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DIFFERENTIAL INVOLVEMENT OF NO AND cGMP SIGNALING PATHWAY IN THE NEUROPROTECTIVE ACTIONS OF SOMATOSTATIN AGAINST AMPA INDUCED EXCITOTOXICITY *IN VIVO*

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CONTENTS

ABBREVIATIONS

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ARMD: Age-Related Macular Degeneration

ARVO: Association for Research in Vision and Ophthalmology

ATP: Adenosine 5’ triphosphate

BIM23014: D-Nal-c-[Cys-Tyr-D-Trp-Lys-Val-Cys]-Thr-NH2 or Lanreotide

bNOS: brain Nitric Oxide Synthase

BSA: Bovine Serum Albumin

cAMP: 3′-5′ cyclic Adenocine Monophosphate

CCK: Cholecystokinin

cGMP: cyclic guanosine monophosphate

CHO cells: Chinese hamster ovary cells

ChAT: Choline Acetyltransferase

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CRAO: Central Retinal Artery Occlusion

DAG: Diacylglycerol

ER : Endoplasmic Reticulum

GABA: gamma-aminobutyric acid

GCL: Ganglion Cell Layer

GH: Growth Hormone

GLAST: Glutamate Aspartate Transporter

Glu: Glutamate

GluR: Glutamate Receptor

GPCRs: G-Protein Coupled Receptors

GTP: Guanosine 5’-triphosphate

IGF: Insulin-like Growth Factor

IgG: Immunoglobulin G (Heavy + Light chains)

iGluR: Ionotropic glutamate receptor

INL: Inner Nuclear Layer

IOP: Intraocular pressure

IP3: Inositol triphosphate

IPL: Inner Plexiform Layer

KA: Kainate

L-NAME: N^{ω}-nitro-L-arginine methyl ester

L-817,818: somatostatin peptidomimetic analog selective for sst5 receptor

mGluR: Metabotropic Glutamate receptor

NADPH: Nicotinamide adenine dinucleotide phosphate

NDS: Normal Donkey Serum

NGS: Normal Goat Serum

NMDA: N-methyl-D-aspartate

nNOS: neuronal Nitric Oxide Synthase

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NR: NMDA Receptor

ODQ: 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one

ONL: Outer Nuclear Layer

OPL: Outer Plexiform Layer

PB : Phosphate buffered

PBS: Phosphate Buffered Saline

PC12 : Rat adrenal pheochromocytoma

PCP: Phencyclidine

PDGF: Platelet-Derived Growth Factor

PFA: Paraformaldehyde

PKG: Protein kinase G or cGMP-dependent protein kinase

ROS: Reactive Oxygen Spieces

RPE: Retinal Pigment Epithelium

rpm: Reverse Transcription-Polymerase Chain Reaction

sGC: soluble Guanylate Cyclase

SRIF: Somatotropin Release Inhibitory Factor

sst: somatostatin receptor subtype

TARPs: transmembrane AMPA receptor regulatory proteins

TBS: Tris Buffer Saline

VEGF: Vascular Endothelial Growth Factor

EAAT: Excitatory Amino Acid Transporter

1. INTRODUCTION

1.1 Anatomy of the eye

The eye is a fluid-filled sphere enclosed by three layers of tissue **(Figure 1.1)**. Most of the outer layer is composed of a tough white fibrous tissue, the sclera. At the front of the eye, however, this opaque outer layer is transformed into the cornea, a specialized transparent tissue that permits light rays to enter the eye. The middle layer of tissue includes three distinct but continuous structures: the iris, the ciliary body, and the choroid. The iris is the colored portion of the eye that can be seen through the cornea. It contains two sets of muscles with opposing actions, which allow the size of the pupil (the opening in its center) to be adjusted under neural control. The ciliary body is a ring of tissue that encircles the lens and includes a muscular component that is important for adjusting the refractive power of the lens, and a vascular component that produces the fluid that fills the front of the eye. The choroid is composed of a rich capillary bed that serves as the main source of blood supply for the photoreceptors of the retina. Only the most inner layer of the eye, the retina, contains neurons that are sensitive to light and are capable of transmitting visual signals to central targets.

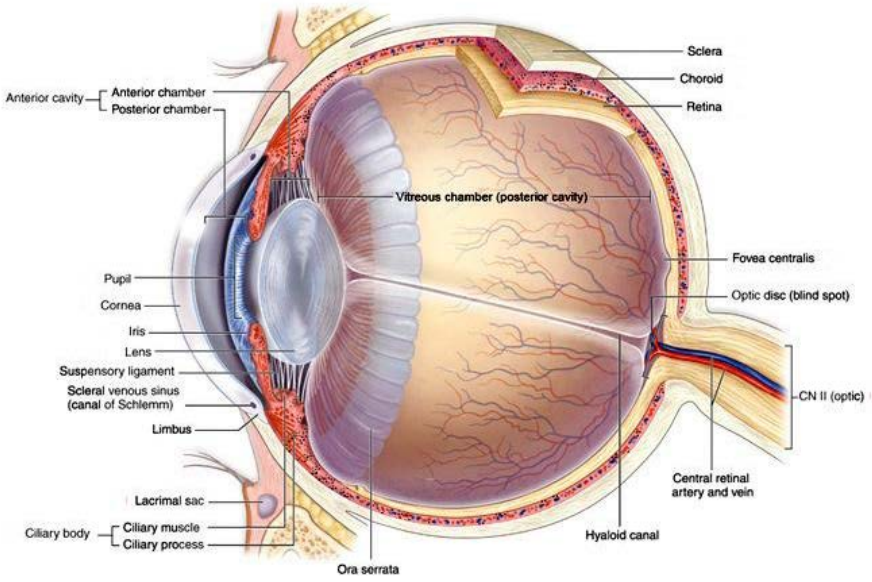


Figure 1.1 Anatomy of the human eye (Screenshot of eye anatomy on McGraw-Hillwebsite - Image courtesy of The McGraw-Hill Companies, Inc. http://www.iris-pharma.com/45-eye-structuresand-ocular-pathologies.html)

En route to the retina, light passes through the cornea, the lens, and two distinct fluid environments. The anterior chamber, the space between the lens and the cornea, is filled with aqueous humor, a clear, watery liquid that supplies nutrients to these structures as well as to the lens. Aqueous humor is produced by the ciliary processes in the posterior chamber (the region between the lens and the iris) and flows into the anterior chamber through the pupil. A specialized meshwork of cells that lies at the junction of the iris and the cornea is responsible for its uptake. Under normal conditions, the rates of aqueous humor production and uptake are in equilibrium, ensuring a constant intraocular pressure. Abnormally high levels of intraocular pressure, which occur in glaucoma, can reduce the blood supply to the eye and eventually damage retinal neurons (Hart, 1992).

The space between the back of the lens and the surface of the retina is filled with a thick, gelatinous substance called the vitreous humor, which accounts for about 80% of the volume of the eye. In addition to maintaining the shape of the eye, the vitreous humor contains phagocytic cells that remove blood and other debris that might otherwise interfere with light transmission.

1.2 Embryological development and general organization of the retina

The retina and optic nerves are derivatives of the forebrain. The optic vesicle develops prior to the fourth week of embryonic life and then inavigates to form the optic cup. This then forms two layers of neuroectodermal cells which contact each other at their apices. The outer layer of neuroectoderm differentiates into the pigmental epithelium which faces the choroid, while the inner layer of neuroectoderm forms the sensory retina (neuroretina). During this time cells of the inner layer (neuroretina) difrerentiate into an outer and an inner neuroblastic layer which are being separated by the non-nucleated transient fiber layer of Chievitz. Cells in both these inner and outer neuroblastic regions develop into retinal neurons and glial cells, those of the outer neuroblastic layer forming rod and cone cells. The inner neuroblastic layer further differentiates into an outer layer composed of five or six rows of Muller, amacrine and horizontal cells and an inner layer composed of two or three layers of cells forming the ganglion cell layer (Dowling, 1970, Dubin, 1974).

In the meantime, pigmentation in the outer layer of the cup increases in the developing pigment epithelium. At the same time the anterior margin of the optic cup grows forward to form the neuroectoderm of the future ciliary body and iris.

The neuroretina thus develops as a thin, delicate transparent tissue lining the inner eye. The neuroretina is loosely attached to the pigment epithelium, this being evident in retinal detachment when the rod and cone cells become separated from the pigmented epithelial layer. The pigment epithelium, however, is attached more securely to Bruch's membrane of the choroid layer.

In common with all other vertebrates, man has an inverted retina where the photoreceptive rod and cone cells lie on the choroid side of the retina and the neuronal cells involved with processing and transmitting neural information to the brain are on the vitreous side of the retina. The net effect is that light must pass through all retinal layers (except at the fovea) before reaching the photoreceptors. The subsequently generated action potentials then pass back via ganglion cells and their axons located in the vitreous nerve fiber layer.

The retina is thickest near the optic disc (0.56 mm) and gradually thins towards the periphery. The retina terminates at the ora serrata where it forms dentate processes

that extend forward into the ciliary body. The retina can be subdivided into two general regions: central and peripheral. The central retina is 5-6 mm diameter and contains the macula, the fovea and the foveola, and it is this area that contains both rod and cone cells. The peripheral retina can be further subdivided into near-periphery, far-periphery and the ora serrata. The cone cells gradually reduce in number from near to far periphery. In the ora serrata the rods gradually disappear.

1.3 Cellular layers of the retina

Except at the fovea centralis, optic disc, and its peripheral margins near the ora serrata, the neuroretina is conventionally divided into ten layers (Bloom et al, 1964). The structure of the retina is represented diagrammatically in **Figure 1.2**

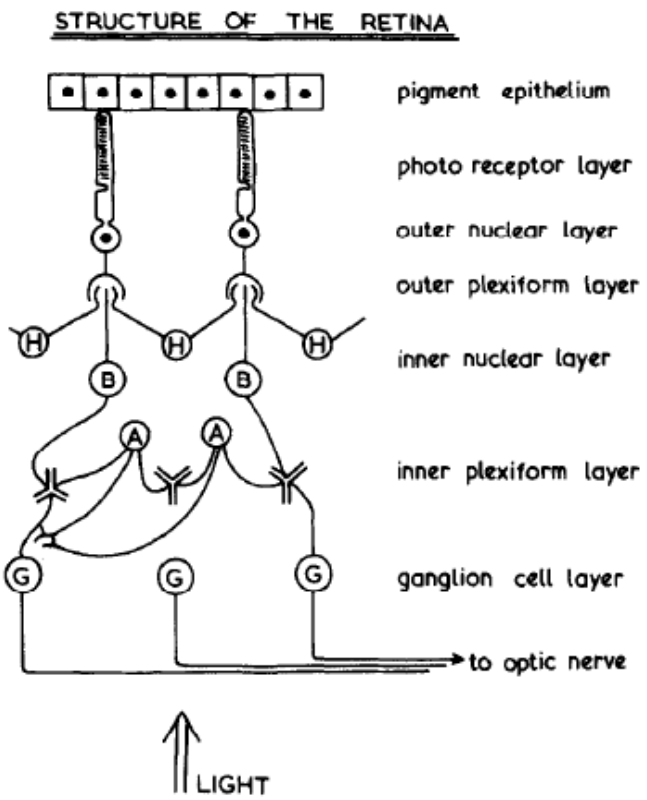


Figure 1.2 Schematic representation of the cellular layers, neuronal elements and interconnections in the mammalian retina.

1.3.1 Pigment Epithelium

The pigment epithelium comprises a single row of prismatic cells which contain a black melanin pigment called fuscin which is contained within granules. The inner surface of these cells send out protoplasmic processes which project between the photoreceptors. The pigment is mainly located within the lower parts of these cells and in the cytoplasmic processes: the choroid side of the pigment epithelium is relatively free of the pigment and contains an oval nucleus. One function of the pigment epithelium is believed to be the absorption of light rays not successful in activating the photopigments in the rods and cones. However most of this light not interacting with the photoreceptors is in fact absorbed by the melanin contained within choroidal melanocytes, and thus any internal reflection on the choroid layer is kept to a minimum with no subsequent activation of photoreceptors elsewhere in the retina resulting in blurring vision.

1.3.2 Photoreceptor Layer

The rods consist of two segments. The outer segment is slender and rod-like and it contains the visual purple pigment (rhodopsin) and the inner segment which is slightly bulbous and transparent. According to Krause (1946), the number of rods in the human retina is $13x10^7$. The cone cells like-wise consist of two segments, their shape varying in different parts of the retina. In general, the outer segment is conical in shape; it contains no visual purple. The whole cone is squatter and more bulbous than the rod. From the body of all cones a smooth inner fiber descends to the middle zone of the outer plexiform layer where it terminates with a thick triangular swelling, the cone pedicle, which is in complex synaptic contact with fibres of bipolar and horizontal cells (Dowling and Werblin , 1971). The number of cones in the human retina is estimated at 6-7 million.

1.3.3 External Limiting Membrane

This is not a true membrane hut rather an illusion of a membrane shown in the light microscope due to staining of the junctions between photoreceptors and Muller cells.

1.3.4 Outer Nuclear Layer

The outer nuclear layer contains the nuclei and central processes of the rods and cones. The central processes of the rods have minute varicosities which may lie within this layer. The central process of the cone is thicker. These three outer layers of the retina are avascular, and nourished by diffusion from capillaries of the choroid.

1.3.5 Outer Plexiform (Molecular) Layer

The outer plexiform layer is acellular and consists of a zone in which the central processes of the photoreceptors synapse with the dendrites of the bipolar and horizontal cells (Dowling and Werblin, 1971).

1.3.6 Inner Nuclear Layer

The inner nuclear layer contains the cell bodies of the bipolar cells, horizontal cells, amacrine cells and Muller's cells. This layer is nourished by capillaries of the retinal artery, and will subsequently undergo avascular necrosis following obliteration of flow in this vessel. In addition, interplexiform cells are present in the inner nuclear layer.

1.3.7 Inner Plexiform Layer

The inner plexiform layer is acellular and contains the synapses between bipolar cells, amacrine cells and ganglion cells.

1.3.8 Ganglion Cell Layer

The ganglion cell layer contains the large cell bodies of the second order neurons.

1.3.9 Nerve Fiber Layer

The nerve fiber layer consists of the axons of the ganglion cells projecting towards the optic disc to enter the optic nerve. The retinal vessels lie for the most part in this layer.

1.3.10 Internal Limiting Membrane

The internal limiting membrane is a thin hyaline membrane between the retina and vitreous. Attached to it are the conjoined expanded bases of the fibers of the Muller cells which penetrate the retina and bind its nervous elements together. Each fiber contains a nucleus in the inner nuclear layer. The fibers pass outwards to the outer limiting membrane.

1.4 Cell Types

1.4.1 Photoreceptors

The structure and localization of the rod and cone cells were described above at 1.3.2.

1.4.2 Horizontal Cells

The horizontal cells are typical interneurons whose cell bodies form the upper regions of the inner nuclear layer. Short dendrites arise from the perikaryon and project into the outer plexiform layer to synapse with one cone pedicle or rod spherule. As a result, horizontal cells receive impulses from one group of rod and cone cells and transmit them to another group of photoreceptors in another location. Two different kinds of horizontal cell have been demonstrated by light microscopy (Dowling, 1970). Type A horizontal cells represent cone horizontals, while type B are believed to represent rod horizontal cells.

1.4.3 Bipolar Cells

The bipolar cells connect photoreceptors with the ganglion cells. Axons thus extend from the outer plexiform layer to the inner plexiform layer: the cell body of these cells lies in the inner nuclear layer (Neal, 1976). Two classes of bipolar cells are

recognized based on their physiological properties. These are the depolarizing bipolar cells and the hyperpolarizing cells.

1.4.4 Ganglion Cells

The ganglion cells are located in the ganglion cell layer. Dendrites spread into the inner plexiform layer to synapse on to bipolar and amacrine cells. From the perikaryon a single axon leaves the retina and projects as an optic nerve fiber to terminate in subcortical visual centers of the brain.

1.4.5 Amacrine Cells

The amacrine cells of the inner plexiform layer are, like the horizontal cells of the outer plexiform layer, oriented parallel to the retinal surface, and at right angles to the axis of the photoreceptors, bipolar cells and ganglion cells. Amacrine cells are interneurons linking certain bipolar cells and certain ganglion cells with a wide horizontal spread within the inner plexiform layer, and probably function to mediate lateral inhibitions within the retinal circuitry. Amacrine cells have also been shown to interact with other amacrine cells.

1.4.6 Glial Cells of the Retina

Muller cells are giant glial cells which occupy the full thickness of the retina from the internal to external limiting membranes. These cells are important for their supportive and nutrient functions, providing the nerve cells with glucose. The nuclei of Muller cells are found almost exclusively in the intermediate portion of the inner nuclear layer. Other glial components found within the retina are fibrous and protoplasmic astrocytes and oligodendrocyte-like cells, normally found in the ganglion cell and inner plexiform layers.

1.5 Neuronal Pathways of the Retina

The photoreceptors are the primary sensory system of the retina. In addition, the retina contains several other types of cells: bipolar cells (or neurons), interneurons (horizontal cells and amacrine cells) and ganglion cells.

The general sequence of neuronal conduction is from activated photoreceptor to bipolar cells (first order neurons) ganglion cells (second order neurones), which in turn project to midbrain and lateral geniculate body (Orr, 1976). The horizontal cells and amacrine cells with wide horizontal spread within the outer and inner plexiform layers respectively, may act to modify lateral interactions between different parts of the retina **(Figure 1.3)**.

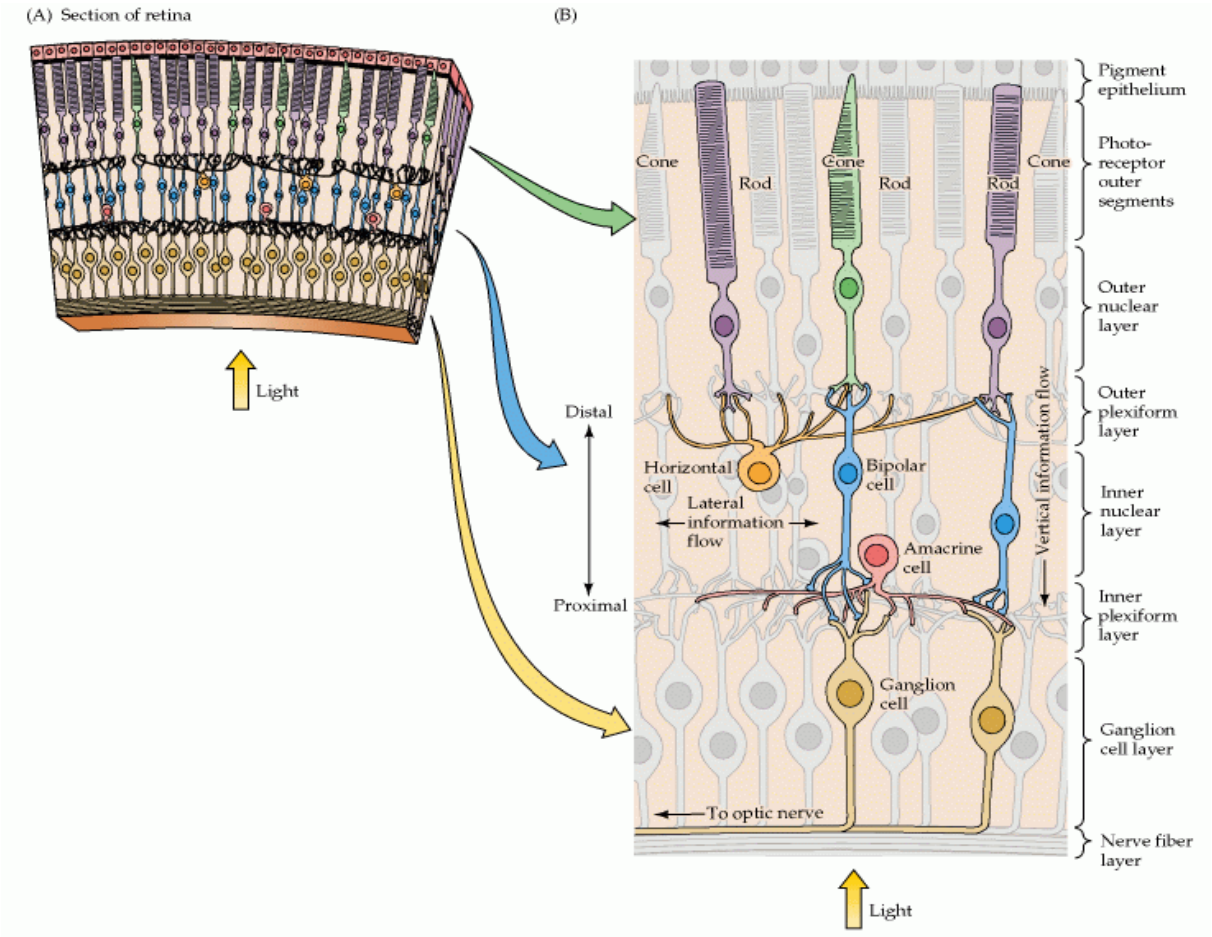


Figure 1.3 **A** Section of retina **B** Schematic representation of the light's direction to the retina (Wassle, 2004)

1.5.1 Retinal Cells and Neurotransmitters

The major direct line of information flow is from photoreceptors to ganglion cells via bipolar cells. In the outer and inner plexiform layers (OPL and IPL), where synaptic contacts are located, horizontal and amacrine cells mediate lateral interactions. It has been well documented that these lateral interactions are

responsible for modulating the activity of the direct line of transmission (Yang, 2004).

