THE ROLE OF GLUTAMATE

IN CORNEAL NOCICEPTION

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Abbreviations

AMPA - 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid

ATP – Adenosine triphosphate

GTP – Guanosine triphosphate

CGRP – Calcitonin gene-related peptide

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

CNS – Central nervous system

D-AP5 - D-(-)-2-amino-5-phosphonopentanoic acid

DMSO – Dimethyl sulfoxide

DRG – Dorsal root ganglion

EAAR – Excitatory amino acid receptor

ED50 – *In vivo* dose of a drug that yields 50% of its maximum response

EPSP – Excitatory post synaptic potential

GLAST – Glutamate-aspartate transporter

GLS – Glutaminase

GLT1 – Glutamate transporter

IR – Immunoreactivity

ID50 – *In vivo* dose of drug that yields 50%of the maximum possible inhibition for that drug

LASIK – Laser assisted in situ keratomileusis

NBQX -2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt

NMDA – N-methyl–D-aspartate

NSF - N-ethylmaleimide sensitive fusion proteins

PBS – Phosphate buffered saline

PI3K – Phosphatidylinositol 3 kinase

PKA – Protein kinase A

PKC – Protein kinase C

PRK – Photorefractive keratectomy

PNS – Peripheral nervous system

SNAP 25 – Synaptosomal associated protein 25

SNARE – Soluble NSF attachment protein receptor

SNAT – Sodium coupled neutral amino acid transporter

SP – Substance P

TG – Trigeminal ganglion

TRPA1 - Transient receptor potential l ankyrin 1

TRPM8 – Transient receptor potential melastatin 8

TRPV1 – Transient receptor potential vanilloid 1

VAMP2 – Vesicular associated membrane protein 2

VGLUT – Vesicular glutamate transporter

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Nociceptors.

The nociceptor is a peripheral sensory neuron that detects noxious (painful) stimuli. A noxious stimulus is one with intensity and quantity of sufficient quality to cause reflex withdrawal, autonomic responses and pain (Sherrington, 1903). Nociceptors are primary sensory neurons that respond to high threshold stimuli (Woolf and Ma, 2007) and are described as having four functional parts: peripheral terminal, axon, cell body and central terminal (synapse; Diag. I). Peripheral sensory axons and terminals also are called peripheral or primary afferents. The neuronal cell bodies are found in peripheral ganglia, e.g., dorsal root and trigeminal ganglia, and the peripheral terminal lies in the target tissue (Woolf and Ma, 2007). The peripheral terminal transduces the external noxious stimulus and initiates an action potential, the axon conducts the action potential, whereas the cell body controls the identity and integrity of the entire neuron. The central terminal participates in the first synapse in the central nervous system (CNS) sensory pathway (Woolf and Ma, 2007). The sensitivity of both the peripheral terminal and central synapse can be altered to augment or diminish nociception. Augmented alterations of nociception at the peripheral terminal and central synapse are called peripheral and central sensitization, respectively. The initial background will focus on aspects common to all nociceptors followed later with a more detailed description of corneal nociceptors.

Trigeminal Ganglion Neuron for Corneal Sensory Innervation

Diagram I. **A trigeminal ganglion neuron for corneal sensory innervation**. Schematic of a primary afferent neuron with its central terminal in the pons and medulla, the cell body in the trigeminal ganglion and a peripheral terminal that innervates the target tissue, e.g., cornea (adapted from Woolf and Ma, 2007).

Nociception is initiated when nociceptive sensory fibers innervating peripheral tissues are activated by potential or ongoing noxious stimuli. This occurs because nociceptive sensory neurons express proteins that respond to specific noxious chemical, mechanical and/or thermal stimuli. These proteins are referred to as transducers (Caterina and Julius, 2001; Julius and Basbaum, 2001) and they respond only to specific, high threshold stimuli. Studies in the skin have shown the presence of a number of ion channels in peripheral sensory terminals that function as transducers of noxious temperature, chemical, and touch sensations (Suguiura et al., 2002; Ferreira et al., 2004; Lumpkin and Caterina, 2007). Among these is the transient receptor potential (TRP) family of cation channels for response to chemical stimulation and noxious and innocuous temperatures. For noxious temperatures in skin, transient receptor potential vanilloid 1 (TRPV1), a noxious heat transducer, responds in the nociceptive range of 46.5°C to 56°C (Caterina et al., 1997; Rau et al., 2007), and transient receptor potential melastatin 8 (TRPM8), a noxious cold transducer, responds to temperatures less than 15°C (Bautista et al., 2007). Transient

receptor potential l ankyrin 1 (TRPA1) also is a noxious cold transducer, responding to temperatures as low as 10°C (Karashimaa et al., 2008). Likewise in the cornea, TRPV1 and TRPM8 have been identified and described (Diag. II; Madrid et al., 2006, Murata and Masuko, 2006, Mergler et al., 2010).

The peripheral terminal of a nociceptor detects noxious or tissue damaging stimuli when the stimulus reaches a threshold to open the transducer ion channel (Woolf and Ma, 2007). An influx of calcium and sodium ions occurs that depolarizes the peripheral terminal membrane (Caterina et al., 1997; Mergler et al., 2010). Nociceptors express specific voltage gated sodium (Na_v) ion channels, Na_v1.7, 1.8 and 1.9, that produce action potentials for the conduction of nerve impulses (Djouhri et al., 2002; Djouhri et al., 2003; Priest et al., 2005). These transmembrane proteins transiently open to permit the rapid influx of sodium ions in response to membrane depolarization. Influx of sodium ions depolarizes the nociceptor axon membrane further generating an action potential that is propagated from the nociceptor peripheral terminal via the axon to the central nervous system (Woolf and Ma, 2007).

With ongoing peripheral stimulation, influx of calcium ions into the peripheral terminal can trigger a number of intracellular signal transduction pathways. For example, activation of protein kinases in the peripheral terminal such as phosphatidylinositol 3-kinase (PI3K), protein kinase A (PKA) or protein kinase C (PKC) causes phosphorylation of proteins such as TRPV1 and sodium channels (Zou et al., 2000; Zou et al., 2002; Zhuang et al., 2004). When there is phosphorylation of TRPV1, the activation threshold is lowered thereby increasing the excitability of the nerve terminal. Also, phosphorylation of sodium channels reduces the resting potential of the membrane and further increases the excitability of the nerve terminal. The net effect forms the basis for peripheral sensitization leading to heightened sensitivity to innocuous (allodynia) and painful (hyperalgesia) stimuli. Peripheral sensitization, therefore, leads to increased action potentials

propagated along the axon toward the CNS (Woolf and Ma, 2007).

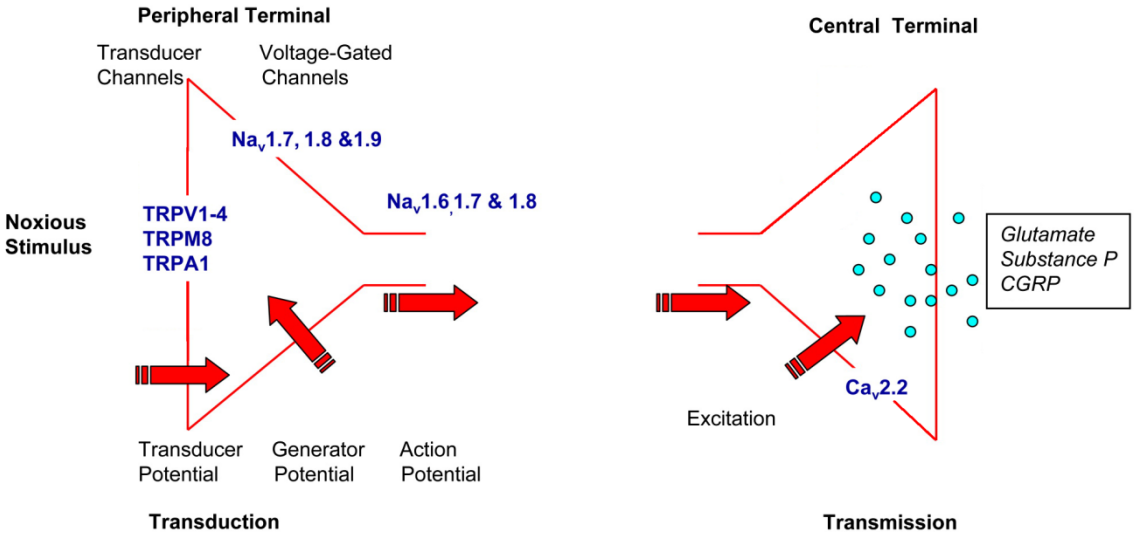


Diagram II. **Schematic of events that occur when a noxious stimulus is applied to a sensory afferent nerve**. Peripheral terminals have transducer channels, TPRV1-4, TRPM8, TRPA1 (1-2 transducer types per neuron), that allow cation entry to initiate generator and action potentials by way of specific sodium channels, Na_v 1.6-1.9. At the central terminal, Ca^{2+} influx by way of voltage-gated calcium channels (Ca_v) initiates neurotransmitter release, i.e., glutamate (modified from Woolf and Ma, 2007).

Arrival of action potential at the central terminal causes the opening of voltage gated calcium ion channels (Diag. II). An influx of calcium ions occurs producing a transient rise in intracellular calcium (Gover et al., 2003) and depolarization-induced, calcium-facilitated neurotransmitter release (Chen et al., 1999; Woolf and Salter, 2000). Neurotransmitters are released by an exocytosis process using t- and v-soluble NSF (N-ethylmaleimide sensitive fusion proteins) attachment protein receptor (SNARE) complexes. For example, synaptobrevin (also called vesicular associated membrane protein 2 [VAMP 2]) is a v-SNARE protein in synaptic vesicles that is inhibited by SNARE-master proteins such as synaptophysin I (Gerst, 2002).

Synaptophysin I, therefore, is very important in regulating neurotransmitter release (Lynch et al., 1994) and is used as a marker for synaptic vesicles (Masliah et al., 1990; Edelmann et al., 1995; Bacci et al., 2001; Sun et al., 2006; Gonzalez-Jamett et al., 2010; Wagget et al., 2010; Bamji et al., 2003). When intracellular calcium levels rise in a terminal, the synaptophysin/synaptobrevin complex dissociates (Reisinger et al., 2004) to allow synaptobrevin to interact with t-SNARES, e.g., syntaxin and SNAP 25, on the plasma membrane (Reisinger et al., 2004). The t- and v-SNARE complex of synaptobrevin, syntaxin and SNAP 25 is a helix protein complex that is required for vesicle/plasma membrane fusion (Diag. III;(Reisinger et al., 2004).

After dissociation from synaptobrevin and exocytosis, synaptophysin I binds to dynamin in a calcium dependent fashion for recovery of vesicular membrane from plasma membrane by endocytosis (Daly et al., 2000). During endocytosis, dynamin assembles to form rings at the base of membrane invaginations, GTP binds to dynamin, and hydrolysis of GTP causes a conformational change of the membrane for vesicle fission (Oh et al., 1998). Synaptophysin I thus has a pivotal role in both endocytosis and exocytosis of synaptic vesicles.

Neurotransmitter release at a nerve terminal

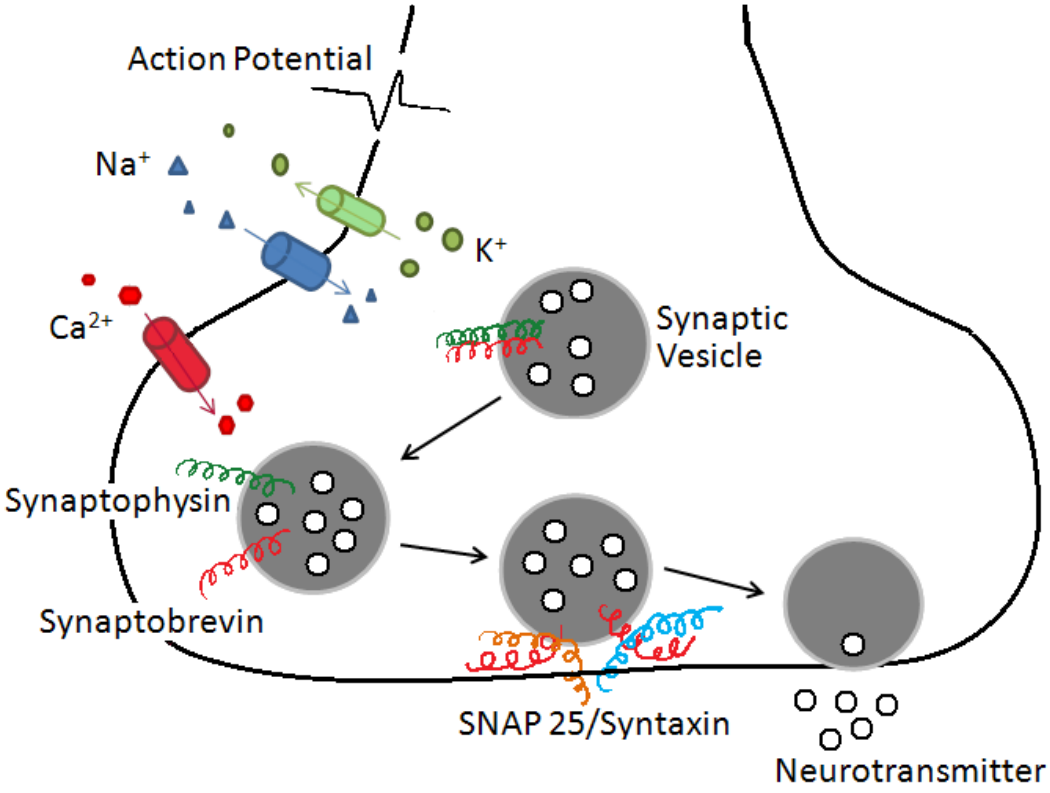


Diagram III. **Schematic showing the series of events that occur with arrival of action potential at a nerve terminal**. Depolarization of the nerve terminals (action potential) occurs with voltage dependent opening of sodium (blue channel in diagram) and potassium (green channel in diagram) channels allowing influx of sodium ions and then efflux of potassium ions. Local depolarization causes voltage gated calcium channels to open (red channel in diagram) allowing influx of calcium ions and increase in intracellular calcium concentration. Intracellular calcium triggers the dissociation of the synaptophysin-synaptobrevin complex thus allowing synaptobrevin to associate with SNAP 25 and syntaxin to form functional SNARE complexes, followed by fusion and exocytosis of neurotransmitters, such as glutamate. After exocytosis, neurotransmitters, such as glutamate, bind to and open receptors on the postsynaptic neurons.

Synaptophysin I has a molecular homologue, synaptoporin (synaptophysin II), with almost 60% sequence homology. Both proteins have four transmembrane domains and two hydrophilic loops within the vesicle, i.e., intravesicular loops. Divergence occurs at their N- and C-terminals that are exposed to the cytoplasmic side. Both synaptophysin I and synaptoporin are expressed in the central and peripheral nervous systems (CNS, PNS). Synaptophysin I is abundantly expressed in the CNS whereas synaptoporin expression is restricted (Masliah et al., 1990, Sun et al., 2006). In the PNS, both synaptophysin and synaptoporin are expressed in nociceptive dorsal root ganglion (DRG) neurons, i.e., small diameter DRG neurons, and their afferent terminals in the dorsal spinal cord (Sun et al., 2006). Some of these DRG neurons coexpress calcitonin gene-related peptide (CGRP), a nociceptive peptide transmitter. Abundant synaptoporin containing nerve fibers occur in the laminae I-II of dorsal horn of spinal segments L_{4-5} while abundant synaptophysin I containing nerve fibers occur in laminae III–IV and ventral lamina II.

1.2 Glutamate in nociceptors

The glutamate-glutamine cycle. Glutamate is the major excitatory neurotransmitter in the vertebrate nervous system and is released from nociceptors at both the peripheral and central nerve terminals. Glutamate metabolism in the PNS occurs via a glutamate-glutamine cycle (Diag. III) where glutamate enters the satellite and Schwann glial cells and is converted to glutamine by glutamine synthetase (Curie and Kelly, 1981; Miller et al., 2002; Miller et al., 2011a). In DRG, neurons take up glutamine and glutaminase converts glutamine to glutamate (Miller et al., 1992; (Hassel et al., 2003). Glutamate also is produced by aspartate aminotransferase by transamination of aspartate to 2-oxoglutarate, yielding glutamate and oxaloacetate (Miller et al., 2002; Miller et al., 2011a).

Following synthesis, glutamate is packaged into synaptic vesicles by vesicular glutamate transporters (Juge et al., 2006, Brumovsky et al., 2007). Vesicular glutamate transporter 1 or 2

(VGLUT1, 2) upload glutamate into synaptic vesicles for the transport and release of glutamate (Aihara et al., 2000; Juge et al., 2006; Moechars et al., 2006; Brumovsky et al., 2007; Miller et al., 2011a). The VGLUTs actively transport glutamate into the vesicles in exchange for hydrogen ions (Bellochio et al., 2000; Juge et al., 2006; Miller et al., 2011a). The electrochemical gradient for glutamate transport is created by hydrolysis of ATP (Bellochio et al., 2000; Juge et al., 2006). This transport is further enhanced in the presence of chloride ions (Bellochio et al., 2000; Juge et al., 2006; Miller et al., 2011a). Thus, vesicular glutamate transporters are considered markers of glutamatergic neurons (Brumovsky et al., 2007; Miller et al., 2011a).

