpressing RGC-5 cells, were exposed to 1 mM glutamate with or without the sigma-1 receptor agonist (+)-SKF10047, antagonist BD1047, and the NMDA receptor antagonist MK801. 10μM glycine, a NMDA receptor co-activator was used in each experiment. Cells were then processed for determination of apoptosis using the calcein/propidium iodide double staining. There were very few propidium iodide positive cells in the control of the three types of RGC-5 cells **(Fig.5-I, Fig. 6-I, Fig.7-I)**. However, treatment with 1mM glutamate increased the number of propidium-positive cells considerably in normal RGC-5, RGC-5-GFP, and RGC-5-S1R by 91.19 ± 1%, 90.00 ± 1%, 40.08 ± 2% **(Fig.5-II, Fig. 6-II, Fig. 7-II)** respectively. The sigma-1 receptor agonist (+)-SKF10047 was able to

reduce the glutamate-induced cell death in normal RGC-5, RGC-5-GFP, and RGC-5-S1R to only 8.31 ± 1%, 12.75 ± 2%, 13.53 ± 5% **(Fig. 5-II, Fig. 6-II, Fig. 7-II)** respectively. To test whether cell protection was being mediated specifically by sigma-1 receptor activation, we exposed the RGC-5 cells to BD1047, a sigma-1 receptor antagonist. BD1047 had little effect on RGC-5 cell death on its own **(Fig.5-V, Fig. 6-V, Fig. 7-V)**, but was able to block the protective effect of (+) SKF10047 **(Fig. 5-VI, Fig. 6-VI, Fig. 7-VI)**. The reduction in the percentage of RGC-5 cells labeled with propidium iodie by MK801, a specific antagonist of NMDA receptors also confirmed that glutamate-induced RGC-5 cell death was mediated through the activation of the NMDA receptor **(Fig. 6-VIII, Fig. 7-VIII)**. These data suggested that acute exposure to high levels of glutamate is toxic to retinal ganglion RGC-5 cells. In addition the calcein/propidium iodide survival assay showed that sigma-1 receptor overexpressing RGC-5 cells had a significant increased resistance to glutamate-induced RGC-5 cell death **(Fig. 7-II, Fig. 8C)**. The calcein/propidium iodide fluorescence images quantitative data is shown **(Fig. 8A)** for normal RGC-5 cells, **(Fig. 8B)** for RGC-5-GFP cells, and **(Fig. 8C)** for Sigma-1 receptor overexpressing RGC-5 cells. Data are shown as (mean percentage ± s.e.m.; n = 6 different fields of cells, where each field contained approximately 60 cells).

Sigma-1 receptor ligand (+)-SKF10047 downregulates Bax in RGC-5 cells.

Increases in Bax expression may lead to mitochondrial depolarization and cytochrome c release resulting in the down-stream activation of executioner caspase to augment apoptosis[20, 34]. In addition, the expression level of Bax has been shown to be affected by glutamate treatment in PC12 cells[22]. Treatment of RGC-5 cells with glutamate led to an increase in Bax protein expression as compared with the control **(Fig. 9)**. To determine

whether glutamate induced increase in Bax levels was mediated by NMDA receptors activation, RGC-5 cells were incubated with the NMDA-specific antagonist MK-801 plus glutamate. MK801 was able to reduce Bax protein levels. Treatment of RGC-5 with glutamate plus the sigma-1 receptor agonist (+)-SKF10047 also resulted in the decrease in Bax protein levels. The effect of (+)-SKF10047 was blocked by BD1047, a sigma-1 receptor antagonist.

The increase of Bax after treatment of BD1047, a sigma-1 receptor antagonist, may be due to the fact that sigma-1 receptor antagonists are capable of inducing apoptosis. Overall, the western blot data showed that sigma-1 receptor agonist protective effect involved the regulation of Bax cellular levels in RGC-5 cells.

Detection of activated caspase-3 in RGC-5 cells treated with glutamate.

The conversion of pro-caspase-3 to active caspase-3 is generally accepted as one of the most reliable indicators of apoptosis[20, 21]. To test whether the activation of caspase-3 is associated with glutamate-induced RGC-5 cell apoptosis, we treated RGC-5 cells with 1mM glutamate for 24 hours with or without sigma-1 receptor agonist (+)-SKF10047 (1μM), and sigma-1 receptor antagonist BD1047 (3μM). Activation of caspase-3 in RGC-5 cells in response to glutamate was confirmed by the APOLOGIX carboxyfluorescein caspase detection kit **(Fig. 11A)** and suggested that glutamate-treated cells were dying by apoptosis. The sigma-1 receptor ligand (+)SKF10047 was again able to reduce the activation of caspase-3 after glutamate treatment. BD1047, a sigma-1 receptor antagonist blocked the effect of (+)-SKF10047. In addition, MK801, The NMDA receptors antagonist, was also able to inhibit significantly caspase-3 activation. The same results were obtained using rat primary retinal ganglion cells **(Fig. 11B)**.

DISCUSSION

The cellular transduction events mediated by sigma-1 receptors are still unclear. Changes in the concentration of free intracellular calcium ions are recognized to be linked to the induction of apoptosis, but the relationship between calcium and its linkage to the apoptotic program is complex, because calcium can be a signal for both life and death pathways [39]. N-methyl-D-aspartate (NMDA)-type of ionotropic glutamate receptor, plays an important role in both the physiology and pathology of neurons[38,39]. It has been shown that repeated NMDA receptor activation is one of the main causes of neurodegenerative disorders [40-42]. The major finding reported in this study is that sigma-1 receptors protect retinal ganglion cells from glutamate-induced apoptosis by regulating the intracellular calcium levels, and the subsequent signaling of apoptotic events including decreasing the expression of Bax levels, as well as limiting caspase-3 activation. The important calcium influx resulting from the hyper-activation of the NMDA receptor complex has been shown to be critically associated with delayed excitotoxic neuronal death [15, 39, 41, 43]. These findings are consistent with our current observations that the toxicity effect of glutamate was mediated through the activation of the glutamate ionotropic NMDA receptors and involved increases in intracellular calcium mobilization in RGC-5 cells. In addition, our results showed that the sigma-1 receptor agonist (+)-N-allylnormetazocine ((+)-SKF10047) inhibited glutamate-induced intracellular calcium mobilization. Similarly, Renaudo et al.[44] reported that the activation rate of volume regulated chloride channels (VRCC) was dramatically delayed in sigma-1 receptor HEK293-transfected cells in the absence of ligands. These findings are in agreement with our studies that sigma-1 receptor overexpressing cells have a lower response to glutamate-induced

calcium mobilization compared to the non-overexpressing sigma-1 receptor retinal ganglion cells.

Changes in intracellular calcium levels appears to be associated with neuroprotective properties of sigma-1 receptors similar to that reported in cancer cells by Spruce et al[45]. The majority of sigma ligands that have been used in different studies also demonstrate that there is altered calcium mobilization[38, 43] in the presence of these sigma-1 receptor ligands. Similar results have been reported by other studies in cells of the central nervous system[35]. Our data also confirm the previous reported data, which showed that the sigma-1 receptor agonist (+)-pentazocine inhibited significantly apoptosis induced by homocysteine or glutamate[46]. The receptor mechanisms mediating sigma-1 receptor neuroprotection comprise complex interactions involving ionotropic, and even voltage-gated calcium signaling processes [47].

In addition to showing that calcium regulation is involved in the neuroprotective mechanism of sigma-1 receptor activation in RGC-5 cells, we also showed that the expression levels of Bax, and caspase-3 activation were decreased by the activation of sigma-1 receptors. Glutamate, the principle excitatory amino acid in the central nervous system, is considered to play an important role in neurotransmission, neuronal development, synaptic plasticity and neuronal degeneration. Glutamate has been shown to upregulate Bax levels in PC12 cells and cortical neurons[21, 22] and to activate caspase-3 in primary cortical neurons[48]. Activation of caspase-3 is an early biochemical marker of general apoptosis in certain types of cells induced by various triggers for apoptosis. The activation of caspase-3 was observed in RGC-5 cells after 24 hours glutamate treatment and sigma-1 receptor agonists can prevent this activation. The same result was

reproduced in primary rat retinal ganglion cells with low glutamate concentration and short time exposure (6 hours).

Overall we have shown that sigma-1 receptor activation protects RGC-5 cells from glutamate-induced excitotoxicity by regulating calcium mobilization in retinal ganglion cells, decreasing Bax expression levels, and by inhibiting caspase-3 activation. The regulation of glutamate-induced calcium mobilization in RGC-5 cells by sigma-1 receptors can activate cell survival signaling pathways and decrease pathways linked to cell death.

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Figures Legends

Figure 1. Morphology differences between undifferentiated and differentiated RGC-5 cells.

Panel I: Morphology of RGC-5 cells: **A).** Undifferentiated RGC-5; **B).** Differentiated RGC-5 cells. **Panel II**: Pseudo color of images of RGC-5: **A).** Undifferentiated retinal ganglion cells. **B).** Differentiated retinal ganglion cells after 30 min incubation with fura-2AM in HBSS) that reflect the levels of [Ca2+]. Colors reflect relative intracellular calcium concentration $[Ca2+]_i$, as follows: **blue**, low; **green/yellow**, intermediate; **red**, high (see calibration bar).

Figure 2. Western blot analysis of sigma-1 receptor protein expression in Normal, GFP, and sigma-1 receptor overexpressing RGC-5 cells.

Cells lysates (20μg of protein) from normal RGC-5, RGC-5-GFP, and sigma-1 receptor overexpressing RGC-5 cells cultured in 10% FBS-DMEM to complete confluence were subjected to 15%-SDS–PAGE followed by immunoblotting using specific antibodies for the sigma-1 receptor. ***(Lane 1)***: normal RGC-5 cells, ***(Lane 2)***: RGC-5 cells transfected with GFP vector (empty Sigma-1 receptor vector) ***(Lane 3)***: RGC-5 overexpressing sigma-1 receptor (clone 4). Sigma-1 receptor can be detected as protein band approximately 25 kDa.

Fig. 3 Dose-response of glutamate induced excitotoxicity in RGC-5 cells.

Cell survival was monitored using the calcein-AM/propidium iodide cell survival assay. Normal RGC-5 cells were treated as follow: **A:** ***1).*** Control (no glutamate) ***2).*** *50*μM glutamate, **3).** 100μM glutamate, **4).** ***250***μM glutamate, **5).** 500 μM glutamate, **6).** 1mM glutamate for 4 days. 10μM of glycine was added in each well. Cells were incubated with 2 μM calcein (Green, live cells) and 2μg/ml of propidium iodide (**PI**)(red, dead cells) for 30 minutes. After the coverslips were mounted on the microscope slide and analyzed under a fluorescence microscope. Scale bar represents 200μm. **B:** Summary of glutamate dose-response calcein-AM/propidium iodide cell survival. fluorescence images quantitative data summary shown as (mean percentage ± s.e.m. n = 6 different fields of cells, where each field contained approximately 60 cells). Image J. Software was used for the quantification.

* statistically significant compared to the control (no glutamate treatment).

Figure 4. NMDA receptor expression is not affected by sigma-1 receptor overexpression.

Cells lysates (20μg of protein) from normal RGC-5, RGC-5 cells transfected with GFP vector, and sigma-1 receptor overexpressing RGC-5 cells were subjected to 7%-SDS–PAGE followed by immunoblotting using specific antibody for the NMDAR1 receptor subunits. ***(Lane 1)***: normal RGC-5 cells, ***(Lane 2)***: RGC-5 transfected with GFP vector, ***(lane 3)***: RGC-5 overexpressing sigma-1 receptor. NMDAR1 can be detected as protein band approximately 120 kDa.

Figure 5. Sigma-1 receptor agonist (+)-SKF10047 protect RGC-5 cells from glutamate induced excitotoxicity.

Cell survival was monitored using the calcein-AM/propidium iodide cell survival assay. Normal RGC-5 cells were treated as follow: ***I).*** Control (no glutamate) ***II).*** *1m*M glutamate, ***III).*** 1μM (+)-SKF10047, ***IV).*** 1mM glutamate + 1 μM (+)-SKF10047, ***V).*** 3μM BD1047, ***VI).*** 1mM glutamate + 3 μM BD1047 + 1 μM (+)-SKF10047 for 4 days. 10μM of glycine was added in each well. Cells were incubated with 2 μM calcein (Green, live cells) and 2μg/ml of propidium iodide (**PI**)(red, dead cells) for 30 minutes. After the coverslips were mounted on the microscope slide and analyzed under a fluorescence microscope. **A** = calcein staining, **B** = Propidium iodide staining, **C** = Merge. **Green** = live cells; **Red** = dead cells. Scale bar represents 20μm.

Figure 6. Sigma-1 receptor agonist (+)-SKF10047 protect RGC-5-GFP (sigma-1 receptor empty vector) cells from glutamate-induced excitotoxicity.

Cell survival was monitored using the calcein-AM/propidium iodide cell survival assay. RGC-5-GFP transfected (sigma-1 receptor empty vector) cells were treated as follow: ***I).*** Control (no glutamate) ***II).*** *1m*M glutamate, ***III).*** 1 μM (+)-SKF10047, ***IV).*** 1mM glutamate + 1 μM (+)-SKF10047, ***V).*** 3 μM BD1047, ***VI).*** 1mM glutamate + 3 μM BD1047 + 1 μM (+)-SKF10047, ***VII).*** 10μM MK801, and ***VIII).*** 1mM glutamate + 10μM MK801 for 4 days. 10μM of glycine was added in each well. Cells were incubated with 2 μM calcein (Green, live cells) and 2 μg/ml of propidium iodide (**PI**)(red, dead cells) for 30 minutes. After the coverslips were mounted on the microscope slide and analyzed under a fluorescence microscope. MK801, a specific NMDA receptor antagonist was able to reduce glutamate-induced increased RGC-5-GFP cell death. This data suggested that glutamate-induced apoptosis was mediated by NMDA receptor activation. **A** = calcein staining, **B** = Propidium iodide staining, **C** = Merge. **Green** = live cells; **Red** = dead cells. Scale bar represents 20μm.

Figure 7. Sigma-1 receptor agonist (+)-SKF10047 protect sigma-1 receptor overexpressing RGC-5 cells from glutamate induced excitotoxicity.

Cell survival was monitored using the calcein-AM/propidium iodide cell survival assay. Sigma-1 receptor overexpressing RGC-5 cells were treated as follow: ***I).*** Control (no glutamate) ***II).*** *1m*M glutamate, ***III).*** 1μM (+)-SKF10047, ***IV).*** 1mM glutamate + 1 μM (+)-SKF10047, ***V).*** 3 μM BD1047, ***VI).*** 1mM glutamate + 3 μM BD1047 + 1 μM (+)-SKF10047, ***VII).*** 10μM MK801, and ***VIII).*** 1mM glutamate + 10μM MK801 for 4 days.

10μM of glycine was added in each well. Cells were incubated with 2μM calcein (Green, live cells) and 2μg/ml of propidium iodide (**PI**)(red, dead cells) for 30 minutes. After the coverslips were mounted on the microscope slide and analyzed under a fluorescence microscope. MK801, a specific NMDA receptor antagonist was able to reduce glutamate induced increase RGC-5 cell death. This data suggested that glutamate-induced apoptosis was mediated by NMDA receptor activation. In addition, sigma-1 receptor overexpressing RGC-5 cells presented a significant resistance to glutamate-induced apoptosis. **A** = calcein staining, **B** = Propidium iodide staining, **C** = Merge. **Green** = live cells, **Red** = dead cells. Scale bar represents 20μm.

Figure 8. Summary of calcein-AM/propidium iodide cell survival assay data.

The calcein/propidium iodide survival assay **(Fig. 3, Fig.4, Fig.5)** fluorescence images quantitative data summary shown as (mean percentage ± s.e.m. n = 6 different fields of cells, where each field contained approximately 60 cells). ***A).*** Normal RGC-5 cells, ***B).*** RGC-5-GFP (sigma-1 receptor empty vector) cells, ***C).*** sigma-1 receptor overexpressing RGC-5 cells. Image J. Software was used for the quantification. * Statistically significant ($P < 0.05$) compared to the control (no treatment), ** Statistically significant ($P < 0.05$) compared to glutamate (1mM), **&** statistically significant ($P < 0.05$) compared to 1mM glutamate treatment in normal RGC-5 cells, and RGC-5-GFP (sigma-1 receptor empty vector) Cells

Figure 9. Western blot analysis of Bax levels in normal RGC-5 cells after glutamate treatment.

Western blot analysis was used to detect the cytosolic levels of Bax protein. RGC-5 cells were treated for 24 hours as follow: ***A). Lane 1).*** Control (no glutamate), ***Lane 2).*** 1 mM glutamate, ***Lane 3).*** 10 μM MK801, ***Lane 4).*** 1mM glutamate + 10 μM MK801, ***Lane 5).*** 1μM (+)-SKF10047, ***Lane 6).*** 1mM glutamate + 1μM (+)-SKF10047, ***Lane 7).*** 3 μM BD1047 and ***Lane 8).*** 1mM glutamate + 3 μM BD1047 + 1 μM (+)-SKF10047. Cells lysates were prepared and 20μg of protein were separated by SDS-12% polyacrylamide gel electrophoresis. Immunoblot analysis of Bax was performed using rabbit polyclonal Bax (N-20) antibody. Beta actin was used as a control for equal loading. ***B).*** Densitometric analysis of the bands was done using the Scion image analysis software (NIH). The quantification of band intensity is represented as a percentage to the value of its corresponding control band on the same membrane and the intensity for Bax are presented as a mean percentage in three separate experiments. * denotes statistical significance ($P < 0.05$) of mean (%) Bax density versus that of control (no treatment). ** denotes statistical significance ($P < 0.05$) of mean (%) Bax density versus that of 1mM glutamate treatment.