While there exists extensive electrical coupling between retinal neurons through gap junctions (Miyachi et al., 1999), chemical transmission mediated by neurotransmitters predominates in the neuronal circuitry of the retina.

Although the retina contains a variety of neurotransmitters, glutamate and γ-aminobutyric acid (GABA) are major excitatory and inhibitory neurotransmitters **(Figure 1.4)**. Glutamate is responsible for the radial flow of visual signal in the retina, and both photoreceptors (rods and cones) and bipolar cells release glutamate in most cases, which induces and/or alters the activity of the post-synaptic neurons (horizontal and bipolar cells for photoreceptors in the outer retina; amacrine and ganglion cells for bipolar cells in the inner retina) by directly changing membrane permeability to ions or by activating intracellular systems through ionotropic and metabotropic glutamate receptors (iGluRs and mGluRs). GABA is used by numerous horizontal cells and amacrine cells in the lateral pathway, modulating synaptic transmission in both synaptic layers. In the OPL horizontal cells receive direct input from photoreceptors and in turn provide negative feedback to cone photoreceptors. Horizontal cells are thought to mediate the responses of the surrounding receptive field of bipolar cells. Input to bipolar cells are from both photoreceptors and horizontal cells. In the IPL bipolar cells and amacrine cells are connected to each other through reciprocal synapses and both types of cells send input to ganglion cells. Amacrine cells may be involved in spatial and temporal integration of visual signals in the IPL (Yang, 2004).

However, nitric oxide (NO), dopamine (DA) acetylocholine (Ach), somatostatin (SRIF) and glycine are also synthesized by amacrine cells and modulate the function of retina. The balanced production and action of these molecules plays an important role in retina's homeostasis and pathological situations response.

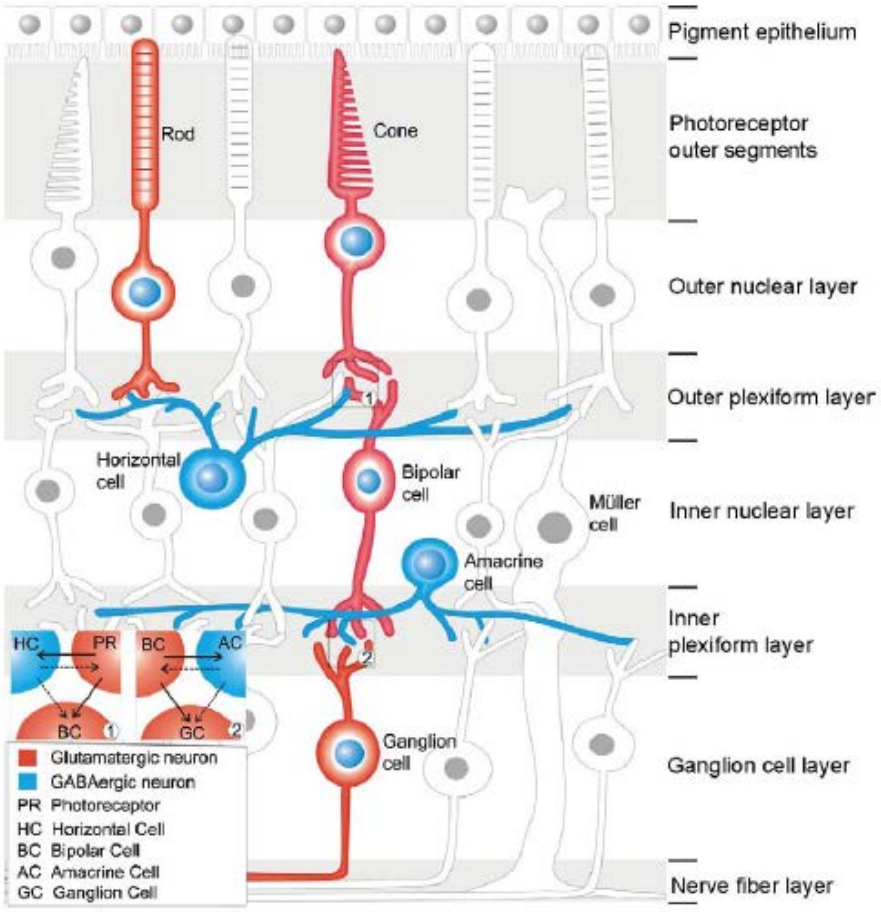


Figure 1.4 A schematic drawing showing cellular layering and organization of the vertebrate retina. Signals generated in photoreceptors (rods and cones) are passed through bipolar cells to ganglion cells, which send retinal output signals to visual centres along the optic nerve. Thus direct line of information flow is modulated by horizontal cells in the outer plexiform layer (OPL) and by amacrine cells in the inner plexiform layer (IPL). Glutamate and GABA are major excitatory and inhibitory neurotransmitters in the retina, respectively. Glutamatergic neurons (photoreceptors, bipolar cells and ganglion cells) are in red, whereas GABAergic neurons are in blue. In the OPL, horizontal cells receive input from photoreceptors and in turn provide negative feedback to cone photoreceptors. Bipolar cells receive inputs from both photoreceptors and horizontal cells. In the IPL, bipolar cells and amacrine cells connect each other through reciprocal synapses and the two types of cells both provide input to ganglion cells. Muller cells are the principal glia, spanning the entire neural retina **(Yang, 2004)**

1.6 Nitric oxide signaling in retina

Two decades after its first detection in the retina, nitric oxide (NO) continues to play an important role in the study of visual signaling pathways. While its liberation by photoreceptors remains controversial, recent evidence supports that amacrine cells are the main source of NO in the inner retina. NO synthesis was shown to depend on light stimulation, and mounting evidence suggests that NO is a regulator of visual adaptation at different signal processing levels. NO modulates light responses in all retinal neuron classes, and specific ion conductances are activated by NO in rods, cones, bipolar and ganglion cells. Light dependent gap junction coupling in the inner and outer plexiform layers is also affected by NO. The vast majority of these effects were shown to be mediated by activation of the NO receptor soluble guanylate cyclase and resultant cGMP elevation.

Nitric oxide (NO) participates in diverse physiological functions within the central nervous system (Garthwaite, 2008). NO is synthesized by conversion of L-arginine to L-citrulline, a reaction that is catalyzed by the enzyme nitric oxide synthase (NOS), in the presence of oxygen and NADPH (Daff, 2010). Vertebrates express three isoforms of NOS: The constitutive calcium-dependent neuronal (nNOS) and endothelial (eNOS) isoforms, and the inducible, calcium independent isoform (iNOS). The three NOS isoforms are expressed to variable degrees in the retina, but nNOS is the main player in visual responses (Kaur et al., 2006).

Very low NO concentrations may exert measurable effects (Batchelor et al., 2010). Under these conditions, the enzyme soluble guanylate cyclase (sGC) is the only established NO receptor which is activated and produces an increase of intracellular cyclic GMP (cGMP) concentrations (Garthwaite, 2005). However, at higher concentrations, reactive oxygen species derived from NO may also modify cysteine and tyrosine residues, thereby modulating the function of a variety of target proteins (Hess et al., 2005).

The NO receptor sGC has been detected in subpopulations of cone bipolar and amacrine cells in the rat retina. To a lower degree, sGC is also regularly expressed by rod bipolar and ganglion cells (Ding and Weinberg, 2007).

sGC activity was shown to be amplified by exogenous NO donors such as sodium nitroprusside (SNP), and by the NO precursor L-arginine and the NOS cofactor NADPH, an effect that could be abolished by the addition of the NO scavenger hemoglobin in the first case, and by the NOS inhibitor l-NMMA in the latter two (Koch et al., 1994).

Retinal acetylcholine signaling through both muscarinic and nicotinic receptors was shown to modulate NO-dependent cGMP-IR in retinal cell types. In rat, carbachol, an agonist of muscarinic acetylcholine receptors, increased retinal cGMP levels, an effect that was sensitive to the sGC inhibitor ODQ and the NOS inhibitor l-NMMA (Borda et al., 2005).

sGC, apart from being activated by NO, can also be stimulated by carbon monoxide (CO), although it is less sensitive to this compound. Interestingly, the underlying mechanism may also depend on NO. In rat, the CO producing enzyme heme oxygenase-2 is expressed only in amacrine and ganglion cells (Cao et al., 2001). In both turtle and salamander, stimulation with CO increased cGMP-IR in the inner retina, but the effect was strongly dependent on endogenous or exogenous NO, suggesting synergistic interactions between NO and CO signaling pathways in the retina (Pong and Eldred, 2009).

1.7 Retinal Ischemia and Cell Death

Retinal ischemia is characterized by the lack of oxygen and glucose supply and the insufficient removal of waste products. These result in cellular energy (ATP) and metabolic failure, alteration of normal neuronal membrane processes **(Figure 1.5)** and ionic homeostasis which leads to membrane depolarization, increase of extracellular glutamate levels and activation of ionotropic glutamate receptors (Lipton and Rosenberg, 1994; Lipton, 1999; Lipton et al., 2001). A subsequent cascade of events involving the rise in intracellular calcium levels and the activation of nitric oxide (NO) formation are believed to be important in cell death (Osborne et al., 2004).

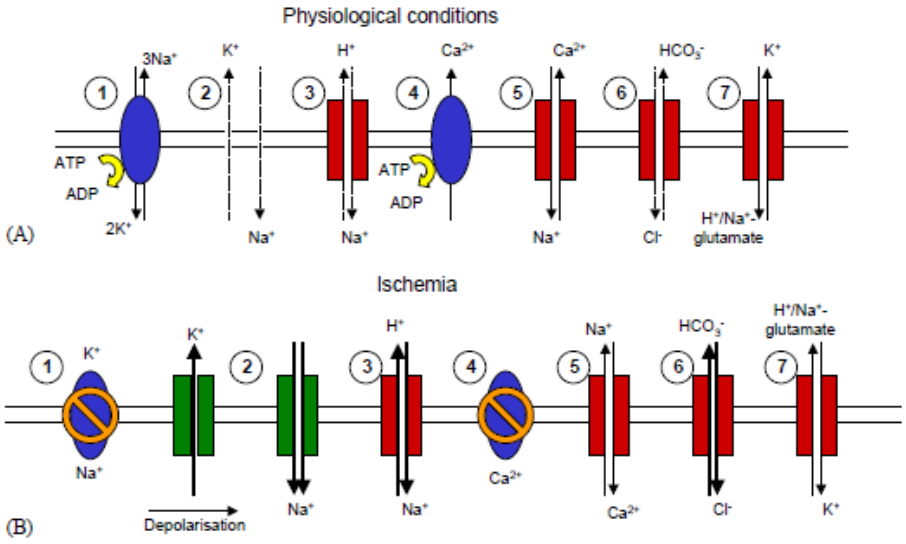


Figure 1.5 Selected neuronal membrane events altered as direct result of cellular energy loss in retinal hypoxia/ischemia. There exist many different membrane bound pumps, channels and exchangers for ions and the functioning of these is often inter-linked. Normal neuronal membrane processes and homeostasis **(A)** are markedly affected by lack of cellular energy that results from an hypoxic or ischemic insult **(B (Osborne et al., 2004)**

It is commonly believed that Ca^{2+} is a major mediator of neuronal cell death in ischemia, where excitotoxic damage secondary to a massive release of glutamate is thought to occur. As a result of ischemia, intracellular calcium levels can increase by several mechanisms: neurotransmitter (e.g. glutamate-gated channels or the ionotropic glutamate receptors of the NMDA or AMPA/kainate types), by reversal of sodium-calcium exchange, through voltage sensitive calcium channels (VSCC) or by stimulation of metabotropic neurotransmitter receptors which can release calcium from intracellular stores. The increased intracellular levels of calcium result in activation of many enzymes which are dependent on this cation for full activity (protein kinase C, calpains, nitric oxide synthase) and these enzymes produce changes which lead to the breakdown of cellular homeostatic mechanisms and structural integrity to cause cell death. Furthermore, when calcium levels are increased in the cytoplasm, mitochondria attempt to take up this cation, but these organelles are also overcome by ischemia-induced elevations and this leads to

mitochondrial permeability transition, cytoplasmic release of cell death promoting factors such as cytochrome C and the initiation of apoptotic cell death **(Figure 1.6)**.

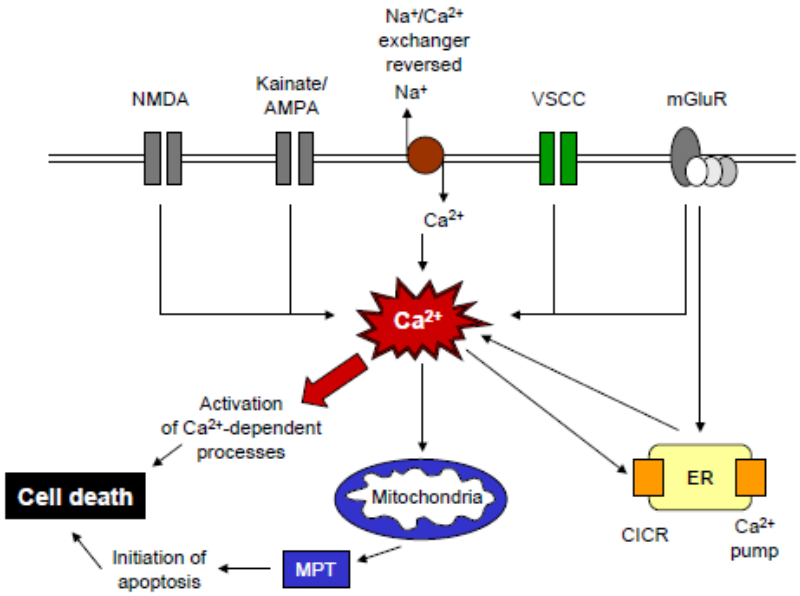


Figure 1.6 The central role of calcium in ischemic neuronal death processes. As a result of ischemia, intracellular calcium levels can increase by several mechanisms. When calcium levels are increased in the cytoplasm, mitochondria attempt to take up this cation, but these organelles are also overcome by ischemia-induced elevation and this leads to mitochondrial permeability transition, cytoplasmic release of cell death promoting factors such as cytochrome C and the initiation of apoptotic cell death **(Osborne et al., 2004)**

Many cascades generated by glutamate and glucose/oxygen deprivation result in the formation of free radicals (Pellegrini et al., 1990) and it has been proposed that free radicals are important mediators in damage caused by retinal ischemia (Bonne et al., 1998). Oxygen-derived and other free radicals are principally formed when reduced compounds, which accumulate during ischemia, are reoxidized **(Figure 1.7)**. There is evidence that this free radical burst, produced during the early stage of reperfusion, overwhelms normal cellular antioxidant defense mechanisms, causing oxidative stress and a variety of types of tissue injury (Gilgun et al., 2002).

There are many ways in which free radicals can be formed during ischemia-reperfusion, but the burst of superoxide radicals (O_2^-) which occurs during the early stage of reperfusion is thought to occur by the following pathway. During ischemia, degradation of ATP leads to the formation of hypoxanthine, and increases in

intracellular calcium in neurones activate the Ca^{2+}- dependent protease calpain. Calpain converts xanthine dehydrogenase into xanthine oxidase and upon reperfusion the latter enzyme oxidizes the accumulated hypoxanthine to uric acid resulting in the release of O_2^-. This reaction is catalyzed by iron, which is released from its protein-bound stores at the low pH generated during ischemia. In addition, O_2^- interacts with nitric oxide (NO), which is produced in considerable amounts following ischemia, leading to the formation of peroxynitrite, nitrosyl radical and eventually OH (Nielsen et al., 1996; Gilgun et al., 2002). Activation of glial cells and infiltrating leukocytes also release inflammatory mediators, such as arachidonic acid, nitric oxide and cytokines, which all play major roles in the formation of free radicals following ischemia.

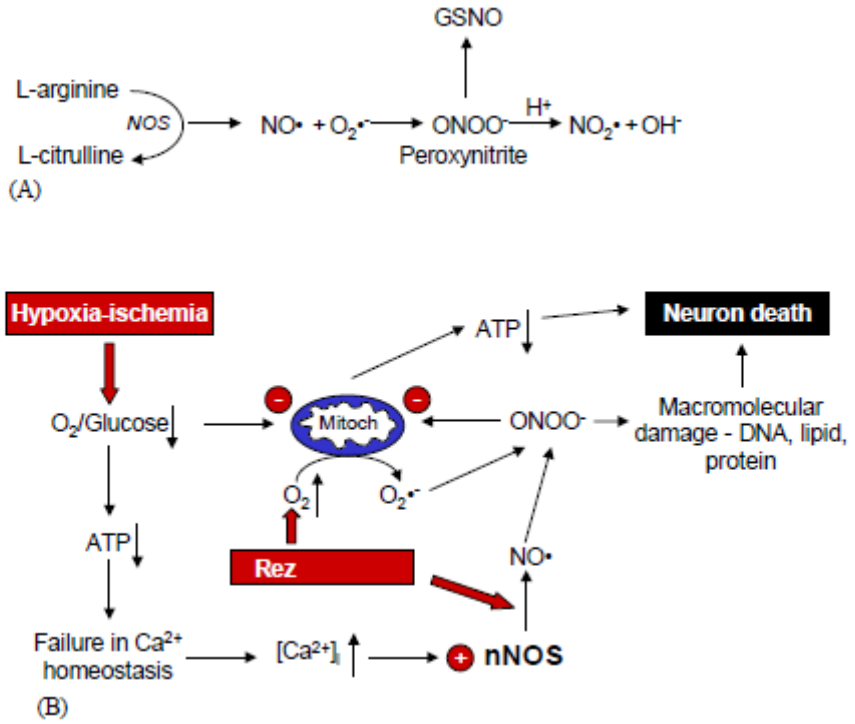


Figure 1.7 Production and reactions of nitrogen-derived radicals. **(A)** Nitric oxide synthase (NOS) produces nitric oxide radicals in the conversion of L-arginine to L-citrulline. This compound acts as an important homeostatic modulating agent under physiological conditions via its vasodilator, antioxidant, antiplatelet and antineutrophil actions. **(B)** Reactions and formation of nitric oxide radicals as a result of ischemia- reperfusion. The combined production of nitric oxide derived radicals and failure of cellular antioxidant defence will lead to widespread macromolecular damage and cell death **(Osborne et al., 2004)**

1.8 Retinal Disease Therapeutics

It is important to note that two main approaches are being taken to develop putative therapeutic strategies: neuroprotection and cell replacement. Cell replacement is a novel therapeutic approach to restore visual capabilities to the degenerated adult neural retina and represents an emerging field of regenerative neurotherapy. The discovery of a population of proliferative cells in the mammalian retina has raised the possibility of harnessing endogenous retinal stem cells to elicit retinal repair. Furthermore, the development of suitable protocols for the reprogramming of differentiated somatic cells to a pluripotent state further increases the therapeutic potential of stem-cell-based technologies for the treatment of major retinal diseases. Stem-cell transplantation in animal models has been most effectively used for the replacement of photoreceptors, although this therapeutic approach is also being used for inner retinal pathologies (Schmeer et al., 2012).

1.9 Animal models of retinal ischemia

A number of in vivo and ex vivo mammalian models **(Table 1.1)** have been developed to study retinal ischemia. Clearly the ability to obtain data from an animal model for the clinical situation requires a model that closely resembles retinal ischemia in humans. Commonly used small laboratory animals are the rat, rabbit and guinea pig, with the rat being the most similar to humans.

Experimental method	Degree of ischemia	Human disease correlate	Selected references
High intraocular pressure	Complete	CRAO, acute angle-closure glaucoma, ophthalmic artery occlusion	Smith and Baird (1952) and Buchi et al. (1991)
Ligature of optic nerve bundle	Complete	CRAO, ophthalmic artery occlusion	Stefansson et al. (1988)
Ligature of ophthalmic vessels	Complete	CRAO, ophthalmic artery occlusion	Otori et al. (1997) and Vidal-Sanz et al. (2001)
Bilateral occlusion of common carotid artery (2-vessel occlusion)	Incomplete	CRAO, ophthalmic artery occlusion, carotid insufficiency	Osborne et al. (1991) and Block et al. (1992)
Bilateral occlusion of vertebral and common carotid arteries (4-vessel occlusion)	Complete	Cardiac arrest/hypovolaemic shock	Osborne et al. (1991) and Block et al. (1992)
Photodynamic ablation	Incomplete	BRAO	Mosinger and Olney (1991) and Daugeliene et al. (2000)
Cardiac arrest	Complete	Cardiac arrest	Feher and Antal (1979) and Strosznajder et al. (1998)
Postmortem ischemia	Complete	—	Johnson and Grierson (1976) and Napper and Kalloniatis (1999)
Intravitreal injection of glutamate receptor agonists	Incomplete?	CRAO?	Schwarcz and Coyle (1977) and Siliprandi et al (1992)

Table 1.1 Animal models of retinal ischemia. **CRAO**= Central Retinal Artery Occlusion. **BRAO**= Branch Retinal Artery Occlusion **(Osborne et al, 2004)**

High IOP-induced ocular ischemia is a frequently used model for retinal ischemia research and has been described in a number of species (Osborne et al., 2002; Gehlbach and Purple, 1994). This method produces pathological features almost identical that seen after Central Retinal Artery Occlusion (CRAO) and may also represent a model of acute angle closure glaucoma. Usually, in this model, the anterior chamber of the rat is cannulated with a needle connected to a reservoir, elevated to produce an IOP of 110 mmHg, such that the IOP is greater than the ocular perfusion pressure. This method produces global ischemia, with and at least 20 min of ischemia are required to reproducibly cause irreversible, functional ischemic injury.