Both VGLUT 1 and VGLUT 2 are expressed by neurons in the DRG (Brumovsky et al., 2007). VGLUT 1 is localized in medium to large sized neurons that mediate proprioception and discriminative touch. VGLUT 2 is localized in small to medium sized nociceptive neurons that contain neuropeptides such as calcitonin gene-related peptide (CGRP) or bind lectins, such as isolectin B4 (IB4; (Brumovsky et al., 2007; Miller et al., 2011a). In the rat skin, VGLUT 2 immunoreactivity in the dermal and intraepidermal plexus is of greater intensity than that DRG neuronal cell bodies (Brumovsky et al., 2007). In the spinal cord, VGLUT2 has a dense and homogenous distribution in the grey matter except in the medial aspect of laminae III–VI where VGLUT1has an intense distribution (Brumovsky et al., 2007).

Glutamate–Glutamine Cycle

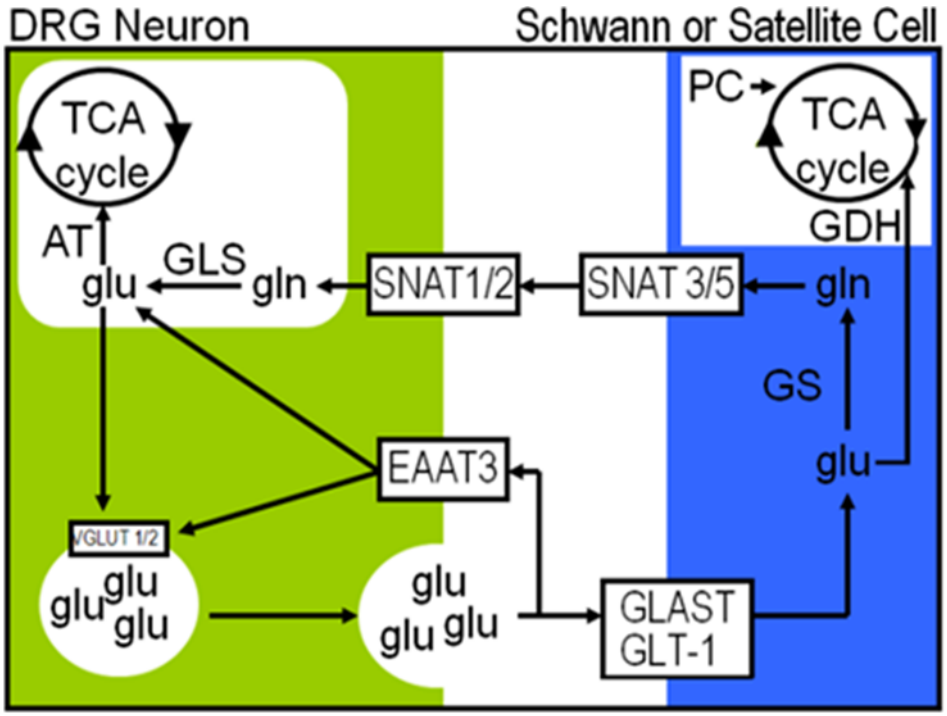


Diagram IV. **Schematic of the glutamate-glutamine cycle in DRG**. In the satellite or Schwann cell, glutamine synthetase (GS) converts glutamate (glu) to glutamine (gln) which is transported by the sodium coupled neutral amino acid transporters (SNAT) into the DRG neuron. Glutaminase (GLS) converts glutamine into glutamate and VGLUT 1 or 2 packages synthesized glutamate into synaptic vesicles. When glutamate is released, it is taken up back into the satellite or Schwann cell by excitatory amino acid transporters, glutamate-aspartate transporter (GLAST) and glutamate transporter-1(GLT-1; from Miller et al., 2011a).

Upon release from a neuronal terminal, glutamate can bind to excitatory amino acid receptors (EAARs), i.e., glutamate receptors. There are 2 major types of EAARs, ionotropic and metabotropic EAARs, and each is comprised of different subunits (Lipton and Rosenberg, 1994; Lin and Kinnamon, 1999). The ionotropic EAARs are membrane ion channels while the

metabotropic EAARs are G-protein coupled receptors that use intracellular second messengers (Lipton and Rosenberg, 1994). In this dissertation, the focus will be on the ionotropic EAARs (Diag. V).

Types of Ionotropic EAARs and their Subunits

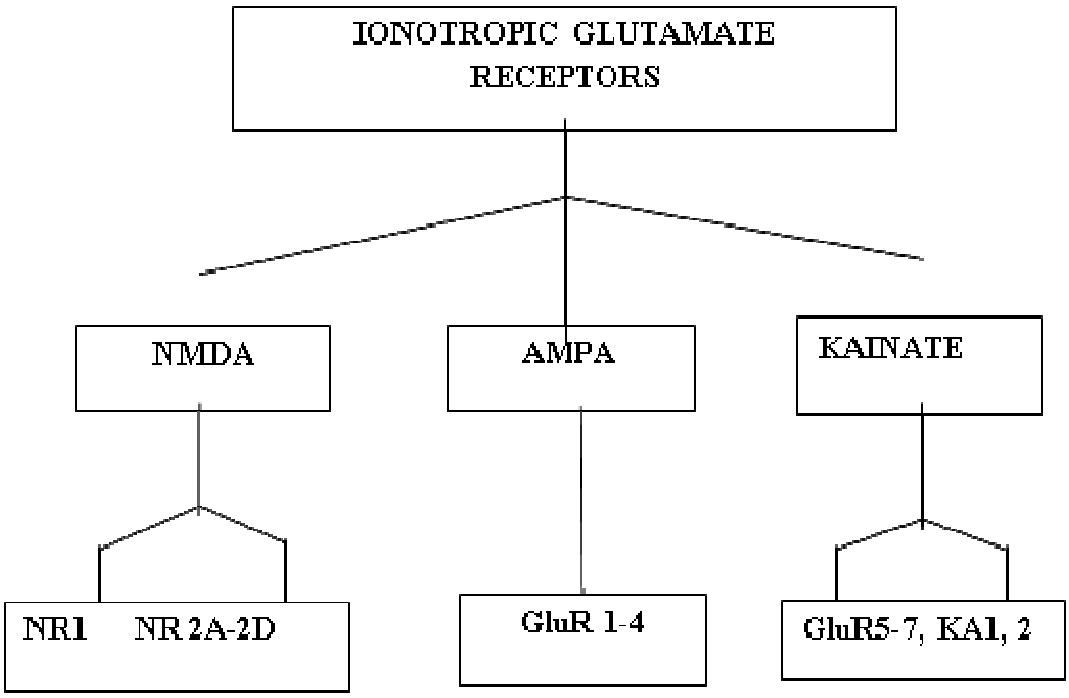
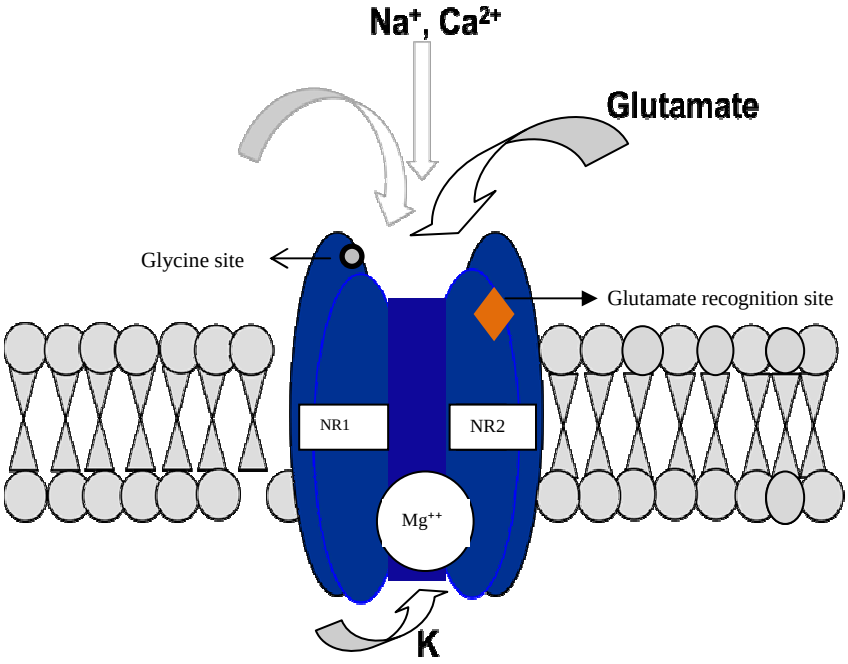


Diagram V. **Schematic of the different types of ionotropic excitatory amino acid receptors, i.e, glutamate receptors, and their subunit composition**. There are 3 types of ionotropic glutamate receptors based on stimulation with specific agonists: NMDA; AMPA (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) Propanoic Acid); kainate. The NMDA has NR1 and NR2A-D subunits, GluR1-4 are the subunits for AMPA receptor, and GluR 5-7 and KA1, 2 are subunits for kainate receptor.

There are three types of the ionotropic glutamate receptors (Diag. V) named for their selective agonists; N-methyl-D-aspartate (NMDA), 2-amino-3-(5-methyl-3-oxo-1, 2- oxazol-4-yl)

propionate (AMPA) and kainate receptors (Wo and Oswald, 1995, Chen et al., 1996, Lu et al., 2009). Each of these receptors has subunits as listed: NMDA subunits - NR1 and NR 2A-2D; AMPA subunits - GluR 1-4; kainate subunits -KA1-2 and GluR5-7 (Wo and Oswald, 1995; Chen et al., 1996; Lu et al., 2009). Ionotropic EAARs exist as tetramers, a four subunit receptor, usually in a heteromeric construct (Diag. VI).

Ionotropic Glutamate Receptor



Diag.VI. **Schematic of the ionotropic NMDA receptor showing the subunits, NR1 and NR2, magnesium block, and glycine and glutamate recognition sites**. Glycine binds to its site where it functions as an activator of the NMDA receptor complex. The magnesium block is voltage dependent and blocks the inflow of ions. Upon membrane depolarization, the block is removed and sodium and calcium cations flow into the cell (adapted from Mayer and Westbrook, 1987, Monyer et al., 1994).

The NMDA NR1 gene has a ubiquitous expression throughout brain development and may reflect

the requirement of NR1 subunit as a part of all NMDA receptor channels. A common NMDA receptor construct consists of two NR1 and two NR2 subunits (Diag. VI; Monyer et al., 1994) The NMDA receptor also expresses glycine binding site, a site needed for channel function (Oliver et al., 1990; Monyer et al., 1994). When glycine binds to this site, it functions as a co-agonist and positive modulator of the NMDA receptor. This ion channel also is blocked by extracellular magnesium ions and removal of magnesium block is voltage dependent (Mayer and Westbrook, 1987; Monyer et al., 1994).

The AMPA receptor subunits exist as tetrameric homomers or heteromers. Unlike heteromers,homomers have not been observed to contribute significantly to the AMPA excitatory postsynaptic potential (EPSP; Lu et al., 2009). AMPA heteromers tend to prefer GluR2 dimers with a dimer either of GluR1, GluR3, or GluR4. Each AMPA subunit has a binding site for glutamate, i.e., four glutamate binding sites per AMPA receptor (Mayer and Armstrong, 2004; Greger et al., 2007).

Both KA1 and KA2 subunits of the kainate receptor have been described as being obligatory subunits for the ionotropic kainate receptor function and are termed the high affinity subunits (Lucifora et al., 2006; Fernandes et al., 2009). Homomers of KA1 or KA2 are nonfunctional (Herb et al., 1992, Fernandes et al., 2009), but, when co-expressed with the GluR5-7 subunits, they form functional heteromeric receptors (Herb et al., 1992; Dingledine et al., 1999; Fernandes et al., 2009).

1.3 Glutamatergic function of peripheral nociceptor terminals

Glutamate release from peripheral terminals may occur following intense stimulation of the primary afferent nerves. Stimulated glutamate release occurs due to mechanical nerve injury (Belmonte et al., 1997; Du et al., 2001; Julius and Basbaum, 2001; Miller et al., 2011a), chemical

activation (Belmonte et al., 1997; Cairns et al., 2001; Du et al., 2001; Julius and Basbaum, 2001; Peng et al., 2011; Miller et al., 2011a), or noxious heat (Du et al., 2001; Julius and Basbaum, 2001). It has been proposed that glutamate release from nociceptor peripheral terminals may sensitize surrounding nerve fibers causing hyperalgesia, heightened sensation to painful stimuli (Cairns et al., 2001; Du et al., 2001; Miller et al., 2011a), and allodynia, painful sensation to non-painful stimuli (Diag. VII).

Glutamatergic Nociceptive Peripheral Terminal

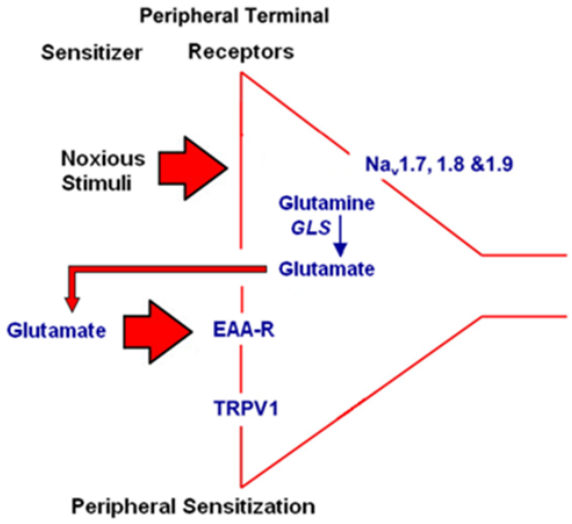


Diagram VII. **Schematic of a peripheral nerve terminal**. Nociceptive peripheral nerve terminals contain excitatory amino acid receptors (EAARs). Glutaminase (GLS) converts glutamine to glutamate in nerve terminals for packaging into synaptic vesicles. Upon noxious stimulus, synaptic vesicles release glutamate from the peripheral terminals. Extracellular glutamate interacts with EAARs on autologous or nearby nerve terminals causing ion channels to open in the membranes. The influx of cations depolarizes peripheral nerve terminal membranes and generates action potentials.

Along with phosphorylation of TRP and sodium channels (described earlier), sensitization also is due to interaction with EAARs on peripheral afferent terminals. Glutamate interacts with EAARs on afferent terminals and directly activates sensory nerve endings (Du et al., 2001; Du et al., 2003; Du and Carlton, 2006; Miller et al., 2011a). Anatomical studies have localized EAARs on unmyelinated axons in the skin (Carlton et al., 1995; Coggeshall et al., 1997; Coggeshall and Carlton, 1998; Du et al., 2003; Miller et al., 2011a). For example, the following EAAR subunits have been demonstrated on unmyelinated nerve fibers in rat hairy and glabrous skinwith electron microscopic immunohistochemistry (Carlton et al., 1995, Coggeshall and Carlton, 1998): NMDA NR1 (21-30% of unmyelinated nerves in rat sural plantar nerves), NR2B, GluR1 (8-17% in rat sural and plantar nerves), GluR2/3 and GluR5-7 (7-44% in rat sural and plantar nerves). Studies also have demonstrated that the glutamate released from nociceptor peripheral terminals participates in neurogenic inflammation (Du et al., 2001, Richardson and Vasko, 2002, Miller et al., 2011a; Hoffman and Miller, 2010). This indicates an interaction of glutamate with EAARs in peripheral tissues (Cairns et al., 2001, Du et al., 2001, Du et al., 2003, Du and Carlton, 2006, Miller et al., 2011a). As in spinal cord, glutamate is co-released along with SP and CGRP and these three neuroactive agents promote the production of other local inflammatory mediators such as prostaglandin and bradykinin (Belmonte et al., 1997; Marfurt et al., 2001; Richardson and Vasko, 2002; Miller et al., 2011a). Prostaglandin and bradykinin also interact with peripheral nerve terminals and the net effect is a reduction in activation threshold of the nociceptors (Du et al., 2001; Miller et al., 2011a).

Evidence for peripheral glutamate release from nociceptors along with localization of EAARs on peripheral afferents may explain why exogenous glutamate or its agonists cause sensitization and pain in experimental animals and humans (Carlton et al., 1995; Coggeshall and Carlton, 1998; Cairns et al., 2001; Du et al., 2001; Du et al., 2003; Du and Carlton, 2006; Miller et al., 2011a). This also is consistent with studies that show up-regulation of glutamate receptors in primary

afferents during inflammation (Peng et al., 2011), such that the sensitized nociceptor has an augmented response on further exposure to glutamate. The phenotypic alterations in primary afferents during ongoing inflammation or nerve injury are thought to form the basis for maladaptive pain, i.e., spontaneous pain, hyperalgesia and allodynia (Diag. VII).