Figure 10: Primary RGCs were grown on 35-mm coverslips for 7 days, fixed, and subjected to immunofluorescent staining for detection of a normally expressed retinal ganglion cell marker, thy-1 (**B**). Cells were incubated with primaries Thy-1.1 monoclonal antibody and sigma-1 receptor polyclonal antibody followed by incubation with secondaries Alexa Fluor 633 and 488). Confocal laser scanning microscope (Zeiss LSM 510) was used to detect Thy-1 (red) DIC: (**A**). Scale bar represents 10μm.

Figure 11 ***A):*** **Glutamate-induced caspase-3 activation in RGC-5 cells**

Normal RGC-5 cells were treated with 1mM glutamate as follow: ***1).*** Control (no glutamate); ***2).*** *1m*M glutamate, ***3).*** 1μM (+)_SKF10047; ***4).*** 1mM glutamate + 1μM (+)SKF10047; ***5)*** 10μM MK801; ***6).*** 1mM Glutamate + 10μM MK801; ***7).*** 3μM BD1047; ***8).*** 1mM Glutamate + 3 μM BD1047 + 1μM (+)SKF10047. After the carboxyfluorescein caspase-3 substrate was added to the cell culture medium for 30 min. The coverslips were subsequently washed once with 1X PBS and mounted on the microscope slide. The caspase-3 activation was detected using confocal laser scanning microscope (Zeiss LSM 410). Scale bars, 20 μm. Fluorescence images quantitative data summary shown as (mean fluorescence ± s.e.m. n = 6 different fields. Image J. Software was used for the quantification. * denotes statistical significance ($P < 0.05$) compared to the control (no treatment). ** denotes statistical significance ($P < 0.05$) compared to glutamate (1mM).

B): **Glutamate-induced caspase-3 activation in rat primary retinal ganglion cells.**

Primary retinal ganglion cells were treated with 250 μM glutamate as follow for 6 hours: ***1).*** Control (no glutamate); ***2).*** 250 μM glutamate, ***3).*** 1μM (+)SKF10047; ***4).*** 250 μM glutamate + 1μM (+)_SKF10047; ***5).*** 10μM MK801; ***6).*** 250 μM Glutamate + 10μM MK801, ***7).*** 3μM BD1047; ***8).*** 250 μM Glutamate + 3μM BD1047 + 1μM (+)SKF10047. After the carboxyfluorescein caspase-3 substrate was added to the cell culture medium

for 30 min. The coverslips were subsequently washed once with 1X PBS and mounted on the microscope slide. The caspase-3 activation was detected using confocal laser scanning microscope (Zeiss LSM 410). Scale bars, 20 μm. fluorescence images quantitative data summary shown as (mean fluorescence ± s.e.m. n = 6 different fields. Image J. Software was used for the quantification. Quantitative data summary of glutamate-induced caspase-3 activation in primary RGC cells. * denotes statistical significance ($P < 0.05$) compared to the control (no treatment). ** denotes statistical significance ($P < 0.05$) compared to glutamate (250 μM).

Figures

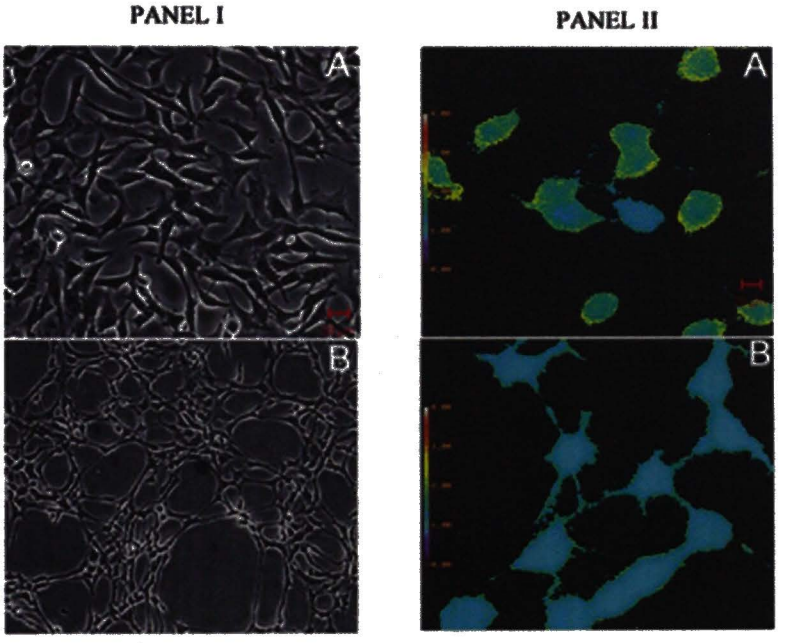


Figure 1.

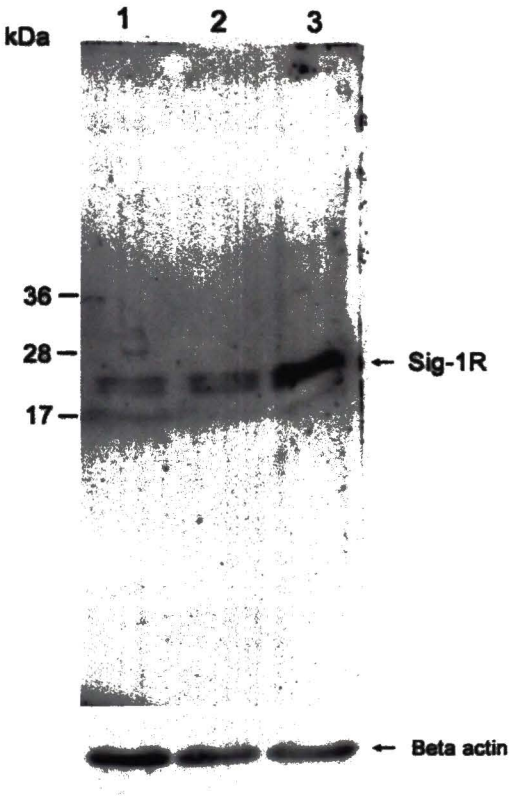


Figure 2.

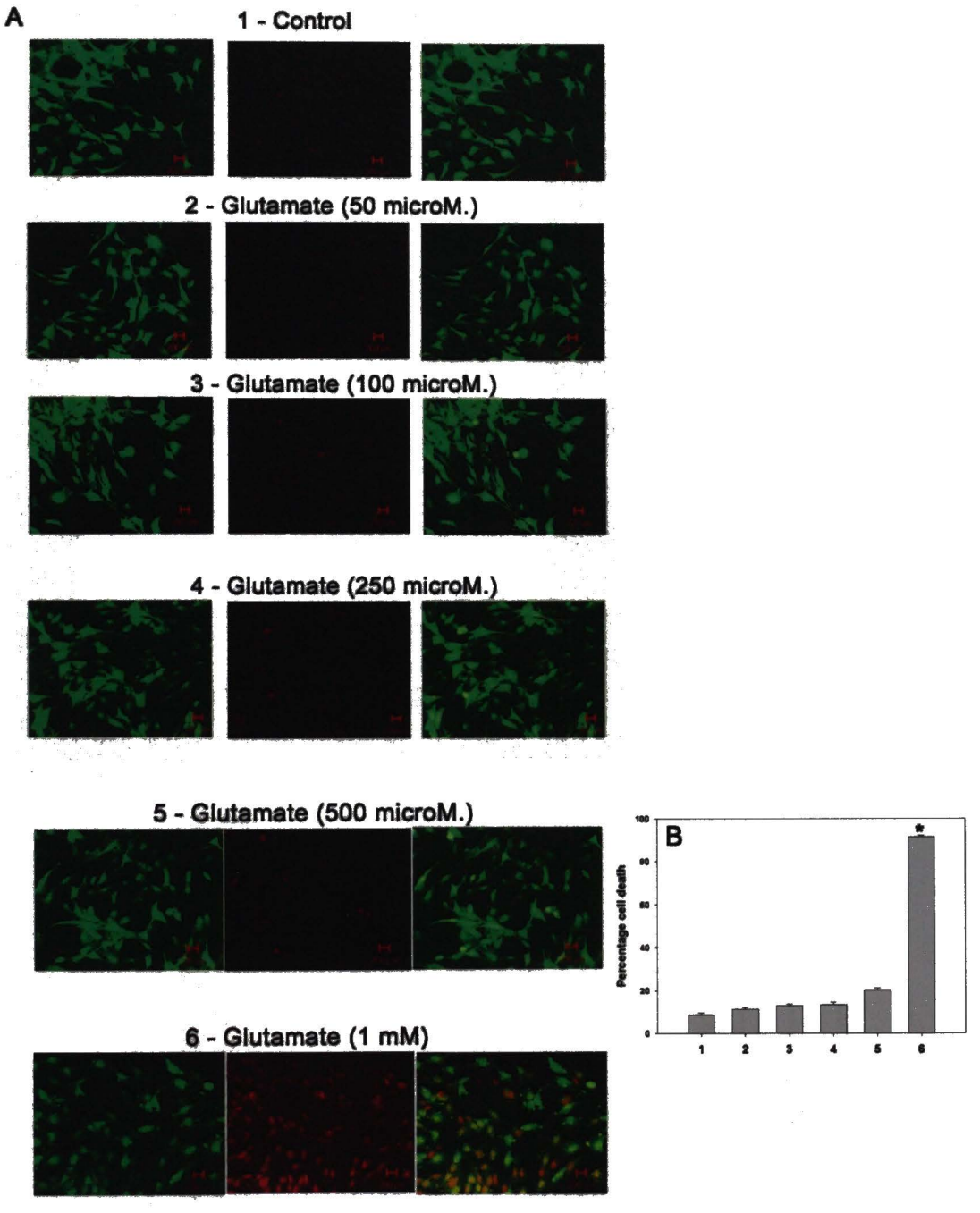

A
1 - Control
2 - Glutamate (50 microM.)
3 - Glutamate (100 microM.)
4 - Glutamate (250 microM.)
5 - Glutamate (500 microM.)
6 - Glutamate (1 mM)
B
Percentage cell death
1
2
3
4
5
6

Figure 3.

Table 1. Concentration dependent elevation of intracellular calcium

Glutamate induced calcium mobilization was measured by fura-2-AM imaging. Cells were pre-incubated for 30 minutes with fura-2 before stimulation with glutamate. Data were analyzed by One Way Analysis of Variance (ANOVA) and all Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) between baseline, peak and one minute post peak. Changes (mean ± SEM).

*Statistically significant (**$p<0.05$**) compared to the baseline.

Treatment	$[Ca^{2+}]_i$ nM, Mean ± SEM	Number of cells (n)
Baseline	68 ± 11	15
Glutamate 50μM	299 ± 63*	15
Baseline	102 ± 6	24
Glutamate 250 μM	1194 ± 177*	24
Baseline	178 ± 24	11
Glutamate 500 μM	4660 ± 1254*	11
Baseline	112.9±14	19
Glutamate 1mM	6011.98 ± 657*	19

Table 2. Concentration dependent inhibition of $[Ca^{2+}]i$ by (+)-SKF10047

Glutamate induced calcium mobilization was measured by fura-2-AM imaging. Cells were pre-incubated for 30 minutes with fura-2 before stimulation with glutamate. Data were analyzed by One Way Analysis of Variance (ANOVA) and all Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) between baseline, peak and one minute post peak. Changes (mean ± SEM).

* Statistically significant (**$p<0.05$**) compared to the baseline. # Statistically significant (**$p<0.05$**) compared to the glutamate (250 μM).

Treatment	$[Ca^{2+}]_i$ nM, Mean ± SEM	Number of cells (n)
Baseline	64 ± 5	44
Glutamate 250 μM	1675 ± 226 *	44
Baseline	80 ± 5	22
Glutamate 250 μM+ (+)-SKF10047 (10 nM)	1090 ± 83 *	22
Baseline	137 ± 56	41
Glutamate 250 μM+ (+)-SKF10047 (1 μM)	639 ± 67 *#	41
Baseline	182 ± 22	13
Glutamate 250 μM + (+)-SKF10047 (10 μM)	268 ± 47 #	13
Baseline	190 ± 17	25
Glutamate 250 μM + (+)-SKF10047 (100 μM)	260 ± 23 #	25

Table 3. Comparison of glutamate-induced intracellular calcium mobilization between retinal ganglion cells line (RGC-5) tansfected with GFP vector and sigma-1 receptor overexpressing RGC-5 cells.

Glutamate induced calcium mobilization was measured by fura-2-AM imaging. Cells were pre-incubated for 30 minutes with fura-2 before stimulation with glutamate. Data were analyzed by One Way Analysis of Variance (ANOVA) and all Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) between baseline, peak and one minute post peak. Changes (mean ± S.EM). *Statistically significant (**p<0.05**) compared to the baseline. **&** statistically significance (**p<0.05**) versus RGC-5 transfected with GFP vector (sigma-1 receptor empty vector) control (glutamate, 250 μM). # Statistically significant (**p<0.05**) compared to the control (glutamate 250 μM).

Treatment	$[Ca^{2+}]_i$ nM, Mean ± SEM	Number of cells (n)
RGC-5 transfected with GFP/sigma-1 receptor empty vector		
Baseline	154 ± 8	21
Glutamate, 250 μM	1086 ± 102*	21
Baseline	90 ± 10	19
Glutamate (250 μM) + (+)-SKF10047 (1 μM)	502 ± 43* #	19
Baseline	83 ±16	19
Glutamate (250μM) + MK801 (10μM)	83 ± 14 #	19
GFP/Sigma-1 receptor overexpressing RGC-5 cells		
Baseline	67 ± 6	16
Glutamate, 250 μM	630 ± 40 *&	16
Baseline	74 ± 4	43
Glutamate (250 μM) + (+)-SKF10047 (1 μM)	199 ± 8 #	43
Baseline	57 ± 2	17
Glutamate (250μM) + MK801 (10μM)	60 ± 2 #	17

Table 4. Inhibition of glutamate-induced calcium influx in RGC-5 cells is mediated through sigma-1 receptor.

Glutamate induced calcium mobilization was measured by fura-2-AM imaging. Cells were pre-incubated for 30 minutes with fura-2 and BD1047 before stimulation with glutamate. Data were analyzed by One Way Analysis of Variance (ANOVA) and all Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) between baseline, peak and one minute post peak. Changes (mean ± SEM).

*Statistically significant (**p<0.05**) compared to the control (Glutamate 250μM).

Treatment	$[Ca^{2+}]_i$ nM, Mean ± SEM	Number of cells (n)
RGC-5 transfected with GFP/sigma-1 receptor empty vector		
Baseline	**122.7 ± 7**	28
Glutamate, 250 μM	**1571 ± 433**	28
Baseline	**109.9 ± 8**	32
Glutamate (250 μM) + (+)-SKF10047 (1 μM)	**666.9 ± 56***	32
Baseline	**100.1 ± 12**	32
Glutamate (250μM) + BD1047 (3μM)	**1520.2 ± 225**	32
Baseline	**49 ± 4**	43
Glutamate (250μM) + BD1047 (3 μM) + (+)-SKF10047 (1 μM)	**1584 ± 237**	43
GFP/Sigma-1 receptor overexpressing RGC-5 cells		
Baseline	**67.9 ± 3**	29
Glutamate, 250 μM	**671.4 ± 68**	29
Baseline	**65.4 ± 3**	43
Glutamate (250 μM) + (+)-SKF10047 (1 μM)	**254.4 ± 14***	43
Baseline	**47.9 ± 3**	42
Glutamate (250μM) + BD1047 (3μM)	**602.9 ± 68**	42
Baseline	**63.1 ± 4**	36
Glutamate (250μM) + BD1047 (3 μM) + (+)-SKF10047 (1 μM)	**633.8 ± 92**	36

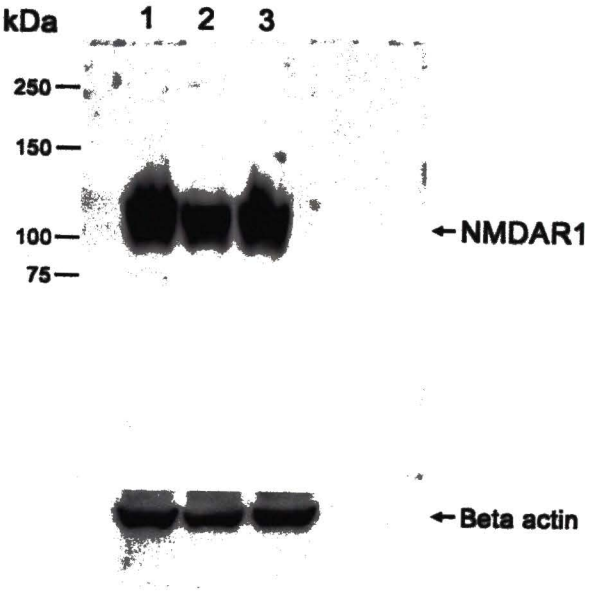
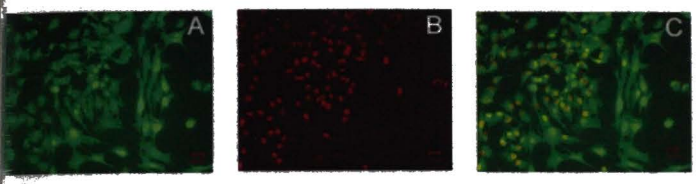


Figure 4.