Vascular ligation is another common method of causing retinal ischemia in rats. In its simplest form it involves placing a suture around the optic nerve bundle, ligating the posterior ciliary vessels. This also constricts the optic nerve, elevates the intraocular pressure (IOP) and probably damages axons. This model can mimic resultant retinal pathology, but the principal problem with this model is that cerebral and optic nerve ischemia is also produced making it difficult to distinguish between the effects of direct retinal ischemia and retrograde degeneration.

Intravitreal injection of excitatory amino acids and especially those of glutamate receptor agonists (NMDA, AMPA and kainate acid), which is the main retina's neurotransmitter, is another widely used *in vivo* model. In severe retinal diseases, such as Diabetic Retinopathy, glaucoma and retinal detachment and in models simulating this diseases also (Barber et al., 1998), glutamate levels are higher compared to those of healthy situations. In addition, the pathological activation of glutamate receptors is considered to be the final common pathway leading to neuronal cell death in many neurodegenerative diseases.

In this study, we used the model of intravitreous injection of AMPA in order to investigate the mechanism involved in the somatostatin's selective receptor ligands-induced neuroprotection.

The neuropeptide somatostatin has been shown to have antiangiogenic actions because it inhibits Growth Hormone (GH), growth factors (IGF-1 and VEGF) and endothelial cell proliferation (Smith et al., 1997; Higgins et al., 2002; Dal Monte et al., 2007; Catalani et al., 2007). In addition, it protects the retina from ischemic and excitotoxic insults (Mastrodimou et al., 2005; Mastrodimou et al., 2008; Catalani et al., 2007; Kiagiadaki et al., 2010).

1.10 Glutamatergic System

1.10.1 Glutamate acid

There is a large number of amino acids which act as excitatory neurotransmitters in the Central Nervous System and lead to quick depolarization of glutamatergic cells (Olney, 1969). Under abnormal conditions, these amino acids provoque the brain's and retina's damage and degeneration (Olney and Ho, 1970). From these early studies, glutamate and aspartate are believed to be the basic excitatory amino acids neurotransmitters.

Glutamate (**Figure 1.8)** is the main excitatory synaptic neurotransmitter in the CNS (Orrego and Villanueva, 1993**)**. It affects the long-lasting changes, the structure and the function of neuronal excitability, the neuronal migration during development and the neuronal cells viability.

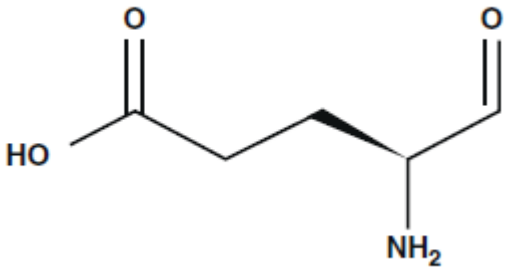


Figure 1.8 Chemical structure of glutamate

Glutamate is a non-essential amino acid which does not cross the blood-brain barrier. It is synthesized in neuron mitochondria from glucose and several precursors. After being synthesized, glutamate is released into the cytoplasm where it accumulates in synaptic vesicles through a process dependent on Mg^{2+}/ATP (Hassel and Dingledine, 2005). The propagation of the nerve impulse towards the axon terminal promotes the release of glutamate from the synapse through a mechanism that depends on intracellular Ca^{2+} by means of exocytosis (Pigino et al., 2005). Glutamate can then interact with its specific receptors.

High levels of excitatory amino acids in the intracellular space lead to neuronal death because of the too high excitability of neurons. The excitation of the glutamate receptors is believed to be the final common pathway for many neurological disorders (i.e epilepsy, Parkinson's disease) (Lipton and Rosenberg, 1994).

Because of its significant neurotoxic effects, the amount of glutamate in the synapse has to be regulated. Much of the released glutamate is taken up by the surrounding glial cells and converted into glutamine. The glutamine is then taken up by the presynaptic axon. Glutamate is deaminated and turned back into glutamate **(Figure 1.9)**. Direct glutamate reuptake by the presynaptic neuron accounts for a small amount of the released glutamate. Another small amount actually escapes from the synaptic space and may have significant peripheral effects (DeVries, 2000). The amount that escapes appears to increase in pathologic conditions.

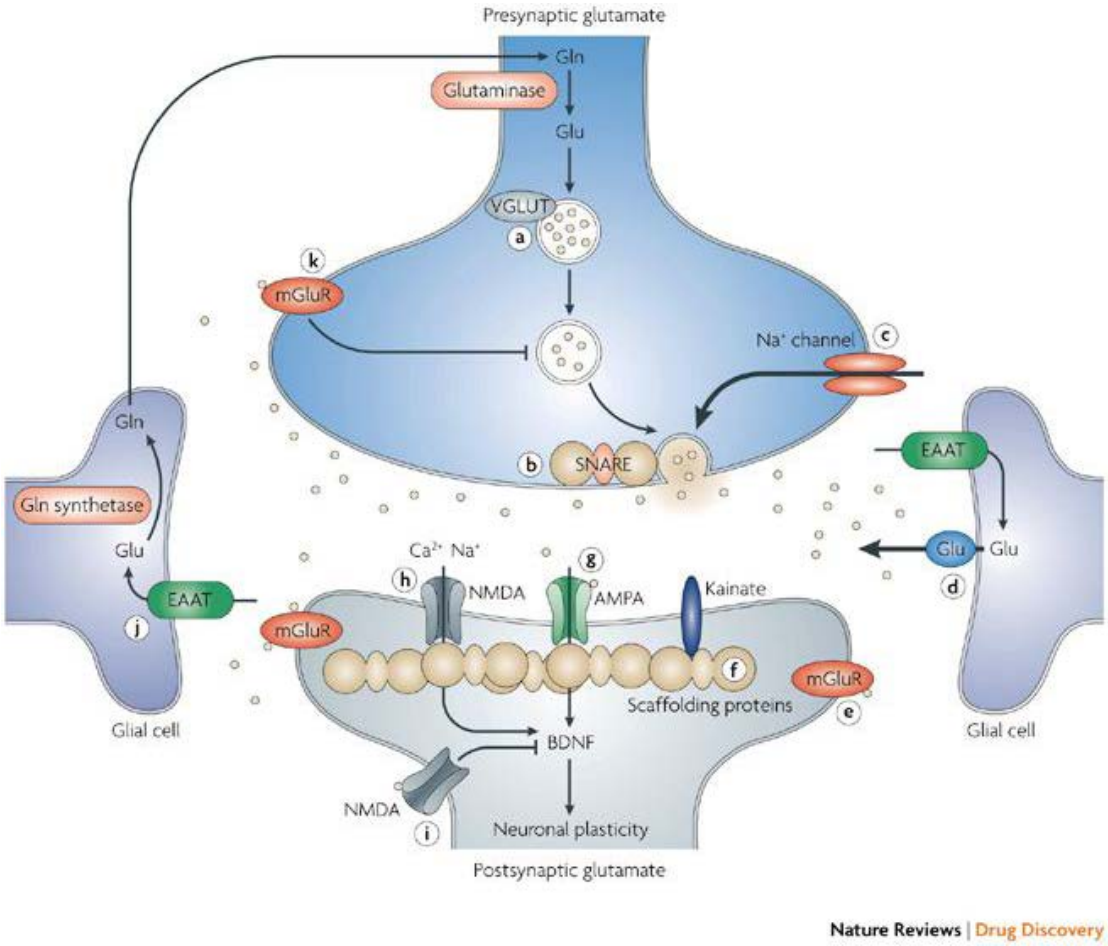


Figure 1.9 Glutamate cycle in the synaptic space **(Sanacora et al., 2008)**

Regarding the glutamate, there are five known high-affinity excitatory amino acid transporters: EAAT1 (GLAST), EAAT2 (GLT-1), EAAT3 (EAAC1), EAAT4 and EAAT5 (Arriza et al., 1997; Fairman et al., 1995; Kanai et al. 1992; Pines et al., 1992). Uptake of glutamate into astrocytes is mediated by GLAST (also found in Muller cells) and GLT1 (or EAAT1 and 2) and into neurons by EAAC1, EAAT4 and EAAT5, of which the last primarily is found in the retinal photoreceptor cells. In addition, there are glutamine transporters that need to be synchronized to transport glutamine from the astrocytes into the neurons.

1.10.2 Glutamate Receptors: Structure and Function

Glutamate receptors **(Figure 1.10)** are classified in two groups: i) ionotropic receptors acting directly via ionic channels (Ozawa et al., 1998) and ii) metabotropic receptors which are included in the G-Protein Coupled Receptors (GPCRs) and act via

secondary messengers (Watkins et al., 1990; Orlando et al., 2002). As with all ligand-gated ion channels, ionotropic glutamate receptors convert the free energy of agonist-binding into a sequence of conformational changes that allow the regulation of ion flux across and the insulation of membrane bilayer. In the case of ionotropic glutamate receptors, the resulting membrane depolarization is a key mediator of intracellular signals in the synapses of the CNS (Madden, 2002). Protein phosphorylation, controlled by the coordinated actions of phosphatases and kinases, is an important regulatory mechanism in synaptic transmission and other neurophysiological processes. Ionotropic and metabotropic glutamate receptors are targets of phosphorylation on serine (iGLURs), threonine (iGLURs) and tyrosine (iGLURs and mGLURs) residues, with functional consequences for cell excitability, plasticity and toxicity (Iacovelli et al., 2012).

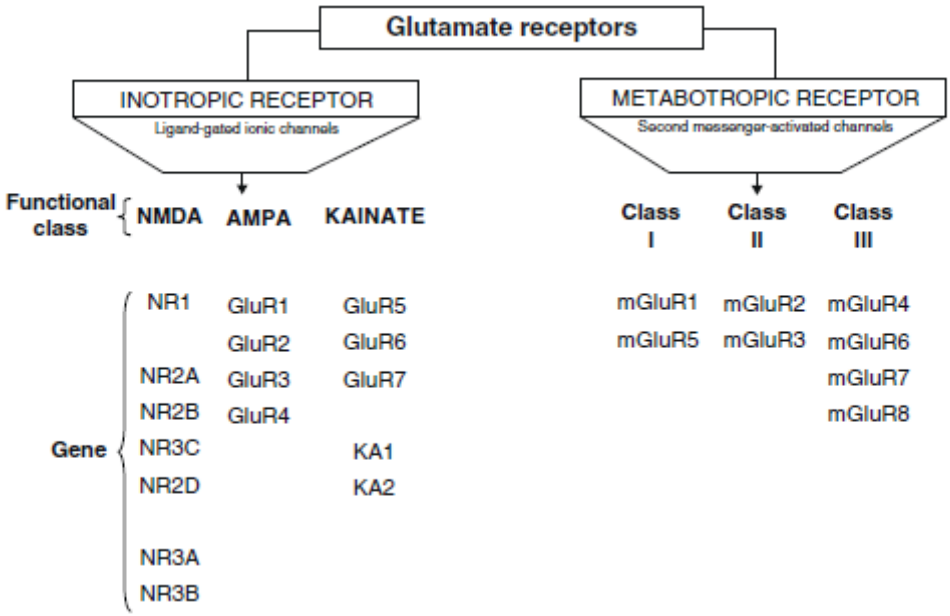


Figure 1.10 Classification of glutamate receptors **(Pulido et al., 2007)**

Ionotropic receptors are further categorized in two subgroups: i) NMDA receptors and ii) non-NMDA receptors, depending on the agonists who activate them and the antagonists who inhibit them. NMDA receptors are activated from the N-methyl-D-aspartate amino acid (NMDA) and their action is inhibited by the (2R)-amino-5-phosphonovaleric acid (APV). Non-NMDA receptors are activated from a-amino-3-

hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) quisqalic acid and are deactivated by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Metabotropic glutamate receptors are activated from 1-Amino-cis-cyclopentane-1,3-diacarboxylic acid (ACPD).

Ionotropic receptors are heteromers constituted by different subunits, which give the receptors different physiological and pharmacological properties **(Figure 1.11)**.

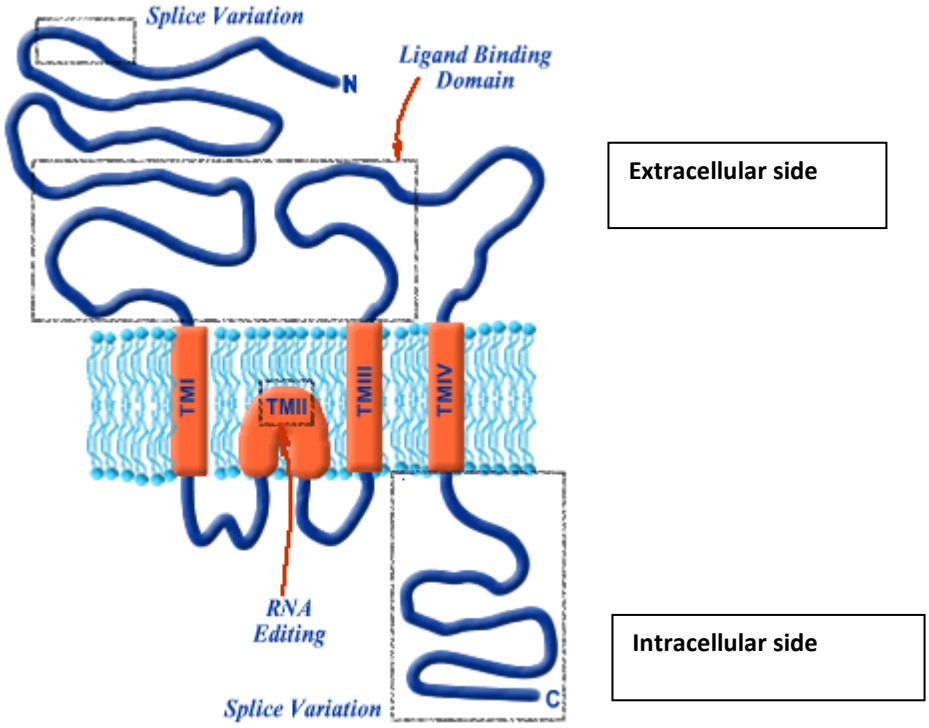


Figure 1.11 General structure of a subunit of an ionotropic glutamate receptor (http://www.bristol.ac.uk/synaptic/receptors/).

AMPA receptors are structured as combinations of GluR1- 4 subunits which form an ion channel permeable to Na^{+}. However, it has been shown that AMPA receptors whose structure does not include a GluR2 subunit are highly permeable to Ca^{2+}. AMPA receptors mediate fast excitatory transmission in the brain and complex with other proteins. These receptors form ligand-gated ion channels through which cations can pass. The four subunits of AMPA receptors are differentially distributed throughout the brain and furthermore, AMPA receptors have also been found on inhibitory interneurons and some glia (Laezza and Dingledine, 2011). This lack of uniform expression of the receptors may allow the possibility of targeting drugs to a

specific brain region without affecting other areas. At most excitatory synapses in the brain, AMPA receptors contain the GluR2 subunit, rendering the channel Ca^{2+}-impermeable (Lu et al., 2009), although some evidence suggest that GluR2-containing receptors may in fact be Ca^{2+}-permeable (Bowie, 2012). Notably, just as synapse components are dynamically regulated, so too are AMPA receptors, shuttling into and out of synapses. As mentioned and earlier, AMPA receptors are complexed with a group of proteins known as transmembrane AMPA receptor regulatory proteins (TARPs) **(Figure 1.12)** (Coombs et al., 2009). The discovery of the first TARP, known as stargazin, came from work on stargazer mutant mice. These spontaneous mutant mice have seizures and cerebellar ataxia, as well as cerebellar granule cells lacking AMPA receptors (Hashimoto et al., 1999).

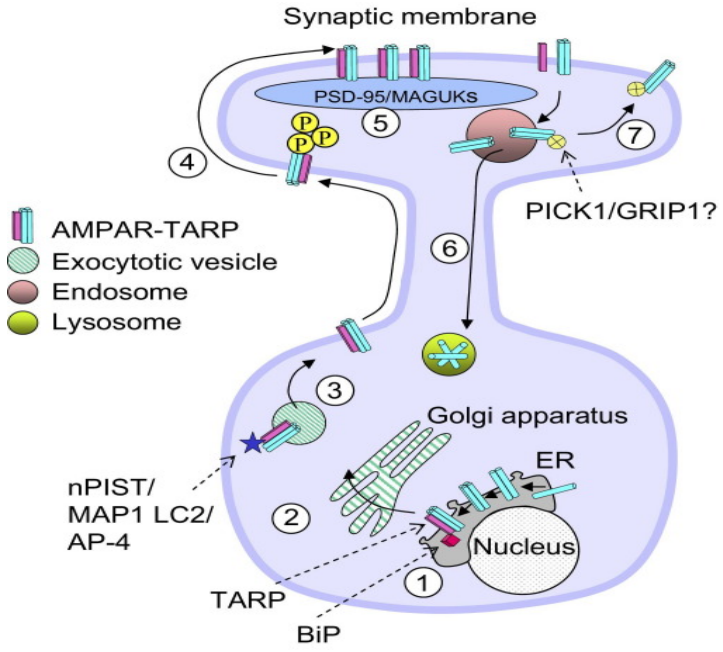


Figure 1.12 Cell trafficking of the TARP-AMPAR assembly. AMPAR biogenesis is initiated in the ER, with formation of dimers (homo or heterodimers) of GluR subunits, followed by tetramerization. TARPs interact with the AMPARs in the ER (1). Within the ER, proteins such as BiP, interact with TARPs to facilitate trafficking of the TARP–AMPAR complex through the TGN (2). In the TGN, TARPs interact with proteins including nPIST, MAP1 LC2 and AP-4, which may assist vesicular trafficking to the cell surface (3). PKC/CaMKII phosphorylate multiple sites on the TARP protein to facilitate membrane mobilization (4) and hence increase the likelihood of AMPARs accumulating at the synapse. AMPARs

are immobilized in the postsynaptic membrane via interaction with MAGUKs, including PSD-95 (5). Following endocytosis, potentially triggered by agonist dependent TARP-AMPAR dissociation, the subsequent fate of the AMPARs may be degradation (6) or possibly recycling to the cell surface by other AMPAR interacting proteins such as PICK1 or GRIP1 (7) **(Coombs and Cull-Candy, 2009)**

Kainate receptors are protein heteromers formed by combinations of the GluR5, GluR6, and GluR7 subunits, together with KA1 and KA2. The combination of KA2 and GluR5 forms a functional receptor that is permeable to Na^{2+}.

The structure of NDMA Receptors (NDMAR) has not been completely explained, although it is hypothesised that they form tetrameric or pentameric structures (Michaelis, 1998). However, it is known that it is formed by combinations of different subunits: NMDAR1 (NR1), NMDAR2 (NR2) and NMDAR3 (NR3) (Hassel et al., 2005). The entire structure forms a Ca^{2+}-permeable ion channel. Functional NDMARs are generally formed by heterotetramers consisting of two dimers composed by NR1—NR2 subunits and these dimmers considered to be the basic functional organisation structure in each receptor.

The structure of mGluRs consists of a protein chain that crosses the membrane 7 times **(Figure 1.13)**. To date, eight units named mGluR1 through mGluR8 have been cloned, and they are classified according to the following: the homology of their amino acids (70% homology among members of the same class, and 45% homology between different classes); in response to their agonists, and the signal paths for second messengers (Hassel et al., 2005).