Although it is clear that glutamate is released from peripheral afferent terminals (Miller et al., 2011a), it is unknown what cellular synaptic proteins are involved in peripheral glutamate release. In the cornea, there is evidence that glutamate and other neurotransmitters are present (Marfurt et al., 2001; Muller et al., 2003; Langford et al., 2010; Miller et al., 2011b), however the glutamate release mechanism and its role in corneal nociception are not fully understood. Glutamate and excitatory amino acid transporters have been identified recently in human superficial corneal epithelial cells and stroma, but no reference has been made to corneal peripheral afferents (Langford et al., 2010). As with skin peripheral afferents, we hypothesize that glutamate in corneal peripheral afferents may play an important role in sensitization of corneal afferent nerves via EAARs.

1.4 Sensory innervation of the cornea

The cornea receives its sensory innervation from the ophthalmic division of the trigeminal ganglion (Muller et al., 1997, Marfurt et al., 2001). The ophthalmic branch divides to form the long and short ciliary nerves that pierce the sclera at the posterior pole of the eyeball and around the optic nerve (Marfurt et al., 2001). The cornea receives its sympathetic innervations from the superior cervical ganglion and parasympathetic innervations from the ciliary and pterygopalatine ganglia (Belmonte et al., 1997, Marfurt et al., 2001). Some corneal sensory nerve fibers are nonmyelinated while others are myelinated (Belmonte et al., 1997), but lose their myelin on entry into the cornea in order to maintain corneal transparency (Muller et al., 2003).

Sensory Innervation of the Cornea

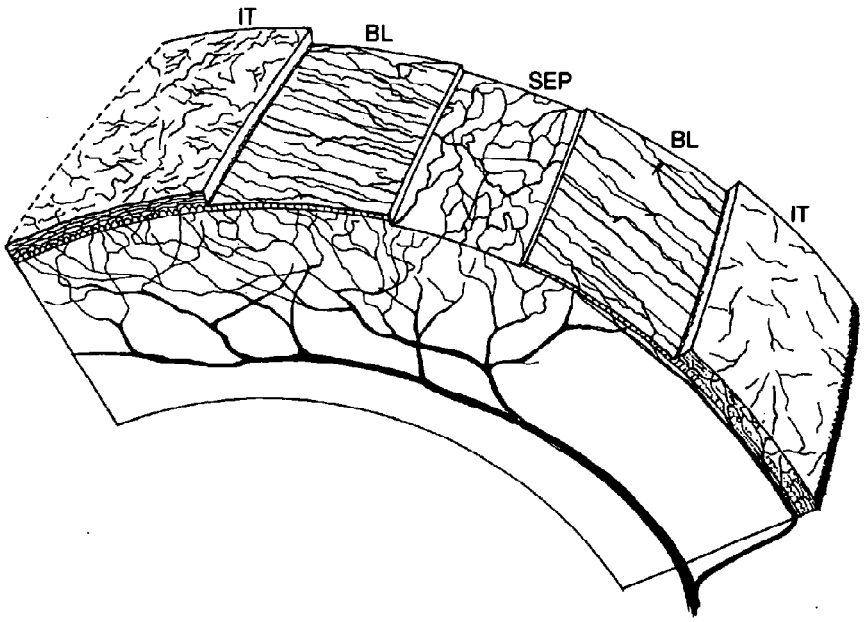


Diagram VIII. **Schematic of corneal innervation**. The ophthalmic division of the trigeminal ganglion branches to form the long and short ciliary nerves. These travel anteriorly in the sclera to enter the corneal stroma in bundles and in a radial fashion. The figure above shows a large stromal bundle that branches extensively to innervate the other parts of the cornea (Chan-Ling, 1989). These branches anastomose to form the subepithelial plexus (SEP). Branching lease fibers from the SEP enter into the epithelium where they terminate as free nerve endings (Beckers et al., 1993). SEP -subepithelial plexus, BL -basal epithelial leashes, IT - intraepithelial terminals. (Diagram from Chan-Ling, 1989)

Within the cornea, the stromal nerve bundles divide to form plexuses that supply the anterior stroma and corneal epithelium (Diag. VIII; Marfurt et al., 2001). A subepithelial nerve plexus is formed and axons from it innervate the superficial corneal epithelial cells (Belmonte et al., 1997).

Studies have demonstrated the presence of corneal epithelial leash formations from the subepithelial plexus (Belmonte et al., 1997, Marfurt et al., 2001, Muller et al., 2003). A leash is composed of 2 to 6 axons occurring in association with the subepithelial plexus (Marfurt et al., 2001). The leashes were described as beaded, running tangential to the corneal surface and are parallel to one another with a common orientation (Marfurt et al., 2001). These terminate within the epithelium as free nerve endings (Diag. VIII; Beckers et al., 1993).

There are 2 types of corneal afferent neurons, the thin myelinated A delta nerve fibers and the unmyelinated C nerve fibers (Belmonte, 1997). The A delta fibers lose their myelin on entry into The cornea (Muller et al., 2003) such that all corneal nerves are devoid of myelin sheath. As in the skin, muscle, and joints, noxious sensing neurons in the cornea are called nociceptors. There is evidence that different types of corneal nociceptors exist (Belmonte et al., 2003): mechanonociceptors, polymodalnociceptors, cold nociceptors and silent nociceptors (Belmonte et al., 2003). Electrophysiological studies indicate that mechanonociceptors discharge in response to damaging mechanical stimuli, whereas polymodalnociceptors discharge in response to a number of stimuli such as carbon dioxide, ATP, endogenous chemicals, low threshold mechanical stimuli and temperatures above 39°C and below 29°C. Cold nociceptors discharge when the temperature of the corneal surface falls below normal temperature of 33°C (Belmonte et al., 2003). Silent nociceptors do not discharge under normal circumstances, but become responsive when they are sensitized (Belmonte et al., 2003). As with other peripheral nociceptive afferents, these sensory neurons express proteins known as transducers to respond to noxious stimuli. For example, TRPV1, the noxious heat transducer, and TRPM8, the noxious cold transducer, have been identified and described in the cornea (Madrid et al., 2006; Murata and Masuko, 2006; Mergler et al., 2010).

1.5 The Role of Glutamate in the Cornea

Our laboratory has shown that primary sensory corneal afferent nerve fibers contain glutamate, glutaminase, and aspartate aminotransferase (Miller et al., 2011b). Furthermore, another study has localized glutamate in the superficial corneal epithelial cells (Langford et al., 2010). Even though there is evidence that glutamate is released into the human cornea (Langford et al., 2010), its mechanism of release, the presence of glutamate receptors in the cornea, and the effect of stimulation of these receptors on behavior is largely unknown. This current dissertation project will identify the presence of molecular machinery for glutamate release in the cornea as a way of demonstrating the potential for vesicular release of glutamate and other neurotransmitters into the cornea. It will focus on immunohistochemistry for synaptophysin, a presynaptic marker for synaptic vesicles, and VGLUT, a marker of glutamatergic synaptic vesicles in corneal sensory afferents. Immunohistochemistry will be verified with a Western blotting study. A retrograde tracing study from the cornea to the trigeminal ganglion (TG) coupled with immunohistochemistry also will be performed to demonstrate that corneal TG neuronal cell bodies produce synaptophysin I and VGLUT2. In order to determine if glutamate can interact with afferents or resident tissue cells, a second study will localize various subunits of NMDA, AMPA, and kainate EAARs in the cornea by immunohistochemistry, specifically the NMDA NRl, the AMPA GluR1 and GluR2/3, and the kainate KA2 and GluR5 subunits. A third study will determine the relative contributions of corneal glutamate receptors on the behavioral response to glutamate and its ionotropic receptor agonists, NMDA, AMPA and kainate. The effect of ionotropic glutamate receptor antagonists on agonist induced corneal nociception will be evaluated using the following antagonists: D-AP5 (D-(-)-2-amino-5-phosphonopentanoic acid [NMDA antagonist]), NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt [AMPA antagonist]) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione disodium salt [AMPA/kainate antagonist]).

1.6 Hypotheses

1. Glutamate release into the cornea occurs via synaptic vesicles.
2. Ionotropic excitatory amino acid (glutamate) receptors are present in the rat cornea.
3. Corneal excitatory amino acid receptors play a role in corneal nociception and EAAR antagonists diminish agonist-induced corneal nociception.

1.7 Aims

1. Determine if molecular machinery for vesicular glutamate release is present in rat cornea with immunohistochemistry. Verify immunohistochemistry with Western blotting. Detect molecular machinery for vesicular glutamate release in corneal trigeminal ganglion neurons. (Chapter 2)
2. Localize excitatory amino acid receptor subunits in the rat cornea with immunohistochemistry. Detect excitatory amino acid receptor subunits in corneal trigeminal ganglion neurons. (Chapter 3)
3. Evaluate the effect of glutamate and ionotropic excitatory amino acid receptor agonists (NMDA, AMPA and kainate) on behavioral responses in the intact cornea. Determine if antagonism of excitatory amino acid receptors reduces agonist-induced behavioral responses. (Chapter 4)

CHAPTER 2

SYNAPTIC PROTEINS AND VESICULAR GLUTAMATE TRANSPORTERS IN RAT CORNEA

Abstract

Aim: To determine if molecular machinery for vesicular glutamate release is present in the rat cornea. Primary afferents in the cornea contain glutamate, substance P, and calcitonin gene-related peptide. It is unclear if these neurotransmitters are released by vesicular mechanisms as in the central nervous system. The aim of this study, therefore, was to describe, in the rat cornea, the localization of proteins involved in synaptic vesicle neurotransmitter release: synaptophysin I and II; vesicular glutamate transporter 1 and 2 (VGLUT1, 2); synaptosomal-associated protein 25 (SNAP-25).

Method: Corneas from Sprague Dawley rats were processed for immunohistochemistry. Whole mounts and tissue sections were incubated in primary antisera against VGLUT2 and synaptophysin I. Also, retrograde tracing from the cornea with Fluorogold was used to identify corneal trigeminal neurons with synaptophysin I and VGLUT2. In addition, corneas were evaluated with Western blots to detect synaptophysin I and VGLUT2.

Results: VGLUT2- and synaptophysin I-immunoreactive afferent nerves were found in the corneal epithelium, stroma and endothelium. These fibers were varicose and exhibited an extensive branching pattern. The presence of VGLUT2 and synaptophysin I in the cornea was

verified further on Western blots. There was also VGLUT2 and synaptophysin I immunoreactivity on corneal trigeminal ganglion neurons.

Conclusion: These data indicate a mechanism for release of glutamate into the cornea. This mechanism may provide a novel outlook on the pharmacological means of regulating corneal physiology during inflammation and nerve injury.

2.1 INTRODUCTION

Glutamate is the excitatory neurotransmitter used by primary sensory neurons located in trigeminal and dorsal root ganglia (Miller et al., 2011a). In addition to use as an excitatory neurotransmitter in the central nervous system, nociceptive primary sensory neurons to the skin and muscle peripherally release glutamate along with substance P (SP) and calcitonin gene-related peptide (CGRP) as part of the neurogenic inflammatory response (Du et al., 2001, Richardson and Vasko, 2002, Du et al., 2003, Miller et al., 2011a). Glutamate's peripheral transport occurs in synaptic vesicles (Juge et al., 2006) containing vesicular glutamate transporters 1 or 2 (VGLUT1 or VGLUT2; Aihara et al., 2000, Bellochio et al., 2000, Moechars et al., 2006, Brumovsky et al., 2007, Miller et al., 2011a). These transporters are responsible for loading synaptic vesicles with glutamate. There also is evidence that glutamate is released into the cornea (Langford et al., 2010; Miller et al., 2011b) along with other neurotransmitters such as substance P (SP) and calcitonin gene-related peptide(Sasaoka et al., 1984; Beckers et al., 1992; Beckers et al., 1993; Marfurt et al., 2001). A mechanism of release for neurotransmitters such as glutamate from corneal afferents has not been studied.

Synaptophysin (I or II) is an integral protein of the synaptic vesicle (Sun et al., 2006) where it plays a role in regulating the amount of neurotransmitter released during exocytosis (Gonzalez-Jamett et al., 2010). Synaptophysin binds synaptobrevin, a vesicle-soluble NSF attachment protein receptor (v-SNARE) protein, to prevent synaptic vesicle docking (Gertz et al., 1999). Upon depolarization, intracellular calcium levels rise in a nerve terminal causing synaptophysin and synaptobrevin to dissociate. Synaptobrevin interacts with target-SNAREs (t-SNARE), such as synaptosomal-associated protein 25 (SNAP-25), for vesicular exocytosis.

Studies show that synaptophysin I and II are localized to neurons in the dorsal root ganglion (Sun et al., 2006) and trigeminal ganglion (Tarsa and Balkowiec, 2009). The peripheral afferents of these sensory neurons are distributed to tissues such as the skin and cornea. If corneal afferents release glutamate from their peripheral terminals, then proteins responsible for release should be localized to the cornea. This current study, therefore, focused on the description of immunohistochemical labeling of synaptophysin I and II, SNAP 25, and VGLUT 1 and 2 in the cornea. The presence of these proteins synaptophysin I and VGLUT2 was confirmed with Western blotting. Retrograde tracing from the cornea was used to verify the presence of synaptophysin I and VGLUT2 in TG.

2.2 METHODOLOGY

2.2.1 Immunohistochemistry

2.2.1.1 *Tissue preparation*

Adult Sprague Dawley rats (n=12) were euthanized with Avertin (i.p.) followed by xylazine (i.p.). The thorax was opened and animals were perfused transcardially via the ascending aorta with 100ml of calcium free Tyrrode's buffer (23°C) followed with 300ml of fixative: 0.8% picric acid, 0.2% paraformaldehyde and 0.1 M phosphate buffer (23°C), pH 7.4 (Hoffman et al., 2010). The bulbus oculi was enucleated and cornea excised. The tissues were incubated for 2 to 3 hours in fixative and transferred to phosphate buffered saline (PBS) in 10% sucrose overnight. Corneas were processed for immunohistochemistry as whole mounts or sagittal tissue sections. For tissue sections, the corneas were frozen in M-1embedding matrix (Lipshaw) and 10µm sagittal sections were cut in the cryostat at -21˚C. Tissue sections were thaw-mounted onto gelatin coated glass slides.

The whole mounts and tissue sections were rinsed three times in PBS. Subsequently, they were processed for immunohistochemistry using rabbit anti-VGLUT 2 (Sigma,HY19, 1/2000), rabbit anti-SNAP 25 (ABD Serotec, 1/20000), rabbit anti-synaptophysin I (Chemicon, 1/5000), and rabbit anti-synaptophysin II (Chemicon, 1/5000). Tissues were incubated on a shaker at 4°C for 120hr. Tissues were rinsed three times with PBS, 10 minutes each, and incubated in biotinylated goat anti-rabbit IgG (Jackson, 1/8000 dilution). The tissues were rinsed three times in PBS, 10 minutes each, and incubated in streptavidin-HRP (Invitrogen, 1/1000 dilution) for 60 minutes. The tissues were rinsed three times in PBS, 10 minutes each, and were incubated for 10 minutes in 200µl of tyramide solution (1/400 dilution) and amplification buffer with 0.3% hydrogen peroxide. Whole mounts were placed on glass slides. Coverslips were apposed using Prolong Gold mounting medium (Vector) and incubated overnight at room temperature. Images were obtained using a Spot camera (Diagnostic Instruments) attached to an Olympus BX51 epifluorescence microscope.

2.2.1.2 *Removal of corneal epithelium with thermolysin*

In order to examine the stromal corneal afferents, corneas were treated with thermolysin (Endo et al., 2004) in the following manner. Adult Sprague Dawleyrats (n = 3)were asphyxiated with carbon dioxide. The bulbus oculi was enucleated and corneas excised. Corneas immediately were incubated in a solution containing 5ml of thermolysin (1:4 dilution in PBS; Sigma) and 5µl of 1M calcium chloride at 4°C for 30 minutes. To stop the enzymatic reaction, the tissues were incubated in 1ml of 0.5M EDTA and 100ml of DMEM (Dubelcco's modification of Eagles medium; Invitrogen) for another 30 minutes. The epithelium was separated from the stroma by gentle scraping with a scalpel blade. The stroma was post fixed in picric acid fixative (0.8% picric acid, 0.2% paraformaldehyde and 0.1 M phosphate buffer at pH of 7.4) and subsequently processed for whole mount immunohistochemistry as described above.

2.2.2 Retrograde tracing:

Adult Sprague Dawley rats (n = 8) were anaesthetized with Isofluorane, the eyelids were parted and 10-20µl of 5% fluorogold was injected with a Hamilton syringe into the cornea using a dissecting microscope. Spills from the injection site immediately were wiped off with cotton tipped applicator and subsequently irrigated with distilled water. Animals were allowed to recover from anesthesia before returning them to their cages. After 5 days, the animals were euthanized with Avertin (i.p.) followed by xylazine (i.p.) and transcardially perfused with fixative as described earlier. Ipsilateral trigeminal ganglia were removed and cryostat sections at 10-20µm were processed for synaptophysin I and VGLUT2 immunoreactivity as described above.