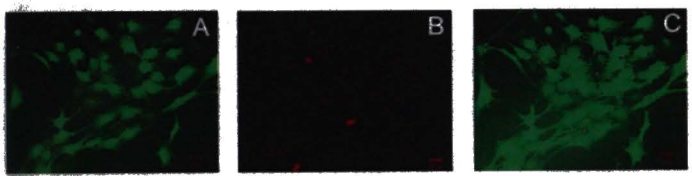
I - Control (No treatment)
(Green = Calcein staining; Red = Propidium iodide)

II - Glutamate (1mM)
(Green= Calcein staining; Red = propidium iodide)



III - (+)-SKF10047 (1microM)
(Green = Calcein staining; Red = Propidium iodide)

IV - ((+)-SKF10047, 1microM + Glutamate, 1mM)
(Green = Calcein staining; Red = propidium iodide)



V- BD1047 (3 microM)
(Green = Calcein staining; Red = Propidium iodide)

VI - BD1047, 3microM + Glutamate, 1mM +
(+)-SKF10047, 1microM)
(Green = Calcein; Red = Propidium iodide)

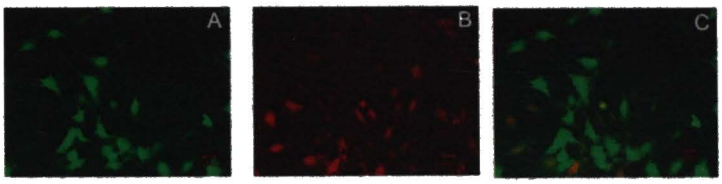
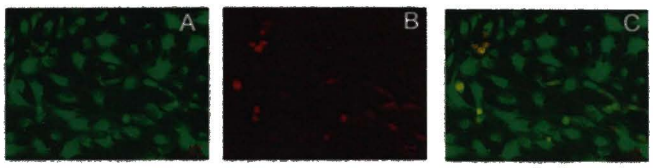


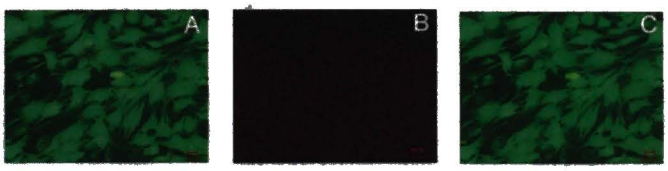
Figure 5.

I -Control (no treatment)
(Green = Calcein staining; Red = Propidium iodide)

II - Glutamate (1mM)
(Green = Calcein staining; Red = Propidium iodide)



III - (+)-SKF10047 (1microM)
(Green = Calcein staining; Red = Propidium iodide)

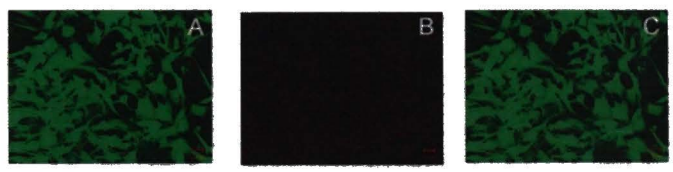


IV - (+)-SKF10047, 1 microM + Glutamate, 1 mM
(Green = Calcein staining; Red = Propidium iodide)

V - BD1047 (3 microM)
(Green = Calcein staining; Red = Propidium iodide)

VI - BD1047, 3 microM + Glutamate, 1mM +
(+)-SKF10047, 1microM
(Green = Calcein staining; Red = Propidium iodide)

VII - MK801 (10 microM)
(Green = Calcein staining; Red = Propidium iodide)

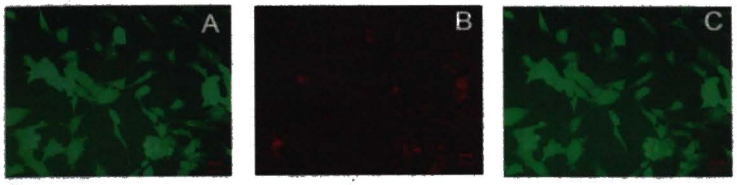


VIII - MK801, 10 microM + Glutamate, 1 mM
(Green = Calcein staining; Red = Propidium iodide)

Figure 6.

I - Control (no treatment)
(Green = Calcein staining; Red = Propidium iodide)

II - Glutamate (1mM)
(Green = Calcein staining; Red = Propidium iodide)

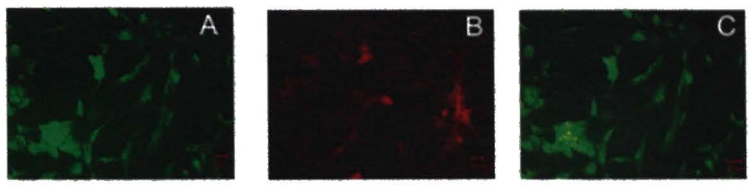


III - (+)-SKF10047 (1 microM)
(Green = Calcein staining; Red = Propidium iodide)

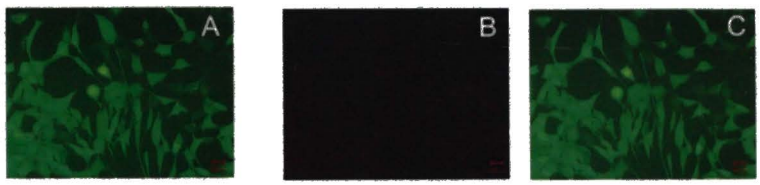
IV - (+)-SK10047, 1microM + Glutamate, 1 mM
(Green = Calcein staining; Red = Propidium iodide)

V - BD1047 (3 microM)
(Green = Calcein staining; Red = Propidium iodide)

VI - BD1047, 3microM + Glutamate, 1mM +
(+)-SKF10047, 1 microM
(Green = Calcein staining; Red = Propidium iodide)



VII - MK801 (10 microM)
(Green = Calcein staining; Red = Propidium iodide)



VIII - MK801, 10 microM + Glutamate, 1 mM
(Green = Calcein staining; Red = Propidium iodide)

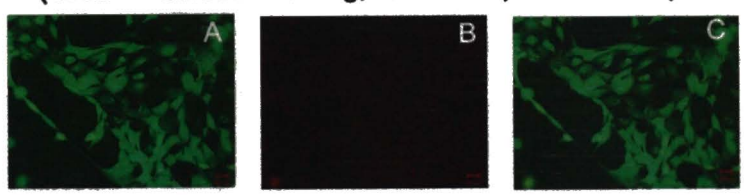
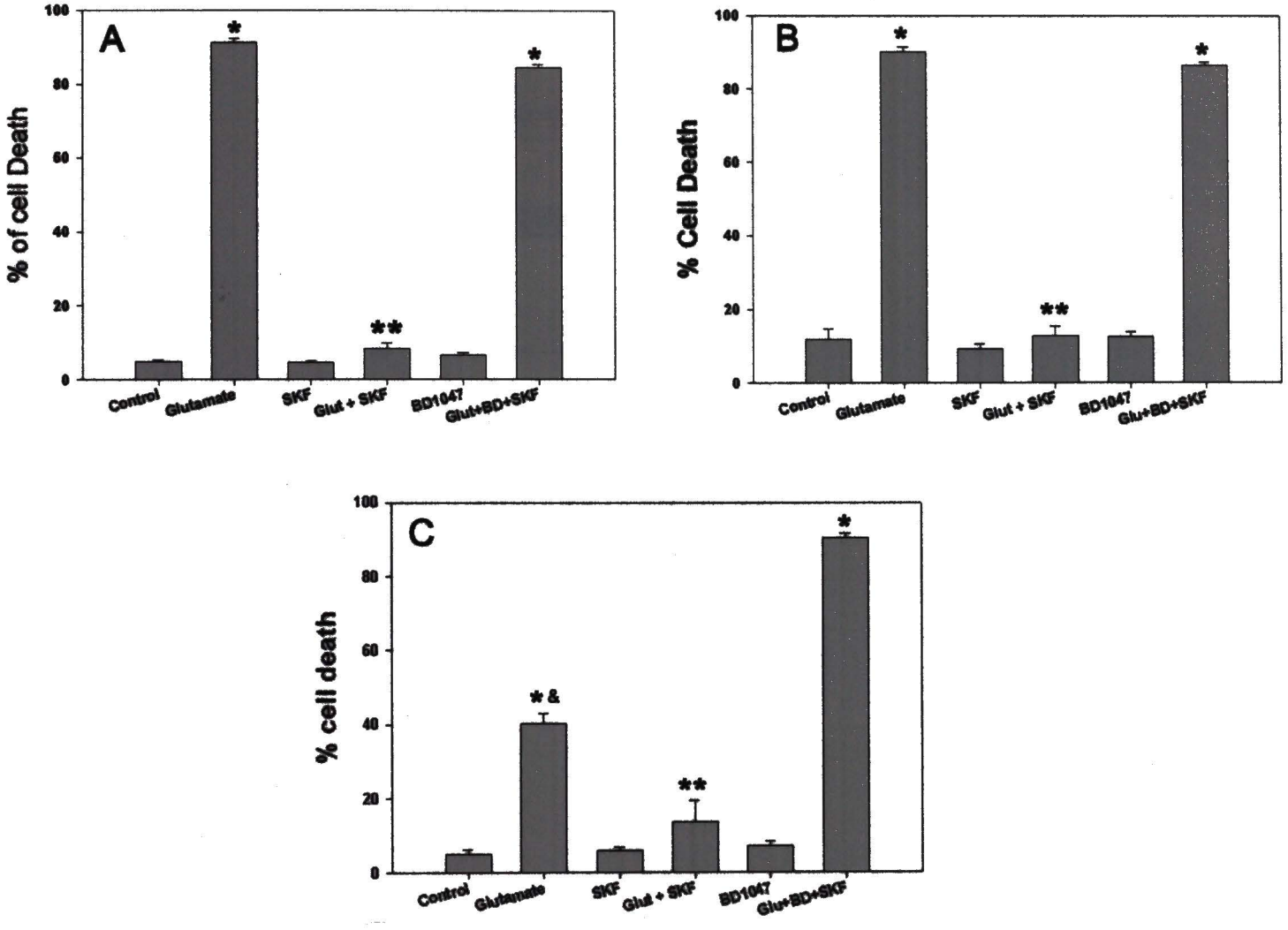


Figure 7.


A
% of cell Death
100
80
60
40
20
0
*
**
*
Control
Glutamate
SKF
Glut + SKF
BD1047
Glut+BD+SKF
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Control
Glutamate
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Glut + SKF
BD1047
Glu+BD+SKF
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100
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Control
Glutamate
SKF
Glut + SKF
BD1047
Glu+BD+SKF

Figure 8.

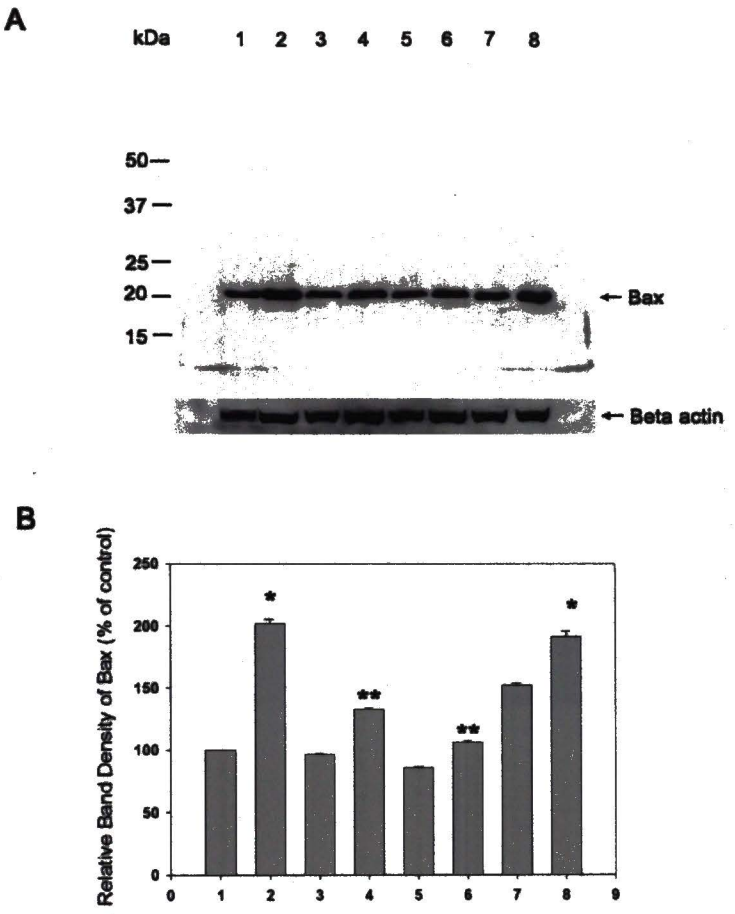
A
kDa
1 2 3 4 5 6 7 8
50—
37—
25—
20—
15—
← Bax
← Beta actin
B
Relative Band Density of Bax (% of control)
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9
*
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Figure 9.

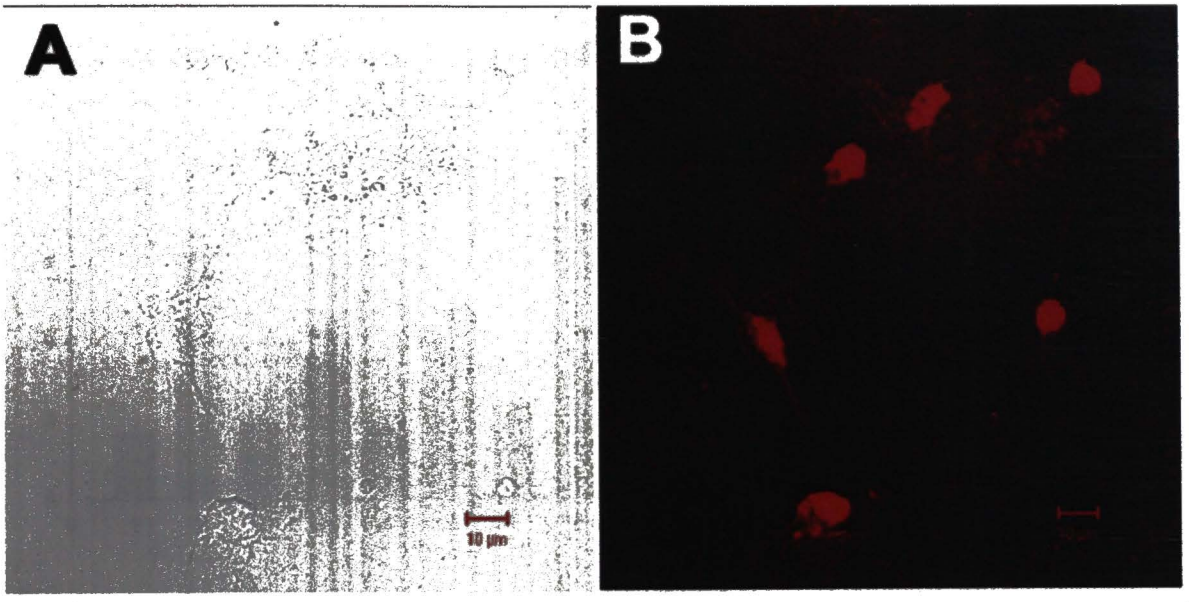

Figure 10.

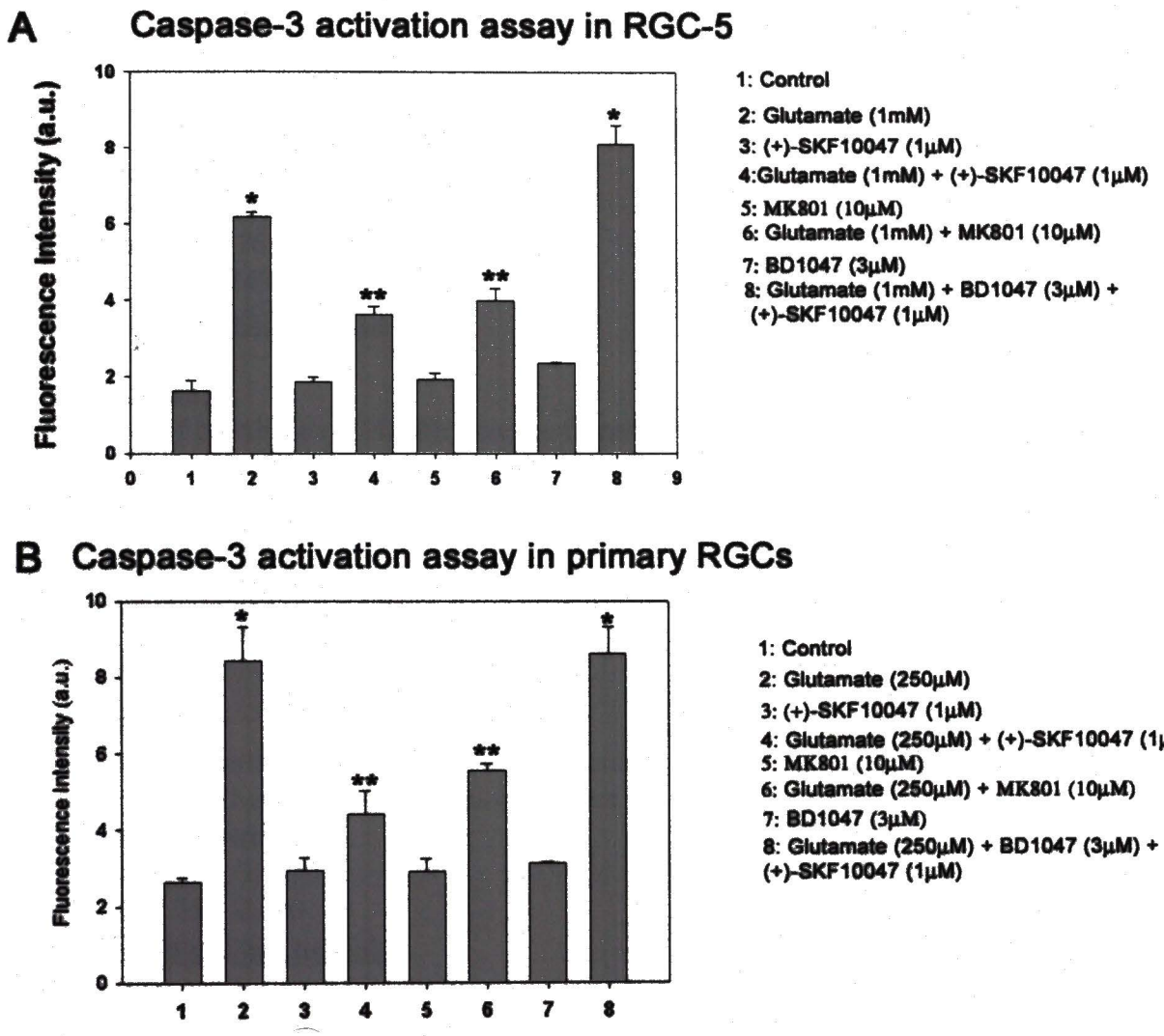
A
Caspase-3 activation assay in RGC-5
Fluorescence Intensity (a.u.)
1: Control
2: Glutamate (1mM)
3: (+)-SKF10047 (1μM)
4:Glutamate (1mM) + (+)-SKF10047 (1μM)
5: MK801 (10μM)
6: Glutamate (1mM) + MK801 (10μM)
7: BD1047 (3μM)
8: Glutamate (1mM) + BD1047 (3μM) + (+)-SKF10047 (1μM)
B
Caspase-3 activation assay in primary RGCs
Fluorescence Intensity (a.u.)
1: Control
2: Glutamate (250μM)
3: (+)-SKF10047 (1μM)
4: Glutamate (250μM) + (+)-SKF10047 (1μM)
5: MK801 (10μM)
6: Glutamate (250μM) + MK801 (10μM)
7: BD1047 (3μM)
8: Glutamate (250μM) + BD1047 (3μM) + (+)-SKF10047 (1μM)

Figure 11.