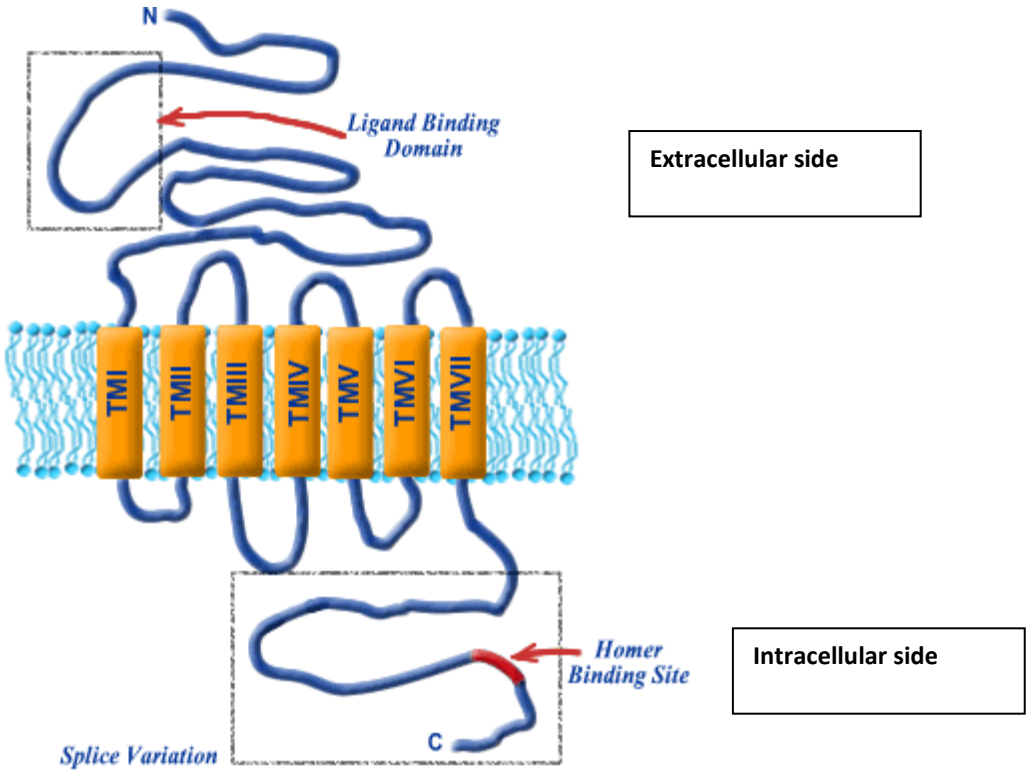


Figure 1.13 General structure of metabolotropic glutamate receptors

Finally, **Figure 1.14** illustrates the most important functional features of ionotropic and metabolotropic glutamate receptors.

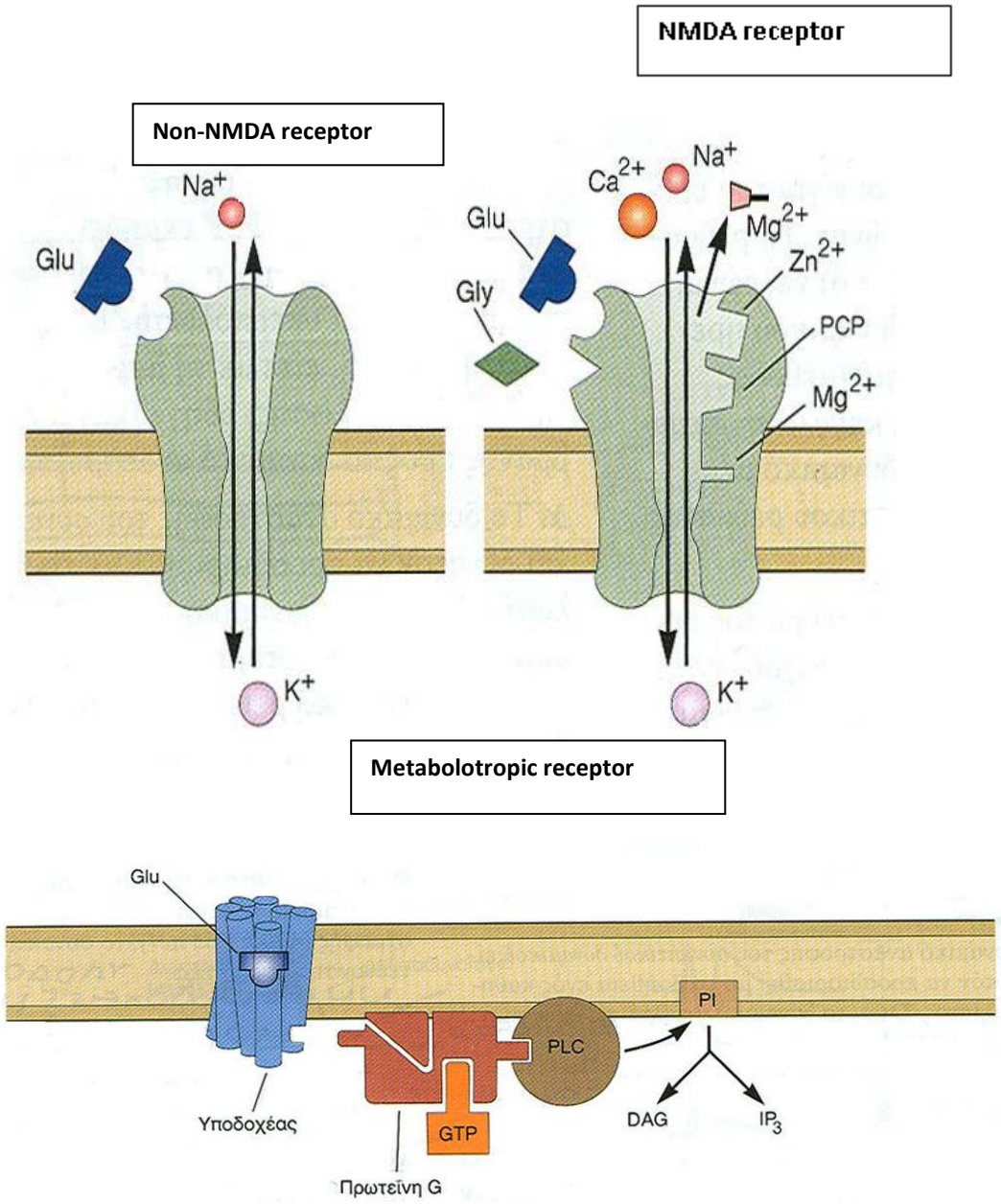


Figure 1.14 Functional features of glutamate receptors **(Kandel et al., 2003)**

1.10.3 Gutamate and GABA Receptors in Retina

Glutamate and γ-aminobutyric acid (GABA) are major excitatory and inhibitory neurotransmitters in the vertebrate retina. Glutamate receptors are important in neural plasticity, neural development and neurodegeneration. N-methyl-d-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors act as glutamate-gated cation channels, whereas metabotropic receptors (mGluRs) modulate the production of second messengers via G proteins.

As it mentioned above, NMDA receptors and mGluRs exist as multiple subunits (NMDAR1 and NMDAR2A-2D) and multiple subtypes (mGluR1-mGluR8). In the visual system, retinal bipolar cells receive glutamate transmission from photoreceptors and contribute to segregating visual signals into ON and OFF pathways. Photoreceptors, generate visual signals and bipolar cells mediate signal transfer from photoreceptors to ganglion cells and both release glutamate, which induces or changes the activity of the post-synaptic neurons (horizontal and bipolar cells for photoreceptors; amacrine and ganglion cells for bipolar cells). Horizontal and amacrine cells, which mediate lateral interaction in the outer and inner retina respectively, use GABA as a principal neurotransmitter.

Recently, glutamate receptors and GABA receptors in the retina have been extensively studied. Both N-methyl-D-aspartate (NMDA) and non-NMDA receptors are expressed in the retina. Photoreceptors possess metabotropic glutamate receptors and several subtypes of GABA receptors (Koulen et al., 1999). Although non-NMDA receptors and metabotropic glutamate receptors are generally thought to be responsible for mediating the transfer of visual signals in the outer retina, there is recent evidence suggesting that NMDA receptors are also expressed in photoreceptors, as well as horizontal and bipolar cells.

In the inner retina, NMDA receptors, in addition to other glutamate receptor subtypes, are abundantly expressed to mediate visual signal transmission from bipolar cells to amacrine and ganglion cells, and could be involved in the modulation of inhibitory feedback from amacrine cells to bipolar cells (Koulen et al., 1999; Hirasawa et al., 2002). Most horizontal cells express AMPA receptors and in addition, these cells also express $GABA_A$ and $GABA_C$ receptors (Yang, 2004).

Signal transfer from photoreceptors to bipolar cells is rather complicated. Whereas AMPA/KA receptors mediate transmission for OFF type bipolar cells, several subtypes of glutamate receptors, both ionotropic and metabotropic, are involved in the generation of light responses of ON type bipolar cells. The molecularly cloned mGluR6 is restrictedly expressed at the postsynaptic site of ON-bipolar cells in both rod and cone systems. Gene targeting of mGluR6 results in a loss of ON responses without changing OFF responses and severely impairs detecting

visual contrasts. Since AMPA receptors mediate OFF responses in OFF-bipolar cells, two distinct types of glutamate receptors effectively operate for ON and OFF responses. mGluR1 and mGluR5 are both coupled to inositol triphosphate (IP3)/calcium signal transduction with an identical agonist selectivity. Single-cell intracellular calcium (Ca^{2+}) recordings indicated that glutamate evokes a non-oscillatory and oscillatory Ca^{2+} response in mGluR1-expressing and mGluR5-expressing cells, respectively. These two closely related mGluR subtypes mediate diverging intracellular signaling in glutamate transmission (Nakanishi et al., 1998). $GABA_A$ and $GABA_C$ receptors are differentially expressed on dendrites and axon terminals of both ON and OFF bipolar cells, mediating inhibition from horizontal cells and amacrine cells (Yang, 2004).

Amacrine cells possess ionotropic glutamate receptors (Masland, 2001), whereas ganglion cells express both ionotropic and metabotropic glutamate receptors (Koulen et al., 1997; Brandstätter et al., 1998; Duvoisin et al., 1995; Hartveit et al., 1995). NMDA receptors are extra-synaptically expressed in ganglion cells and may play physiological roles in a special mode.). $GABA_A$ receptors also exist in amacrine and ganglion cells. There are studies suggesting that $GABA_C$ receptors may be involved in the activity of these neurons and moreover that responses of these retinal third order neurons are modulated by $GABA_B$ receptors, and that in ganglion cells exist several subtypes of $GABA_B$ receptors (Yang, 2004).

Finally, **Table 1.2** provides a general overview for the presence of glutamate receptors in the different retinal cell types.

Retinal neuron	Receptor type			
	GluRs			
	iGluRs			mGluRs
	AMPA	KA	NMDA	
Photoreceptor	?	?	?	Group II, Group III (+)
Horizontal cell	+	–	–	Group I (+), Group III (?)
Bipolar cell				
ON	+	?	?	Group I, Group III (+)
OFF	+	+	–	Group I, Group III (+), Group II (?)
Amacrine cell	+	+	+	Group I, Group II, Group III (+)
Ganglion cell	+	?	+	Group I (+), Group III (?)

Table 2 (+) Strong positive evidence for the expression; (-) Strong negative evidence for the expression; (?) Evidence is inconsistent and/or there are only reports about the existence in a sparse neuron population or in some special species **(Yang, 2004)**

1.10.4 Excitotoxicity

Neuronal death caused by excessive liberation of Glutamate and overactivation of Glutamate receptors is known as excitotoxicity (Olney, 2002). Excitotoxicity is associated with a number of pathological states of the Central Nervous System, such as epilepsy (Lapouble et al., 2002), hypoxia and/or ischaemia and trauma. It is also thought to be involved in Huntington, Alzheimer and Parkinson diseases (Nishizawa, 2001; Mattson, 2000).

Overactivation of glutamate receptors, mainly NDMARs, is one of the processes involved in the neurodegeneration and cell death that are present in a number of diseases (Martin et al., 1998). This produces an increase in intracellular Ca^{2+}, thereby promoting lipid peroxidation (LP) of the cell membrane, Endoplasmic Reticulum and mitochondria (Mattson et al., 2000). This LP is due to the production of nitric oxide (NO) and superoxide radicals (O_2^-), which form peroxynitrites. There is also production of 4-hydroxynonenal (HNE), which alters the activity of membrane transporters and ionic channels when lipids in the membranes are peroxydated (Mattson et al., 2000). LP also damages ATPase Na^+/K^+, glucose transporters and glutamate as part of the excitotoxic process and disturbs ionic homeostasis in the Endoplasmic Reticulum (ER) and mitochondria, which supply the ATP energy **(Figure 1.16)**. The increase in intracellular Ca^{2+} concentration elicits the activation of intracellular signal pathways related to cellular apoptosis, such as the activation of different Ca^{2+}-dependent enzymes (proteases, nucleases, and phospholipases). In addition, excitotoxicity is correlated with MAPK activation (Kawasaki et al., 1997).

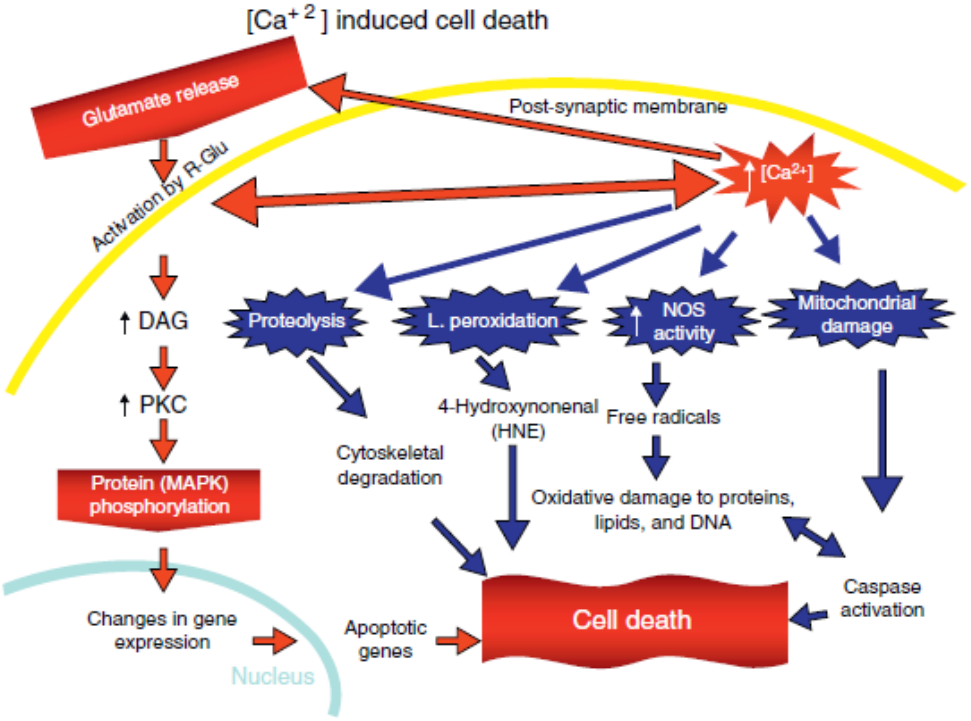


Figure 1.16 Excitotoxic cell death **(Flores et al., 2012)**

1.11 Somatostatin

1.11.1 Characteristics and Physiology

The neuropeptide somatostatin (somatotropin release inhibitory factor, SRIF) is a cyclic tetradecapeptide, which is widely distributed in the peripheral and central nervous system. The presence of immunoreactive somatostatin in rat retina was first discovered by Shapiro et al in 1979. The expression and the localization of SRIF and of its receptors in mammalian retinas, including humans, have been reviewed in recent years (Cervia et al., 2008; Thermos, 2003). There is a general agreement that such a distribution reflects the pleiotropic functions of retinal somatostatin as a result of the multiple signaling actions of its receptors. In general, SRIF immunoreactivity is localised to sparsely distributed, wide-field amacrine and/or displaced amacrine cells and, in some instances, in a subset of ganglion cells. In particular, in the mouse (Cristiani et al., 2002), rat (Larsen et al., 1990;), guinea pig (Tornqvist et al., 1982) and human retina (Tornqvist and Ehinger, 1988), SRIF-containing cells are detected in both the inner nuclear layer and the ganglion cell

layer. In the mouse retina, the population of SRIF-containing amacrine cells is sparsely distributed to all retinal regions, while the population of displaced amacrine cells is confined to the ventral retina (Cristiani et al., 2002). In the rabbit, cat and primate retina, most SRIF immunoreactive cells are displaced amacrine cells that are predominantly distributed to the ventral retina (Sagar and Marshall, 1988; Sagar, 1987). In spite of its very sparse distribution, somatostatin processes extensively in the Inner Plexiform Layer of all retinal regions. SRIF immunoreactive ganglion cells have been identified as a small subset of OFF-center alpha ganglion cells, mostly localised to the inferior retina (White et al., 1991). Finally, a transient population of SRIF containing ganglion cells has been discovered in the rat retina during postnatal development (Xiang et al., 2001).

Two biologically active forms arise from the C-terminal portion of a single pro-peptide **(Figure 1.17)**: somatostatin-14 (SST-14), originally described in the hypothalamus and the amino-terminally extended somatostatin-28 (SST-28), discovered later in the gut and are derived by tissue specific proteolytic processing of prosomatostatin, the 92-amino acid precursor of somatostatin-14 and -28. SST-14 and SST-28 are predominantly produced by neurones and secretory cells in the central and peripheral nervous system and the gastrointestinal tract. However, they are also expressed in the placenta, the kidney, the retina and in cells of the immune system (Olias et al., 2004).

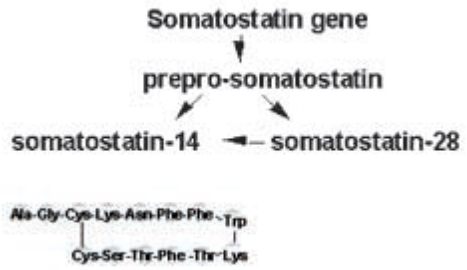


Figure 1.17 Schematic representation of somatostatin apparented peptides **(Olias et al., 2004)**

Release of somatostatin from neurons and secretory cells usually depends on membrane depolarization or increasing cytosolic Ca^{2+} concentrations and is triggered

by ions, hormones, growth factors and nutrient. The major actions of the neuropeptide include inhibition of hormone secretion from the pituitary, most notably that of Growth Hormone (GH), the pancreas and other endocrine sites and inhibition of exocrine secretion in a number of various organs. Moreover, somatostatin exerts antiproliferative actions and is a widely distributed neurotransmitter substance in the brain and peripheral nerve cells (Olias et al., 2004).

SRIF has been shown to modulate K^+ and Ca^{2+} currents in neurons, endocrine cells, and some cell lines. Many classes of K^+ currents are reported to be increased by SRIF, including a K^+ leak current (Schweitzer et al., 1998), an inward rectifier (Takano et al., 1997), a delayed rectifier K^+ current (Wang et al., 1989), and a Ca^{2+}-activated K^+ current (White et al., 1991). The effect of SRIF on Ca^{2+} current is inhibitory, and it acts on high voltage-activated Ca^{2+} currents of the L-type (Rosenthal et al., 1988) and N-type (Shapiro et al., 1993). In axonal terminals, the combined action of SRIF on K^+ and Ca^{2+} currents has been reported to reduce transmitter release. Zalutsky and Miller (1990) reported that SRIF was excitatory to most ganglion cells tested in rabbit retina, but whether the peptide acted directly on ganglion cells or via circuitry that is presynaptic to ganglion cells was not unequivocal. More recently, sst_{2A} immunostaining was localized to a variety of rat and rabbit retinal neurons, including bipolar cells and cone photoreceptors (Johnson et al., 1998, 2000). The location of sst_{2A} receptors on photoreceptor terminals suggested a possible role of SRIF in regulating the release of glutamate, the identified neurotransmitter of photoreceptors (Marc et al., 1990). Akopian et al. (2000) showed that in the salamander retina, both rods and cones express sst_{2A} receptors found that SRIF reduced the Ca^{2+} current of rods but increased that of cones and in addition, this peptide increased the delayed rectifier K^+ current of rods and cones. In a later study, Petucci et al. (2001) showed that somatostatin modulates the K^+ currents and the Ca^{2+} influx in isolated rod bipolar cells.

1.11.2 Somatostatin receptors: structure and function in retina

Somatostatin acts in retina via the receptors localized in the plasma-membrane of cell-targets and it mediates a diverse array of physiological actions by interacting with specific receptors found in the plasma membrane (Thermos and Reisine, 1988; Thermos et al., 1989). In the retina, there were many reports showing somatostatin immunoreactivity in amacrine, associational ganglion, and interplexiform cells and suggested SRIF as a neurotransmitter or neuromodulator in this organ (Liapakis and Thermos 1992; Liapakis et al., 1993; Vasilaki et al., 2003).

Somatostatin receptors are G-Protein Coupled Receptors (GPCRs) **(Figure 1.18)**. These receptors mediate the inhibitory effects of SRIF on adenylate cyclase activity and Ca^{2+} conductance (Wang et al., 1990), as well as its potentiation of K^{+} conductance and other intracellular systems (Kleuss et al., 1992).

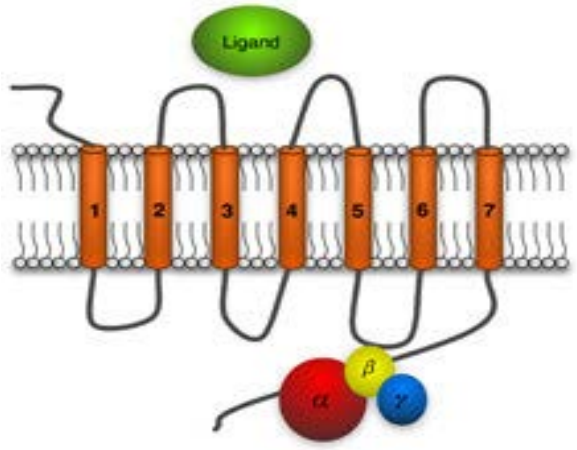


Figure 1.18 GPCRs structure

Five somatostatin receptor subtypes have been cloned and are named sst_1, sst_2, sst_3, sst_4 and sst_5 (Yamada et al., 1992; Hoyer et al., 1995). Especially, sst_2 receptor in mice and rats, undergoes in two isoforms, sst2A and sst2B, resulting from alternative splicing of sst_2 gene's mRNA.