2.2.3 Western blot

Fresh cornea and trigeminal ganglia (positive control) were obtained from adult Sprague Dawley rats (n =3)and homogenized using lysis buffer (8M urea, 50mM TrisHCl, 1mM dithiothreitol and 1mM EDTA,pH 7.4, 4°C) and phosphatase inhibitor cocktail I and II and protease inhibitor (Sigma). Homogenates were centrifuged (70,000 rpm, 20 minutes) and the protein concentration of the supernatant was measured (BCA Protein Assay Kit, Pierce, Rockford, IL) to normalize the samples. Rabbit anti-VGLUT2 antibody (Sigma, HY19) and rabbit anti-synaptophysin I (Chemicon) were conjugated M-280 Dynabeads (Schechter et al., 2005); Miller et al., 2012). Equal amounts of total protein (75mg/ml) were exposed to rabbit anti-synaptophysin I antibody-beads or rabbit anti-VGLUT2 antibody-beads (16 hr, 4°C) for protein purification (Schechter et al., 1998). Samples were exposed to a magnet to collect the bead-antibody-protein complex. The purified VGLUT-2 or synaptophysin I was eluted from the beads using Lammelli buffer (10mM Tris Base, 1mM EDTA, and 2.5% SDS%, 3β-mercaptoethanol and 0.01% bromophenol blue) and heating the samples at 100°C for five minutes.

Gel electrophoresis was performed using a 12.5% polyacrylamide gel (GE Healthcare; Phast-System, Promega) along with molecular weight standards (Novagen; Schechter et al., 2005). Separated proteins were transferred to a nitrocellulose membrane using the PhastSystem in a buffer of 25 mMTris, 192 mM glycine and 20% methanol, pH 8.0 at 25mA for 20 minutes. Immunoblotting was performed using the Protoblot II AP System (Promega). Membranes were dried at 37°C, rinsed in 20 mMTris-HCL, 150 mMNaCl and 0.05% Tween 20, pH 7.5 (TBST), washed in 1% bovine serum albumin (BSA) in TBST and incubated in rabbit anti-synaptophysin I (1:1000) or rabbit anti-VGLUT2 (1:2000) antisera for 1 hour at room temperature. Membranes were washed in TBST followed by incubation in alkaline phosphatase conjugated goat anti-rabbit antibody for 30 minutes and washed twice in TBST and TBS. Membranes were incubated in Western Blue stabilized substrate for alkaline phosphatase (Promega; 5-bromo-4-chloro-3-indolyl-phosphate, nitro blue tetrazolium) for immunoreactive detection.

2.3 RESULTS

2.3.1 Immunohistochemistry

Immunoreactivity was not observed for synaptophysin II or VGLUT 1 in corneal whole mounts or tissue sections. Corneal afferent nerves were immunoreactive (IR) for VGLUT 2. The whole mounts show intense VGLUT2 immunoreactivity in the epithelium such that it was difficult to delineate the layers of the cornea. For this reason, the corneal epithelium was removed with thermolysin. Following the removal of the corneal epithelium, numerous nerve fibers were visualized traveling through the stroma (Fig. 2.1.1). These nerve bundles displayed extensive branching pattern. On the sagittal sections (Fig.2.1.2), VGLUT2-IR nerve fibers were found in the epithelium (Fig 2.1.2 and 2.1.3), stroma and endothelium (Fig. 2.1.2). In the epithelium and endothelium, VGLUT 2-IR afferents fibers exhibit varicosities along their trajectories and were

seen as scattered punctae throughout the stroma. Within the endothelium (Fig2.1.2), varicose IR fibers travel along most of the entire length of the endothelium. Retrograde tracing indicated that corneal trigeminal ganglion neurons were labeled and that small to medium diameter neurons had VGLUT 2 immunoreactivity (Fig. 2.2).

Synaptophysin I-IR varicose nerve fibers were localized in corneal whole mounts and tissue sections (Fig. 2.3). There were numerous synaptophysin I-IR beaded nerve fibers in the epithelium, endothelium and stroma (Fig. 2.3.2 and 2.3.3). Some synaptophysin I-IR corneal epithelial nerves fibers initially travel parallel to the corneal surface, but turn abruptly, almost at right angles, to terminate within the superficial corneal epithelium. Within the stroma, varicose synaptophysin I-IR nerve bundles were observed. In the endothelium, synaptophysin I-IR corneal nerve fibers could be seen along the entire length of the endothelium. Retrograde tracing demonstrated labeled corneal trigeminal ganglion neurons with synaptophysin I immunoreactivity (Fig. 2.4).

In corneal whole mounts, there were numerous varicose SNAP 25-IR corneal nerve fibers (Fig. 2.5). The SNAP 35-IR nerve fibers in the stroma had extensive branching before entering the subepithelial plexus.

2.3.2 Western blots

Western blotting was used to verify the presence of VGLUT2 and synaptophysin I in intact rat cornea. A single VGLUT 2-IR band from the cornea occurred at 65 kiloDaltons (kDa;Fig. 2.6). A single synaptophysin I-IR band at 38 kDa was obtained from the rat cornea (Fig. 2.7). Trigeminal ganglia were used as positive controls and showed comparable bands at 65Kd for VGLUT2 and 38Kd for synaptophysin I.

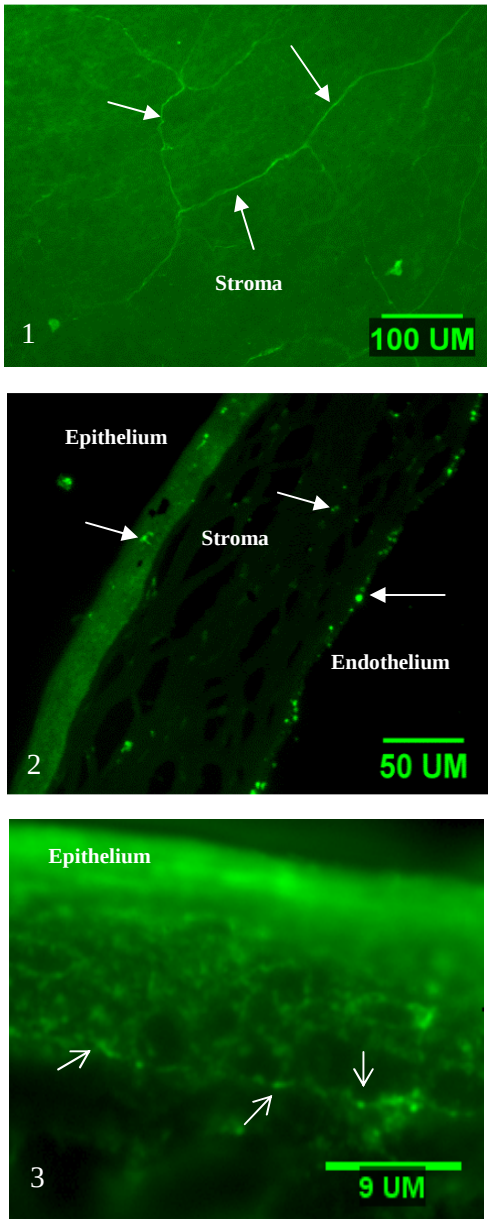


Figure 2.1.**VGLUT2 Immunohistochemistry in the rat cornea.**In figure2.1.1 (corneal whole mount), the epithelium was been removed to reveal the underlying VGLUT2-IR stromal nerve bundles with their extensive pattern of branching (white arrows). Sagittal section (10µm) of the rat cornea (Fig 2.1.2 and 2.1.3). The white arrows show VGLUT2-IR nerve fibers in the corneal epithelium, stroma and endothelium (Fig 2.1.2). Intensely labeled VGLUT2-IR nerves can be seen within the corneal epithelium (Fig.2.1.3).

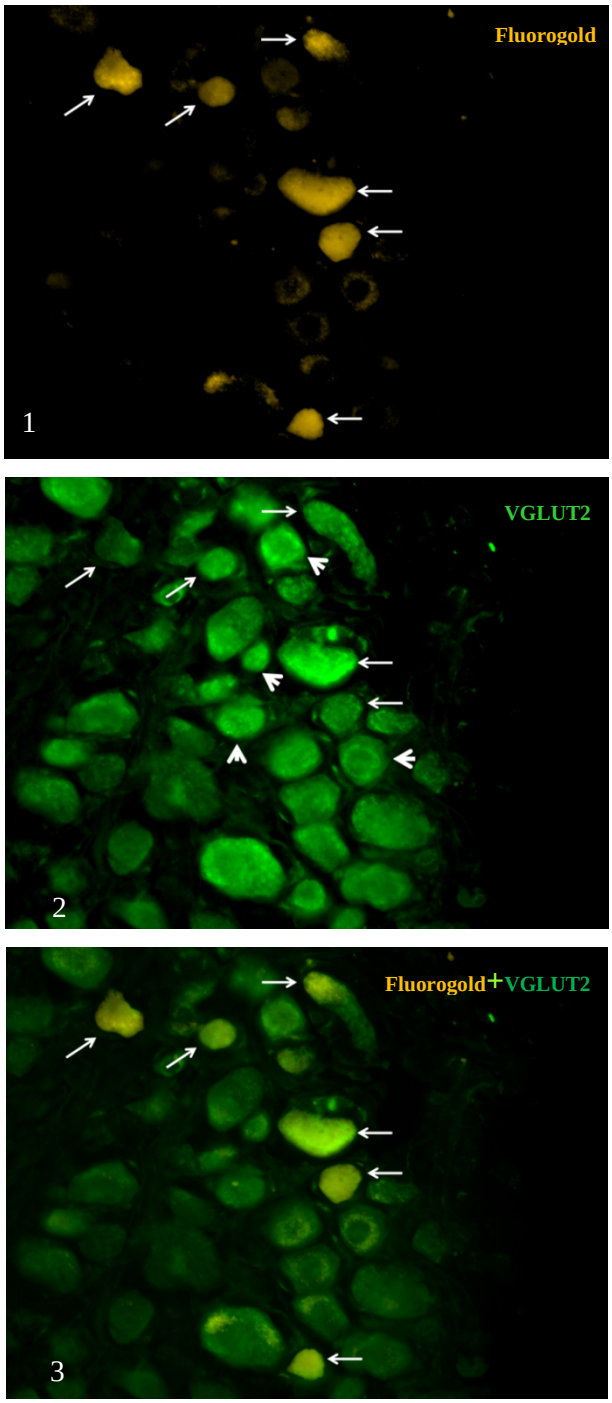


Figure 2.2. **Retrograde tracing study for VGLUT 2**. Sections from the trigeminal ganglion. Trigeminal ganglion neurons were retrogradely labeled with fluorogold (Fig 2.2.1, arrows). Neuronal cell bodies in the trigeminal ganglion were IR for VGLUT2 (Fig 2.2.2, arrows & arrowheads). Small-medium diameter fluorogold neurons contained VGLUT 2 immunoreactivity (Fig 2.2.3, arrows).

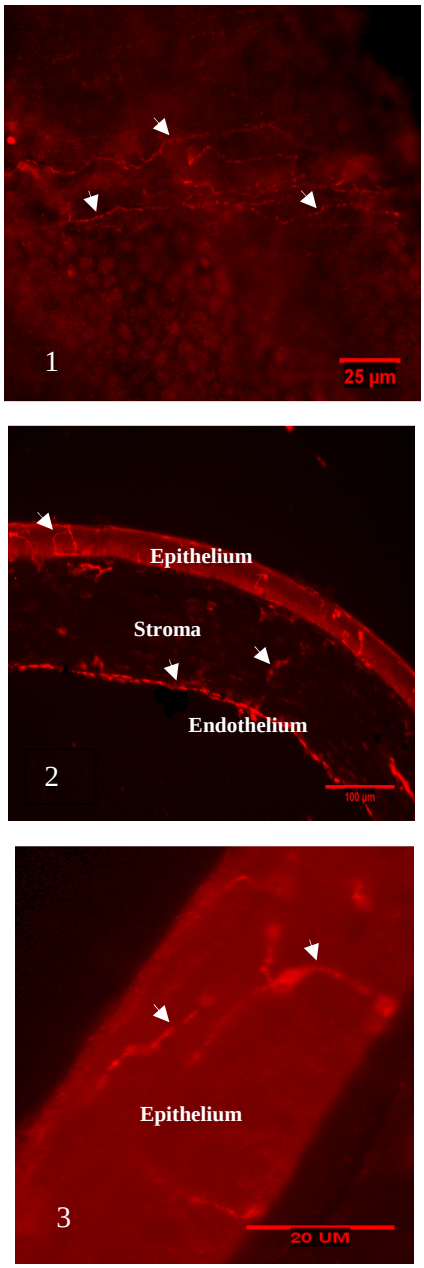


Figure 2.3. **Synaptophysin I immunohistochemistry in the rat cornea.**Corneal whole mount showing synaptophysin I-IR corneal afferents (Fig 2.3.1) as indicated by the white arrows. Sagittal section (10µm) of the rat cornea shows IR afferents in the epithelium, stroma and endothelium (Fig 2.3.2). In figure 2.3.3, the white arrows show varicose synaptophysin I IR afferents within the corneal epithelium.

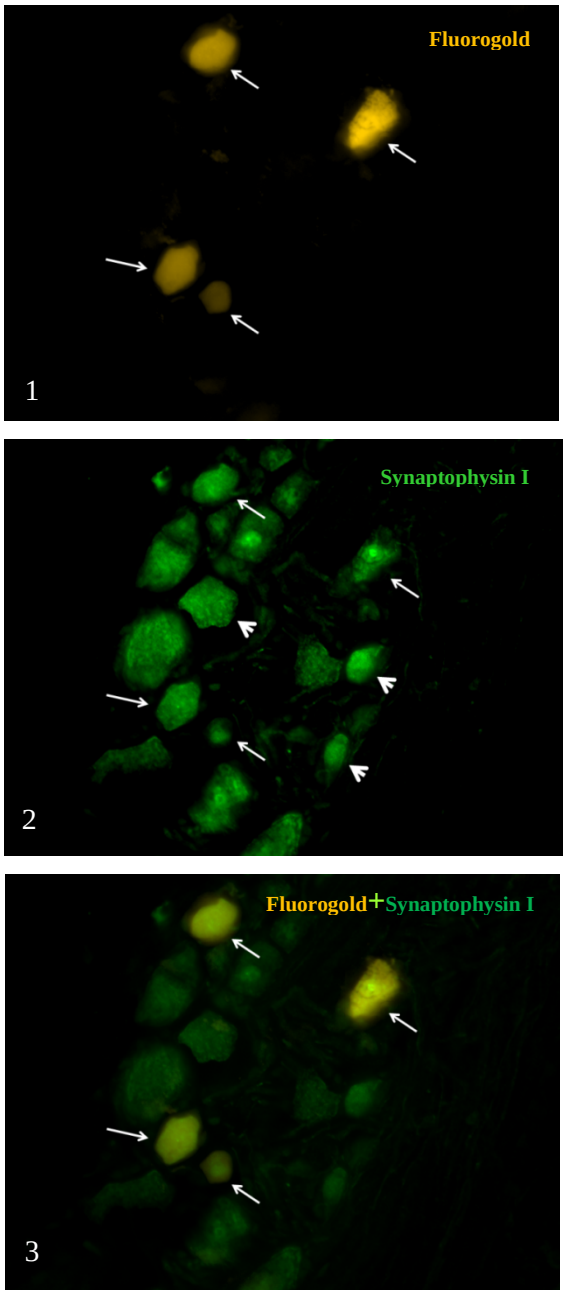


Figure2.4. **Retrograde tracing study for Synaptophysin I**. Sections from the trigeminal ganglion. Trigeminal ganglion neurons were retrogradely labeled with fluorogold (Fig 2.4.1, arrows). Neuronal cell bodies in the trigeminal ganglion were IR for synaptophysin (Fig 2.1.2, arrows and arrowheads). Fluorogold labeled neurons contained synaptophysin I immunoreactivity (Fig 2.1.3, arrows).

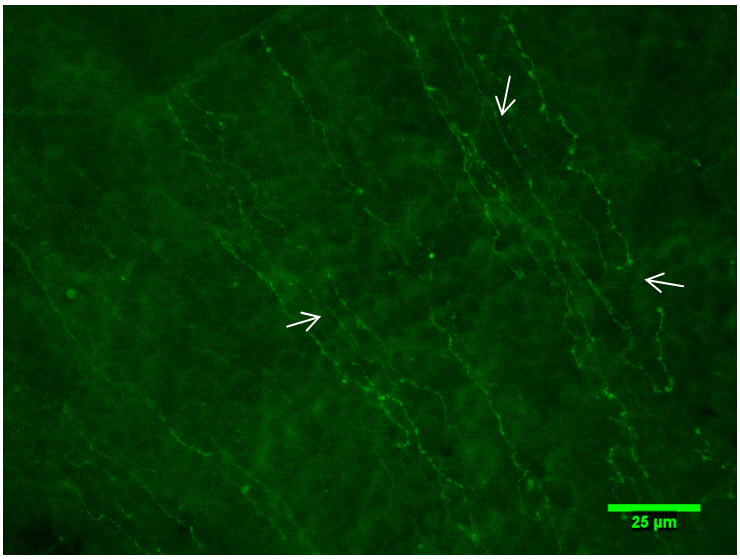


Figure2.5. **SNAP25 immunoreactivity in rat cornea**. In this cornea whole mount, the white arrows point to SNAP25-IR nerve fibers within the cornea. These varicose nerve fibers are numerous and branched within the stroma of the cornea.