CHAPTER 4

Title: Sigma-1 receptor regulation of voltage-gated calcium channels involves a direct interaction between sigma-1 receptors and voltage-gated L-type calcium channel.

Abbreviated title: Sigma-1 receptor and calcium regulation

Authors:

Kissaou T. Tchedre
University of North Texas Health Science Center
Department of Biomedical Sciences
3500 Camps Bowie Blvd
Fort Worth, TX 76107
Phone: 817 735 2490
Email: ktchedre@hsc.unt.edu

Ren-Qi Huang
University of North Texas Health Science Center
Department of Pharmacology& Neurosciences
3500 Camps Bowie Blvd
Fort Worth, TX 76107
Phone: 817 735 -2095
Email: rhuang@hsc.unt.edu

Adnan Dibas
University of North Texas Health Science Center
Department of Pharmacology& Neurosciences
3500 Camps Bowie Blvd
Fort Worth, TX 76107
Phone: 817 735 -2490
Email: adibas@hsc.unt.edu

Raghu R. Krishnamoorthy
University of North Texas Health Science Center
Department of Pharmacology& Neurosciences
3500 Camps Bowie Blvd
Fort Worth, TX 76107
Phone: 817 735 -0139
Email: rkrishna@hsc.unt.edu

Glenn H. Dillon
University of North Texas Health Science Center
Department of Pharmacology& Neurosciences
3500 Camps Bowie Blvd

Fort Worth, TX 76107
Phone: 817 735 -2055
Email: gdillon@hsc.unt.edu

Corresponding author:

Thomas Yorio
University of North Texas Health Science Center
Department of Pharmacology& Neurosciences
3500 Camps Bowie Blvd
Fort Worth, TX 76107
Phone: 817 735 -0258
Email: yoriot@hsc.unt.edu

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Sigma-1 receptor regulation of voltage-gated calcium channels involves a direct interaction between sigma-1 receptor and voltage-gated L-type calcium channel.

Kissaou T. Tchedre[1], Ren-Qi Huang [2], Adnan Dibas[2], Raghu R. Krishnamoorthy[2], Glenn H. Dillon[2], and Thomas Yorio[2]

[1]Department of Biomedical Sciences; [2]Department of Pharmacology & Neuroscience, University of North Texas Health Science Center, Fort Worth TX 76107

ABSTRACT

Purpose: The sigma-1 receptor is a recently discovered family of transmembrane proteins that are expressed in the central nervous system including the eye that mediate the regulation of ion channels. The exact function of sigma receptors remains to be elucidated. The purpose of this study was to investigate the effect of sigma-1 receptor ligands on calcium homeostasis in a retinal ganglion cell line (RGC-5), and rat primary retinal ganglion cells.

Methods: Calcium imaging was used to assess the effect of sigma-1 receptor agonist (+)-N-allylnormetazocine ((+)-SKF10047) on potassium chloride (KCl)-induced calcium influx in RGC-5 cells. The whole cell patch clamp technique was used to analyze the effect of (+)-SKF10047 on calcium currents in primary retinal ganglion cells. Co-immunoprecipitation assessed the interaction between sigma-1 receptor and voltage gated L-type calcium channel.

Results: The sigma-1 receptor agonist (+)-SKF10047 inhibited potassium chloride (KCl)-induced calcium influx. The sigma-1 receptor antagonist, BD1047, reversed the inhibitory effect of (+)-SKF10047. The whole-cell patch clamp of rat cultured primary retinal ganglion cells demonstrated that (+)-SKF10047 inhibited calcium channel currents. We report for the first time that there is an association between L-type calcium channels and sigma-1 receptors and sigma-1 receptor ligands may indirectly influence voltage-gated calcium channels by interacting with the sigma-1 receptor associated voltage-gated calcium channel complex. **Conclusion:** These results suggest that sigma-1 receptor activation can regulate calcium homeostasis and signaling in retinal ganglia. Regulation of calcium influx in retinal ganglion cells by sigma-1 receptor ligands may represent in part the neuroprotective effect of sigma-1 receptors.

INTRODUCTION

Sigma-1 receptors (accession number NP 005857) were first described as one of the subtypes of opioid receptors. However, further studies have led to the distinction between sigma receptors from opioid receptors [1]. Sigma-1 receptor was first cloned from guinea pig liver[2]. Using a sequence homology approach, human [3], mouse, and rat sigma-1 receptors were cloned using guinea pig sigma-1 receptor cDNA [3-6]. Sigma-1 receptors are localized on both the endoplasmic reticulum and the plasma membrane. In addition, it was shown that sigma-1 receptors translocate from the endoplasmic reticulum-associated reticular network to the cell periphery upon stimulation [7]. Sigma receptors are non-opiate and non-phencyclidine (PCP) membrane-bound protein receptors, which are expressed in different tissues, including liver, endocrine glands, central nervous system[8]. The endogenous ligand of sigma-1 receptor is not known. However, it has been suggested that

neurosteroids might be the endogenous ligand of sigma receptors [9]. In the eye, the lachrymal gland, retina, iris-ciliary body, cornea and lens also express sigma-1 receptors [10-12].

Two sigma receptor subtypes have been identified by ligand binding assays and photoaffinity labeling. The Sigma-1 receptor has a molecular weight of approximately 25 kDa and the sigma-2 receptor has a molecular weight of ~ 18-21 kDa [2, 13]. Various pharmacological and physiological functions have been proposed for sigma-1 receptors and include: antipsychotic activity, modulation of opioid analgesia[14] and neuroprotection.[15-17] Sigma-1 receptor ligands inhibit the NMDA receptor-ion channel complex,[17,18, 19] voltage-gated calcium channels in intact neuronal cells[20], and Kv1.4 potassium channels[21, 22]. It has been shown that knock down of sigma-1 receptor expression by siRNA increases cell death in lens cells [23]. Sigma-1 receptor antagonists have also been shown to induce apoptotic cell death that can be rescued by caspase-3 inhibitors[24] or by sigma-1 agonists (Tchedre et al. 2008 *IOVS* in press).

We have shown in this research that sigma-1 receptor activation can attenuate calcium influx in retinal ganglion cell by regulating voltage-gated calcium channels. We report for the first time that there is a physical interaction between L-type calcium channels and sigma receptors and these results indicate a novel relationship similar to that described for the Kv1.4 potassium channels and sigma receptors[21]. In addition, we also demonstrate that the sigma-1 receptor agonist (+)-SKF10047 inhibits voltage-gated calcium channel currents. These results suggest that sigma-1 receptors can modulate calcium homeostasis in the rat retinal ganglion RGC-5 cells, and primary rat retinal ganglion cells by regulating calcium levels and calcium signaling. This regulation of

calcium concentration may represent in part the neuroprotective mechanism of sigma-1 receptors.

Materials and Methods

Nifedipine was purchased from Sigma Chemical Co. (St. Louis, MO). (+)-N-allylnormetazocine ((+)-SKF10047), and ([2-(3,4-dichlorophenyl) ethyl]-*N*-methyl-2-(diamino) ethylamine (BD1047) were purchased from Tocris bioscience (Ellisville, Misouri, USA). The Exacta Cruz™ (F: sc-45043) immunoprecipitation kit was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Sigma-1 receptor was a gift from Dr. Hayashi, Teruo (NIH/NIDA/IRP, Baltimore, MD). Rabbit normal IgG was purchased from sigma chemical Co. Secondary antibodies including donkey anti-rabbit IgG and donkey anti-mouse IgG conjugated to HRP were purchased from Amersham Biosciences, Piscataway, NJ. Thy-1.1 polyclonal antibody was purchased from Santa Cruz biotechnology (Santa Cruz, CA). Monoclonal anti-neurofilament-200 and voltage-dependent L-type α1C subunit antibody (anti-human α1C) were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescent probes including goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 633 and fura-2-AM were purchased from Molecular Probes, Eugene, OR.

Retinal ganglion (RGC-5) cells culture.

The RGC-5 cell line was developed specifically to establish a permanent rat retinal ganglion cell culture [25]. RGC-5 cell cultures were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 U/ml of penicillin and 100 μg/ml of streptomycin and incubated at 37°C in 5% CO_2

and air. Retinal ganglion cells (RGC-5) were differentiated as described by Dauphin et al. (Rachel Dauphin et al., 2007).

Primary rat retinal ganglion cell isolation: Modified immunopanning protocol.

All procedures were performed in accordance with the ARVO guidelines for care and use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee at the UNT Health Science Center. Wistar Kyoto rats (3-4 wks old, either sex) were purchased from Harlan Laboratory (Indianapolis, IN). The rats were anesthetized with isoflurane and then decapitated. The eyes were enucleated and the retina were gently peeled off with fine forceps and placed in sterile phosphate buffer saline (PBS). Retina were collected in DMEM and incubated at 37°C in 3ml of dispase for 1 hour. After 1-hour incubation, the retinas were gently washed once with complete neurobasal medium. Retinal ganglion cells were obtained by trituration of the retina in neurobasal medium (GTBCO, Grand Island, NY) with a fire-polished Pasteur pipette. The cell suspension collected was seeded on coverslips previously coated first with poly-D-lysine (overnight) and second coated with Thy 1.1 monoclonal antibody (Chemicon International, CA) for 2 hours. After 30 minutes incubation, the coverslips were gently washed once with complete neurobasal medium, then the coverslips were covered with 2 ml of complete neurobasal medium supplemented with B27 and Pen-Strep (Gibco, Carlsbad, CA), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic growth factor (CTNF) (biosource PHC 7074), and forskolin (Sigma-Aldricht F6886). Cell culture dishes were subsequently incubated in a 5% CO2 incubator at 37°C for up to 7 days *in vitro* (7 DIV). Isolated retinal ganglion cells were characterized by

immunocytochemistry for a normally expressed retinal ganglion cell marker, thy-1 [26], and neurofilament-200.

Intracellular Calcium ($[Ca^{2+}]_i$) measurement

Intracellular calcium in retinal ganglion cells (RGC-5) was measured at 37 °C by the ratiometric technique using fura-2AM (excitation at 340 nm and 380 nm, emission at 510 nm) according to Prasanna et al[27]. Intracellular calcium ($[Ca^{2+}]_I$) in nanomolar (nM) was calculated using the Grynkiewicz equation [28]. In calcium imaging studies, cells were pretreated with antagonist drugs such as BD1047, and nifedipine for 30 minutes before stimulating the cells with KCl.

Preparation of stably transfected RGC-5 Cells

Stably transfected with sigma-1 receptor RGC-5 cells was established as previously described by Tchedre et al. (Tchedre et al. 2008 *IOVS* in press). Briefly, A rat sigma-1 receptor cDNA cloned in the pSPORT-1 plasmid vector was a gift from Dr. Ganapathy (Medical College of Georgia, Augusta, GA). E. Coli competent strain SURE-2 (Stratagene, CA) cells were transformed with a plasmid pSPORT-Sigma-1R. Transformed cells were plated on ampicillin (100 μg/ml) containing Luris-Bertani (LB, 10g Bacto-tryptone, 5g yeast extract, 10g NaCl, 15g agar in 1 liters of dH_2O) plates. Many colonies were obtained after overnight incubation. Colonies were picked and grown in Luria-Bertani (LB, 10g Bacto-tryptone, 5g yeast extract, 10g NaCl in 1 liters of dH_2O, Adjust pH to 7.5 with NaOH) media for large-scale preparation of plasmid DNA. Plasmid DNA was purified through the equilibrium cesium chloride gradient method. After the plasmid preparation, the purity of the DNA was checked on agarose gel. The lipofectamine ™ 2000 (Invitrogen) method was used for transfection of retinal ganglion

(RGC-5) cells. Since RGC-5 cells are neomycin (G418) resistant, co-transfection of pSPORT-Sigma-1 and pZeoSV2 (Invitrogen) was used. After transfection, zeocin resistant clones were selected and the expression of sigma-1 receptors was assessed by western blot.

Preparation of cell lysate and western blotting of proteins

Non-overexpressing and sigma-1 receptor overexpressing RGC-5 cells lysates were prepared as described by Bu et al.[29] Protein concentrations of cell lysates were determined using a BioRad DC protein kit (Bio-Rad Laboratories, Hercules, CA, USA). The samples were run on SDS-PAGE gel and immunoblot analysis was carried out using standard methods as described by Laemmli[30] and Towbin et al.[31] using anti-sigma-1 receptor rabbit polyclonal antibody. The blots were developed using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Immunofluorescence microscopy:

Primary retinal ganglion (7 DIV) cells were grown on coverslips and treated for 30 min. Coverslips were fixed with 4% paraformaldehyde and blocked with blocking solution (5% BSA + 5% normal goat serum) for 1 hour at room temperature. The blocking buffer was discarded and the coverslips were washed three times with 1X PBS, before the addition of the affinity-purified Sigma-1 receptor polyclonal antibody (1/200 dilution) (Santa Cruz Biotechnology, Inc, CA.) and voltage-dependent L-type α1C subunit antibody (1/200) (Sigma-Aldrich (St. Louis, MO)) or Thy1.1 monoclonal antibody (1/500 dilution) (Santa Cruz, CA) and monoclonal anti-neurofilament-200 (Sigma-Aldrich (St. Louis, MO)) with 1% normal goat serum and 5% bovine serum albumin (Sigma-Aldrich, St Louis, MN). The incubation was performed overnight at 4°C. Coverslips were

washed three times and 1/500 secondary antibodies (Alexa Fluor 633) and (Alexa fluor 488) was added and the coverslips were incubated for 1 hour in the dark. After the incubation, the coverslips were washed again three times with 1X PBS. The mounting was done on glass slides using ProlongR Gold antifade reagent with DAPI (Invitrogen , CA) and allowed to dry for 20 minutes in the dark. Cells were viewed with confocal laser scanning microscope (Zeiss LSM 510).

Whole cell Patch Clamp

Macroscopic calcium currents were measured using whole-cell patch clamp[32]. Patch pipettes of borosilicate glass (1B150F, World Precision Instrument, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 5-7 MΩ. The pipette solution contained (mM): 140 CsCl, 10 EGTA, 4 Mg-ATP, and 0.2 Na_3-GTP, pH 7.2. The external bath solution contained (in mM) 125 NaCl, 1 MgCl, 10 HEPES, 5 $CaCl_2$, 10 glucose, 0.5 TTX (μM), pH 7.3. Coverslips containing cells were placed in a small chamber (~ 1.5 ml) and superfused continuously (7-10 ml/min). The cells were visualized using an upright, fixed stage microscope (Nikon Optiphot-2UD) with 12 V-100 W halogen lamp equipped with standard Hoffman modulation contrast (HMC) optics and video camera system (Sony model XC-75 CCD video camera module, Tandy video monitor). The whole-cell Ca^{2+} currents were obtained using a patch clamp amplifier (PC-505B, Warner Instruments, Hamden, CT) equipped with 201B headstage. The currents were low-pass filtered at 2 kHz, sampled at 50 kHz and stored on a computer (pCalmp6.0.2, Axon Instruments) for subsequent analysis. 60-80% series resistance compensation was applied at the amplifier. Leak current was subtracted using a P/4 protocol. Cells were voltage clamped at -90 mV. Currents were

evoked with voltage step from -90 to 0 mV for 55 or 200 ms. all recordings were carried out at room temperature (20-21°C).

Co-immunoprecipitation of sigma-1 receptor and voltage-gated L-type calcium channels:

Non-overexpressing and sigma-1 receptor overexpressing retinal ganglion (RGC-5) cells were collected and lysed with ice-cold non-denaturing lysis buffer (1% (w/v) Triton X-100, 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02% (w/v) sodium azide). Immediately before use, fresh protease inhibitors (20 μg/ ml leupeptin, 20 μg/ ml aprotinin, 20 μg/ ml soybean trypsin inhibitor and 40 μg/ml PMSF) were added. The co-immunoprecipitation was achieved using The Exacta Cruz™ (F: sc-45043) immunoprecipitation kit method (as described in Exacta Cruz™ datasheet: http://datasheets.scbt.com/sc-45043.pdf). After the co-immunoprecipitation, the samples were resolved on a SDS-PAGE gel and immunoblotted via standard methods using anti-sigma-1 receptor antibody (1/500) or anti-human α1C antibody (1/500). The blots were developed using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

RESULTS

Sigma-1 receptors are expressed in RGC-5 cells.

Sigma-1 receptors are expressed in many organs including the eye [11]. In this study, using immunoblot analysis we examined the expression of sigma-1 receptors both in control and sigma receptor-1 receptor overexpressing RGC-5 cells. The western blot showed the expression of sigma-1 receptor in RGC-5 cells (**Fig.1, *lanes 1&2***), and sigma-1 receptor over-expression in RGC-5 (clone 4, **Fig.1, *lane 3***, see method section for the preparation of stably transfected RGC-5) cells respectively (**Fig.1**).