The SRIF receptors homology is 70%. Based on their sequence homology, the receptor subtypes can be divided in two subgroups: SRIF1 and SRIF2. The SRIF1 receptor group comprises sst_2, sst_3 and sst_5 (SRIF1A, SRIF1B and srif1C) while sst_1 and sst_4 (SRIF2A and SRIF2B) belong to the SRIF2 group. This classification is based on structural features and strongly supported by their pharmacological properties.

SRIF1 but not SRIF2 receptors bind readily to the classical octapeptide or hexapeptide analogues, octreotide, lanreotide, seglitide and vapreotide (Vanetti et al., 1992, Bruns et al., 1995, Schindler et al., 1998).

Pasireotide is able to activate sst_3 and sst_5 receptors but is only a partial agonist at the sst_2 receptor. Octreotide exhibits potent agonistic properties at the sst_2 receptor but produces very little sst_5 receptor activation. Like octreotide, somatoprim is a full agonist at the sst_2 receptor. Unlike octreotide, somatoprim is also a potent agonist at the sst_5 receptor (Kliewer et al., 2012)

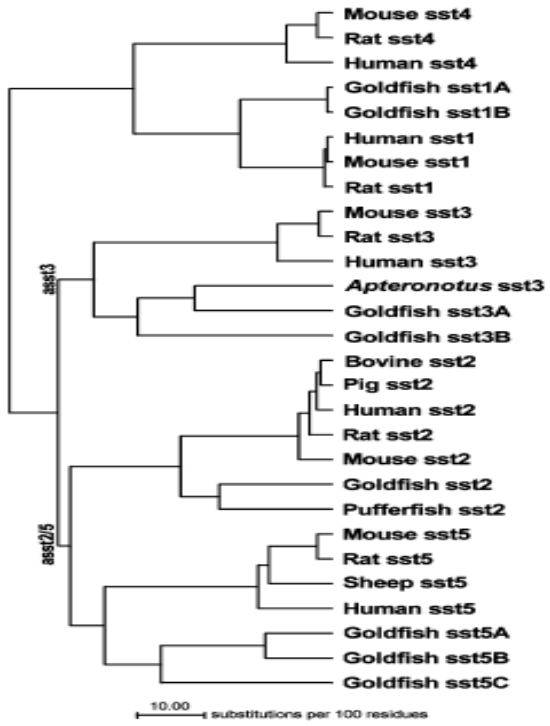


Figure 1.19 Phylogenetic tree based on the alignement of amino acid sequences of the known full-length somatostatin receptor in vertebrates **(Olias et al., 2004)**

While the presence of sst_1, sst_2 and sst_4 receptors on specific retinal neurons has been established in the rat (Thermos, 2003), the location of the sst_5 subtype was reported in rat retina initially by Ke and Zhong (2007) and provided evidence for the presence of sst_5 receptors on retinal GABAergic, dopaminergic and cholinergic amacrine cells. A more recent study showed that the activation of sst_5 receptor protects retina from AMPA-induced excitotoxicity (Kiagiadaki et al., 2010). sst_5

belongs to the SRIF 1 receptor group which comprises subtypes sst_2, sst_3 and sst_5 (Olias et al., 2004).

A study by Xiao-Hua Wu et al. (2012), conducted a comparative study of the expression of sst_5 in mouse and bullfrog retinas by immunofluorescence double labeling. The expression profiles of sst_5 in the retinas of the two species were similar. In the inner retina sst_5 was localized to dopaminergic and cholinergic amacrine cells, stained by tyrosine hydroxylase (TH) and choline acetyl transferase (ChAT) respectively, and cells in the ganglion cell layer, whereas in the outer retina immunostaining for sst_5 was observed in horizontal cells. However, a more widespread, abundant distribution of labeling for sst_5, as compared to mouse retina, was seen in bullfrog retina. Strong labeling for sst_5 was diffusely distributed in both outer and inner plexiform layers (OPL and IPL) in the bullfrog retina, but the labeling was only observed in the IPL of the mouse retina. In addition, bullfrog photoreceptors, both rods and cones, but not mouse cones, were labeled by sst_5. Finally, **Figure 1.20** summarizes the distribution of SRIF receptors in the retina.

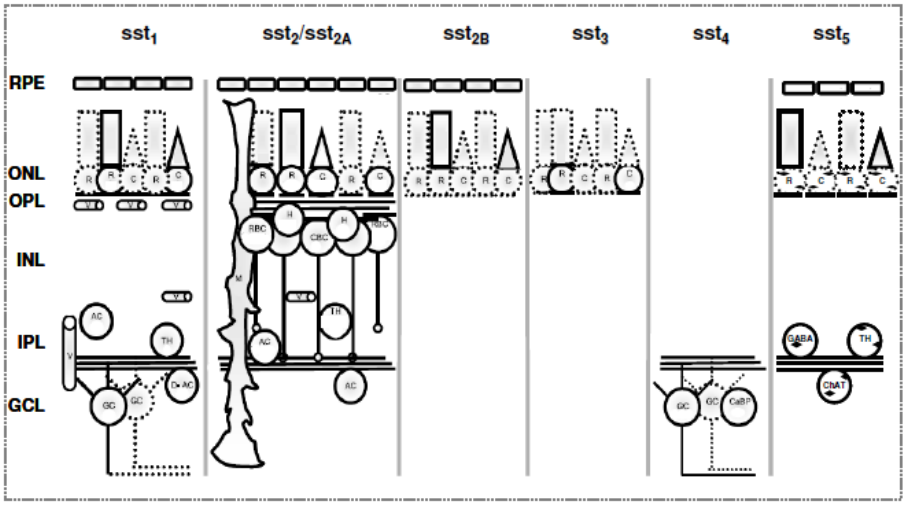


Figure 1.20 Mapping of ssts in the retina. This figure summarizes the results of different investigations on the localization of somatostatin receptor subtypes in retinal circuitry and the Retinal Pigmental Epithelium (RPE) **(Thermos, 2003)**.

2. AIM OF THE STUDY

The neuropeptide somatostatin and selective analogues for the sst_2 and sst_5 receptor subtypes provided neuroprotection against AMPA induced retinal toxicity in vivo (Kiagiadaki and Thermos, 2008; Kiagiadaki et al., 2010; Kokona et al., 2012). Previous work in our lab showed that in rat retinal explants, somatostatin increased NO basal levels (Vasilaki et al., 2002) and cGMP levels (Mastrodimou et al., 2006) in a concentration dependent manner via an sst_2 dependent mechanism and also that NO/peroxynitrate and cGMP are mediators in the protection of retinal neurons from chemical ischemia (Mastrodimou et al., 2008). The aim of the present study was to examine the mechanism mediating this $sst_{2/5}$-mediated protection, and more specifically, to investigate the possible involvement of the nitric oxide (NO) and/or cGMP signaling pathway.

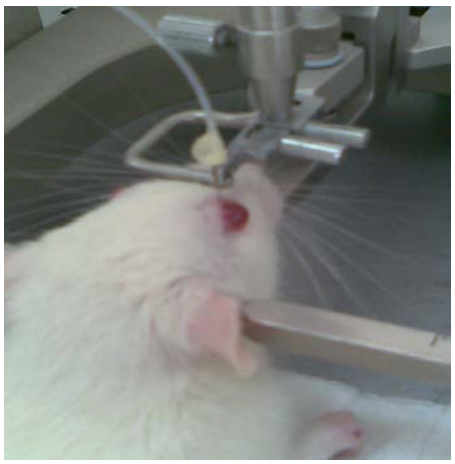
3. EXPERIMENTAL PROCEDURE

3.1 Animals

Adult male and female Sprague–Dawley rats, weighting 250–300 g, were used for all experiments. Animals were housed one to two animals per cage in a room maintained at 22°C, with an alternating 12-hours light–dark cycle. Food and water were available *ad libitum*. Euthanization was performed with ether inhalation. All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with Greek national laws (Animal Act, P.D. 160/91).

3.2 AMPA Excitotoxicity and Neuroprotection

The in vivo model of AMPA excitotoxicity was employed according to Kiagiadaki and Thermos (2008). The animals were anesthetized with intramuscular injections of xylazine (14.5 mg/kg) and ketamine (200 mg/kg) and were placed in a stereotaxic apparatus to stabilize the head and facilitate intravitreal administration. Injections were made with 27- or 30-gauge needles connected to a 1ml Hamilton syringe adapted to a minipump. The tip of the needle was inserted behind the sclera-cornea border into the vitreous humor. Each intravitreal injection was performed with a flow rate of 1μL/min for 5 minutes.



One eye received phosphate-buffered saline 50Mm (PBS, 50mM K2HPO4/NaH2PO4, 0.9% NaCl, pH=7.4) as control and the other eye received AMPA

(Tocris) 42nmoles/eye diluted in PBS 50mM. Experiments for the neuroprotection involved the injection of AMPA (42nmol/eye) in combination with lanreotide/BIM23014 (sst_2/sst_5 selective analog, 10^{-4}M, Bachem). To examine the possible involvement of nitric oxide (NO) in the neuroprotecive effects of lanreotide, the nitric oxide synthase inhibitor L-NAME (Sigma, 3 or 30nmol) was co-injected with AMPA (42nmol/eye) and lanreotide (10^{-4}M). To examine the possible involvement of cGMP in the neuroprotective effects of lanreotide, the soluble guanylate cyclase inhibitor ODQ (Tocris, 10nmol) was co-injected with AMPA (42nmol) and lanreotide (10^{-4}M). In addition, to confirm the neuroprotective effects of cGMP against AMPA excitotoxicity, a membrane permeable cGMP analogue 8-Bromo-cGMP (Tocris, 1mM) was co-injected with AMPA (42nmol). Furthermore, according to a recent study (Kiagiadaki et al., 2010) we confirmed that the activation of the sst_5 receptor can be protective against AMPA insult. The selective ligand for sst5 receptor L-817,818 (10^{-4}M) was co-injected with AMPA (42nmol). In order to investigate the possible involvement of NO and cGMP in the sst_5 receptor's actions, L-NAME (3 or 30 nmol) was co-injected with AMPA (41nmol) and L-sst5 (10^{-4}M) and ODQ (10nmol) was co-injected with AMPA (42nmol/eye) and L-sst5 (10^{-4}M) respectively. All animals were killed 24hrs after treatment.

3.3 Immunohistochemical Studies

3.3.1 Tissue Preparation

Twenty four hours after treatment, rats were humanely killed, the eyes were removed and the eyecups were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer for 1 hour at 4°C. After fixation, eyecups were rinsed in phosphate buffer and were incubated in 30% sucrose overnight at 4°C for cryoprotection. Tissues were frozen in isopentanium at -45°C for 1 minute and kept at -80°C until further use. Eyecups were sectioned vertically at 10-μm thickness using a cryostat (Superfrost; Erie Scientific, Portsmouth, NH), and stored at -20°C. Nine slices were put on every slide.

3.3.2 Immunohistochemistry

Previous studies in our laboratory (Kiagiadaki & Thermos, 2008, Kiagiadaki et al., 2010) showed that intravitreal AMPA administration led to the loss of ChAT, bNOS and calbindin immunoreactivity, but it did not affect photoreceptor, rod bipolar and ganglion cells. Therefore antibodies only against the two of these retinal markers were employed in the present study. A goat polyclonal antibody raised against ChAT (Choline acetyl transferase; 1:400, MILIPORE), and a rabbit polyclonal antibody raised against bNOS (1:1000, Sigma, St. Louis, Missouri, USA) were employed as markers for acetylocholine (ChAT) and neuronal nitric oxide synthase (NOS) containing retinal cells, respectively.

Cryostat retinal sections were employed and treated according to Kiagiadaki & Thermos (2008). The sections were incubated in 0.1M Tris-HCl buffer; TBS, pH 7.4 containing 3.3% normal goat or normal donkey serum for 30min, washed in 0.1M TBS and incubated with the primary antibodies in 0.1M TBS containing 0.3% Triton X-100 and 0.5% normal goat (for bNOS) or normal donkey (for ChAT) serum at room temperature (NGS and NDS respectively). The sections were washed again and incubated for 1 and a half hour with the appropriate secondary antibody Alexa Fluor546 goat anti-rabbit IgG (H+L) (1:400, Molecular Probes, Eugene, U.S.A) for the polyclonal antibodies or Alexa Fluor546 donkey anti-goat IgG (H+L) (1:1000, Molecular Probes, Life technologies, U.S.A) for the polyclonal antibodies ChAT and bNOS respectively. Finally, the sections were washed and cover slipped with mounting medium with DAPI (VECTOR Laboratories, Burlingame, CA). Negative controls were included by omitting the primary antibody.

3.4 Determination of retinal cell death

Caspase Immunoreactivity

To determine cell loss, a polyclonal antibody raised against caspase-3 for the labeling of cells which undergo apoptotic cell death (1:300, Cell Signaling, MA, USA). The sections were incubated in 0.1M Tris-HCl buffer; TBS, pH 7.4 containing 3.3% normal goat serum (NGS) for 30min, washed in 0.1M TBS and incubated with the

primary antibodies in 0.1M TBS containing 0.3% Triton X-100 and 0.5% normal goat serum at room temperature. The sections were washed again and incubated for 1 and a half hour with the appropriate secondary antibody Alexa Fluor546 goat anti-rabbit IgG (H+L) (1:400, Molecular Probes, Eugene, U.S.A). This retinal cell loss was observed by confocal microscopy.

3.5 Microscopy

Light microscopy images were taken with a camera (Axioncam with Plan-Neofluar Carl Zeiss, Oberkochen, Germany). Optic sections were taken with a z-axis resolution of 1.1 µm through the immunolabeled cells. Light and contrast adjustments of images were processed with the use of commercial software (Photoshop ver. 7.0; Adobe Systems, San Jose, CA).

3.6 Quantification studies

For the quantification of bNOS and ChAT-expressing retinal cells, each section of control or treated retinas was studied by means of a x 32/0.75 lens (Axioskop with Plan-Neofluar ×32/0.75; Carl Zeiss, Oberkochen, Germany). The total number of bNOS and ChAT immunoreactive neurons in each retinal section was counted. As mentioned earlier, each slide contained nine slices. Given that some slices were not appropriate for cell counting (e.g., because of tissue folding or damage during sectioning), the number of sections used for the quantification studies was six to nine for each slide examined. The number of slides (retinas) used for the quantification studies was equal to or more than 2 for each treatment. Therefore the minimum number of sections that could be examined was n=2 (number of retinas) x6.

3.7 Preparation of solutions

PB 0,2M: 2,76gr NaH_2PO_4 + ddH_2O in final volume 100ml (sol. A)

17,42gr K_2HPO_4 + ddH_2O in final volume 500ml (sol. B)

sol. A + sol. B → 600ml PB 0,2M

PBS 0,1M: 50ml PB 0,2M + 0,9gr NaCl + ddH_2O in final volume 100ml.

PBS 50mM: 25ml PB 0,2M + o,9 gr NaCl in final volume 100ml.

TBS 0,1M: 6,055gr Trisma Base + 4,5gr NaCl + ddH_2O in final volume 500ml. addition of serried HCl while stirring and measuring pH 7.4.

PFA 4%: (40ml ddH_2O + 4gr PFA → stir at 550C) + 4 drops NaOH 4% + ddH_2O in final volume 50ml. More addition 50ml PB 0,2M and filtration.

Sucrose solution: 3gr sucrose + 10ml PB 0,1M

4. RESULTS

4.1 Effect of Lanreotide in the AMPA Model of Retinal Excitotoxicity -ChAT Immunoreactivity (IR)

The AMPA excitotoxicity model was employed to examine the neuroprotective effects of Lanreotide in rat retina. Choline acetyl transferase (ChAT) is a marker for cholinergic cells. ChAT-IR was localized in amacrine cells in the Inner Nuclear Layer (INL), in displaced amacrine cells in the Ganglion Cell Layer (GCL) and in processes in the Inner Plexiform Layer (IPL) of PBS-treated retinas (control) (n=5, **Fig. 4.1A**). Intravitreal administration of the excitatory amino acid AMPA (42 nmol/eye) caused almost a complete loss of ChAT-immunoreactive neurons (n=5, **Fig. 4.1B**). Lanreotide ($sst_{2/5}$ receptor analogue) co-injected with AMPA, afforded a partial recovery of ChAT-IR (n=5, **Fig. 4.1C**). Neuroprotection was afforded at the concentration of 10^{-4}M (n=5, **Fig.4.1C**). This is in agreement with previous findings reported by Kiagiadaki and Thermos (2008).

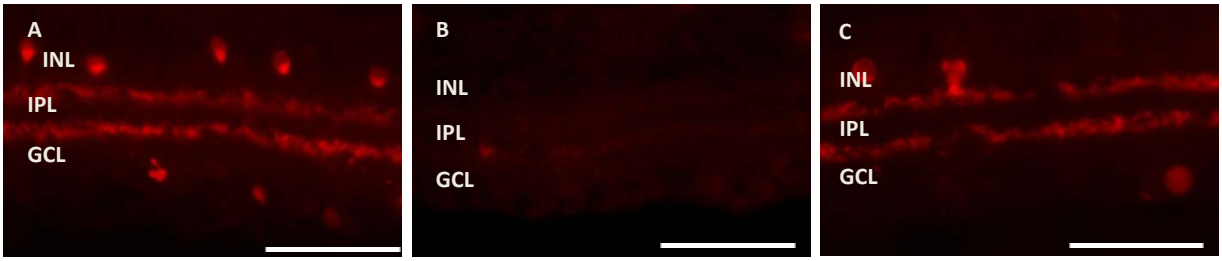


Figure 4.1 Effect of lanreotide on AMPA excitotoxicity. ChAT immunoreactivity in **A.** PBS- (control, n=5) and **B.** AMPA- (42nmol, n=5) treated rat retinas. **C** Co-injected with AMPA (42nmol), the $sst_{2/5}$ selective ligand lanreotide (10^{-4}M, n=5) protected cholinergic amacrine neurons. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar: 50μm**.

4.2 Effect of blockade of NO synthesis and sGC activity on the neuroprotective effects of Lanreotide -ChAT immunoreactivity

To examine whether NO is involved in lanreotide's neuroprotective action, a non-selective NOS (nitric oxide synthase) blocker, L-NAME at the concentrations of 3 and 30 nmol, was used. Co-administration of the NOS inhibitor L-NAME (3nmol, n=4) with AMPA and lanreotide (10^{-4}M) reversed the somatostatinergic neuroprotection in cholinergic cells (**Fig.4.2D**).

In addition, we examined the effect of soluble guanylate cyclase inhibitor ODQ (10nmol, n=4) on the neuroprotective effects of lanreotide. As shown in **Fig.4.2E**, ODQ coinjected with AMPA and lanreotide, greatly reduced the lanreotide's neuroprotective effects.

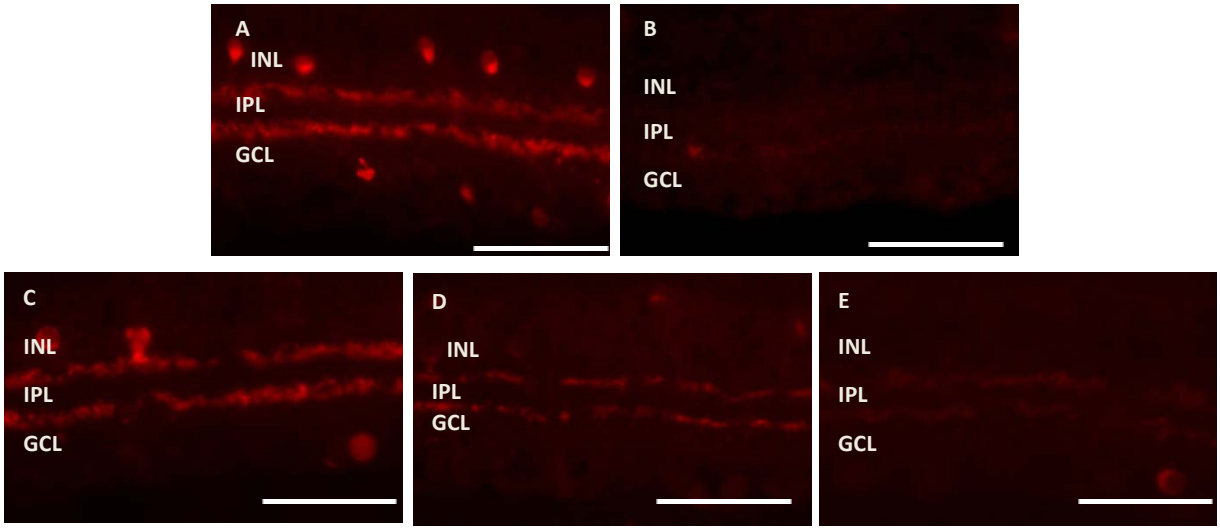
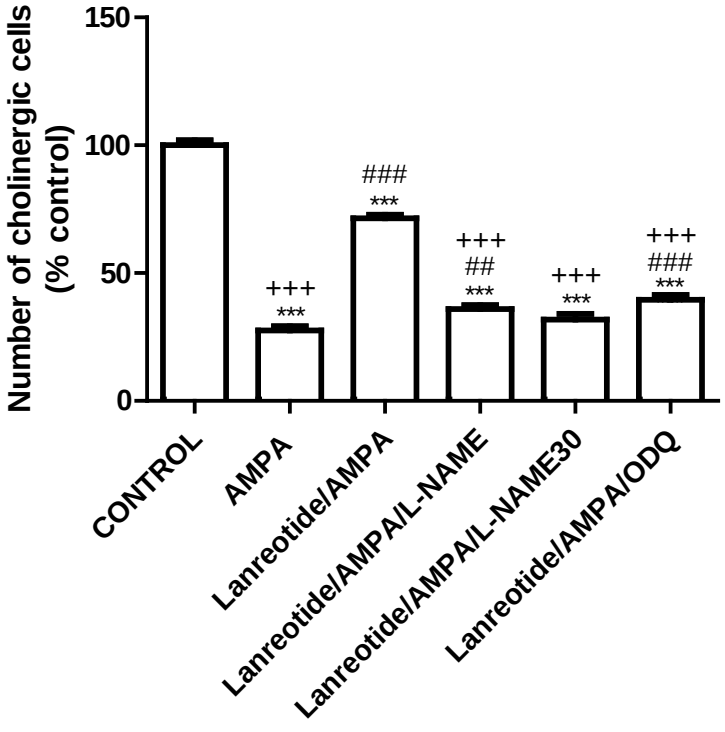


Figure 4.2 Effect of blockade of NO synthesis and sGC activity on the neuroprotective actions of lanreotide. ChAT immunoreactivity in **A.** PBS- (control, n=5) and **B.** AMPA- (42nmol, n=5) treated rat retinas. **C.** Coinjected with AMPA (42nmol), the $sst_{2/5}$ selective ligand lanreotide (10^{-4}M, n=5) partially protected cholinergic amacrine neurons. **D** Blockade of NO synthesis by coinjecting the NOS inhibitor L-NAME (3nmol, n=4) with AMPA and lanreotide reversed the protective action of lanreotide. **E.** Blockade of the soluble guanylate cyclase activity by coinjecting the soluble guanylate cyclase inhibitor ODQ (10nmol, n=4) reversed the neuroprotective effects of lanreotide. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar: 50μm**.