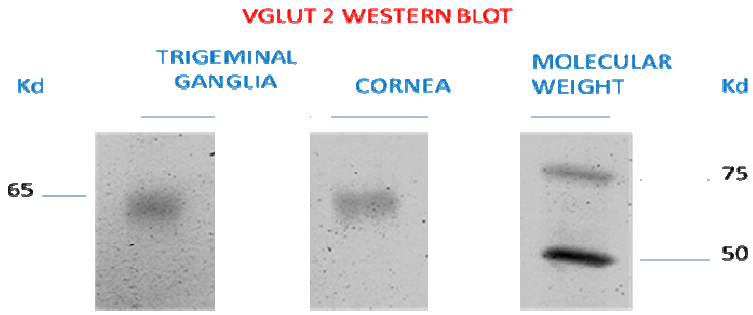


Figure 2.6. **Western blot detection of VGLUT2 in the rat cornea**. At 65 kDa, a single VGLUT 2-IR band was detected from the trigeminal ganglia and cornea. Numbers on the right indicate positions of molecular weight markers in kiloDaltons (kDa).

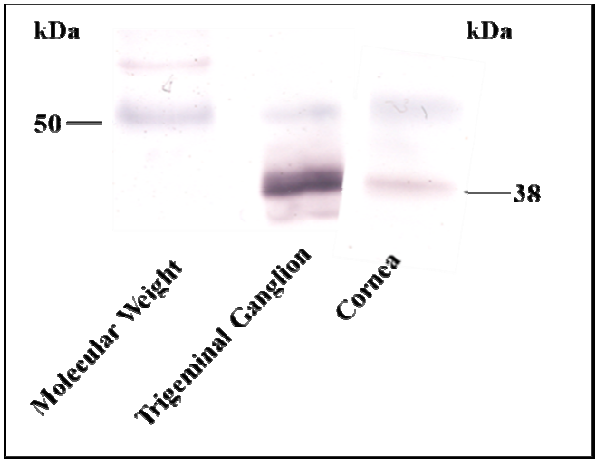


Figure2.7. **Western blot detection of synaptophysin I in the rat cornea**. At ~38 kDa, a single synaptophysin I-IR band was detected from the trigeminal ganglia and cornea. Numbers on the right indicate positions of molecular weight markers in kiloDaltons (kDa).

2.4 DISCUSSION

In skin and joint, glutamate, SP, and CGRP are released from Aδ and C peripheral sensory nerve terminals to sensitize surrounding nerve terminals and cells and initiate neurogenic inflammatory cascades (Woolf and Ma, 207; Miller et al., 2011a). Peripheral glutamate release appears to require depolarization of primary afferent terminals with high threshold stimulation (Miller et al., 2011a). In the cornea, there is evidence that glutamate, SP, and CGRP are contained in corneal nerve fibers (Beckers et al., 1992, Beckers et al., 1993, Marfurt et al., 2001; Miller et al., 2011b), but a mechanism for peripheral release has not been explored. In this study, we identified synaptic vesicle proteins, synaptophysin I and VGLUT2, and a t-SNARE protein, SNAP 25, in primary afferent immunoreactive nerve fibers in the cornea. The presence of synaptophysin I and VGLUT 2 was verified with Western blotting. Their origin from Aδ and C primary afferent

neuronal cell bodies (small-medium diameter; Hoffman et al., 2010) was determined by retrograde tracing coupled with immunohistochemistry for synaptophysin I and VGLUT 2.

VGLUTs are required for synaptic vesicle loading and storage of glutamate for neurotransmission (Bellochio et al., 2000; Juge et al., 2006; Moechars et al., 2006; Brumovsky et al., 2007; Miller et al., 2011a). Primary sensory neurons in DRG and TG express VGLUT1 or 2 and mouse sciatic nerve axons and intraepidermal afferents contain VGLUT2 (Brumovsky et al., 2007). Glutamate and other neurotransmitters are released from nerve terminals by an exocytotic process using complexes of t- and v-SNAREs. For example, the v-SNARE of synaptic vesicles, synaptobrevin, is inhibited tonically by synaptophysin via a calcium mediated process (Gerst, 1999). Upon nerve terminal depolarization, elevated intracellular calcium levels cause the synaptophysin-synaptobrevin complex to dissociate and synaptobrevin is able to interact with syntaxin and SNAP 25, t-SNARES, on the plasma membrane (Reisinger et al., 2004). The synaptobrevin, syntaxin and SNAP 25 helix complex is required for vesicle/plasma membrane fusion (Reisinger et al., 2004). Our results demonstrated the presence of SNAP 25, VGLUT2 and synaptophysin I in rat corneal afferents. One interpretation of these results would be that glutamate is released into the cornea by vesicular exocytosis, potentially from high threshold sensory stimuli (Miller et al., 2011a).

Previous studies show that branches of the long and short ciliary nerves enter the corneal stroma in bundles and in a radial fashion (Chan-Ling, 1989; Muller et al., 1997; Marfurt et al., 2001). These stromal nerve bundles divide to pierce Bowman's membrane and form the subepithelial plexus. Branches from this plexus, termed leash fibers, enter into the epithelium and they terminate as free nerve endings (Chan-Ling, 1989; Muller et al., 1997; Marfurt et al., 2001). This previously reported sensory innervation pattern of the cornea matches with localization of VGLUT2 and synaptophysin I in this study. Varicosities in nerve fibers located in the peripheral

and central nervous systems are potential sites of “en passage” neurotransmitter release (Burnstock, 2007; Fuxe et al., 2010). The presence of VGLUT2 and synaptophysin I in varicose stromal and leash fibers may indicate release in the stroma and epithelium. The presence of immunoreactive varicose stromal and leash fibers may indicate that they have the potential for glutamate release, as well as epithelial free nerve endings.

In skin, joint, and muscle, glutamate causes sensitization of peripheral afferents and other cell types in both normal and inflamed tissues (Du et al., 2001; Miller et al., 2011a). Intense stimuli trigger vesicular release of glutamate from primary afferent nerves into peripheral tissues, such as the skin (Brumovsky et al., 2007; Miller et al., 2011a). Glutamate, released from nerve terminals, interacts with excitatory amino acid receptors (EAARs) on surrounding afferent nerves to lower their activation threshold, thereby causing peripheral sensitization (Du et al., 2001; Miller et al., 2011a). It is not known, however, if glutamate has similar sensitizing effects in the cornea via EAAR’s. The identification of SNAP25, synaptophysin I and VGLUT2 in corneal peripheral afferents provides a foundation for determining the mechanism for peripheral glutamate release in the cornea. Understanding the peripheral release of glutamate in the cornea may provide insight into corneal physiology during inflammation and tissue damage.

CHAPTER 3

IMMUNOLOCALIZATION OF IONOTROPIC GLUTAMATE RECEPTORS IN THE RAT CORNEA

Abstract

Aim: Immunohistochemical localization of select ionotropic excitatory amino acid receptor (EAAR; glutamate) subunits in intact rat cornea.

Methods: Corneas from adult Sprague Dawley rats were fixed with paraformaldehyde/picric acid and processed for immunohistochemistry. Whole mounts and saggital tissue sections were incubated in primary antisera against EAAR subunits: 1. NMDA receptor subunit NR1 and phosphorylated NR1 (pNR1); 2.AMPA receptor subunits GluR1 and GluR2/3, 3. Kainate receptor subunits KA2. In addition, retrograde tracing from the cornea with Fluorogold was used to identify corneal trigeminal neurons with NR1, KA2, and GluR5 immunoreactivity.

Results: Rat corneas were immunoreactive for NR1, pNR1, GluR1, GluR2/3, and KA2 subunits. Immunoreactivity for pNR1 occurred on the membrane of corneal epithelial cells and as scattered punctae within the epithelial cell nucleus. In addition, corneal epithelial nerve fibers were immunoreactive for the NR1 receptor subunit. Corneal epithelial cells were immunoreactive for GluR1, as well as GluR1 immunoreactive nerve fibers within the subepithelial plexus. Intense GluR2/3 immunoreactive granules were localized in the cytoplasm of corneal epithelial cells, as well as perinuclear clustering of immmunoreactive punctae. Epithelial cells were immunoreactive

for KA2 receptor subunit with scattered cytoplasmic punctae. Retrograde tracing from the cornea localized NR1, KA2, and GluR5 immunoreactivity in trigeminal neurons.

Conclusion: These results indicate that ionotropic EAARs are localized to both rat cornea epithelial cells and corneal primary afferents. Glutamate released from corneal epithelial cells and/or afferents could produce sensitization of the cornea via NMDA, AMPA and kainate EAARs.

3.1 INTRODUCTION

Glutamate is an excitatory neurotransmitter in central and peripheral axons of trigeminal and dorsal root ganglion neurons (TG, DRG; Miller et al, 2011a). Trafficking and release of glutamate in these sensory neurons occurs via vesicular glutamate transporter 1 or 2 (VGLUT1, 2; Aihara et al., 2000, Bellochio et al., 2000, Moechars et al., 2006, Brumovsky et al., 2007, Miller et al., 2011a; Chapter 2 - Ibitokun, 2012). Glutamate is released peripherally from primary afferent nerve fibers in skin, muscle and joint as part of the neurogenic inflammatory response where it exerts a sensitizing effect on surrounding afferents and resident and migrating inflammatory cells (Cairns et al., 2001, Du and Carlton, 2006, Miller et al., 2011a). This sensitizing effect occurs via ionotropic and metabotropic excitatory amino acid (glutamate) receptors (EAARs; (Du et al., 2001; Du et al., 2003; Du and Carlton, 2006; Peng et al., 2011; Miller et al., 2011a). It is not known, however, whether EAARs are located in the cornea or if glutamate has a similar effect in the cornea as in other peripheral tissues.

In the cornea, glutamate is present in human superficial epithelial cells and stroma (Langford et al., 2010) and rat corneal afferents (Miller et al., 2011b). Rat corneal afferents have VGLUT 2 for glutamate transport and storage (Chapter 2 - Ibitokun, 2012) and the release of glutamate from corneal afferents appears to occur via vesicular exocytosis using synaptophysin I and SNAP 25 (synaptosomalassociated protein 25) (Chapter 2 - Ibitokun, 2012). If glutamate is released from afferents into the cornea, then sensitization of corneal afferents and cells may occur by interaction with iontotropic and/or metabotropic EAARs similar to what has been described in the rodent skin, joint, and muscle (Cairns et al., 2001; Du et al., 2001; Du et al., 2003; Du and Carlton, 2006; Brumovsky et al., 2007; Miller et al., 2011a). The current study, therefore, will focus on localization of iontropic EAARs in the rat cornea.

Ionotropic glutamate receptors are classified into three types based on selective agonists: N-methyl-D-aspartate (NMDA); 2-amino-3-(5-methyl-3-oxo-1, 2- oxazol-4-yl) propionate (AMPA); kainate (Wo and Oswald, 1995, Chen et al., 1996, Lu et al., 2009). These ionotropic receptors are comprised of subunits: NMDA - NR1, NR 2A-2D subunits; AMPA -GluR 1-4 subunits; Kainate- KA1-2, GluR5-7 subunits (Wo and Oswald, 1995; Chen et al., 1996; Lu et al., 2009). Previous immunohistochemical studies have localized EAARs on unmyelinated axons in the rat skin. For example, EAAR subunits (NR1, NR2B, GluR1, GluR2/3, GluR5-7) have been demonstrated on unmyelinated nerve fibers in rat hairy and glabrous skinwith electron microscopy (Carlton et al., 1995, Coggeshall et al., 1997, Coggeshall and Carlton, 1998, Du et al., 2003, Du and Carlton, 2006). The NMDA NR1 subunit also has been localized to human and rat skin keratinocytes (Fischer et al., 2004a; Fischer et al., 2004b), Miller and Schechter – unpublished observations). To further understand the potential role of glutamate in the cornea, this study aims at determining EAAR expression and localization in the rat cornea by immunohistochemistry and with retrograde tracing from the cornea to the TG coupled with EAAR immunohistochemistry.

3.2 METHODOLOGY

3.2.1 Immunohistochemistry.

Adult Sprague Dawleyrats (n = 12)were euthanized with Avertin (i.p.) followed by xylazine (i.p.). The thorax was opened and animals were perfused transcardially via the ascending aorta with 100ml of calcium free Tyrrode's buffer (23°C) followed with 300ml of fixative: 0.8% picric acid, 0.2% paraformaldehyde and 0.1 M phosphate buffer (23°C), pH 7.4 (Hoffman et al., 2010). The globe was enucleated and cornea excised. The tissues were incubated for 2 to 3 hours in fixative and transferred to phosphate buffered saline (PBS) in 10% sucrose overnight. Corneas were processed for immunohistochemistry as whole mounts or sagittal tissue sections. For tissue

sections, the corneas were frozen in M-1 embedding matrix (Lipshaw) and 10µm sagittal sections were cut in the cryostat at -21˚C. Tissue sections were thaw-mounted onto gelatin coated glass slides.

The whole mounts and tissue sections were rinsed three times, 10 minutes each in PBS. Subsequently, they were processed for immunohistochemistry using the following antisera: rabbit anti-phosphoNR1 (recognizing phosphosphorylated Serine 897, 1/500, Upstate), rabbit anti-NR1 (1/250, Epitomics), rabbit anti-GluR1 (1/2000, AbDSerotec), rabbit anti-GluR2/3 (1/250, Chemicon), rabbit anti-KA2, (1/1000, Millipore) and rabbit anti-GluR5 (1/1000, Novus). The antisera concentrations were determined after doing a series of dilution curves for each antibody on cornea, TG, and DRG. Tissues were incubated in primary antisera on a shaker at 4°C for 120hr (Hoffman et al., 2010). Tissues were rinsed three times in PBS, 10 minutes each and incubated in Alexafluor 488 conjugated goat anti-rabbit IgG (1/2000) for 60-90 minutes. Tissues were rinsed three times in PBS, 10 minutes each. Whole mounts were placed on glass slides. Coverslips were apposed using Prolong Gold mounting medium (Vector) and incubated overnight at room temperature. Images were obtained using a Spot camera (Diagnostic Instruments) attached to an Olympus BX5I epifluorescence microscope.

3.2.2 Retrograde tracing.

Adult Sprague Dawley rats (n = 5) were anaesthetized with isofluorane, the eyelids were parted and 10µl of 5% Fluorogold was injected with a Hamilton syringe into the cornea using a dissecting microscope. The injection site was wipedimmediately with cotton tipped applicator and irrigated with distilled water. Animals were allowed to recover from anesthesia before returning them to their cages. After 5 days, the animals were euthanized and transcardially perfused with fixative as described above. Ipsilateral trigeminal ganglia were removed and cryostat sections at

10-20μm were processed for NR1, GluR1, KA2, and GluR5 immunoreactivity as described above.

3.3 RESULTS

On corneal whole mounts (Fig 3.1), there was intense NMDA pNR1 immunoreactivity on the corneal epithelial cell membrane and as scattered puntae within the nucleus. On sagittal sections (Fig. 3.2), NMDA NR1 immunoreactive (IR) varicose nerve fibers were identified that coursed through the corneal epithelium.

GluR1 immunoreactivity was localized to the subepithelial corneal nerve plexus on corneal whole mount (Fig. 3.3). The nerve fibers were numerous and they had an extensive branching pattern. GluR2/3 immunoreactivity occurred in corneal epithelial cells within the cytoplasm and perinuclear area (Fig. 3.4).

On whole mounts, corneal epithelial cells contained scattered KA2-IR granules in the cytoplasm (Fig. 3.5).

Numerous neurons in the TG were identified with Fluorogold labeling (Fig. 3.6). NR1 immunoreactivity was localized to all retrogradely labeled neurons (Fig. 3.7), whereas KA2 and GluR5 immunoreactivity was only identified in small-medium diameter neurons (Hoffman et al., 2010) that were retrogradely labeled (Fig. 3.8-3.9).