Characterization of KCl-induced calcium influx in RGC-5 Cells:

Sigma-1 receptor agonist (+)-SKF10047 inhibits KCl-induced calcium influx in RGC-5 cells.

Intracellular calcium mobilization in retinal ganglion cells (RGC-5) was measured by a ratiometric technique using fura- 2-AM. Depolarization with KCl (20, 40 and 80 mM) dose dependently induced calcium influx in RGC-5 cells **(Fig. 2)**. The sigma-1 receptor agonist (+)-SKF10047 (10nM, 1, 10 and 100μM) was able to significantly inhibit the KCl-induced calcium influx peak dose dependently (**Fig. 3*A***). 1μM of (+)-SKF10047 inhibited KCl-induced calcium influx peak from 100% to 22% (**Fig. 3 *B&D***). Nifedipine, a well-known L-type calcium channel blocker, was used to determine whether the calcium response observed is mainly mediated through L-type calcium channels. Nifedipine significantly inhibited the KCl induced calcium influx (**Fig. 3C**). BD1047 had no significant effect on KCl-induced calcium influx in RGC-5 cells on its own, but was able to block the effect of (+)-SKF10047 (**Fig. 3*D***). In addition, we tested whether or not extracellular calcium was required for the KCl-induced calcium influx. No calcium response was seen in the absence of extracellular calcium. But when cells were stimulated with KCl in the presence of 2.5 mM calcium a peak of calcium response was seen **(Fig. 4)**. This experiment shows that extracellular calcium is required for the KCl-induced calcium influx in RGC-5 cells. Taken together, the calcium data suggest that activation of sigma-1 receptors leads to the inhibition of calcium channels.

KCl-induced increase in $[Ca^{2+}]_i$ could also result from inhibition of Ca^{2+} reuptake into SR and/or stimulation of Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} stores[33].

We therefore, used thapsigargin to investigate whether the KCl-stimulated increase in $[Ca^{2+}]_i$ in RGC-5 cells was due to mobilization from IP_3 sensitive Ca^{2+} stores. Addition of thapsigargin to RGC-5 cells resulted in an increase in $[Ca^{2+}]i$, followed by a drop to a new higher steady-state calcium level. When the cells were subsequently stimulated with KCl after addition of thapsigargin, there was an increase in $[Ca^{2+}]_i$ with a profile normally observed in KCl-treated cells (data not shown). These data suggest that KCl-induced calcium influx is mainly mediated through extracellular calcium. Overall the calcium data suggests that activation of sigma-1 receptors, through regulation of L-type calcium channels may be playing an important role in retinal ganglion cells.

Expression of Sigma-1 receptor and Voltage gated L-type calcium channels in rat primary RGC.

Sigma receptor and voltage gated calcium channels have been shown to be expressed in the central nervous [20] system, in the eye[11], and in many other systems [13]. Using the immunocytochemistry we confirmed the expression of both receptor in our system (**Fig. 5).**

Sigma-1 receptor ligand (+)-SKF10047 inhibits voltage-gated calcium channel currents in the rat primary retinal ganglion cells.

To directly assess the modulation of voltage gated-calcium channels (VGCCs) by sigma-1 receptors, whole-cell calcium currents were measured using the patch clamp technique on rat cultured retinal ganglion cells (7 DIV). Currents were evoked with voltage steps from -90 to 0 mV for 55 ms. The average current amplitude was 284 ± 33 pA (n=38). Extracellular application of the sigma-1 receptor agonist, (+)-SKF10047 (0.3-10 μM) inhibited calcium current amplitude in a dose-dependent manner **(Fig. 6A&C).**

The onset of the effect was relatively rapid, approximately 3 min after (+)-SKF10047 perfusion. Furthermore, the (+)-SKF10047 effect was typically reversible in 3-5 min after washout.

In order to study the effects of (+)-SKF10047 on activation properties of calcium currents, current-voltage relationships (I-V curves) was determined from the amplitude of current elicited by 200 ms-lasting voltage ramp from a holding potential of -80 mV to 50 mV. As can be observed in the I-V curve **(Fig. 6B)**, at potentials more positive than -30 mV, an inward Ca^{2+} current was evoked. The maximum peak amplitude was obtained at approximately -10 mV and the amplitude was reduced at more positive potentials. The inward Ca^{2+} current was reduced by (+)-SKF10047 (10 μM) at all values of membrane potentials. However, (+)-SKF10047 did not significantly modify the properties of I-V curves. On the average, the voltage for half-maximal activation ($V_{1/2max}$), maximal activation (V_{max}), half-maximal inactivation ($V_{1/2imax}$) and reversal potential did not differ significantly between control and (+)-SKF10047 treatment (n=5, $p>0.05$, paired t-test). These data suggest that (+)-SKF10047 did not alter the I-V relationships of VGCCs.

BD1047 has already been identified as a selective sigma-1 receptor antagonist [14]. To determine whether the observed effect of (+)-SKF10047 on VGCCs is mediated by sigma-1 receptors, we tested (+)-SKF100047 effect in the presence of BD1047. BD1047 (3 μM) alone had no effect on calcium channel currents (current amplitude 105 ± 7.8% of control, n=5, $p>0.05$, paired t-test, compared to the control). The Ca^{2+} current amplitude was reduced to 80 ± 2.3% of control by 1 μM (+)-SKF10047 alone **(Fig. 6*C*)**. However, as shown in **(Fig. 7*A&B*)**, pretreatment with BD1047 completely abolished the effect of (+)-SKF10047 (96 ± 5.3% of control, n=5, $p>0.05$, paired t-test, compared to control).

These data suggest that the observed (+)-SKF10047 inhibitory effect on voltage-gated Ca^{2+} current is mediated by activation of sigma-1 receptors.

In order to characterize the Ca^{2+} channel subtypes inhibited by (+)-SKF10047, we examined the effect of (+)-SKF10047 in the presence of nifedipine, a selective blocker of L-type Ca^{2+} channels. **Fig. 8*A*** shows individual Ca^{2+} current traces in one representative experiment, before, 4 min after nifedipine (10 μM) and after 3 min (+)-SKF10047 (3 μM) in the presence of nifedipine. **Fig. 8*B*** shows the percentage inhibition of (+)-SKF10047 in presence of nifedipine compared to (+)-SKF10047 alone. Nifedipine alone reduced Ca^{2+} current amplitude by 26 ± 6.7% (n=4, $P<0.01$, paired t-test, compared to control). (+)-SKF10047 (3 μM) alone reduced Ca^{2+} current by 24 ± 2.1% while it only inhibited Ca^{2+} currents by 11 ± 4.7% in the presence of nifedipine (n=4, $p<0.05$, unpaired t-test). The effect of (+)-SKF10047 was significantly attenuated when L-type channel were pharmacologically blocked, suggesting that sigma-1 receptor not only inhibit L-type voltage gated calcium channels, but also other voltage-gated calcium channels.

Sigma Receptor-Voltage gated L-type calcium channels (L-VGCC) co - immunoprecipitation

We performed co-immunoprecipitation experiments to detect whether sigma-1 receptors were associated with voltage-gated L-type calcium channels. Membrane lysates were prepared from both RGC-5 cells and sigma-1 receptor overexpressing RGC-5 cells and immunoprecipitated with rabbit antibodies against L-VGCC or sigma-1 receptor. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on cell lysates, then the proteins were transferred to a nitrocellulose membrane and probed with rabbit antibodies against L-VGCC or sigma-1 receptor. Bands were visualized with an HRP-conjugated

antibody and enhanced chemiluminescence, as described in the method section. Probing the membrane with antibody against the sigma-1 receptor revealed a distinct band of ~25 kDa in samples immunoprecipitated with anti-human L-type calcium channels (α1C) antibody (**Fig. 9*A*, Lanes 1 & 2**) or anti-sigma-1 receptor (**Fig. 9*A* Lane 3**). Probing the membrane with anti-human α1C antibody indicated a distinct band of ~ 205 kDa in samples immunoprecipitated with anti-human α1C antibody (**Fig. 9*B*, Lanes 1 & 2**) or anti-sigma-1 receptor (**Fig. 9*B*, Lane 3**). These results suggest a direct physical interaction between sigma-1 receptor and voltage-gated L-type calcium channels.

DISCUSSION

Although the sigma-1 receptor was cloned almost a decade ago[2], the molecular characterization of the sigma-1 receptor is just beginning to be assessed. The sigma receptor itself is ubiquitously distributed in vertebrates, and has been implicated in a wide range of biological functions[34, 35]. Sigma-1 receptors are membrane-bound protein receptors, which are expressed in many tissues, such as liver, endocrine glands, lachrymal gland, retina [11, 12], and in the central nervous system[5, 36]. In the central nervous system neurons, sigma receptors have been shown to produce various cellular effects including inhibition of calcium influx through voltage activated calcium channels in rat sympathetic and parasympathetic neurons[20].

In the present studies, we showed for the first time using co-immunoprecipitation that there is a physical interaction between voltage-gated L-type calcium channels and sigma-1 receptors. These results indicate a novel relationship similar to that described for the Kv1.4 potassium channels and sigma receptors.[21] This effect of sigma-1 receptors on voltage-gated calcium L-type calcium channels supports the hypothesis that protein-

protein interactions are characteristic of sigma receptor signal transduction. This form of signal transduction has already been proposed in previous studies, in which it was shown that sigma receptor-mediated signal transduction does not require G-protein activation or protein phosphorylation [37]. Retinal ganglion cells express L-type calcium channels, as well as other type of voltage-gated calcium channels. Since we focused on interaction of sigma-1 receptors with L-type calcium channels, we cannot rule out the possibility that sigma-1 receptors directly interact with other types of voltage-gated calcium channels. Further studies will focus on the direct interaction of other voltage-gated calcium channels and sigma-1 receptors. Indeed, some inhibitory effect of (+)-SKF10047 on voltage-gated calcium channels was noted in the presence of the L-type inhibitor nifedipine. Thus, other subtypes of voltage-gated calcium channels may be modulated by sigma-1 receptor activation. This possibility is consistent with the finding that sigma-1 receptors inhibit multiple classes of Ca^{2+} channels in guinea pig ileum longitudinal muscle[38]. Further studies will be needed to assess this possibility definitively in retinal ganglion cells. While we cannot rule out the involvement of sigma-2 receptors in the calcium influx inhibition in our system, we focused our studies on the sigma-1 receptor, which is already cloned and well studied. Therefore, it is possible that there are some contributions of sigma-2 receptors in our system, but it was not tested. Differential regulation of calcium channels and NMDA receptors have been shown to determine the survival or death in rat primary cortical neurons.[39, 40] Therefore, in addition to regulating proapoptotic genes,[24] (Tchedre et al., 2008 *IOVS*, in press), sigma-1 receptors prevent calcium overload, in retinal ganglion cells by regulating L-type calcium channels activation.

In summary we have shown that in retinal ganglion cells, sigma-1 receptors control in part calcium homeostasis through the regulation of calcium influx. We report for the first time that there is a physical interaction between L-type calcium channels and sigma-1 receptors. By controlling excess calcium influx in the cell, sigma-1 receptor ligands may contribute to the control of calcium overload in retinal ganglion cells, which is shown to induce cells death[41, 42].

Acknowledgment

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Figures legends:

Figure 1. Western blot analysis of sigma-1 receptor proteins expression in RGC-5 cells.

Cells lysates from the non-overexpressing and sigma-1 receptor overexpressing RGC-5 cells were subjected to SDS–PAGE followed by immunoblotting using specific antibodies for the sigma-1 receptor. ***(Lane 1 & 2)***: retinal ganglion cells (RGC-5); ***(lane 3)***: RGC-5 overexpressing sigma-1 receptor (clone 4). The western blot results also showed the expression and over-expression of sigma-1 receptor in RGC-5 cells, and clone 4 RGC-5 cells respectively.

Figure 2. Potassium Chloride (KCl)-induced calcium influx in RGC-5 cells dos-dependently.

KCl-induced calcium influx was measured by fura-2-AM imaging. Cells were pre-incubated for 30 minutes with fura-2 before stimulation with KCl. KCl-induced calcium influx in RGC-5 cells was concentration- dependent Data were analyzed by One Repeated Way Analysis of Variance (ANOVA) and all Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) between baseline, peak and one minute post peak. Changes (mean ± SEM). *Statistically significant (**$p<0.05$**) compared to the baseline.

Figure 3. Sigma-1 receptor ligand (+)-SKF10047 effect on KCl-induced calcium influx in RGC-5 cells:

KCl-induced calcium influx was measured by fura-2-AM imaging. Cells were pre-incubated for 30 minutes with fura-2 before stimulation with 20mM KCl. ***A).*** Sigma-1 receptor agonist (+)-SKF10047 inhibited KCl-induced calcium influx in a concentration-

dependent manner. Data were analyzed by One Repeated Way Analysis of Variance (ANOVA) and all Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) between baseline, peak and one minute post peak. Changes (mean ± SEM). *Statistically significant (**p<0.05**) compared to the baseline. ***B).*** Representative $[Ca^{2+}]_i$ trends in response to KCl in RGC-5 cells: KCl (20 mM) induces calcium influx in retinal ganglion (RGC-5) cells ***(Solid line curve)*** and (+)-SKF10047, 1μM inhibits KCl-induced calcium influx in RGC-5 cells ***(Dot line curve). C).*** Representative $[Ca^{2+}]_i$ trends in response to KCl in the presence of nifedipine. Nifedipine significantly inhibited the KCl-induced calcium influx similarly as (+)-SKF10047. ***D).*** Summary of KCl-induced calcium influx measured by fura-2-AM imaging in retinal ganglion (RGC-5) cells. Cells were pre-incubated with fura-2-AM for 30 minutes with the sigma-1 receptor antagonist (BD1047) before stimulation with KCl. Data were analyzed by One Repeated Way Analysis of Variance (ANOVA) and all Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) between baseline, peak and one-minute post peak. Values are the mean ± S.EM of ((**n = 44** for KCl, 20 mM); (**n = 44** for (+)-SKF10047, 1μM); (**n =36**, for BD1047, 3μM); (**n =30**, for KCL + BD1047 + (+)-SKF10047)).

*Statistically significant (**p<0.05**) compared to the Control KCl (20 mM).

Figure 4. Extracellular calcium is required for the KCl induced calcium influx in RGC-5 cells

Representative $[Ca^{2+}]_i$ trends in response to KCl. The calcium response is measured by fura2-AM imaging. RGC-5 cells were pre-incubated with fura-2AM in a calcium free HBSS for 30 min before stimulation with KCl alone without or with calcium (2.5 mM).

Figure 5. Expression of Sigma-1 receptor and Voltage gated L-type calcium Channels in primary rat retinal ganglion cells.

Immunocytochemistry. Primary RGC were grown on 35-mm coverslips for 7 days, fixed, and subjected to immunofluorescent staining for sigma-1 receptors and voltage gated L-type calcium channels. Cells were incubated with primaries anti-goat sigma-1 receptor polyclonal antibody and anti-rabbit voltage-dependent L-type α1C subunit antibody followed by incubation with secondary Alexa Fluor 488 donkey anti goat and Alexa fluor 633 goat anti rabbit. Confocal laser scanning microscope (Zeiss LSM 510) was used to detect sigma-1 receptor (green) and voltage gated L-type calcium channels (red). **a).** DIC; **b).** Sigma-1 receptor; **c).** Voltage gated L-type channels; **d).** Merge. Scale bar represents 10μm.

Figure 6: Effect of sigma-1 receptor agonist (+)-SKF10047 on voltage-gated Ca^{2+} channels in rat RGC. ***A).*** Representative traces showing that the sigma-1 receptor agonist (+)-SKF10047 (SKF) inhibited voltage-gated Ca^{2+} currents in a concentration-dependent manner. 5 mM Ca^{2+} was added to external solution as the charge carrier. Whole-cell Ca^{2+} currents were recorded using patch clamp on cultured RGC (2 DIV) prepared from adult rats. Currents were evoked with a depolarization voltage step from -90 to 0 mV for 55 ms. Ca^{2+} currents were measured in the absence (as control) or presence of SKF for 3 minutes and during 3 minutes of washout. ***B),*** The current-voltage relationship was measured using a single voltage ramp from -80 mV to +50 mV of 200 ms duration (0.65 mV/ms). The I-V curves exhibited similar properties in the control and in (+)-SKF10047 treatment. ***C),*** Concentration-dependent inhibition of Ca^{2+}

currents by (+)-SKF10047. All currents are normalized to the control (assigned as 100%). Each column indicates the mean of 4-6 observations.

Figure 7: (+)-SKF10047 effect on Ca^{2+} currents is mediated by sigma-1 receptors.

A), Representative traces were recorded in the control, at 3 min- perfusion with BD1047 (BD) (3 μM) or BD (3 μM) + SKF10047 (SKF) (1 μM). Treatment with BD1047 alone had little effect on whole-cell Ca^{2+} currents but prevented (+)-SKF10047- induced inhibition ***B),*** Summary of BD1047 blockade of (+)-SKF10047-induced inhibition of Ca^{2+} channels currents. The currents are normalized to the control (100%). Each bar in the graph point represents mean value of 5 cells. ***C, D, E).*** Modified immunopanning protocol for RGCs isolation (see method section for technique detail). Cells were characterized by immunocytochemistry for a normally expressed retinal ganglion cells markers thy-1 [26] and neurofilament-200. **a).** Thy-1 labeling (red); **b*).*** Neurofilament-200 (green); **c).** DIC picture, **d*)***. Merge. As shown on the figures, both methods yield very pure primary retinal ganglion cell cultures. Scale bar represents 10μm.

Figure. 8: Sigma-1 receptor inhibits calcium currents in RGCs.