Quantification data involving six groups namely control, AMPA, AMPA+Lanreotide, AMPA+Lanreotide+LNAME 3nmol, AMPA+Lanreotide+L-NAME 30nmol and AMPA+Lanreotide+ODQ 10nmol are shown in **Graph 4.1** AMPA reduced the number of cholinergic cells by approximately 70% while Lanreotide (10^{-4}M) reversed the attenuation providing an approximate 70% neuroprotection compared to control, which was significantly different from both control ($^{***}p<0.001$) and AMPA values ($^{\#\#\#}p<0.001$). This neuroprotection was inhibited by the presence of the L-NAME (3 nmol) bringing the ChAT-IR cells to almost what was observed in the presence of AMPA ($^{\#\#}p<0.01$). L-NAME (30 nmol) fully reversed the neuroprotection to AMPA levels. Additionally, the presence of ODQ (10nmol) also diminished the neuroprotective effects of lanreotide in cholinergic cells but without bringing the number of ChAT-IR cells to what was observed in the presence of AMPA ($^{\#\#\#}p<0.001$).



Graph 4.1 Effect of the blockade of NO synthesis and sGC activity on the neuroprotective action of lanreotide on cholinergic retinal cells. The $sst_{2/5}$ selective ligand lanreotide protected cholinergic retinal neurons, while the guanylate cyclase inhibitor ODQ caused the decrease in the number of cell somata. The NOS inhibitor L-NAME (3nmol, n=4 or 30nmol, n=3) also reduced the number of cholinergic cells. Neuman-Keuls Multiple Comparison Test, ***p<0.001 compared to control group,

$^{###}$p<0.001 compared to AMPA-treated group, $^{##}$p<0.01 compared to AMPA-treated group, $^{+++}$p<0.001 compared to AMPA + lanreotide- treated group.

4.3 Effect of Lanreotide in the AMPA Model of Retinal Excitotoxicity – bNOS Immunoreactivity (IR)

bNOS –IR was also examined in the retinas of all groups. bNOs-IR is used to identify amacrine cells that express the enzyme nitric oxide synthase responsible for the synthesis of nitric oxide (NO). bNOS –IR was localized in amacrine cells in the INL and GCL and in processes in the IPL of PBS-treated retinas (control, n=5, **Fig. 4.3A**). Intravitreal administration of the excitatory amino acid AMPA (42 nmol/eye) caused an attenuation of bNOS-IR (n=5, **Fig. 4.3B**). Lanreotide (10^{-4}M, n=5), co-injected with AMPA, afforded partial recovery of bNOS-IR also (**Fig.4.3C**) as previously reported (Kiagiadaki and Thermos, 2008).

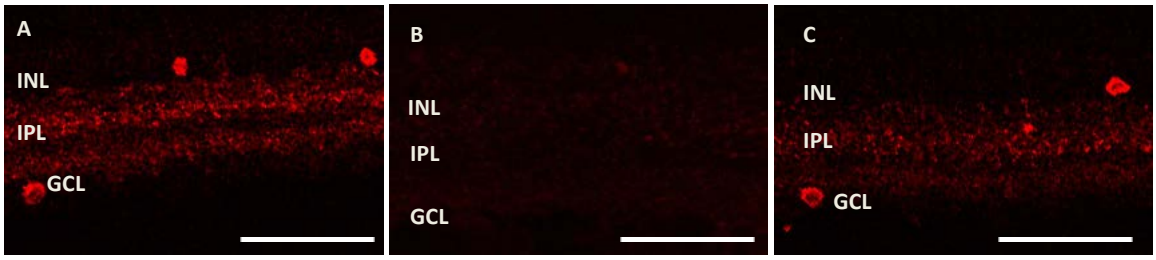


Figure 4.3 Effect of Lanreotide on AMPA excitotoxicity. bNOS immunoreactivity in **A** PBS- (control, n=5) and **B** AMPA- (42nmol, n=5) treated rat retinas. **C** Coinjected with AMPA (42nmol), the sst2/5 selective ligand lanreotide (10^{-4}M, n=5) partially protected bNOS expressing neurons. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar: 50μm**.

4.4 Effect of blockade of NO synthesis and sGC activity on the neuroprotective effects of Lanreotide -bNOS Immunoreactivity

bNOS immunoreactivity was examined in the presence of the two inhibitors, L-NAME (3 or 30nmol) and ODQ (10nmol). Blockade of NO synthesis by coinjecting L-NAME (3nmol, n=4, **Fig.4.4D** or 30nmol, n=3, **Fig.4.4E**) with AMPA (42nmol/eye) and lanreotide (10^{-4}M) reversed also the neuroprotective effects of lanreotide and reduced the number of bNOS containing neurons in the three retinal cell layers (INL,

IPL, GCL). Similarly, the coinjection of ODQ (10nmol, n=4, **Fig.4.4F**) caused a loss in the number of bNOS expressing retinal cells, especially those in the inner plexiform layer (IPL) and in the ganglion cell layer (GCL).

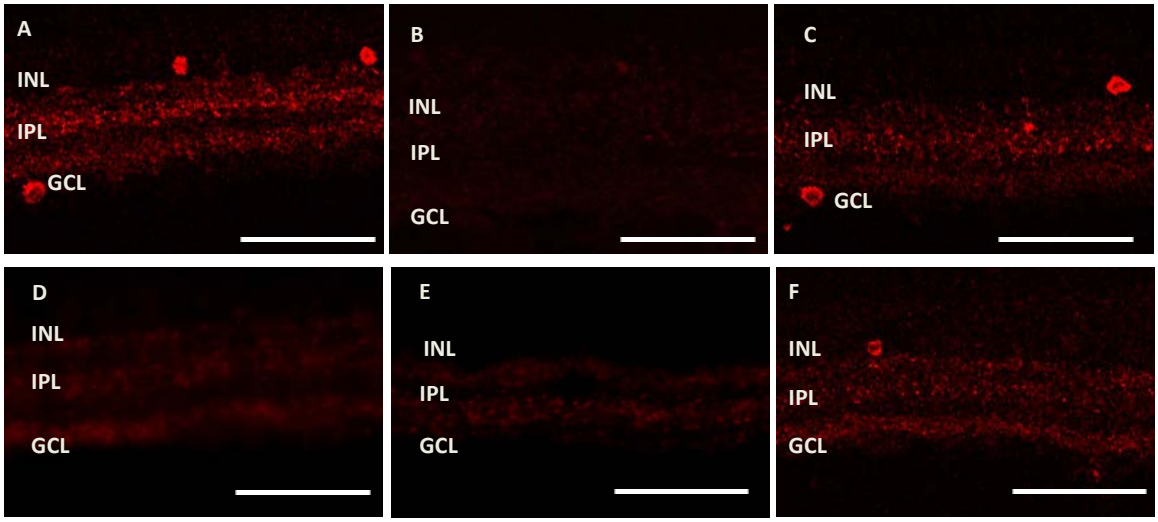
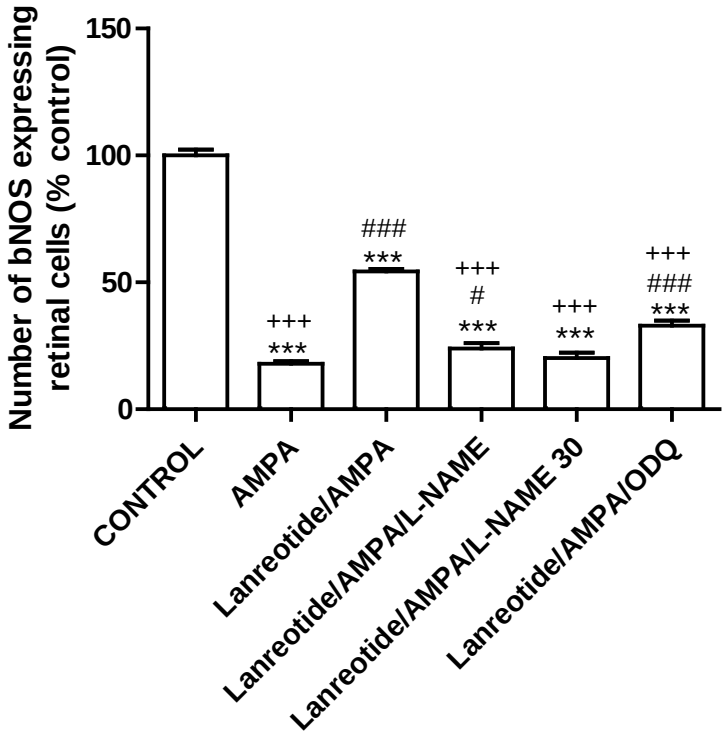


Figure 4.4 Effect of blockade of NO synthesis and soluble guanylate cyclase activity on the neuroprotective effects of lanreotide. bNOS immunoreactivity in **A** PBS- (control, n=5) and **B** AMPA- (42nmol, n=5) treated rat retinas. **C** Coinjected with AMPA (42nmol), the sst2/5 selective ligand lanreotide (10^{-4}M, n=5) partially protected bNOS expressing neurons. Blockade of NO synthesis by coinjecting the NOS inhibitor L-NAME **D** (3nmol, n=4) or L-NAME **E** (30nmol, n=3) with AMPA and lanreotide reversed the protective action of lanreotide. **F** Blockade of guanylate cyclase activity by coinjecting the guanylate cyclase inhibitor ODQ (10nmol, n=4) also reversed the neuroprotective effects of lanreotide. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar: 50μm**.

Quantification analysis of the same groups, as mentioned above, for the bNOS expressing retinal cells was also obtained and is shown in **Graph 4.2** Lanreotide protected the retina from the AMPA excitotoxicity. Lanreotide's protection (approximately 60%) was observed at the dose of 10^{-4}M, as shown by the statistically significant results ($^{###}$ p<0.001 compared to the AMPA group). L-NAME (3 or 30nmol) reversed this protection in a dose dependent manner with a reduction of approximately 30% compared to the Lanreotide group. ODQ also reversed the neuroprotection producing a reduction of 40% approximately compared to lanreotide's neuroprotection ($^{+++}$p<0.001).



Graph 4.2 Effect of the blockade of NO and cGMP synthesis on neuroprotective action of lanreotide on bNOS expressing retinal cells. The $sst_{2/5}$ selective ligand lanreotide partially protected cholinergic retinal neurons, while the soluble guanylate cyclase inhibitor ODQ caused the decrease of cell somata number. The NOS inhibitor L-NAME (3nmol, n=4 or 30nmol, n=3) reduced also the number of bNOS expressing cells in a dose-dependent manner. Neuman-Keuls Multiple Comparison Test, ***$p<0.001$ compared to control group, $^{###}p<0.001$ compared to AMPA-treated group, $^{##}p<0.01$ compared to AMPA-treated group, $^{#}p<0.05$ compared to AMPA-treated group, $^{+++}p<0.001$ compared to AMPA + lanreotide- treated group.

4.5 Effect of Lanreotide in the AMPA Model of Retinal Excitotoxicity and the possible involvement of NO/sGC/cGMP signaling pathway in this effect -Caspase-3 Immunoreactivity (IR)

The neuroprotective role of Lanreotide in the AMPA excitotoxicity model was also substantiated by the caspase-3 IR. Caspase–3 IR was used to assess retinal cell death by AMPA (42 nmol/eye) and neuroprotection by Lanreotide. Caspase-IR was

detected in the Outer Plexiform Layer (OPL) and the Ganglion Cell Layer (GCL). The results shown in **Figure 4.5** confirm the excitotoxic effects of AMPA (n=3, **Fig.4.5B**). Caspase immunoreactivity labeling apoptotic retinal cells was increased in the AMPA treated retina compared to control (n=3, **Fig.4.5A**). Lanreotide (10^{-4}M, n=3) reduced caspase staining in the Outer Plexiform Layer (OPL) and much more that in the Ganglion cell Layer (GCL) (**Fig.4.5C**) compared to AMPA group. In contrast with Lanreotide, L-NAME at the concentration of 3nmol (n=3) increased the number of caspase immunoreactive apoptotic cells in the two retinal cell layers and especially that in the Ganglion Cell Layer (GCL) (**Fig.4.5D**) whereas at the concentration of 30nmol (n=3) this NO synthase inhibitor produced a significant increase of the caspase labeling apoptotic retinal cells in the Outer Plexiform Layer (OPL) and a less increase of the apoptotic staining in the Ganglion Cell Layer (GCL) (**Fig.4.5E**). Finally, the coinjection of ODQ (10nmol, n=3) also icreased the caspase labeled apoptotic cells in the Outer Plexiform Layer (OPL) and in the Ganglion Cell Layer (GCL) compared to Lanreotide (**Fig.4.5F**).

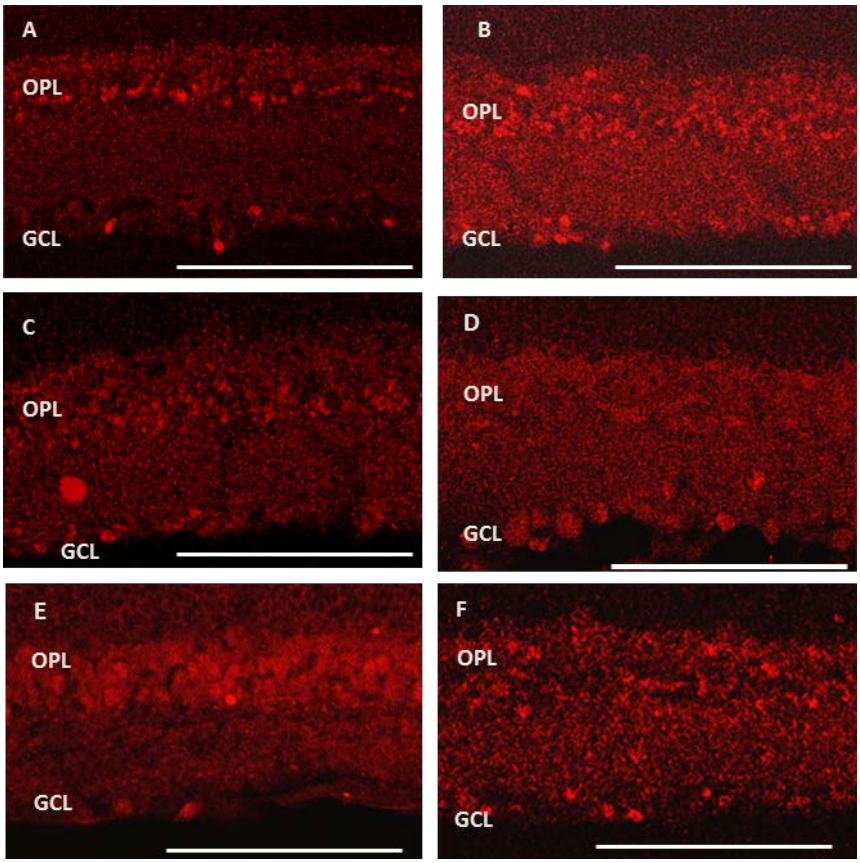


Figure 4.5 Effect of blockade of NO synthesis and sGC activity on the neuroprotective actions of lanreotide against AMPA-induced retinal apoptosis. Caspase-3 immunoreactivity in **A** PBS-control (n=3), **B** AMPA 42nmol (n=3), **C** AMPA 42nmol +lanreotide 10^{-4}M (n=3), **D** AMPA 42nmol +lanreotide 10^{-4}M +L-NAME 3nmol (n=3), **E** AMPA 42 nmol + lanreotide 10^{-4}M + L-NAME 30nmol (n=3). AMPA increased the caspase labeled apoptotic cells compared to control. Lanreotide partially protected the retina from AMPA-induced apoptosis. Blockade of cGMP production reversed the protective actions of lanreotide and blockade of NO synthesis reversed the neuroprotective effect of this analogue in the inner retina in a dose-dependent manner **F** AMPA 42nmol +lanreotide 10^{-4}M +ODQ 10nmol (n=3), **OPL**: Outer Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar: 50μm**.

4.6 Neuroprotective role of cGMP against retinal excitotoxicity -ChAT Immunoreactivity

To examine whether cyclic GMP alone could provide neuroprotection of retinal cholinergic neurons against the AMPA insult, a membrane permeable cGMP analog named 8-Bromo-cGMP (1mM, n=5) was coinjected with AMPA (42nmol/eye). As shown in **Fig.4.6C**, a recovery of cholinergic signal was observed and that amounted to approximately 80% compared to the control group (***p<0.001) **(Graph 4.3).**

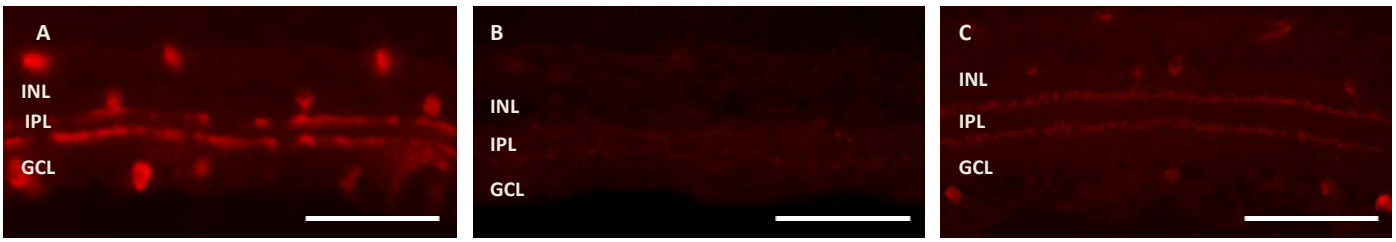
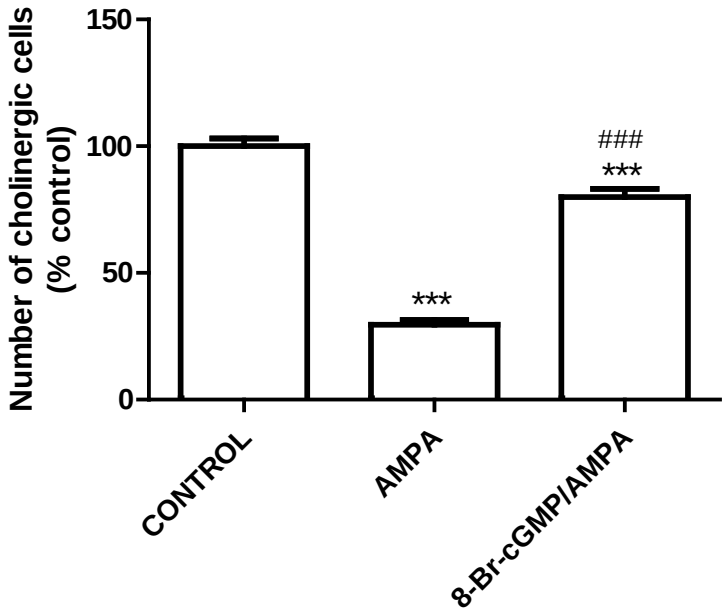


Figure 4.6 Neuroprotective effects of cGMP against AMPA- induced retinal excitotoxicity. ChAT immunoreactivity in **A** PBS-(control, n=5) and **B** AMPA 42nmol- (n=5) treated retinal tissue. Coinjected with AMPA the membrane permeable cGMP analogue 8-Br-cGMP 1mM (n=5) **C** partially protected cholinergic neurons. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar: 50μm**.