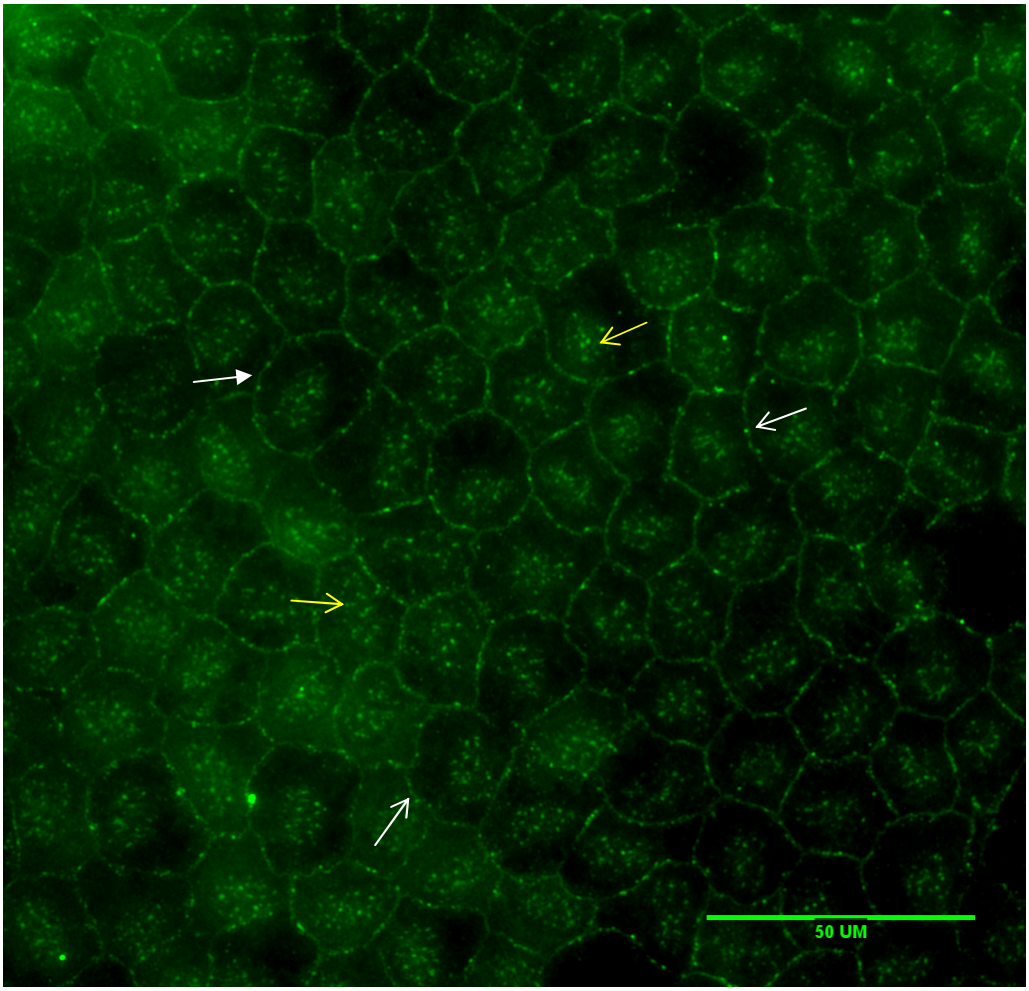


Figure3.1. **NMDA pNR1 immunoreactivity in cornea (whole mount)**. Corneal epithelial cell membranes are immunoreactive for NMDA pNR 1 (white arrows) and as well as scattered pNR1 immunoreactive granules within the nucleus (yellow arrows).

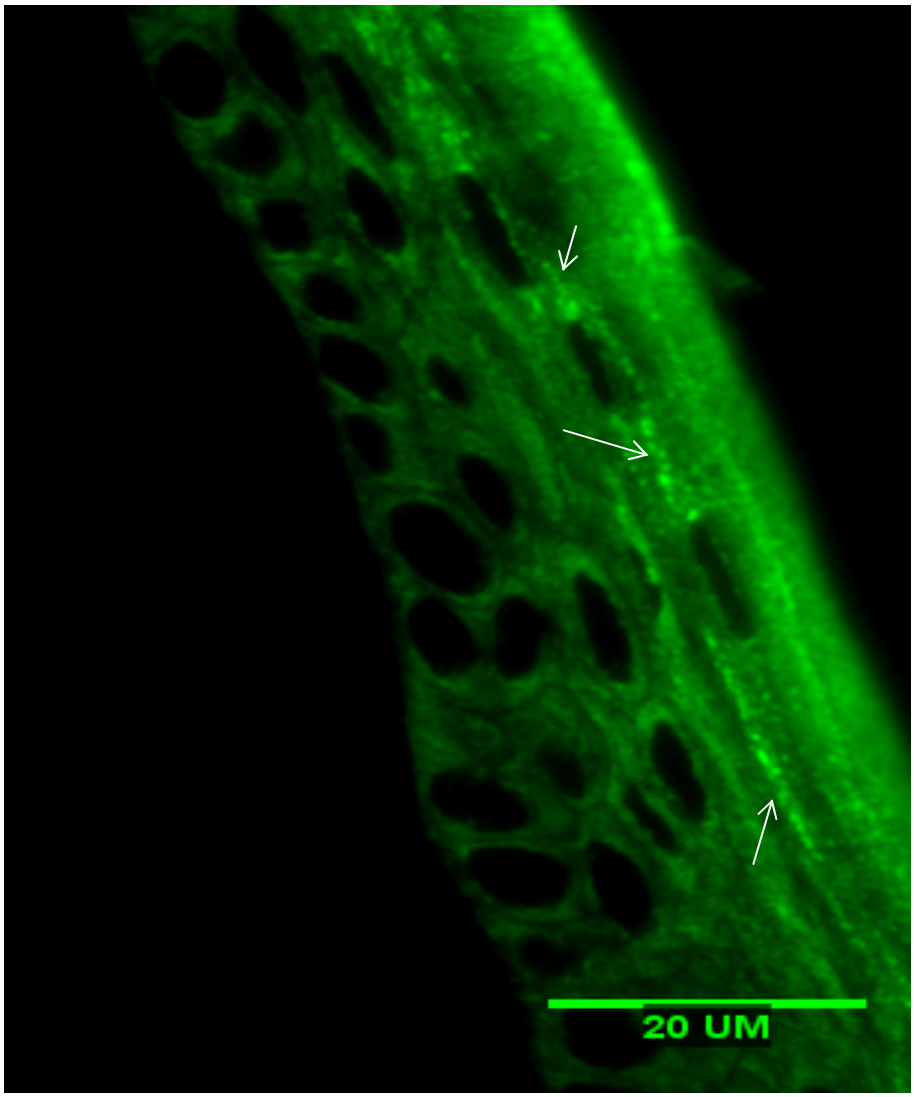


Figure3.2; **NMDA NR1immunoreactivity in the rat cornea (10µm sagittal section).** There was intense NMDA NR1 immunoreactivity on corneal epithelial nerve fibers (white arrows). These corneal afferent nerve fibers exhibited varicosities and traveled between the superficial corneal epithelial cells.

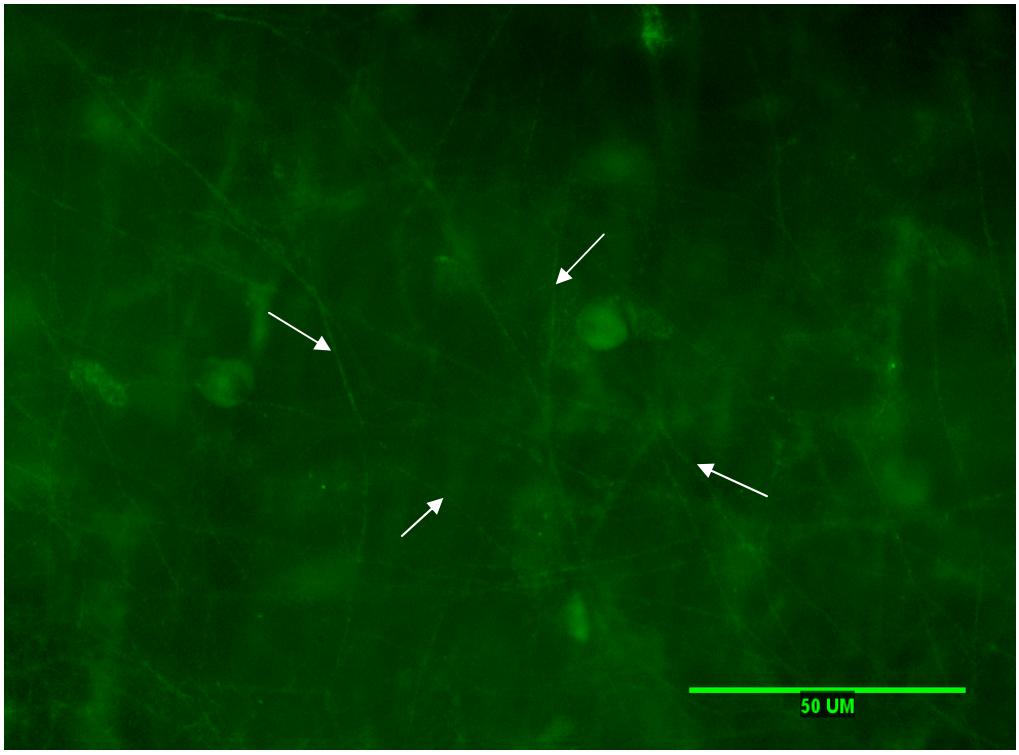


Figure3.3; **AMPA GluR1 immunoreactivity in cornea (whole mount).** Whole mount of the cornea shows GluR1 immunoreactivity on subepithelial plexus as indicated by the white arrows.

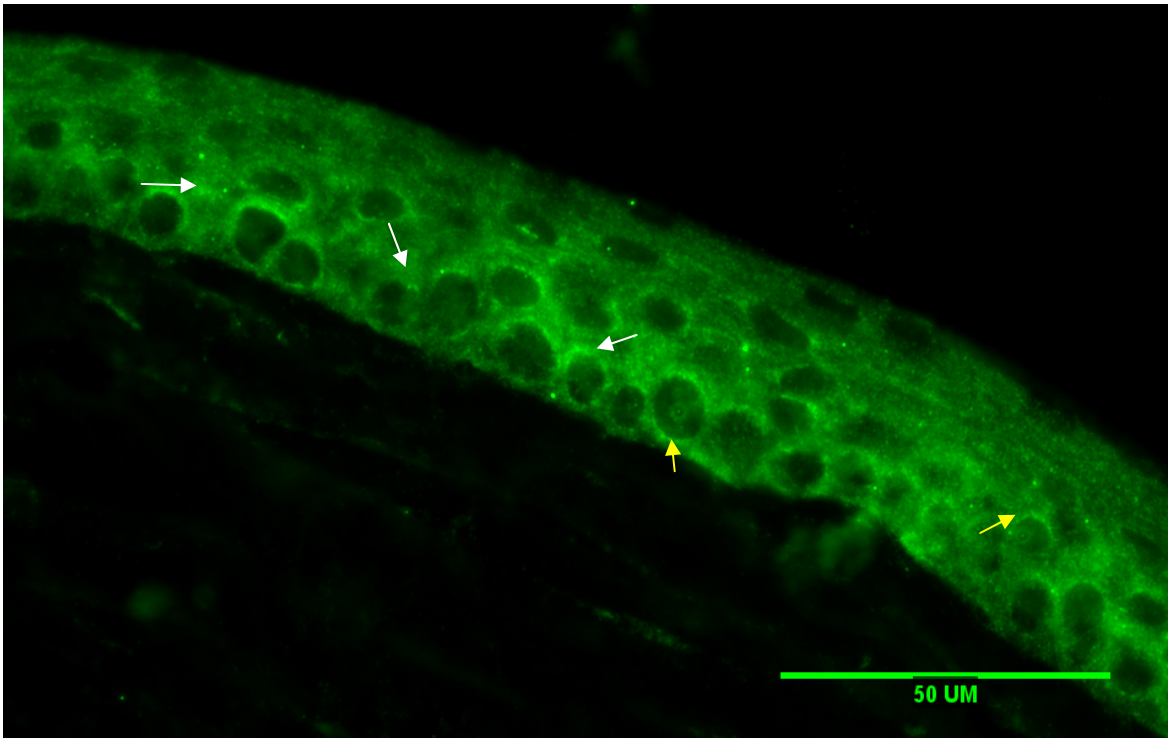


Figure3.4; **AMPA GluR2/3 immunoreactivity in the rat cornea (10µm sagittal section)**. GluR2/3 immunoreactivity occurred in corneal epithelial cells. The distribution is mainly within the cytoplasm (white arrows) of the epithelial cells with a clustering around the nucleus (yellow arrows).

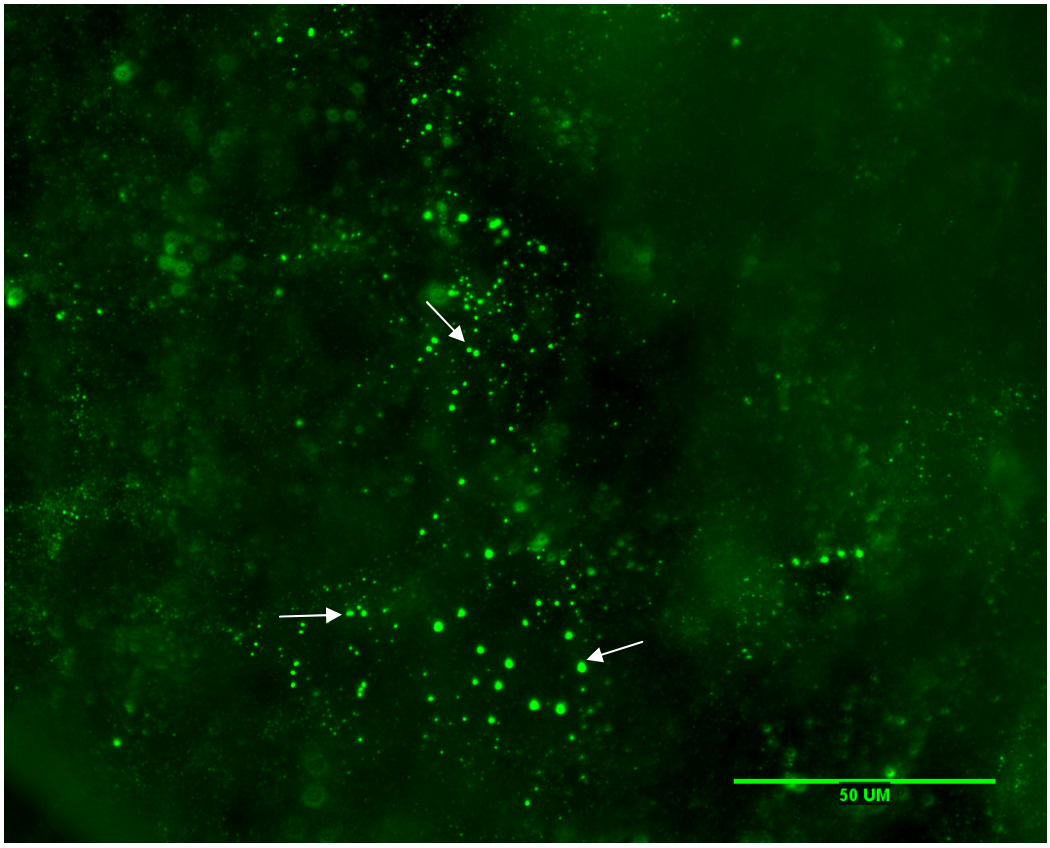


Figure3.5; **Kainate KA2 immunohistochemistry in cornea (whole mount)**. There were numerous scattered immunoreactive granules in corneal epithelial cells for KA2 receptor subunit as indicated by the white arrows.

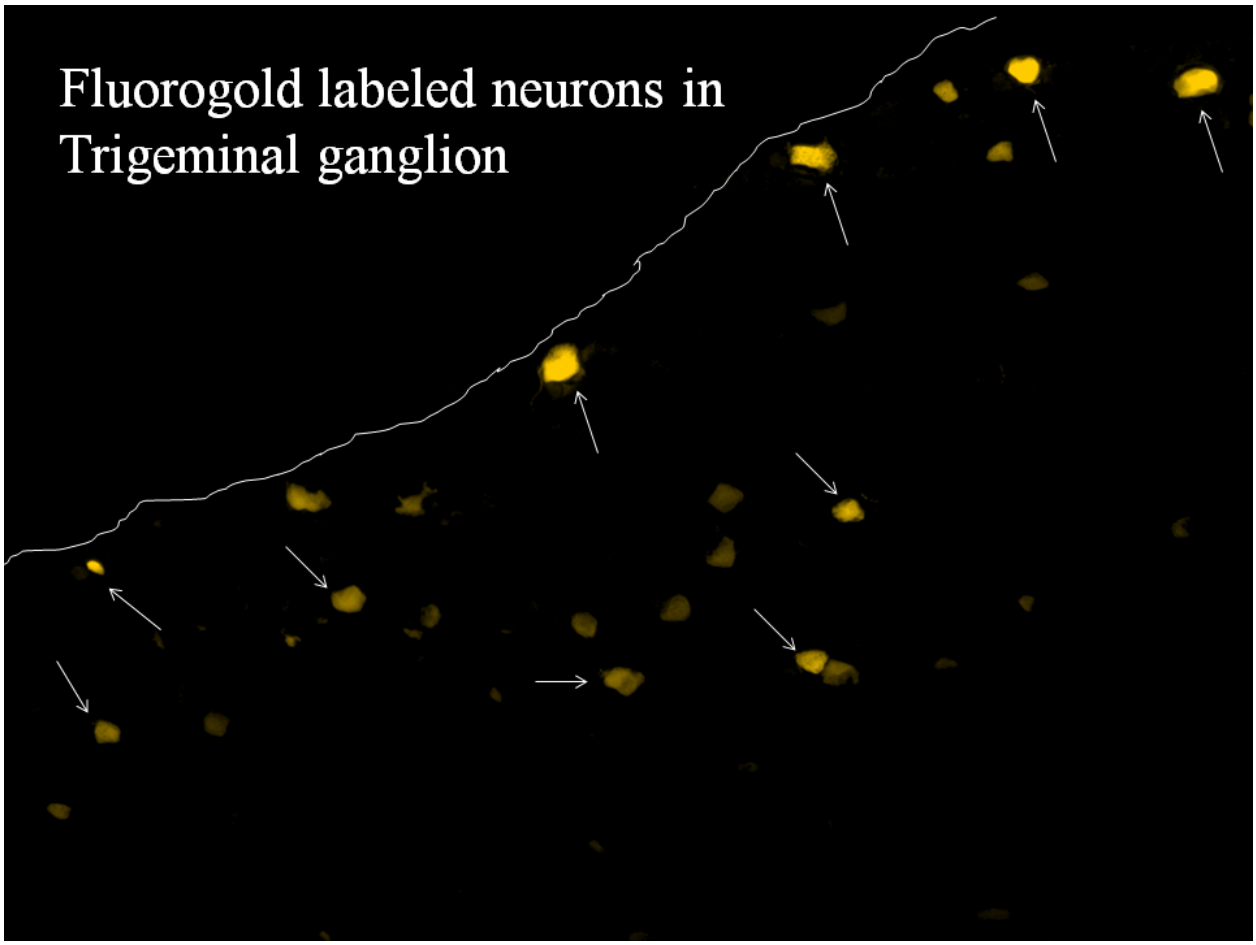


Figure 3.6. **Retrograde labeling of trigeminal neurons from cornea**. Fluorogold was injected into the cornea and, after five days, the trigeminal ganglion was processed for fluorescence microscopy. Numerous Fluorogold neurons, arrows point out examples, were retrogradely labeled from the cornea.