Effect of nifedipine, a L-type Ca^{2+} channel blocker on (+)-SKF10047- induced inhibition of Ca^{2+} currents in rat cultured RGC. ***A),*** Whole-cell Ca^{2+} currents were recorded in the control, perfusion with nifedipine (Nif, 10 μM) or Nif (10 μM)+SKF (3 μM). The solid lines indicate the duration of perfusion. The arrows indicate the time point when the current was measured. Note that SKF- induced inhibition was rapidly reversible after washout (indicated with *). ***B),*** Blockade of L-type Ca^{2+} channels with nifedipine significantly reduced SKF-induced inhibition ($p<0.05$, unpaired t-test). For

direct comparison, the SKF (3μM) data were re-plotted from **Fig. 1C**. All currents are normalized to the control prior to SKF treatment.

Figure 9. Co-immunoprecipitation of Sigma-1 receptor (Sigma-1R) and voltage gated L-type calcium channel (L-VGCC).

Co-immunoprecipitation of Sigma-1 receptor (Sigma-1R) and voltage gated L-type calcium channel. Cell lysates were prepared from the rat non-transfected and sigma-1 receptor cDNA transfected retinal ganglion (RGC-5), and immunoprecipitated with either anti-human α1C antibody, or sigma-1 receptor antibody. Eluates from the co-immunoprecipitation were resolved with SDS-PAGE: ***lanes 1*** represents (RGC-5 non transfected cells), ***lanes 2, 3*** and ***4*** represent (RGC-5 overexpressing the sigma-1 receptor). Immunoprecipitated samples were run on duplicate gels and the blots probed with either anti-sigma-1 receptor ***(A)*** or anti-human L-type calcium channels (α1C) ***(B)*** antibodies. The co-immunoprecipitation results show a direct physical interaction between sigma-1 receptor and voltage gated L-type calcium channels.

Figures

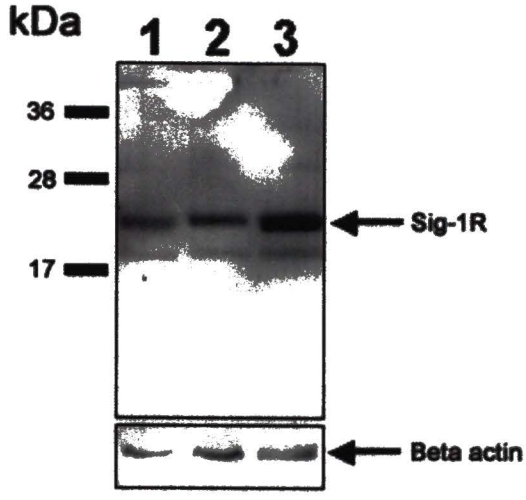
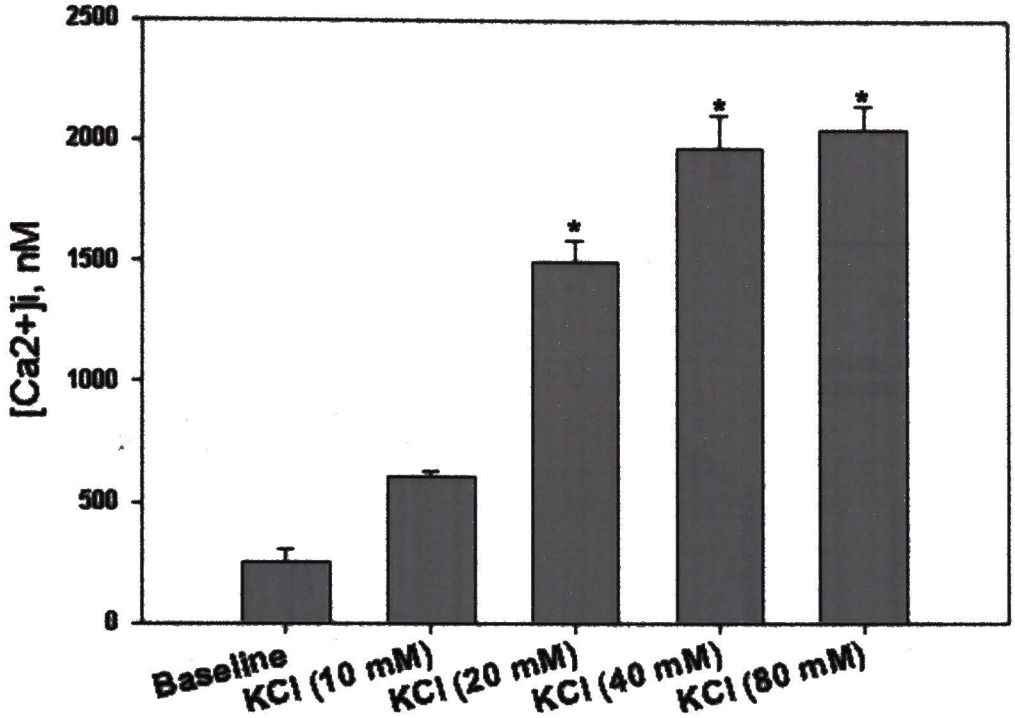


Figure 1.


2500
2000
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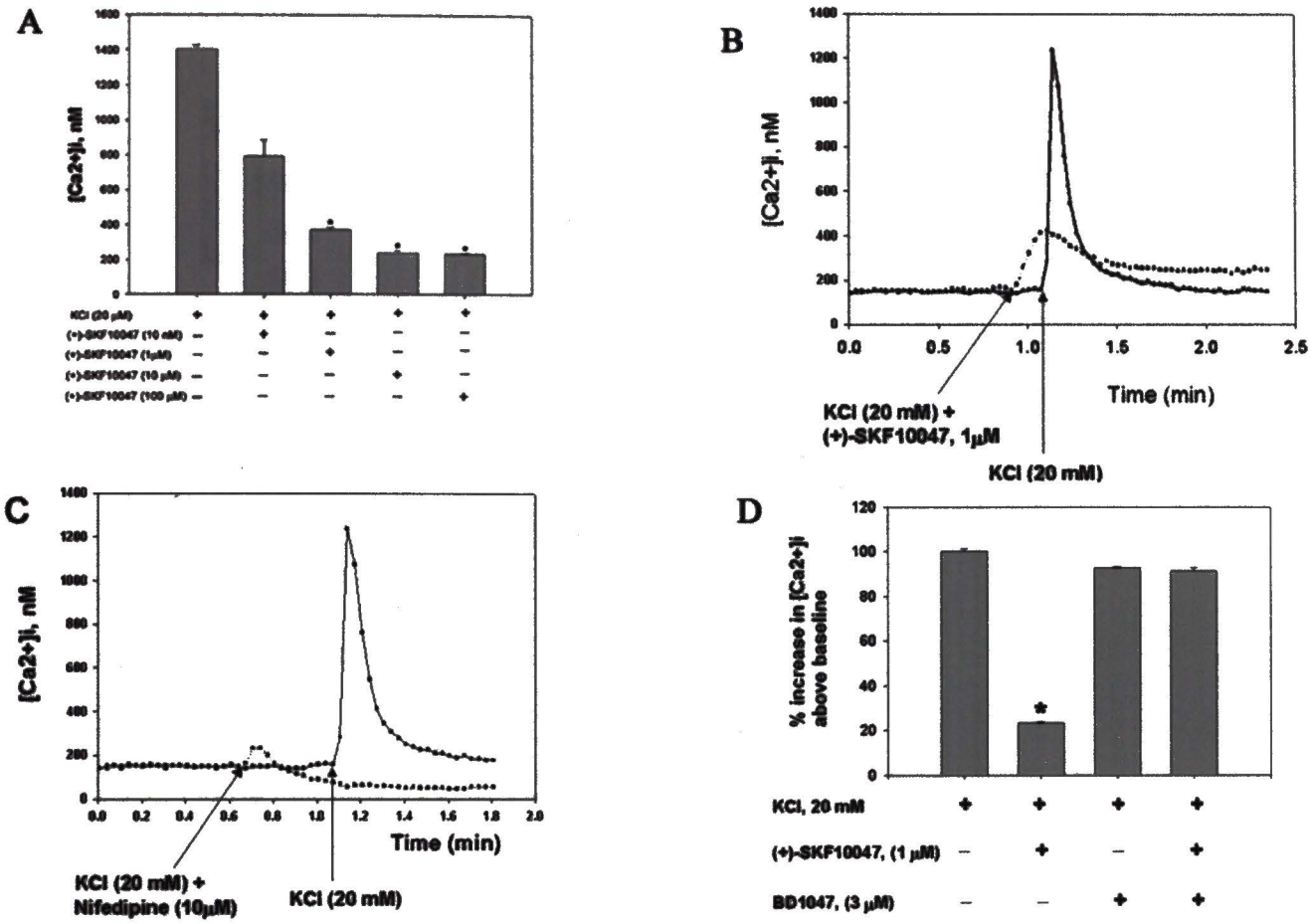
1000
500
0
[Ca2+]i, nM
*
*
*
Baseline
KCl (10 mM)
KCl (20 mM)
KCl (40 mM)
KCl (80 mM)

Figure 2.


A
[Ca2+]i, nM
KCl (20 μM)
(+)-SKF10047 (10 nM)
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(+)-SKF10047 (10 μM)
(+)-SKF10047 (100 μM)
B
[Ca2+]i, nM
Time (min)
KCl (20 mM) +
(+)-SKF10047, 1μM
KCl (20 mM)
C
[Ca2+]i, nM
Time (min)
KCl (20 mM) +
Nifedipine (10μM)
KCl (20 mM)
D
% increase in [Ca2+]i
above baseline
KCl, 20 mM
(+)-SKF10047, (1 μM)
BD1047, (3 μM)

Figure 3.

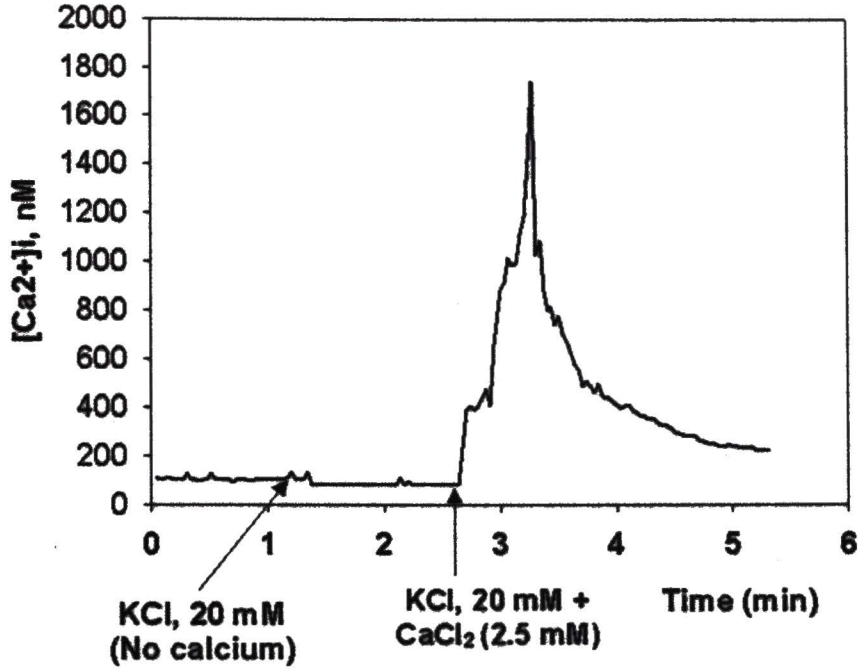
2000
1800
1600
1400
1200
1000
800
600
400
200
0
[Ca2+]i, nM
0
1
2
3
4
5
6
Time (min)
KCl, 20 mM
(No calcium)
KCl, 20 mM +
CaCl2 (2.5 mM)

Figure 4

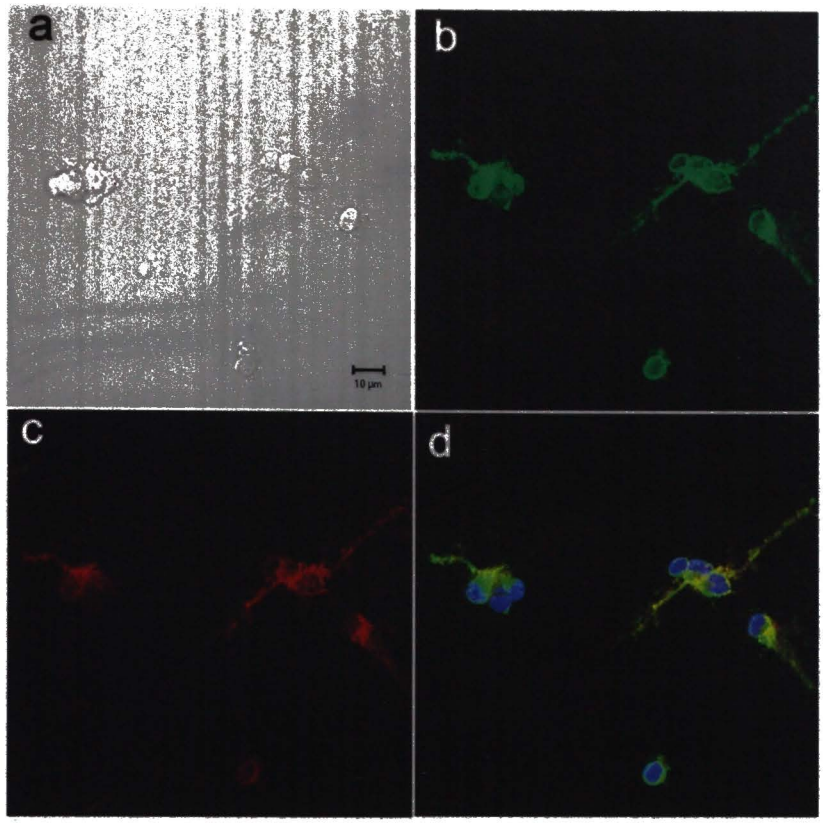

Figure 5.

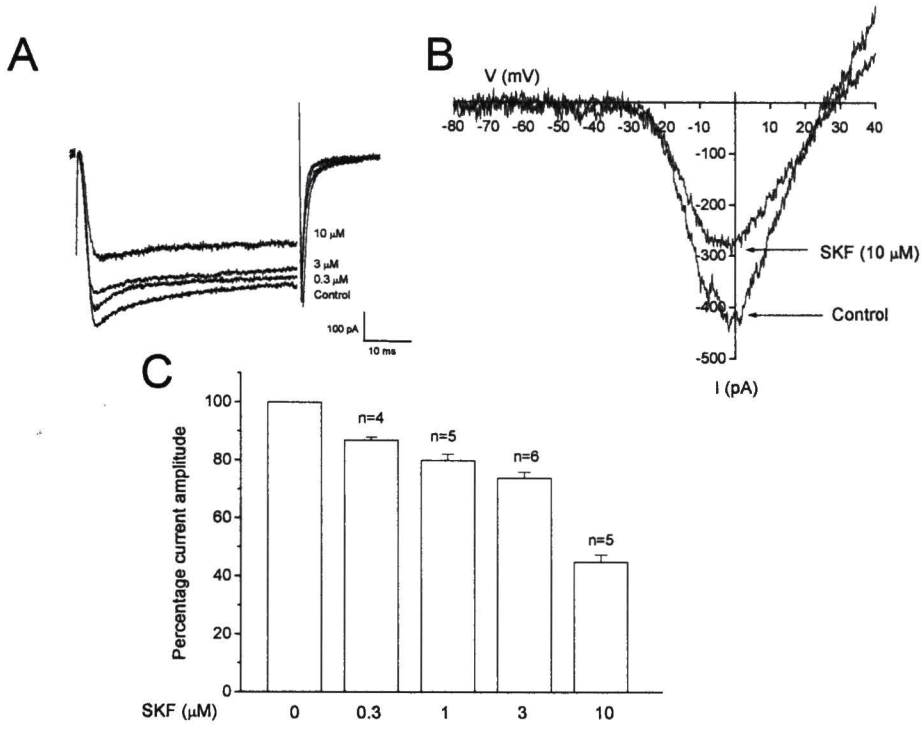
A
10 μM
3 μM
0.3 μM
Control
100 pA
10 ms
B
V (mV)
-80 -70 -60 -50 -40 -30 -20 -10 10 20 30 40
-100
-200
-300
-400
-500
SKF (10 μM)
Control
I (pA)
C
Percentage current amplitude
100
80
60
40
20
0
n=4
n=5
n=6
n=5
SKF (μM)
0
0.3
1
3
10

Figure 6.

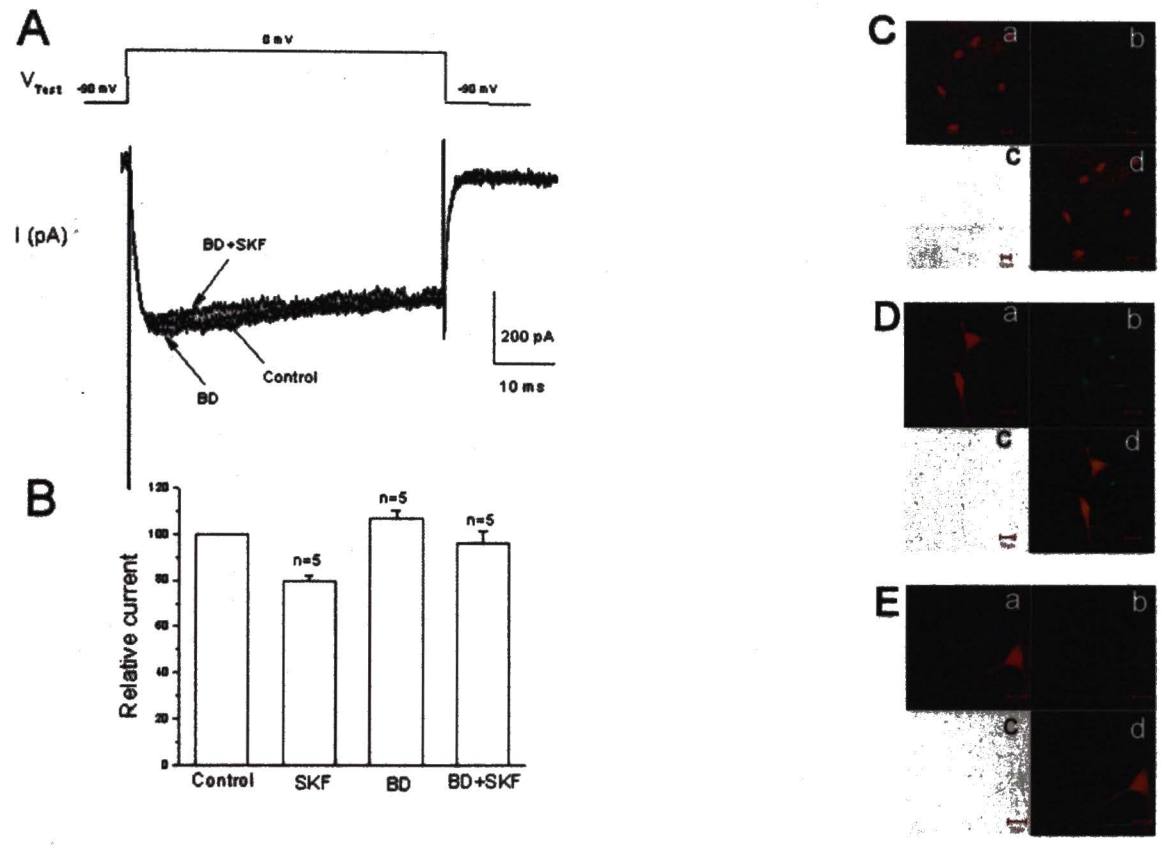
A
B
C
D
E
I (pA)
BD+SKF
Control
BD
200 pA
10 ms
Relative current
n=5
Control
SKF
BD
BD+SKF
a
b
c
d

Figure 7.