Graph 4.3 Neuroprotective effects of cGMP against AMPA excitotoxicity on amacrine cholinergic retinal cells. 8-Br- cGMP protected significantly this cell type of retinal cells. ***p<0.001 compared to control group, ###p<0.001 compared to AMPA-treated group.

4.7 Neuroprotective role of cGMP against retinal excitotoxicity -bNOS Immunoreactivity

The number of bNOS expressing retinal cells was also increased significantly after the coinjection of AMPA and 8-Bromo-cGMP implicating cGMP signaling in the neuroprotection against AMPA excitotoxicity (**Fig.4.7C**). The presence of 8-Br-cGMP increased the number of bNOS containing retinal neurons by approximately 80% compared to control (***p<0.001), a result similar to the observation for cholinergic cells (**Graph 4.4**).

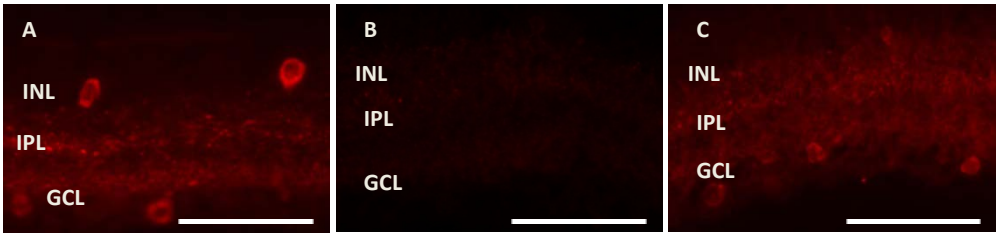
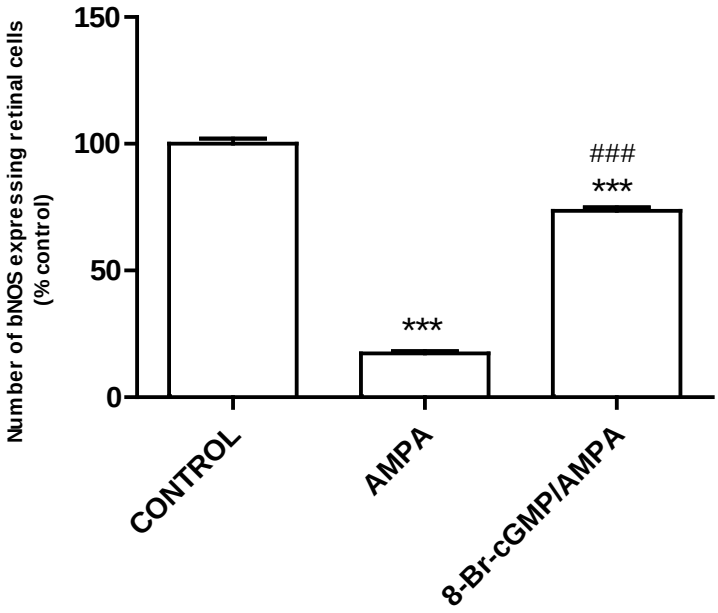


Figure 4.7 Neuroprotective effects of cGMP against AMPA- induced retinal excitotoxicity. bNOS immunoreactivity in **A** PBS-(control, n=5) and **B** AMPA 42nmol- (n=5) treated retinal tissue. Coinjected

with AMPA the membrane permeable cGMP analogue 8-Br-cGMP 1mM **C** at least partially protected bNOS expressing neurons. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer.**Scale bar: 50μm**.



Graph 4.4 Neuroprotective effects of cGMP against AMPA excitotoxicity on bNOS expressing retinal cells. 8-Br- cGMP partially protected this cell type of retinal cells. ***p<0.001 compared to control group, ###p<0.001 compared to AMPA-treated group.

4.8 Effect of L-817,818, an sst5 receptor selective agonist, in the AMPA Model of Retinal Excitotoxicity – ChAT Immunoreactivity

To examine whether sst5 receptor activation alone could provide neuroprotection of retinal neurons against AMPA excitotoxicity, a selective analog for the sst5 receptor, L-817,818, was coinjected with AMPA. In agreement with a recent study (Kiagiadaki et al., 2010) and as shown in **Fig.4.8C**, L-817,818 (10^{-4}M, n=5) partially protected the cholinergic cells during AMPA toxicity.

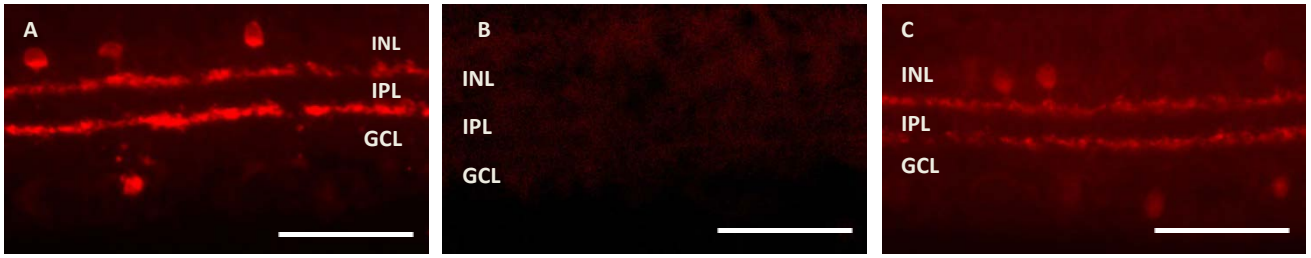


Figure 4.8 Effect of L-817,818 on AMPA excitotoxicity. ChAT immunoreactivity in **A** PBS- (control, n=5) and **B** AMPA- (42nmol, n=5) treated rat retinas. **C** Coinjected with AMPA (42nmol), the sst_5 selective ligand L-817,818 (10^{-4}M, n=5) protected the cholinergic amacrine neurons. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar :50μm**.

4.9 Effect of blockade of NO synthesis and sGC activity on neuroprotective effects of activation of sst5 receptor -ChAT Immunoreactivity

In contrast with the results of lanreotide experiments, coinjection of L-NAME (3nmol, n=5) with AMPA (42nmol/eye, n=5) and L-817,818 (10^{-4}M, n=5) did not reduce significantly the number of cholinergic amacrine cells (**Fig.4.9D**). On the other hand, coinjection of ODQ (10 nmol, n=3) with AMPA (42nmol/eye) and L-817,818 reduced the number of cholinergic cells and especially the displaced amacrine cells in the Ganglion Cell Layer (GCL) (**Fig.4.9E).**

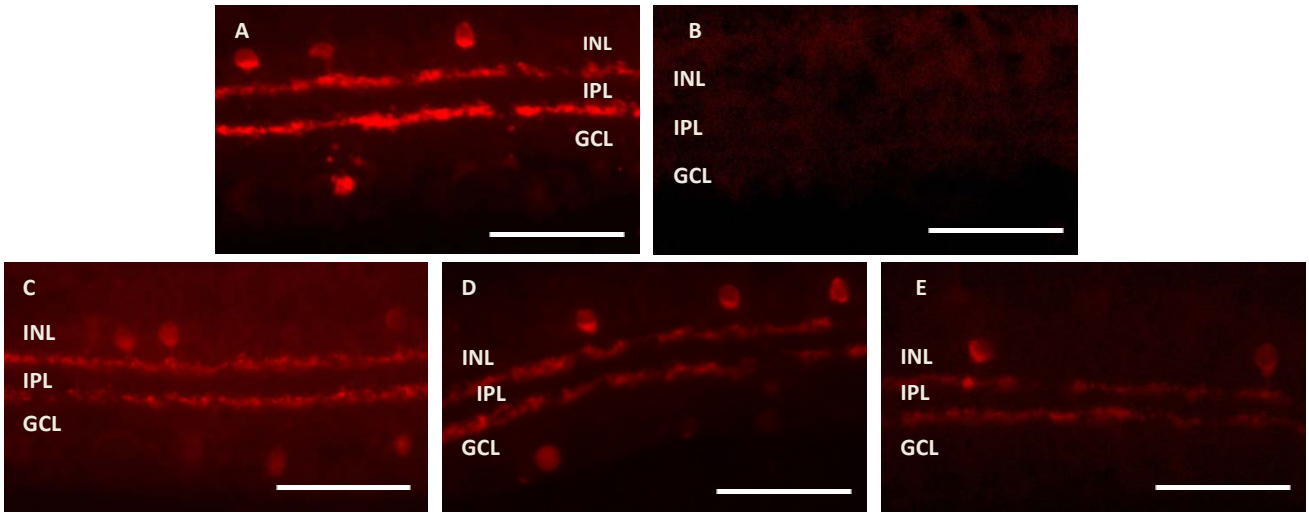
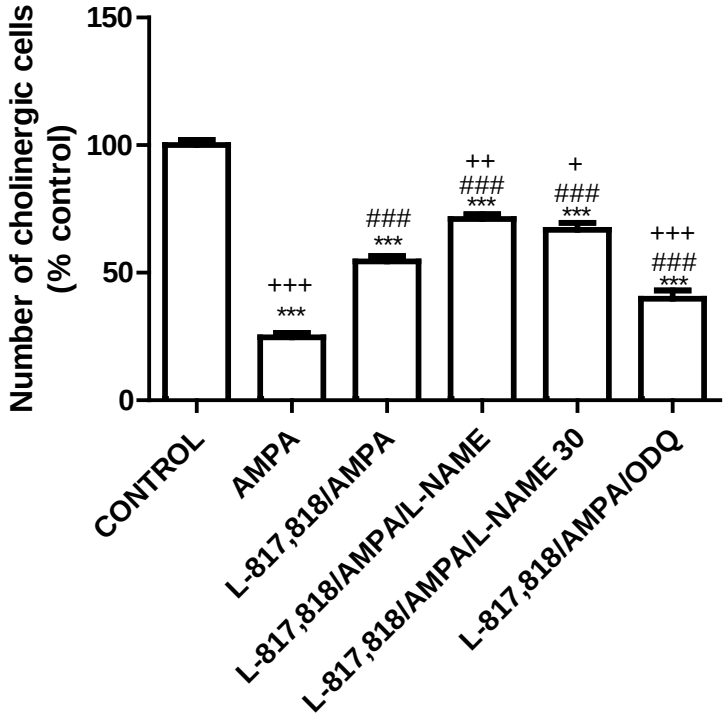


Figure 4.9 Effect of blockade of NO synthesis and sGC activity on the neuroprotective effects of L-817,818. ChAT immunoreactivity in **A** PBS- (control, n=5) and **B** AMPA- (42nmol, n=5) treated rat retinas. **C** Coinjected with AMPA (42nmol), the sst_5 selective ligand L-817,818 (10^{-4}M, n=5) protected cholinergic amacrine neurons. **D** Blockade of NO synthesis by coinjecting the NOS inhibitor L-NAME (3nmol, n=5) with AMPA and L-817,818 did not reduce significantly the number of cholinergic retinal cells. **E** Blockade of soluble guanylate cyclase activity by coinjecting its inhibitor ODQ (10nmol, n=3) reversed the neuroprotective effects of L-817,818. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar :50μm**.

Quantification data involving six groups namely control, AMPA, AMPA+L-817,818, AMPA+L-817,818+L-NAME 3nmol, AMPA+L-817,818+L-NAME 30nmol and AMPA+L-817,818+ODQ 10nmol are shown in **Graph 4.5**. AMPA reduced the number of cholinergic cells by approximately 60%. L-817,818 (10^{-4}M) reversed this reduction providing an approximate 50% protection compared to control, which was significantly different from both control (***p>0.001) and AMPA values ($^{###}$p<0.001). L-NAME at the concentration of 3nmol did not influence the L-817,818 neuroprotection and even brought the ChAT immunoreactive cells to a level approximately 20% higher than this of L-817,818 ($^{++}$p<0.1). At the concentration of 30nmol L-NAME also did not show a significant action in the cholinergic amacrine cells. The L-817,818 neuroprotection was inhibited by the presence of the ODQ (10nmol) which reduced the cholinergic retinal cells approximately 20% compared to L-817,818 ($^{+++}$p<0.001).



Graph 4.5 Effect of the blockade of NO and cGMP synthesis on neuroprotective action of L-817,818 on cholinergic retinal cells. The sst_5 selective ligand L-817,818 partially protected cholinergic retinal neurons, while the NOS inhibitor L-NAME (3nmol, n=5 or 30nmol, n=2) did not reduce the number of cholinergic cells. The guanylate cyclase inhibitor ODQ decreased the number of cholinergic cell somata. Neuman-Keuls Multiple Comparison Test, ***p<0.001 compared to control group, $^{###}$p<0.001

compared to AMPA-treated group, ##p<0.01 compared to AMPA-treated group, $^{+}$p<0.1 compared to AMPA + L-817,818- treated group, $^{++}$p<0.01 compared to AMPA + L-817,818- treated group.

4.10 Effect of L-817,818 in the AMPA Model of Retinal Excitotoxicity - bNOS Immunoreactivity

L-817,818 (10^{-4}M, n=5), as it shown in **Fig.4.10C** also afforded neuroprotective effects in the bNOS expressing retinal cells, especially in those in the Ganglion Cell Layer (GCL), against AMPA excitotoxicity.

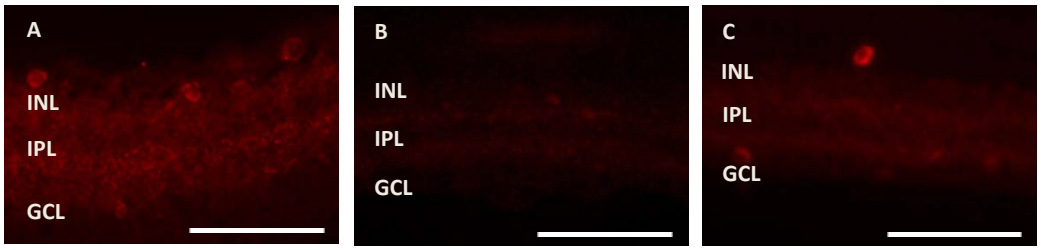


Figure 4.10 Effect of blockade of NO and sGC activity on the neuroprotective effects of L-817,818. bNOS immunoreactivity in **A** PBS- (control, n=5) and **B** AMPA- (42nmol, n=5) treated rat retinas. **C** Coinjected with AMPA (42nmol), the sst5 selective ligand L-817,818 (10^{-4}M, n=5) protected bNOS expressing neurons. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar: 50μm**.

4.11 Effect of blockade of NO synthesis and sGC activity on neuroprotective effects of activation of sst5 receptor -bNOS Immunoreactivity (IR)

Blockade of NO synthesis by coinjecting L-NAME (3nmol, n=5 **Fig.4.11D** or 30nmol, n=3 **Fig.4.11E**) did not reduce significantly neither the bNOS immunoreactive retinal cells. Whereas as it shown in **Fig.4.11F** the coinjection of ODQ (10nmol, n=3) reversed the neuroprotective effects of L-817,818 especially in the Ganglion Cell Layer (GCL).

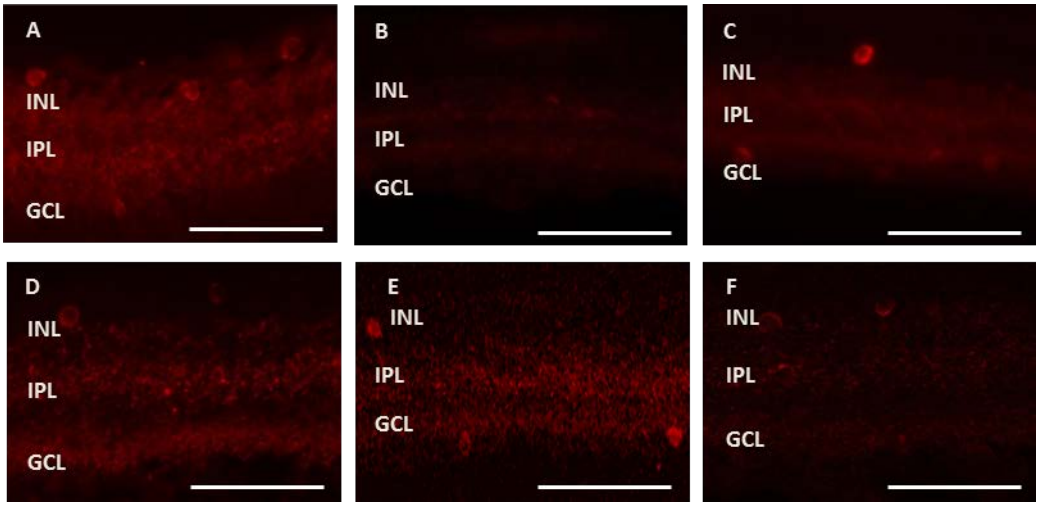
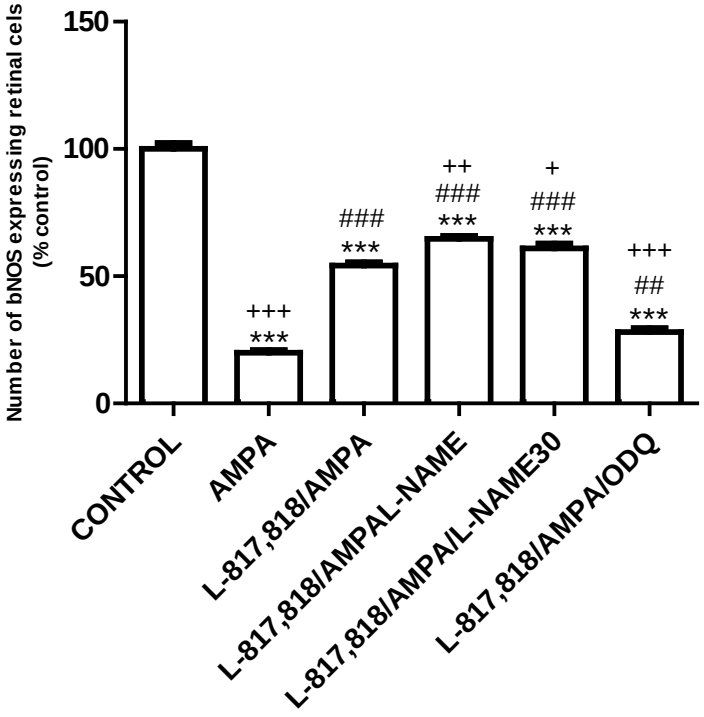


Figure 4.11 Effect of blockade of NO synthesis and sGC activity on the neuroprotective effects of L-817,818. bNOS immunoreactivity in **A** PBS- (control, n=5) and **B** AMPA- (42nmol, n=5) treated rat retinas. **C** Coinjected with AMPA (42nmol), the sst5 selective ligand L-817,818 (10^{-4}M, n=5) protected bNOS expressing neurons. The blockade of NO synthesis by coinjecting the NOS inhibitor L-NAME **D** (3nmol, n=5) or L-NAME **E** (30nmol, n=3) with AMPA and L-817,818 did not reverse the protective actions of L-817,818. **F** Blockade of soluble guanylate cyclase activity by coinjecting the guanylate cyclase inhibitor ODQ (10nmol, n=3) reversed the neuroprotective effects of L-817,818. **INL**: Inner Nuclear Layer, **IPL**: Inner Plexiform Layer, **GCL**: Ganglion Cell Layer. **Scale bar: 50μm**.

Quantification analysis of the same groups, as mentioned above, for the bNOS expressing retinal cells was also obtained and is shown in **Graph 4.6**. L-817,818 protected the retina from the AMPA excitotoxicity. This protection (approximately 50%) was observed at the dose of 10^{-4}M, as shown by the statistically significant results ($^{###}$ $p<0.001$ compared to the AMPA group). L-NAME, at the concentration of 3 or 30nmol, increased the number of bNOS expressing retinal cells approximately 10% compared to L-817,818 ($^{++}p<0.1$) while ODQ reversed significantly the L-817,818 neuoprotection compared to the L-817,818-treated group ($^{+++}p<0.001$).



Graph 4.6 Effect of the blockade of NO synthesis and sGC activity on theneuroprotective action of L-817,818 on bNOS retinal cells. The sst_5 selective ligand L-817,818 partially protected cholinergic retinal neurons. The NOS inhibitor L-NAME (3nmol or 30nmol) did not reduce the number of bNOS expressing retinal cells. while the soluble guanylate cyclase inhibitor ODQ caused the decrease of bNOS expressing cells. Neuman-Keuls Multiple Comparison Test, ***p<0.001 compared to control group, $^{###}$p<0.001 compared to AMPA-treated group, $^{##}$p<0.01 compared to AMPA-treated group, $^{+++}$p<0.001 compared to AMPA + L-817,818- treated group, $^{+}$p<0.1 compared to AMPA + L-817,818-treated group.

5. DISCUSSION

The aim of the present study was to investigate the neuroprotective effects of selective ligands for the somatostatin receptors sst_2 and sst_5 on AMPA-induced excitotoxicity and the mechanism involved in this neuroprotection. The main finding of this study is that somatostatin selective analogs for sst_2 and sst_5 receptors, intravitrealy injected, provide neuroprotection on AMPA-induced neurotoxicity *in vivo* in rat retina and that this neuroprotection might be mediated by the nitric oxide (NO) and/or cGMP signaling pathway.