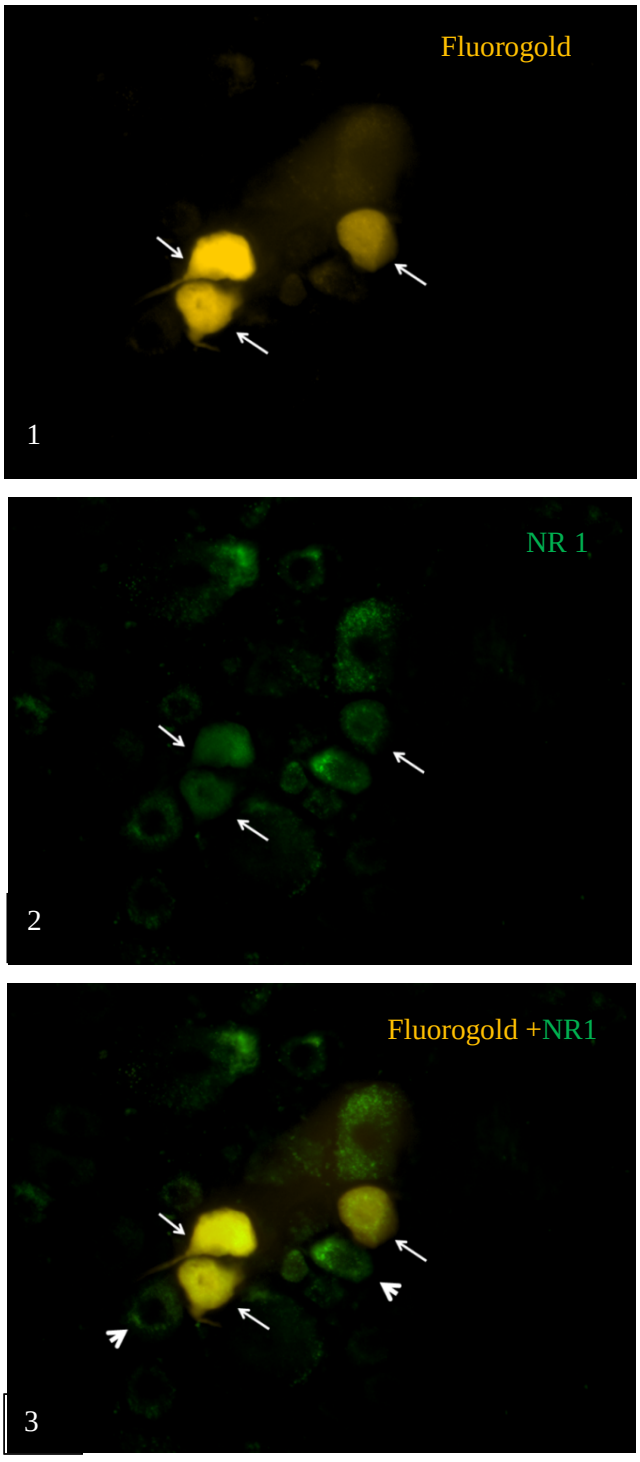


Figure3.7; **Retrograde tracing study for NR1**. NR1 immunoreactivity (arrows, arrowheads) was localized to all retrogradely labeled neurons (arrows).

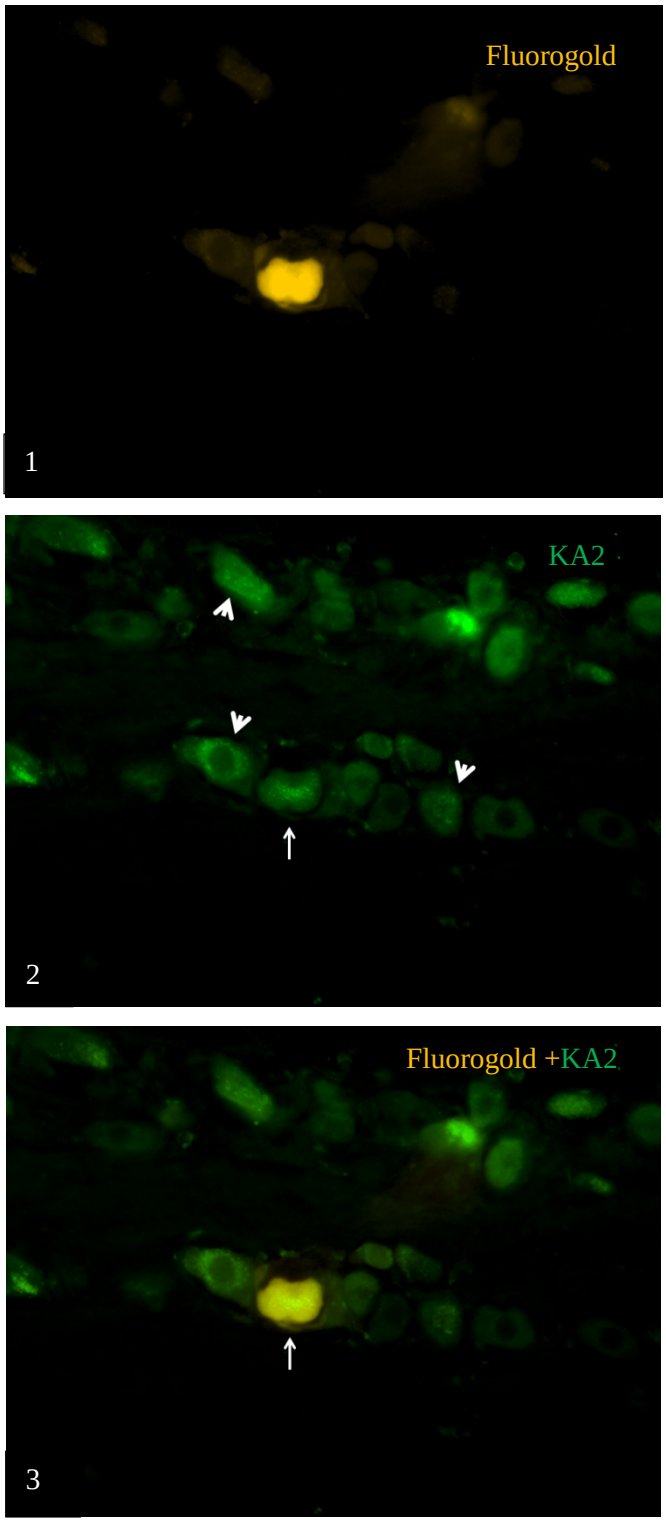


Figure 3.8; **Retrograde tracing study forKA2**. KA2 immunoreactivity (arrows, arrowheads) was localized to all retrogradely labeled neurons (arrows).

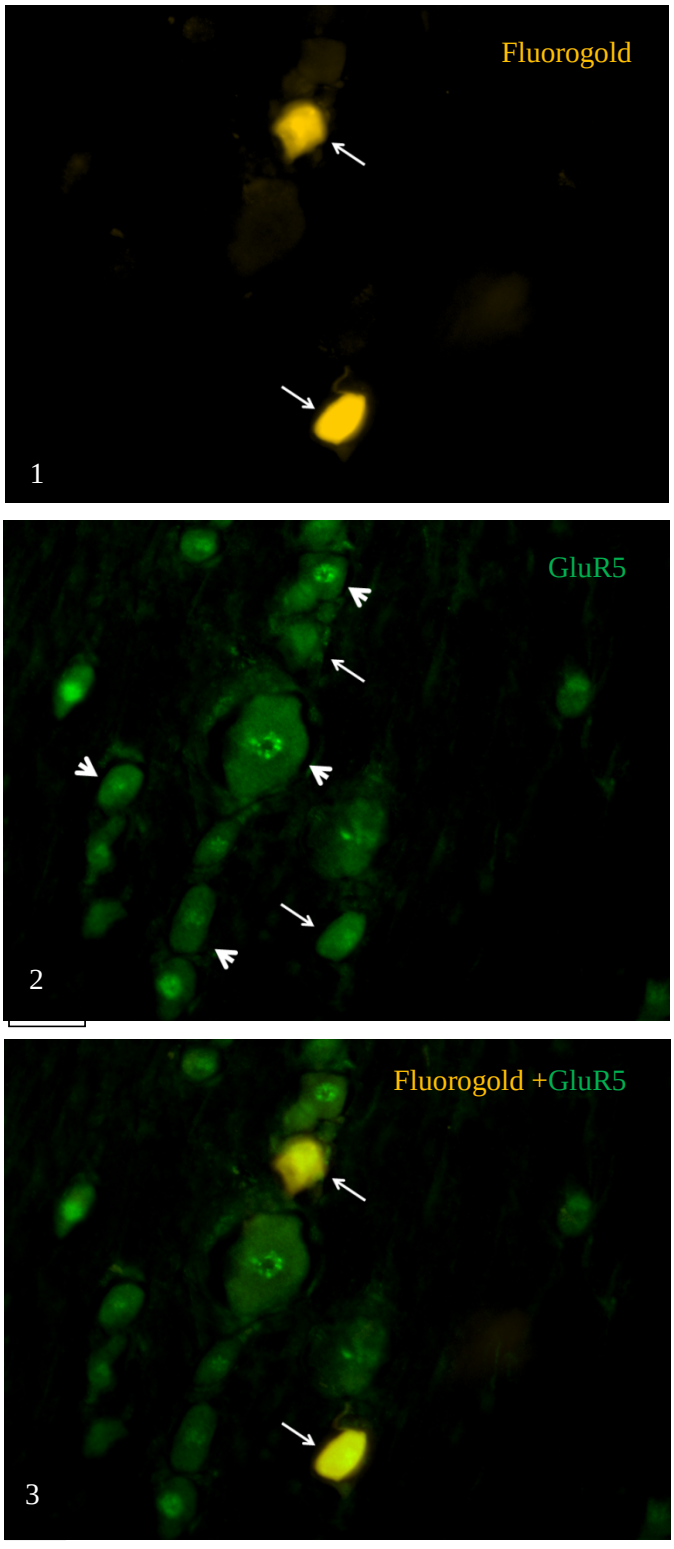


Figure3.9; **Retrograde tracing study forGluR5**. GluR5 immunoreactivity (arrows, arrowheads) was localized to all retrogradely labeled neurons (arrows).

3.4 DISCUSSION

The present study demonstrates that NR1 (NMDA), GluR1 (AMPA), and GluR5 (kainate) EAAR subunits are present in corneal afferent nerves and/or corneal trigeminal neuronal cell bodies. These findings are consistent with previous studies that localized ionotropic EAARs to unmyelinated axons in the rat dermis (Carlton et al., 1995, Coggeshall et al., 1997, Coggeshall and Carlton, 1998, Du et al., 2003). For example, immunohistochemical studies localized EAAR subunits in rat sural and plantar nerves at the ultrastructural level: NMDA NR1 (21-30% of unmyelinated nerves), NR2B, GluR1 (8-17%), GluR2/3 and GluR5-7 (7-44%). In the rat facial skin, most cutaneous afferents have NR2B immunoreactivity (Gazerani et al., 2010a), as well as muscle afferents in female rat temporalis muscle (Gazerani et al., 2010b). In the human hairy skin, unmyelinated axons (~25%) below the dermal–epidermal junction contain NR1, GluR 2/3, GluR5/6/7 immunoreactivity (Kinkelin et al., 2000), whereas afferents in human tendon contain NR1 immunoreactivity (Alfredson et al., 2001a).

To our knowledge, this is the first demonstration of EAAR subunit immunoreactivity in nerve fibers among epithelial cells. Corneal afferents are both unmyelinated and myelinated, but myelination is not maintained upon axonal entry into the cornea stroma for corneal transparency (Belmonte et al., 1997; Muller et al., 2003). Within the cornea, nerve bundles divide to form plexuses to supply the anterior corneal stroma and epithelium (Marfurt et al., 2001). Afferents form a subepithelial nerve plexus and corneal epithelial leashes, comprised of 2-6 axons, from the plexus innervate superficial corneal epithelial cells (Belmonte et al., 1997; Marfurt et al., 2001, Muller et al., 2003). The epithelial leashes are varicose fibers tangential to the corneal surface, parallel to one another, and terminate in the epithelium as free nerve endings (Marfurt et al., 2001; Beckers et al., 1993). In the current study, NR1 and GluR1 immunoreactivity occurred in nerve fibers of the stroma, subepithelial plexus, leashes, and/or free nerve endings. Glutamate release in the cornea from epithelial cells or peripheral nerve terminals potentially could interact

with nearby nerve endings in either the stroma or the epithelium.

Glutamate released in the cornea also could interact with corneal epithelial cells. In the current study, epithelial cells contained immunoreactivity for NR1 (NMDA), GluR2/3 (AMPA), and KA2 (kainate) EAAR subunits. Other investigators have identified EAAR subunits in cells in the stratum granulosum of the skin(Fischer et al., 2004b), buccal epithelium (Fischer et al., 2004a), olfactory epithelium (Borgmann-Winter et al., 2009) and renal tubular epithelium (Bozic et al., 2011). Furthermore, there is evidence that glutamate is present in corneal epithelial cells (Langford et al., 2010). Stimulation of EAARs on epithelial cells is thought to sensitize resident cells and surrounding afferents. It also has been shown that EAARs play a role in regulating calcium homeostasis in keratinocytes and calcium induced keratinocyte differentiation (Fischer et al., 2004a; Fischer et al., 2004b; Fischer et al., 2009).

Glutamate is released peripherally from nociceptive afferent nerve terminals in response to high threshold stimuli in skin, joint, and muscle (Cairns et al., 2001; Du et al., 2001; Miller et al., 2011a). Glutamate interacts with EAARs on peripheral afferent nerve terminals to cause nocifensive behavior and sensitization in both normal and inflamed tissues (Cairns et al., 2001; Du et al., 2001; Miller et al., 2011a). Examples of EAAR that have been reported to produce sensitization in peripheral tissues include NMDA, AMPA and kainate receptors (Du et al., 2003; Du and Carlton, 2006; Miller et al., 2011a). The presence of glutamatergic nerve fibers (Miller et al., 2011b; Ibitokun – Chapter 2) and EAARs in rat cornea suggests that glutamate may have a similar effect in the cornea. The nocifensive role of EAARs in the rat cornea will be addressed in the next chapter.

CHAPTER 4

IONOTROPIC GLUTAMATE RECEPTORS IN THE RAT CORNEA: BEHAVIORAL EVALUATION

Aim: To determine the relative contribution of excitatory amino acid receptors (EAARs) on behavioral responses to glutamate, ionotropic EAAR receptor agonists, and EAAR antagonists.

Methods: The number of cornea nocifensive or irritation behaviors (blinking, wiping and head shaking movements) were counted up to 30 seconds after instillation of increasing concentrations of: monosodium glutamate , NMDA (N-methyl-D-aspartate), AMPA (2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid) and kainate. The effect of increasing concentrations of EAAR antagonists were determined on respective EAAR agonists, D-AP5 [D-(-)-2-Amino-5-phosphonopentanoic acid, (NMDA antagonist)], NBQX [2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt, (AMPA antagonist)], and CNQX [6-cyano-7-nitroquinoxaline-2,3-dione disodium, (AMPA/kainate antagonist)].

Results: Glutamate, AMPA and kainate independently induced corneal irritation behaviors in a dose dependent fashion. The effects were blocked with complementary EAAR antagonists. NMDA did not induce significant nocifensive responses, however, D-AP5, NMDA antagonist, did produce a dose dependent inhibition of glutamate-induced behavior.

Conclusion: These data indicate that EAARs can contribute to corneal irritation or nocifensive behavior. Data also show that antagonism of corneal glutamate receptors can inhibit EAAR-induced corneal irritation behavior. EAARs may be important pharmacological targets for controlling corneal pain/irritation.

4.1 INTRODUCTION

Glutamate is an excitatory neurotransmitter released from peripheral Aδ and C sensory nerve terminals (Miller et al., 2011a). The peripheral transport from trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons occurs in synaptic vesicles containing vesicular glutamate transporter 1 or 2 (VGLUT1/2; Aihara et al., 2000, Bellochio et al., 2000, Moechars et al., 2006, Brumovsky et al., 2007, Ibitokun, 2012 – Chapter 2). Peripheral release of glutamate from nociceptive primary afferent nerve terminals in skin, muscle and joint occurs upon strong noxious stimulation. Glutamate released from peripheral nerve terminals exerts a sensitizing effect on surrounding afferents and resident cells and participates in the neurogenic inflammatory response (Cairns et al., 2001, Du and Carlton, 2006, Miller et al., 2011a). The effect of glutamate on the cornea, however, is not known.