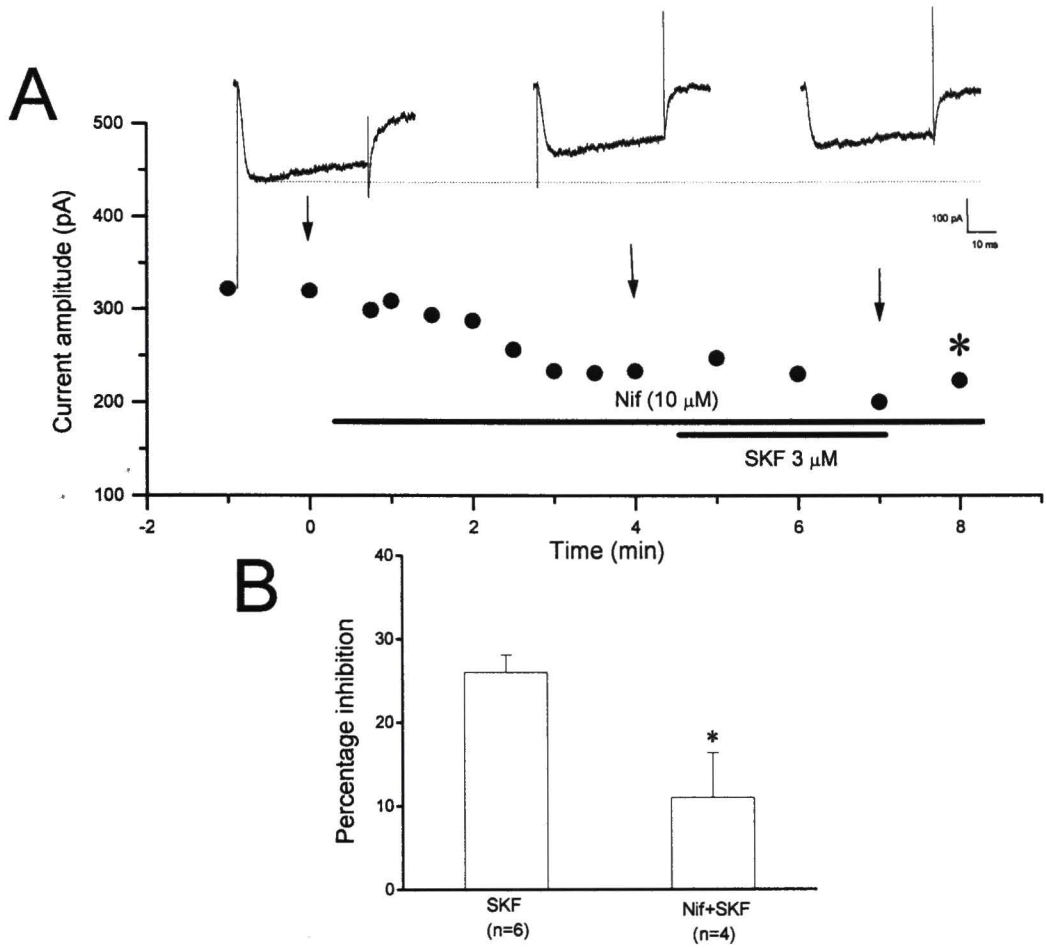
A
500
400
300
200
100
Current amplitude (pA)
100 pA
10 ms
Nif (10 μM)
SKF 3 μM
*
-2
0
2
4
6
8
Time (min)
B
40
30
20
10
0
Percentage inhibition
*
SKF
(n=6)
Nif+SKF
(n=4)

Figure 8.

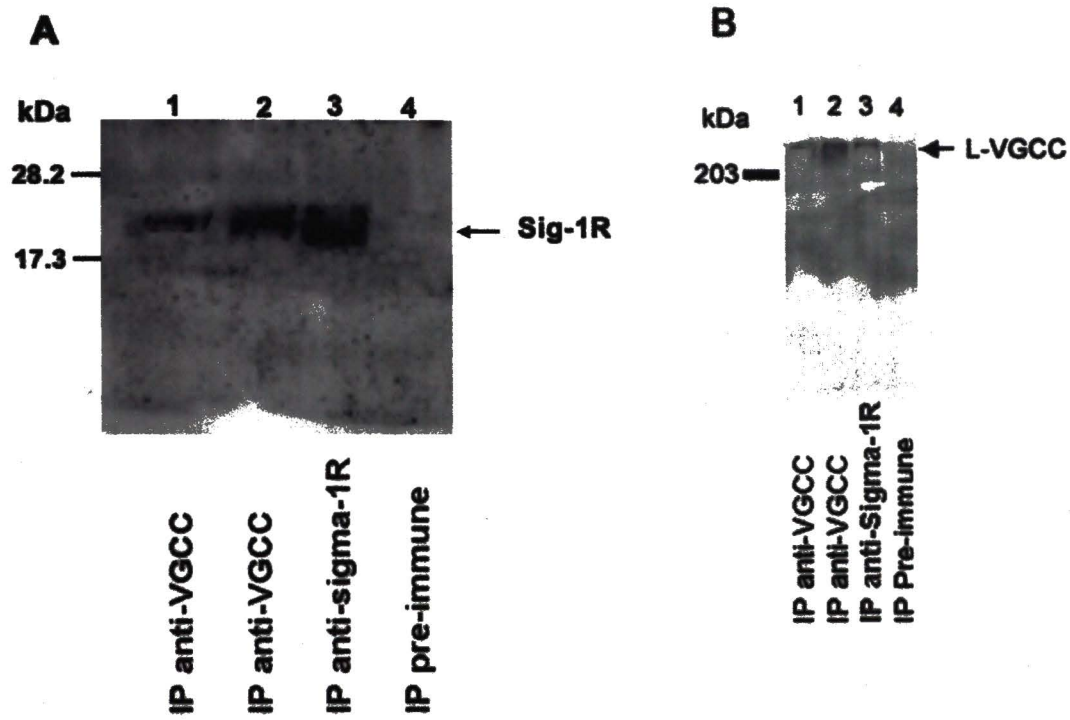
A
kDa
1
2
3
4
28.2
17.3
Sig-1R
IP anti-VGCC
IP anti-VGCC
IP anti-sigma-1R
IP pre-immune
B
kDa
1 2 3 4
203
L-VGCC
IP anti-VGCC
IP anti-VGCC
IP anti-Sigma-1R
IP Pre-immune

Figure 9.

Chapter 5

CONCLUSIONS AND FUTURE DIRECTIONS

The characterization of sigma-1 receptors in the eye in this study provided insight into the neuroprotection mechanism of action of sigma-1 receptor activation particularly in relation to retinal ganglion cell survival. Using the calcium imaging and the whole cell patch clamp techniques, we assessed both the actions of sigma-1 receptor activation on calcium influx and on intracellular calcium mobilization in both primary retinal ganglion cells and retinal ganglion cells lines (RGC-5). These results clearly demonstrated that the sigma-1 receptor agonist (+)-N-allylnormetazocine ((+)-SKF10047) inhibited potassium chloride (KCl)-induced calcium influx and calcium channel currents. The sigma-1 receptor antagonist, [2-(3,4-dichlorophenyl) ethyl]-*N*-methyl-2- (diamino) ethylamine (BD1047) reversed the inhibitory effect of (+)-SKF10047. Similarly, (+)-SKF10047 was able to inhibit glutamate-induced intracellular calcium mobilization. We confirmed the *in vitro* calcium studies data by overexpressing sigma-1 receptor in retinal ganglion cell lines. Our data showed that the overexpressing RGC-5 (RGC-5-S1R) cells had lower glutamate-induced intracellular calcium mobilization compared to normal RGC-5 cells. In addition, the sigma-1 receptor ligand (+)-SKF10047 reduced the glutamate calcium response in normal and RGC-5-S1R cells.

Using the cell survival assay, we also showed that sigma-1 receptor activation protected RGC-5 cells from glutamate-induced cell death. RGC-5-S1R cells showed a significant resistance to glutamate-induced apoptosis compared to the control RGC-5 cells. BD1047, a sigma-1 receptor antagonist, blocked the protective effect of (+)-SKF10047. In addition, western blot analysis showed that (+)-SKF10047 inhibited the increase in Bax

and caveolin-1 expression after glutamate treatments. Addition of glutamate resulted in cell death and appeared to involved activation of caspase-3. Sigma-1 receptor activation prevented an increase in caspase-3 expression.

Using the co-immunoprecipitation technique, we reported for the first time the association between L-type calcium channels and sigma-1 receptors. Thus sigma-1 receptor ligands may indirectly influence the voltage-gated calcium channels by interacting with the sigma-1 receptor associated voltage-gated calcium channel complex.

The lower response of glutamate-induced calcium mobilization observed in sigma-1 receptor overexpression RGC cells may be due to the fact that sigma-1 receptors are also activated by neurosteriods, which can be present in trace amounts in the fetal bovine serum (FBS) used in the cell culture media DMEM. From the results of this research project it is clear that sigma-1 receptor regulates many calcium entry paths in retinal ganglion cells. We suggest that by regulating calcium homeostasis in retinal ganglion cells, sigma-1 receptors directly or indirectly regulate cell death pathways linked to calcium. NMDA receptor overstimulation by glutamate, in addition to increasing intracellular calcium, leads to the depolarization of the plasma membrane, which can also activate voltage-dependent calcium channels. Together these channels aggravate the intracellular calcium overload. Excessive calcium accumulation is the key observed process leading to neuronal death or injury [1]. Calcium overload will induce the expression level of Bax [2] and this increase in Bax expression may lead to mitochondrial depolarization and cytochrome c release resulting in the down-stream activation of executioner caspase to augment apoptosis [3,4]. Glutamate-induced calcium mobilization also leads to the upregulation of caveolin-1. Studies have shown that

caveolin-1 inactivates several survival molecules such as Erk [5, 6], MEK1 [5, 6], EGFR [6]. This sugest that an increase in caveolin-1 expression can augment cell death. In addition to these previous studies, it has been shown that reduced expression of caveolin-1 in NIH/3T3 fibroblast increases their resistance to apoptosis [7]. *Diagram 2* depicts a summary of the cellular signaling pathways involved in sigma-1 receptor neuroprotection actions.

Overall we have shown that sigma-1 receptor activation protected retinal ganglion cells from cell death by controlling calcium levels and pro-apoptotic genes such as Bax, caveolin-1, and caspase-3 activation. The regulation of calcium channels involved a direct interaction between sigma-1 receptors and voltage gated L-type calcium channels. The regulation of calcium levels in retinal ganglion cells by sigma-1 receptors can activate cell survival signaling pathways and decrease pathways linked to cell death.

Future directions

Sigma receptor pharmacology is still in its infancy compared with our knowledge of more conventional receptor systems. The natural sigma ligands still remain unknown, although sterols are likely candidates. The signaling mechanisms underlying sigma receptor actions are also very poorly understood. It is, however, established that the receptor resides mainly in the ER where it has a primary role in influencing calcium signaling [8]. Future studies will not only address the role of receptor trafficking to and from the ER in sigma receptor signaling but it will also be directed to answering the specific roles of sigma receptors in tissues. So far only the Sigma-1 receptor has been cloned and sequenced and there is evidence from binding studies in the retina at least that the elusive sigma-2 receptor is also present. The three-dimension (3-D) structure or the

cloned sigma-2 receptor is yet to be determined. Once the necessary molecular tool kits are available, it is likely that this receptor system will be of considerable interest to researchers in a wide range of ocular tissues.

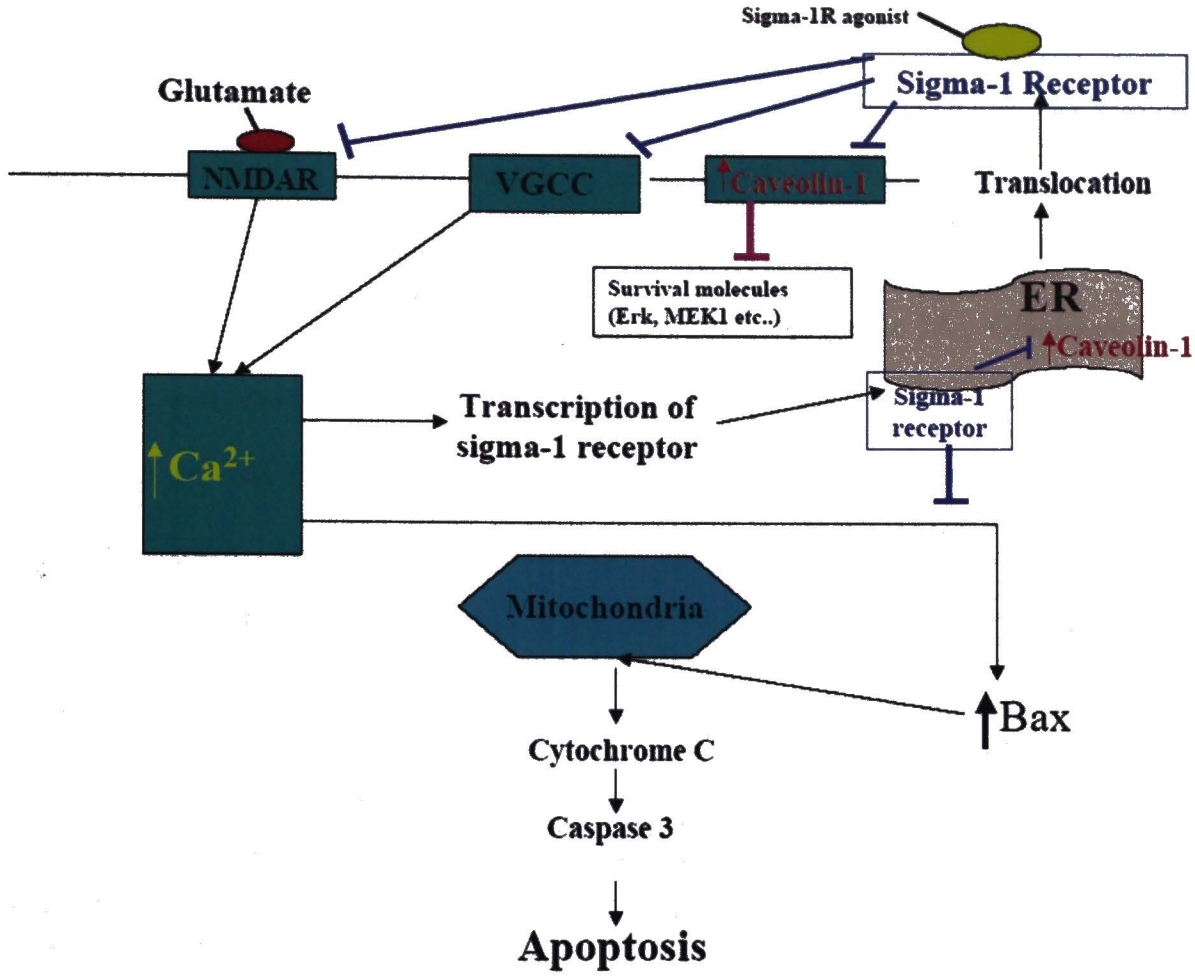

Diagram-2. Summary of the cellular signaling pathways involved in sigma-1 receptor neuroprotection actions.

Activation of N-methyl-D-aspartate receptors (NMDAR), or L-type voltage-gated calcium channels (L-VGCCs) allows the influx of calcium. Increased intracellular calcium levels may lead to the transcription and/or translocation of sigma-1 receptors from the ER to the plasma membrane where it decreases the excitatory neurotransmitter-induced calcium influx. Sigma-1 receptors activation also controls the upregulation of Bax, caveolin-1, and the activation of caspase-3. By controlling calcium levels, and pro-apoptotic genes (Bax, caveolin-1, and caspase-3), sigma-1 receptors promote retinal ganglion cell survival.

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APPENDIX

The following results were part of the dissertation that were not included as part of the chapters, however, these results provide some information on what to focus on in the future work in order to fully understand the mechanism of action of sigma-1 receptors.