Excitotoxicity is the basic cause of ischemia induced cell death. Excitatory amino acids such as AMPA, NMDA and kainate have been used as models for inducing ischemia and discovering novel potential antiischemic and neuroprotective agents for the treatment of retinopathies (Lipton, 1999; Osborne et al., 2004).

In the present study, we employed the *in vivo* model of AMPA excitotoxicity to examine the mechanisms involved in the neuroprotective properties of somatostatin selective analogs. Recently, it was shown that AMPA eliminated ChAT-IR in a dose-dependent (21, 42, 84 nmoles) manner, 24 hours after its intravitreal administration. The most effective dose was that of 42nmoles. Similarly, AMPA reduced the number of bNOS expressing amacrine cells (Kiagiadaki and Thermos, 2008; Kiagiadaki et al., 2010). No effect was observed to the photoreceptors, bipolar and ganglion cells, suggesting the absence or low levels of the AMPA receptors in these neurons (Kiagiadaki and Thermos, 2008). These results suggested that AMPA excitotoxicity is a good model of retinal cell loss, useful in the understanding of the early events underlying the pathophysiology of ischemia and for testing new agents as neuroprotectants against AMPA toxicity.

Based on the above observations, we used as immunohistochemical retinal markers polyclonal antibodies against the choline acetyl transferase (ChAT), the synthase of nitric oxide (bNOS) and the caspase-3. Injection of AMPA reduced significantly the immunoreactivity of cholinergic and bNOS containing retinal cells, whereas this injection increased significantly the caspase immunoreactivity. These results are in agreement with the recent study by Kiagiadaki et al. (2010).

The above results can be justified by earlier studies which have showed the differential expression of AMPA receptors and their subunits (GluR1 to GluR4) in the retina (Brandstatter et al., 1998; Yang, 2004; Jacobs et al., 2007). In addition, Liu and Zukin (2007) have shown that AMPA receptors lacking GluR2 have an increased permeability to intracellular calcium ions, making the neurons that express these receptors more vulnerable.

Studies have shown that ChAT immunoreactivity is abolished after the intravitrealy injection of kainate receptor agonists (Osborne et al., 1995) and of quisqalic acid receptor agonists (Fischer et al., 1995). As it concerns the bNOS reactive retinal cells, there are no immunohistochemical data to support the localization of AMPA receptors on NOS-containing amacrine cells in the retina. An earlier study, examining the effect of excitotoxicity in the rabbit retina, indicated that NADPH-diaphorase (marker for NOS) amacrine cells contained NMDA and kainic acid receptors (Sagar SM, 1990).In addition, in turtle retina, NOS inhibitors antagonized the kainate acid increase in cGMP-like immunoreactivity, suggesting that the activation of kainic acid receptors in NOS containing neurons stimulate NO release (Blute et al., 1998).

All the findings above suggested that AMPA is a good model of amacrine retinal cell loss and support its use for the testing of agents as neuroprotectants of retinal neurons against toxicity. Subsequently, using this *in vivo* model of AMPA excitotoxicity, we further investigated whether specific somatostatin selective ligands for the sst_2 and sst_5 receptors could be useful in the reversal of the actions of AMPA and which mechanisms may be involved in this reversion. Somatostatin is released from a subclass of amacrine cells in the retina (Shapiro et al., 1979; Mastrodimou, 2004) and activates its receptors sst_1, sst_2, sst_4 and sst_5, found in different retinal neurons (Johnson et al., 1998; Thermos, 2003).

The mechanisms via somatostatin and its analogs reveal their neuroprotective effects are still under investigation. SRIF was initially characterized for its adenyl cyclase and pertussis toxin sensitive actions (Mahy et al., 1988) and now is known to activate and other pathways that may mediate its neuroprotective effects (Olias et al., 2004). Activation of sst_2 and sst_5 receptors inhibited the voltage-gated Ca^{2+} channels (Tallent et al., 1996) and this inhibition was induced by a cGMP-dependent

protein kinase (Meriney et al., 1994). In addition, earlier studies of our lab showed that SRIF increases NO (Vasilaki et al., 2002) and cGMP levels in rat retinal explants (Mastrodimou et al., 2006) and also that NO NO/peroxynitrate and cGMP are mediators in the protection of retinal neurons from chemical ischemia (Mastrodimou et al., 2008).

After the coinjection of lanreotide (sst_2/sst_5 ligand) with AMPA, we observed that this selective analog reversed significantly the AMPA-induced loss of ChAT and bNOS immunoreactivity and also largely abolished the caspase immunoreactivity in the retinas treated with Lanreotide. These results are in agreement with previous studies which have shown that Lanreotide (sst_2/sst_5 ligand) and L-779,976 (sst_2 ligand,) afforded protection, while the selective sst_1 and sst4 ligands had no effect (Kiagiadaki and Thermos, 2008).

The main aim of this study was to investigate the mechanism involved in the $sst_{2/5}$ ligand neuroprotection. As mentioned and above, previous studies in our lab supported the involvement of NO/ cGMP signaling in the function of somatostatin and its analogues in the retina (Thermos, 2008). In retinal explants, SRIF increased nitric oxide (NO) basal levels (Vasilaki et al., 2002) and cGMP levels (Mstrodimou et al., 2006) in a concentration dependent manner via an sst_2 dependent mechanism. In a chemical ischemia model in the rat retina, NO donors and the membrane permeable cGMP analog, 8-Br- cGMP protected the retina in a concentration dependent manner, whereas NMMA (NOS inhibitor) and ODQ (sGC inhibitor) reversed the lanreotide's neuroprotective effects. These findings implicate the NO/sGC and cGMP pathways in this protection (Mastrodimou et al., 2008).

NO has been shown to influence channel proteins by two effector pathways, namely in a cGMP-dependent and cGMP-independent manner in which NO acts directly on channel proteins through S-nitrosylation. In other paradigms, NMDA receptors were shown to be down-regulated by NO-induced S-nitrosylation (Lipton et al., 2001). In the retina, AMPA receptors also have been shown to be negatively regulated by cGMP. In retinal horizontal cells, the function of kainate receptors was depressed by cGMP and by NO in a sGC-protein kinase G (PKG) dependent manner (McMahon and Ponomareva, 1996).

Based on all the above mentioned observations, we investigated whether NO/cGMP signaling pathway is also involved in lanreotide's and L-817-818 neuroprotective effects against AMPA induced toxicity *in vivo*. Co-administration of lanreotide with the inhibitor of the synthase of nitric oxide (NO), L-NAME prevented its neuroprotection against AMPA toxicity in a dose dependent manner. This was confirmed by ChAT, bNOS and caspase immunoreactivity studies. Similarly, coinjection of lanreotide and the inhibitor of the soluble guanylate cyclase, ODQ reduced the lanreotide neuprotection of the cholinergic and bNOS containing retinal neurons and this was also confirmed by caspase labeling. We also confirmed the involvement of cGMP in the protection of the retina from excitotoxicity by coinjecting the cell-permeable analog of cGMP, 8-Br- cGMP with AMPA, which showed to produce protection both in cholinergic amacrine and bNOS expressing retinal cells.

Till recently, the sst_1, sst_2 and sst_4 receptors were believed to be responsible for SRIF's actions in mammalian retina. However, Ke and Zhong (2007) recently reported the presence of sst_5 receptors in retinal amacrine cells that express GABA, dopamine and acetylcholine. Agonists such as octreotide and Lanreotide (BIM23014) have high affinity for both sst_2 and sst_5 receptors (Olias et al., 2004) and therefore there is a concern in which receptor is responsible for the actions of the $sst_{2/5}$ ligands. A recent study revealed that the sst_5 peptidomimetic selective analog L-817,818 afforded partial protection to the retina against AMPA toxicity (Kiagiadaki et al., 2010), suggesting that this receptor is also involved in the neuroprotective actions of the $sst_{2/5}$ ligands. In the present study, coinjection of L-817,818 with AMPA also revealed a partial neuroprotection for the cholinergic and bNOS expressing retinal cells against AMPA insult in these type of retinal neurons.

Furthermore, having the knowledge that the activation of somatostatin receptor (sst_5) protects the rat retina from AMPA-induced neurotoxicity, we investigated the possibility that the protection mediated by the sst5 receptor is also via the NO/cGMP signaling pathway. Immunohistochemistry studies using anti-retinal neuron markers for choline acetyl transferase (ChAT) and NO synthase (bNOS) were performed to examine the cause of this retinal cell loss and protection. As mentioned above, L-

817,818 protected the cholinergic and bNOS expressing retinal cells from the AMPA toxicity while coinjection of L-NAME with L-817,818 had no significant effect on the sst5 neuroprotection when administered at the concentrations of 3 and 30nmol. In contrast to L-NAME, the coinjection of ODQ at the concentration of 10nmol with L-817,818 reduced the protection of the cholinergic and bNOS containing retinal cells.

These results for the coinjection of L-NAME and ODQ respectively, implicate the soluble guanylate cyclase (sGC)/cGMP signaling pathway in the L-817,818 neuroprotective effects, as well L-NAME did not appear to have significant anti-neuroprotective action in the L-817,818 effects. Therefore, the neuroprotection due to sst_5 activation may be a NO-independent event. There are reports revealing that sGC, apart from being activated by NO, can also be stimulated by carbon monoxide (CO), although it is less sensitive to this compound. Cao et al. (2000) reported that in turtle, the CO producing enzyme heme oxygenase-2 is expressed in all photoreceptors and certain bipolar, amacrine and ganglion cells, while in rat, it is only detected in amacrine and ganglion cells. Other studies in turtle and salamander, also showed that stimulation with CO increased cGMP-IR in the inner retina, but the effect was strongly dependent on endogenous or exogenous NO, suggesting synergistic interactions between NO and CO signaling pathways in the retina (Cao and Eldred, 2003; Pong and Eldred, 2009).

It is also important to report that NO, in ischemic or excitotoxic conditions, reacts with O^{2-} and is quickly converted to peroxynitrate ($ONOO^-$) (Osborne et al., 2004). Subsequently, $ONOO^-$ has been shown to modulate voltage-gated Ca^{2+} channels (Okhuma et al., 2001) as well as neurotransmitters release (Okhuma et al., 1995). In addition, a recent study revealed that NO, cGMP and SIN-1 (peroxynitrate donor) trigger GABA release in retina (Yu and Eldred., 2005). Therefore, SRIF may trigger GABA release via the sst_5 activation and subsequently, GABA being an inhibitory neurotransmitter, suppressing the glutamate levels in the retinal cells. This might be a possible somatostatin's neuroprotective mechanism via the excitotoxicity's elimination.

However, results regarding the regulation of acetylcholine signaling by NO, are conflicting. In rat retina, acetylcholine is synthesized and released exclusively by

starburst amacrine cells, which do not produce NO. In rat retina, NO donors inhibited depolarization-induced acetylcholine release and this effect was mediated by enhanced GABA signaling on cholinergic neurons (Okada et al., 2001). However, another study in rabbit found that the NO donor SNAP increased light induced acetylcholine release, through a mechanism involving NO-dependent inhibition of glycine signaling by amacrine cells (Neal et al., 1997). Possibly, both effects could be reconciled by the observation that NO regulates retinal GABA and glycine signaling in opposite ways (Yu and Eldred, 2005b).

There is adequate evidence suggesting that retinal NO modulates GABA and glycine signaling. In the retina, inhibitory neurotransmitters are liberated by amacrine cells, of which a subpopulation also releases NO. In cultured rat amacrine cells, NO donors and membrane-permeable cGMP analogues inhibit $GABA_A$ receptor signaling through a mechanism dependent on PKG (Wexler et al., 1998), while basal GABA release from amacrine and ganglion cells was inhibited by endogenous NO in chicken retina (Maggesissi et al., 2009). On the other hand, an increase of $GABA_A$ receptor currents was caused by stimulation with the NO donor NOC-12 in amacrine cells of the same species, and in turtle, exogenous NO provoked an increase of GABA release from amacrine cells, while glycine signaling was inhibited (Yu and Eldred, 2005b).

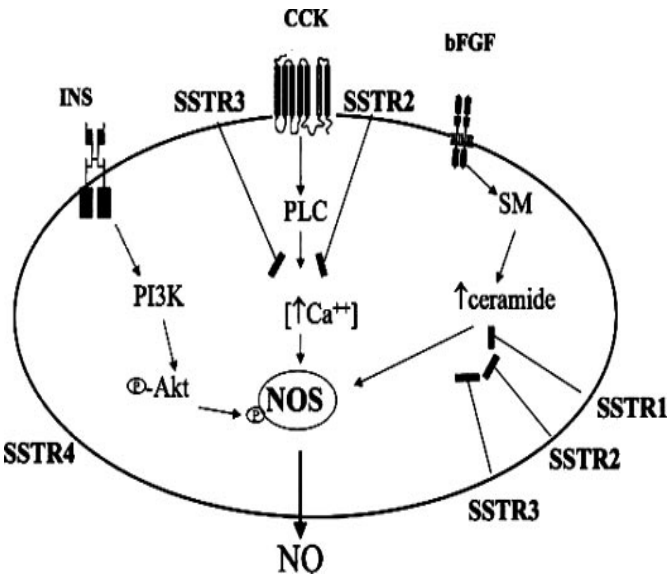
Further retinal neuromodulators that are affected by NO signaling are dopamine, somatostatin and melatonin. Bugnon et al. (1994) showed that NOS inhibition increased basal dopamine levels in bovine retina, through a mechanism independent from cGMP. Furthermore, somatostatin release was also shown to be increased by both exogenous NO and the NO precursor L-arginine in rat, while melatonin synthesis by photoreceptors was inhibited by NO donor application (Wellard and Morgan, 2004). These reports may provide, at least to a point, an explanation for the results of our study where the levels of nitric oxide synthase were not affected significantly by the inhibition of this enzyme whereas the soluble guanylate cyclase activity was reduced.

Studies have shown that somatostatin receptor subtypes are coupled to distinct signal transduction pathways. sst_1 and sst_2 mediate the stimulation of a tyrosine

phosphatase activity, which is involved in the antiproliferative effects (Buscail et al., 1994; Florio et al., 1994). In another study, using CHO cells expressing sst_5, RC-160 (sst_5 selective analog) inhibited the proliferation induced by serum or by CCK-8 (Buscail et al., 1995). The inhibitory effect of RC-160 was not abolished by specific inhibitors of tyrosine and serine-threonine phosphatases, such as orthovanadate or okadaic acid, respectively, indicating that a protein phosphatase was not involved in the negative growth signal coupled to sst_5. Cordelier et al. (1997) showed that the proliferation of CHO cells stably expressing sst_5 receptor is possibly modulated by CCK and that the sst_5 mediated inhibitory effect of somatostatin on mitogen-induced increases in intracellular cGMP levels, a result in agreement with our study.

Another study suggested that somatostatin actions through the sst_5 receptor could inhibit NO production originating from nNOS and showed that nNOS is constitutively associated with sst_5 in CHO/sst5 cells and that sst_5- nNOS complex formation is up-regulated after somatostatin binding to sst_5 (Cordelier et al., 2006). In addition, studies from our lab showed that an sst_5 selective agonist did not regulate the release of NO in retinal explants whereas sst_2 agonists afforded an increase in NO levels (Vasilaki et al., 2003).

As depicted in the figure below, Arena et al., (2006) suggest that somatostatin is able to affect NO production but that its activity depends on the signaling context that leads to different intracellular pathways and on the somatostatin receptor subtypes activated (the sst_5 is not shown).



[64]

Figure. Diagrammatic Representation of the Subtype-Specific Effects of SSTRs on NO Production. Intracellular pathways involved in the bFGF-, CCK-, and insulin (INS)-induced NOS activation in CHO-k1 cells and the interference of SST via the different SSTR subtypes. Insulin modulates NOS activity via the PI3K/Aktdependent phosphorylation of eNOS; CCK induces a PLC-mediated release Ca^{2+} from the intracellular stores responsible for the classical Ca^{2+}-dependent activation of nNOS; bFGF regulates eNOS activity via the activation of sphingomyelinase (SM) and the generation of ceramide. SST via the SSTR subtypes 1, 2, and 3 inhibits the bFGF effects acting downstream from the ceramide production and via SSTR2 and -3 antagonizes the Ca^{2+}-dependent NO production induced by CCK. Insulin activation of eNOS is not modified after SST treatment. SSTR4 is the only SSTR subtype tested that was ineffective on all the intracellular pathways leading to NO production **(Arena et al., 2006)**.

According to our data, we can assume that somatostatin analogs protect the retina from the AMPA induced neurotoxicity by activating sst_2 receptors located on retinal neurons with the subsequent increase in NO/cGMP levels that leads to the negative regulation of cGMP-dependent Ca^{2+} channels, the attenuation of intracellular Ca^{2+} levels and the prevention of retinal cell death. Activation of sst_5 receptors also leads to retinal neuroprotection via a cGMP mediated mechanism that is NO–independent or it is modulated indirectly via the nitric oxide (NO), something that needs further investigation. In agreement with our results, Tallent et al. (1997) showed that both sst_2 and sst_5 receptors were implicated in SRIF's inhibition of voltage-gated calcium channels and therefore sst_5 may afford neuroprotection by attenuating intracellular calcium levels.

Several downstream pathways have been proposed to mediate the anti-apoptotic effects of elevating cGMP levels, including induction of Bcl-2 mRNA and protein (Genaro et al., 1995), suppression of the gene expression of the pro-apoptotic Bcl-2 binding protein BNIP3 gene (Zamora et al., 2001), prevention of cytochrome C release from the mitochondria and activation of c-Src and subsequent induction of tyrosine phosphorylation of Bcl-2 (Tejedo et al., 2001).

In conclusion, these results emphasize the neuroprotective effects of sst_2 and sst_5 receptors against AMPA induced neurotoxicity *in vivo* via a mechanism including NO and/or cGMP signaling pathway while it is necessary the identification of intracellular molecules, selective for the somatostatin receptor subtypes and able to

affect nitric oxide synthesis and the downstream signaling pathways activated by this molecule.

In general and based on all the above findings, we can assume that the neuroprotective effects of the somatostatinergic system against AMPA-induced excitotoxicity *in vivo* are mediated :

i. via the activation of sst_2 receptors and the subsequent increase of NO. NO, in turn activates cGMP production and via this molecule closes the cGMP-dependent Ca^{2+} ion channels, thus reducing the Ca^{2+} levels or

ii. via cGMP, peroxynitrate or GABA and thus influencing neurotransmitters release, like glutamate.

6. ABSTRACT

The neuropeptide somatostatin and its selective analogs for the sst_2 and sst_5 receptor subtypes provided neuroprotection against AMPA induced retinal excitotoxicity in vivo (Kiagiadaki and Thermos, 2008; Kiagiadaki et al. 2010). The aim of the present study was to examine the mechanism mediating this protection with emphasize in the investigation of the possible involvement of the nitric oxide (NO)/ cGMP signaling pathway.

Adult female and male Sprague Dawley (250-300 g) rats were employed. The animals received intravitrealy PBS (50mM), AMPA (42nmol/eye), AMPA (42nmol) in combination with Lanreotide ($sst_{2/5}$ selective ligand, 10^{-4}M), AMPA (42nmol) in combination with L-817,818 (sst_5 selective ligand, 10^{-4}M), AMPA (42nmol) in combination with Lanreotide (10^{-4}M) and L-NAME (nitric oxide synthase inhibitor,3, 30 nmol) or ODQ (soluble guanylate cyclase inhibitor, 10nmol) and AMPA (42nmol) in combination with L-817,818 (10^{-4}M) and L-NAME (3, 30 nmol) or ODQ (10 nmol) or AMPA (42nmol) in combination with 8-Bromo-cGMP (membrane permeable cGMP analog, 1mM).

Immunohistochemistry studies using retinal markers against ChAT and bNOS, for cholinergic and neuronal NOS containing amacrine cells respectively, and caspase-3 immunoreactivity studies were employed to examine retinal cell loss and protection. Lanreotide and L-817,818 protected cholinergic and bNOS containing retinal cells from the AMPA insult. The inhibition of nitric oxide synthase (NOS) by L-NAME attenuated Lanreotide's neuroprotective effect in a dose-dependent manner. The inhibition of soluble guanylate cyclase (sGC) activity by ODQ also reduced the neuroprotective actions of Lanreotide. These results suggest the involvement of NO/cGMP pathway in lanreotide's neuroprotective actions. In addition, 8-Br-cGMP alone protected the retina from AMPA toxicity. In contrast with the Lanreotide's inhibition by L-NAME, the L-817,818 neuroprotective actions did not reversed significantly by L-NAME. Whereas, the inhibition of sGC activity by ODQ attenuated the L-817,818 neuroprotective action, suggesting the involvement of a NO-independent sGC/cGMP signaling pathway in L-817,818 neuroprotective actions.

The above results indicate that a NO and/or sGC/cGMP mechanism is involved in the neuroprotective effects of the activation of somatostatin sst_2 and sst_5 receptor subtypes after AMPA induced excitotoxicity in retina and it would be in scientific interest the elucidation of the downstream pathways involved in this neuroprotection.

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