Sigma-1 receptor and lipids rafts

Caveolin-1, a highly conserved membrane-associated protein, forms the foundation of cholesterol-rich membrane microdomains, called caveolae, within which caveolin-1 regulates the functions of various signaling proteins. An increase in caveolin-1 expression has been shown in macrophages undergoing apoptosis [1]. In addition, the endoplasmic reticulum has been shown to be the site of cholesterol- induced apoptosis in microphages [1, 2]. Recently, a complex formation has been shown between sigma-1 receptors at the mitochondrion-associated ER membrane (MAM) with a chaperone BiP. Sigma-1 receptors dissociate upon ER Ca^{2+} depletion or via ligand stimulation, from BiP. This dissociation leads to prolonged Ca^{2+} signaling into mitochondria via IP3 receptors [3]. Under ER stress or calcium overload, sigma-1 receptors can translocate to the plasma membrane and this translocation of sigma-1 receptors in cells counteracts the initial ER effect. Since glutamate can induce apoptosis in retinal ganglion cells [4] and regulate caveolin-1 expression in rat hippocampal neurons [5], We performed both immunocytochemistry and western blot analysis in order to determine if sigma-1 receptor activation can control the level of expression of caveoline-1 in RGC-5. The data showed that the sigma-1 receptor agonist (+)-SKF10047 inhibited the upregulation of glutamate-mediated increase caveolin-1 expression **(figure 1 & 2)**. Moreover, the sigma-1 receptor antagonist, BD1047 was able to block the effect of (+)-SKF10047. Sigma-1 receptors

apparently not only control the endoplasmic reticulum lipid compartmentalization and export as shown by Hayashi et al.[6], But also control the expression levels of caveolin-1 in lipids rafts to promote RGC-5 cell survival.

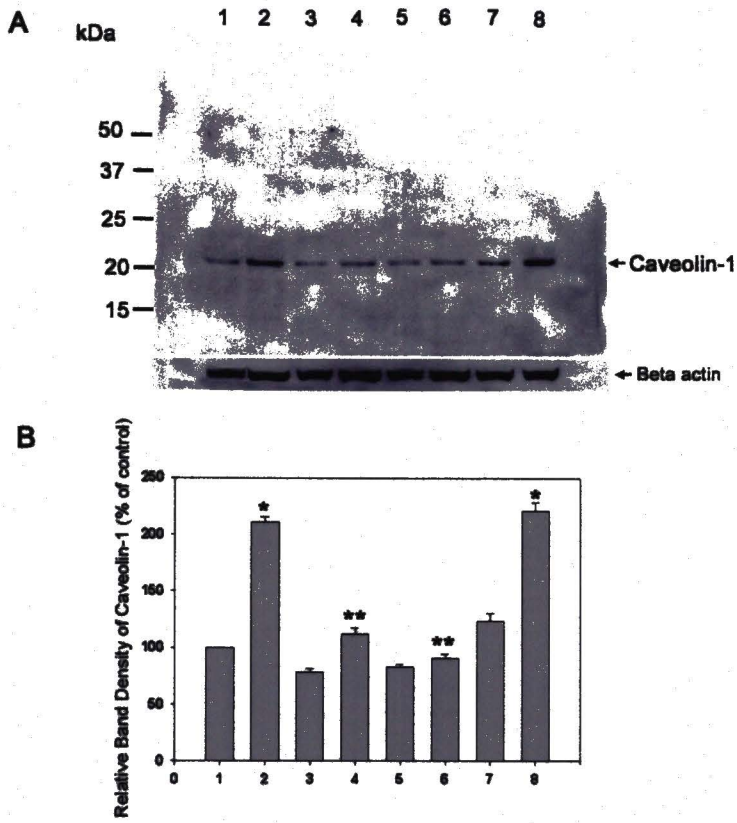

Figure 1. Western blot analysis of caveolin-1 levels in normal RGC-5 cells after glutamate treatment.RGC-5 cells were treated for 24 hours as follow: ***A). Lane 1).*** Control (no glutamate)**;** ***Lane 2).*** 1 mM glutamate; **Lane** ***3).*** 10 μM MK801; ***Lane 4).*** 1mM glutamate + 10 μM MK801; ***Lane 5).*** 1μM (+)-SKF10047; ***Lane 6).*** 1mM glutamate + 1μM (+)-SKF10047; ***Lane 7).*** 3 μM BD1047; and ***Lane 8).*** 1mM glutamate + 3 μM BD1047 + 1 μM (+)-SKF10047. ***B).*** Densitometric analysis of the bands was done using the Scion image analysis software (NIH). The quantification of band intensity

is represented as a percentage to the value of its corresponding control band on the same membrane and the intensity for caveolin-1 are presented as a mean percentage in three separate experiments. * denotes statistical significance ($P < 0.05$) of mean (%) caveolin-1 density versus that of control (no treatment). ** denotes statistical significance ($P < 0.05$) of mean (%) caveolin-1 density versus that of 1mM glutamate treatment.

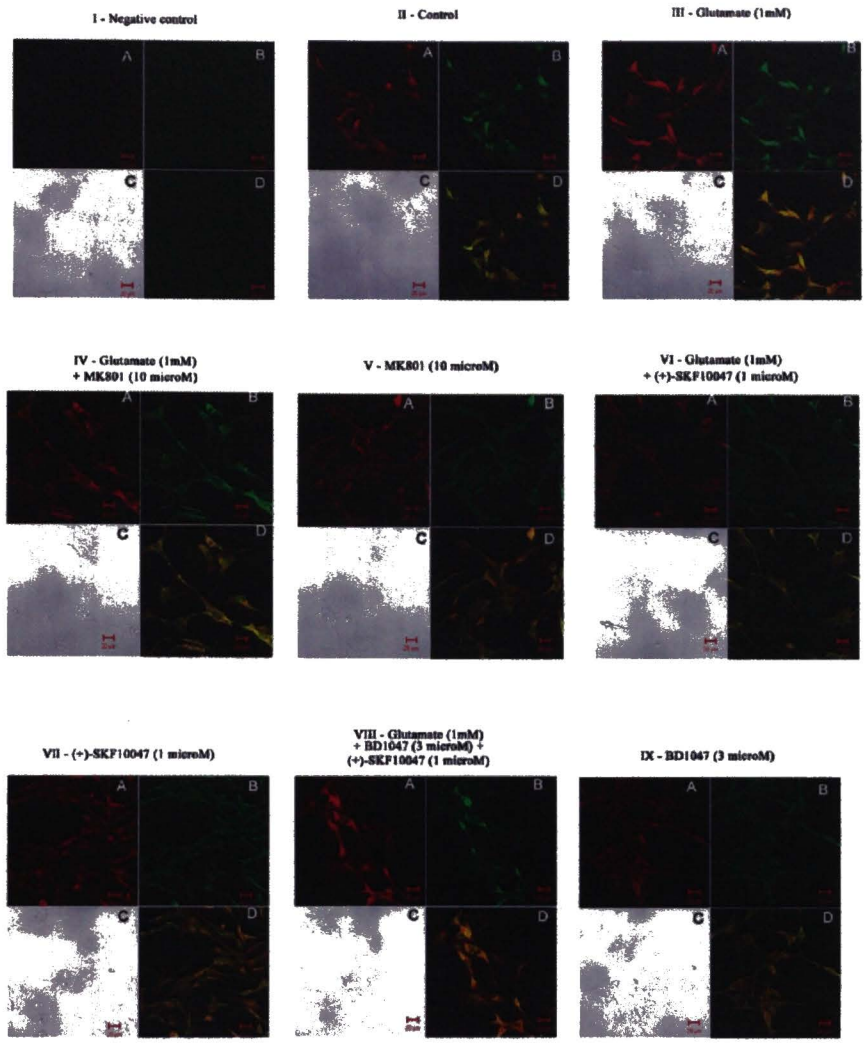

Figure 2. Immunocytochemistry analysis of caveolin-1 levels in normal RGC-5 cells after glutamate treatment.

Sigma receptor and transcription factors

Activation of sigma receptors leads to important neuroprotection actions in the eye and the central nervous system. Using a transcription factor search software program, we found that the upstream 5' site of the sigma receptor gene promoter contains calcium responsive element binding sites for many transcription factors including stimulator factors (*USF 1 and 2)* **(see figure 3. for more details)**. USFs have been shown to bind to calcium responsive elements (CaRE2) within the BDNF promoter III *in vivo*. USFs have also been shown to be regulated by calcium influx into neurons, suggesting that they are likely to function as transcriptional regulators [7]. Since the sigma-1 receptor upstream 5' site contains calcium responsive element binding sites for many transcription factors, calcium levels in the cells might be one of the major factors that lead to the activation and *de novo* synthesis of sigma-1 receptors in neuronal cells. Activation of sigma-1 receptor may also affect the expression levels of some cell survival factors (such as CREB, C/EBPβ, MEF2) as well as Kinases, such as the extracellular signal-regulated kinase (ERK), CaMKIV, and phosphatases such as calcineurin. The regulation of these factors may be involved in sigma-1 receptor-mediated cell survival and neuroprotection. Therefore, future studies will be focused on the mechanism by which USFs mediate Ca2+ dependant transcription of sigma receptors. Understanding the cellular action of sigma-1 receptors would be critical in understanding the functional role sigma-1 receptors have in the eye and perhaps in the central nervous system.

Sigma-1 Receptors and CAAT-Enhancer Binding Protein (CEBP)

Differential regulation of calcium channels and NMDA receptor activities can control neuronal survival by regulating transcription factors such as C/EBPs, calcium- dependent kinases such as CaMKIV, and calcium-dependent phosphatases such as Calcineurin. In addition, it has been shown that glutamate excitotoxicity is mediated by a rapid increase of C/EBPβ and the increase activity of calcineurin. To determine whether sigma-1 receptor activation affected the levels of C/EBP, we performed an immunocytochemistry analysis using retinal ganglion cells (RGC-5) as shown on **figure 4.** The immunocytochemistry analysis showed that sigma-1 receptor agonist *N*-allylnormetazocine (SKF10047), inhibited CAAT-Enhancer Binding Protein (CEBP) phosphorylation and translocation into the nucleus. The significance of this finding is not clear a this time. Therefore, further analysis is need to clarify such actions.

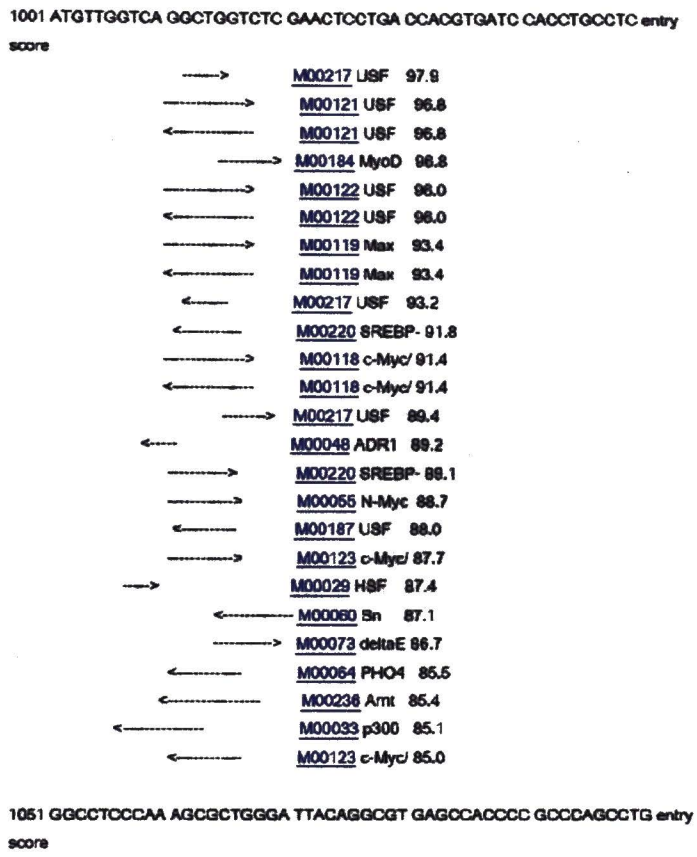

Figure 3. Transcription factors binding sites between 1001 – 1051 nucleotides of sigma 1 receptor (GeneBank, Accession No. AF001975), using transcription factor search software program: http://www.cbrc.jp/research/db/TFSEARCH.html

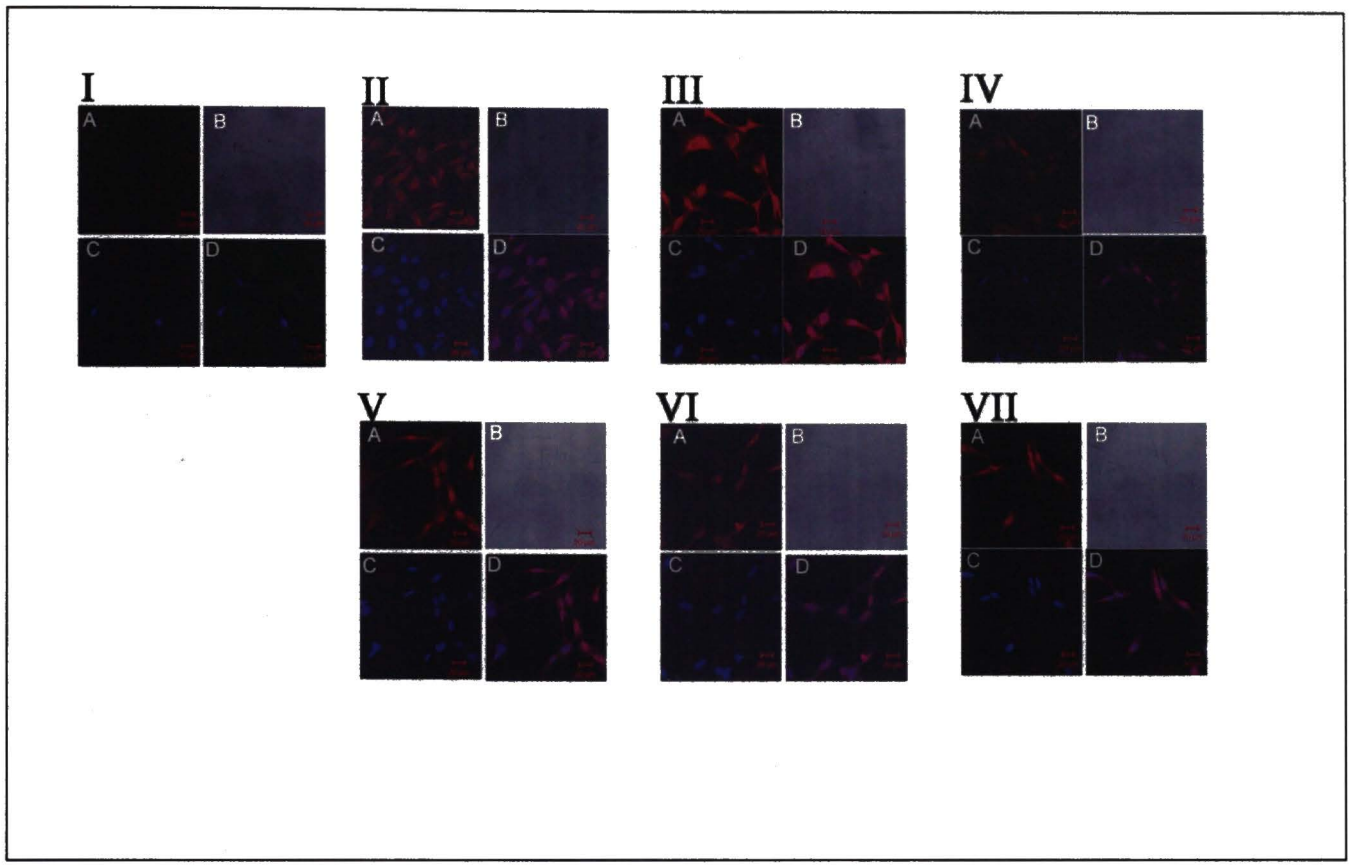

Figure 4. (+)SKF10047 inhibits CAAT-Enhancer Binding Protein (CEBP) phosphorylation and translocation into the nucleus.

Differentiated RGC-5 cells were treated as follow: ***I)***. Negative control; ***II)***. Control (no glutamate); ***III)***. *G*lutamate (250mM); ***IV)***. Glutamate (250mM) + MK801 (10mM); ***V)***. glutamate (250mM) + (+)-SKF10047 (1μM); ***VI)***. (+)-SKF10047 (1μM), ***VII)***. MK801 (10mM); Glutamate induces phosphorylation and translocation of C/EBP from the cytoplasm to the nucleus.

Sigma-1 receptors and calcium overload stress response

Acute glutamate neurotoxicity is mediated by NMDA receptors [8-10]. In addition to its pathological effects in the eye, endothelin-1 has also been shown to mediate intracellular calcium mobilization through endothelin A receptors (ETA) [11]. Based on these previous observations we investigated the role of sigma receptors during the oxidative damage by western blot analysis. Preliminary data from the western analysis showed that sigma-1 receptors were upregulated in retinal ganglion cells after endothelin-1 and glutamate treatment (24 hours) **(figure 5)**. The apparent increase of sigma-1 receptors appears to result from an increase in translocation of sigma-1 receptor from the ER to the cell membrane.

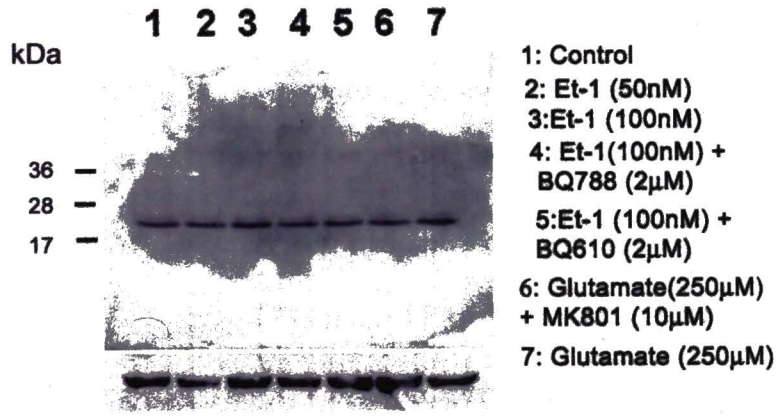

Figure 5. Effects of glutamate, and endothelin-1 on the protein expression of the sigma- 1 receptors in RGC-5 cells. ***A).***

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