There is evidence that glutamate is present in human corneal superficial epithelial cells and stroma and in corneal afferents (Langford et al., 2010; Miller et al., 2011b). Substance P (SP) and calcitonin gene-related peptide (CGRP), neurotransmitters often co-released with glutamate, have been described in corneal primary afferent nerve fibers and terminals (Sasaoka et al., 1984, Beckers et al., 1993, Belmonte et al., 1997, Marfurt et al., 2001, Muller et al., 2003). We previously identified synaptic vesicle-related proteins, vesicular glutamate transporter 2 (VGLUT2), synaptophysin I and SNAP 25 (synaptosomal associated protein 25), in nerve fibers of the rat cornea (Ibitokun – Chapter 2). These results indicated the potential release of glutamate from corneal primary afferent nerve terminals, similar to afferents in other peripheral tissues (Brumovsky et al., 2007; Miller et al., 2011a). In addition, subunits of excitatory amino acid receptors (EAARs) have been identified immunohistochemically in corneal primary afferents and epithelial cells (Ibitokun, 2012 – Chapter 3). Stimulation of EAARs in skin, joint, and muscle produces nocifensive behavior and pain responses in rodents and humans, respectively (Cairns et

al., 2001, Du and Carlton, 2006, Miller et al., 2011a). The presence of corneal glutamate and EAARs suggests that similar events may occur from glutamatergic interactions in the cornea. To further understand the role of glutamate in the cornea, the present study aims at investigating the contribution of peripheral EAARs in cornea to irritative/nocifensive behavior.

4.2 METHODS

4.2.1 Animals

All experiments were approved by the Oklahoma State University Animal Care and Use committee. A standardized corneal nociceptive animal model was employed to determine if stimulation of corneal glutamate receptors contribute to corneal irritation/nocifensive behavior (Gonzalez et al., 1993; Kárai et al., 2004; Bates et al., 2010). Adult Sprague Dawley rats (250 to 450g) were used in all experiments. Rats were housed in groups of 2 to 3 in plastic cages with soft bedding under a 12-hour light/dark cycle. They had access to food and water ad libitum. Steps were taken to minimize the number of animals used and their discomfort. Some animals were used more than once in this study, but this only occurred 7 days after initial use. All corneal stimulations were limited to 30 sec and eyes were irrigated with water following stimulation.

4.2.2 Dose response assay for EAAR agonists

In order to detect nocifensive behavioral responses from EAAR stimulation, the following solutions were prepared: 1. monosodium glutamate (Sigma) - 0.01, 0.03, 0.1, 0.3, 0.5, 1.0M; 2. AMPA (2-amino-3-(5-methyl-3-oxo-1, 2- oxazol-4-yl) propanoic acid; (Tocris)) - 0.1, 0.3, 1.0, 3.0, 10 mM; 3. Kainate (Sigma) - 0.001, 0.033, 0.1, 0.33, 1.0, 3.3 and 10 mM; 4. NMDA(N-methyl-D-aspartate; (RBI)) - 0.1, 0.33, 1.0, 3.3, 10mM. The concentrations for the EAAR agonists were based on previous studies using other nociceptive tests in rodent skin, joints, and muscle (Miller et al., 2011a). The vehicle of instillation for glutamate, NMDA, AMPA and

kainate was phosphate buffered saline (PBS, pH = 7.4; (Carlton et al., 1995; Liu et al., 2002; Du et al., 2003; Du and Carlton, 2006; Miller et al., 2011a). Each animal was removed from its cage, restrained by loosely wrapping it with a surgical towel and the eyelid was parted by hand. Monosodium glutamate, NMDA, AMPA or kainate solution was topically instilled (10µl) into the cornea (n=3-6/ treatment group). Rats were quickly removed from the towel and placed on top of a table with plexiglass walls for observation. The number of nocifensive/irritative behaviors, i.e., blinking, wiping, scratching, and head shaking movements, were counted up to 30 seconds after instillation and compared with animals that received only vehicle (Gonzalez et al., 1993, Karai et al., 2004, Bates et al., 2010).

4.2.3 Antagonism of EAAR agonists

In order to antagonize EAAR-agonist induced behavioral responses, the following solutions were prepared: 1. NBQX (2, 3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt; AMPA antagonist; Tocris) - 0.033, 0.1, 0.33, 1 µg/µl; 2. CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt; AMPA/kainate antagonist; Tocris) - 0.001, 0.01, 0.033, 0.1, 0.33, 1 mM. The concentrations for the EAAR antagonists were based concentrations used in previous studies (Miller et al., 2011a). The vehicle of instillation for CNQX was phosphate buffered saline (PBS, pH = 7.4; (Liu et al., 2002; Miller et al., 2011a) while PBS with 1% Dimethyl sulfoxide (DMSO) was the vehicle for NBQX (Lin et al., 1999; Miller et al., 2011a). Solutions were made in vehicle with or without their respective agonist: AMPA (0.1mM) or kainate (0.1mM). All the animals received their drugs by topical instillation into the cornea as described above. Animals in each group were pretreated with 10µl NBQX or CNQX alone. After 10 minutes pretreatment with antagonist, animals were co-treated with 10µl of a mixture of AMPA/NBQX or kainate/CNQX solutions. The pretreatment concentrations of the antagonists were the same as the co-treatment concentrations. Agonist concentration was determined byED_{50} or the minimum dose that induced corneal irritation on the dose response assay. Nocifensive

responses were counted up to 30 seconds after instillation. These responses were compared with animals that received only agonist or vehicle.

4.2.4Antagonism of glutamate with D-AP5

The NMDA receptor may be activated following AMPA and/or kainate receptor activation and membrane depolarization with glutamate, the endogenous neurotransmitter. In order to antagonize NMDA-related behavioral responses using glutamate, the following solutions were prepared: D-AP5 (D-(-)-2-amino-5-phosphonopentanoic acid; NMDA antagonist; Tocris) - 0.1, 0.3, 1.0, 3.0 and 10µg/µl. Solutions were made in vehicle with or without monosodium glutamate (0.5M). Animals were pretreated with 10µl D-AP5 alone and, after 10 minutes pretreatment, animals were co-treated with 10µl of a mixture of glutamate/AP5 solutions. Nocifensive behaviors were counted up to 30 seconds after instillation. These responses were compared with animals that received only agonist or vehicle.

4.2.5 Data Analysis and Statistics

Values were reported as means ± SEM. Statistical significance was determined using one way ANOVA and Newman Keul's post hoc multiple comparison test. P values ≤ 0.05 was considered as significant. For comparison with glutamate response, data were converted into a percentage of maximal-minimal responses for determining dose response curves. A maximal behavioral response of 24 with glutamate stimulation was considered 100% and minimal vehicle response of 1 was considered 0%. All data were converted into a percentage of maximal-minimal responses for determining dose response curves (Miller et al., 2011b). Statistical tests were performed with GraphPad Prism software version 5.0 (San Diego, California USA).

$$\text{Maximum percent effect} = \frac{\text{(Post drug latency – Baseline)}}{\text{Cut off – Baseline}} \times 100$$

4.3 RESULTS

4.3.1 Glutamate-induced corneal irritation

Topical instillation of monosodium glutamate solution at 0.01-0.1M into intact rat cornea produced no significant behavioral responses compared to responses with vehicle. Topical application of 0.3M glutamate was the first dose that produced the irritative behaviors of wiping, blinking, scratching, and head shaking and the behaviors peaked at 1.3M glutamate (Fig. 4.1.1, $P \leq 0.05$). The glutamate-induced behavior, therefore, occurred in a dose dependent fashion with an ED_{50} of 0.5M (Fig.4.2.1).

4.3.2 AMPA-induced corneal irritation

AMPA-induced corneal nocifensive behavior only occurred at the dose of 1.0mM AMPA (Fig. 4.1.2; $P \leq 0.05$) and this was approximately 30% of the maximal response from glutamate (Fig. 4.2.2). Lower (0.1-0.3mM) and higher (3-10mM) doses did not produce responses different from vehicle (Fig. 4.1.2). Curve fitting of the dose responses, therefore, produced an "inverted U-shaped dose-effect curve" (IUSDEC; Fig. 4.1.2; (Baldi and Bucherelli, 2005). From the 0.1-1.0mM dose response, an ED_{50} of 0.5mM for AMPA was determined.

4.3.3 Kainate-induced corneal irritation

Topical application of kainate induced corneal irritation only at the 1mM concentration (Fig. 4.1.3, $P \leq 0.05$) and this was approximately 20% of the maximal response from glutamate (Fig. 4.2.3). Lower (0.001-0.3mM) and higher (3-10mM) doses did not produce responses different from vehicle (Fig. 4.1.3). As was observed with AMPA, curve fitting of kainate responses produced an IUSDEC (Fig. 4.1.3; (Baldi and Bucherelli, 2005). From the 0.001-1.0mM dose response, an ED_{50} of 0.1mM for kainate was determined.

4.3.4 NMDA-induced corneal irritation

Instillation of the cornea with NMDA solutions in the concentration range of 0.1-10mM did not induce significant different nociceptive responses compared to vehicle (Fig. 4.1.4).

4.3.5 Blockade of AMPA-induced corneal irritation

To block AMPA induced stimulation, a fixed concentration of 1.0mM AMPA was used with varying concentrations of NBQX, a specific AMPA antagonist. At low concentration of 0.033µg/µl NBQX, the nocifensive behavioral responses were not significantly different when compared with animals that received 1.0mM AMPA (Fig. 4.3.1). At higher concentrations of 0.1-1µg/µl, NBQX reduced the corneal irritation that was induced by 1.0M AMPA (Fig. 4.3.1; $P \leq 0.05$). Antagonism dose response curves determined an $ID_{50}$of 0.03µg/µl for NBQX inhibition of 1.0mM AMPA (Fig. 4.4.1).

4.3.6 Blockade of kainate-induced corneal irritation

With the co-treatment of 0.1mM kainate and increasing concentrations of CNQX, kainate/AMPA antagonist, the nocifensive responses were significantly reduced in a dose dependent fashion (Fig. 4.3.2). The highest concentrations of CNQX used in the study (0.033, 0.1, 0.33 and 1mM) reduced the kainate-induced responses ($P \leq 0.05$). Antagonism dose response curves determined an $ID_{50}$of 0.02µg/µl for CNQX inhibition of 0.1mM kainate (Fig. 4.4.2).

4.4.7 Blockade of glutamate-induced corneal irritation

Glutamate activation of AMPA and kainate receptors depolarizes neuronal cellular membranes and this depolarization removes the Mg^{2+} block of NMDA receptors, allowing activation by glutamate (Nowak et al., 1984). To explore the role of NMDA receptors in glutamate-induced nociceptive behavior, NMDA antagonism with D-AP5 was used in conjunction with glutamate (0.5M). Doses from 0.1-3.3µg/ml D-AP5 had no effect on glutamate, but 10µg/µl D-AP5

reduced glutamate induced corneal irritation (Fig. 4.3.3, $P \leq 0.05$). Antagonism dose response curves determined an ID_{50} of 3µg/µl for D-AP5 inhibition of 0.5M glutamate (Fig. 4.4.3).

Fig. 4.1.1 Glutamate dose response study

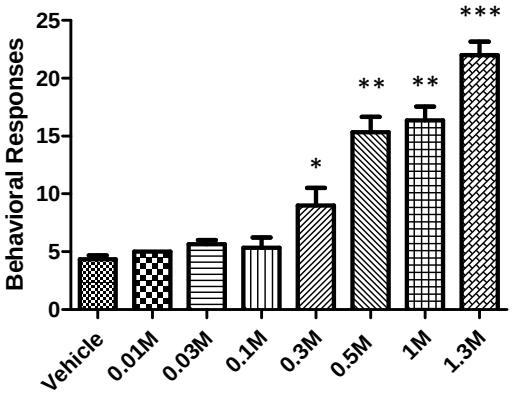


Fig. 4.1.2 AMPA dose response study

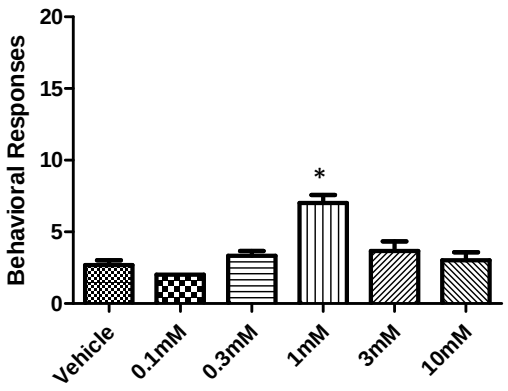


Fig. 4.1.3 Kainate dose response study

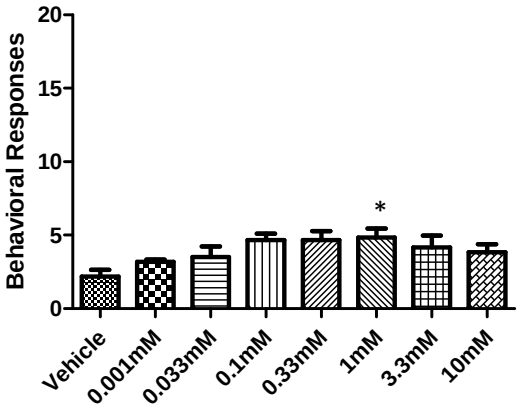


Fig4.1.4 NMDA dose response study

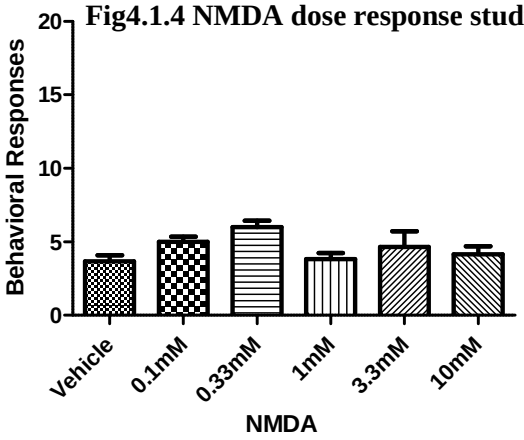


Figure4.1 **Nocifensive behavioral assay – EAAR agonists**. Monosodium glutamate, AMPA and kainate (Fig. 4.1.1– 4.1.3) induced corneal irritation in a dose dependent fashion. NMDA did not produce significant behavioral responses (Fig. 4.1.4). * Indicates a significant difference, one way ANOVA. $P < 0.05$.n = 3-6 for each treatment group.

Fig 4.2.1 Glutamate dose response curve

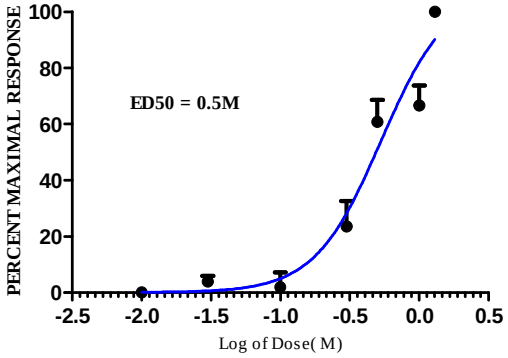


Fig 4.2.2 AMPA dose response curve

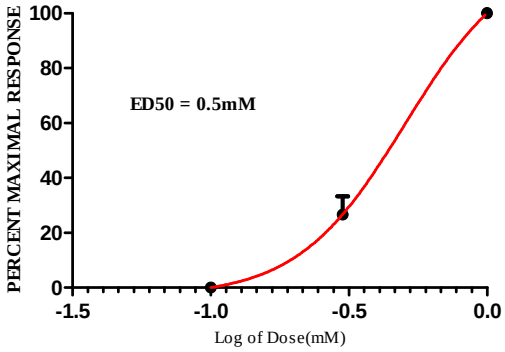


Fig 4.2.3 Kainate dose response curve

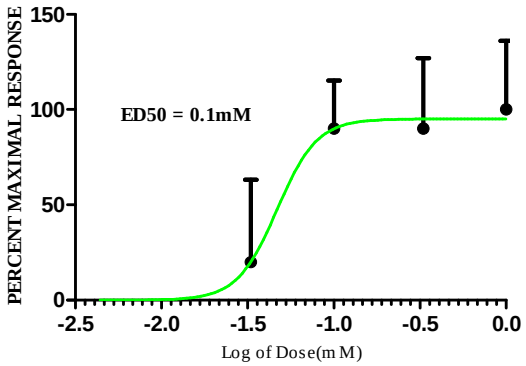


Figure 4.2. **Agonist dose response curves**. Fig. 4.2.1-4.2.4 are dose response curves for glutamate (Fig. 4.2.1), AMPA (Fig, 4.2.2) and kainate (Fig. 4.2.3). Glutamate, AMPA and kainate elicited significant nocifensive responses in a dose dependent fashion (ED_{50} = 0.5M, 0.5mM and 0.1mM respectively). There was no significant response with NMDA.

Fig. 4.3.1 AMPA Inhibition with NBQX

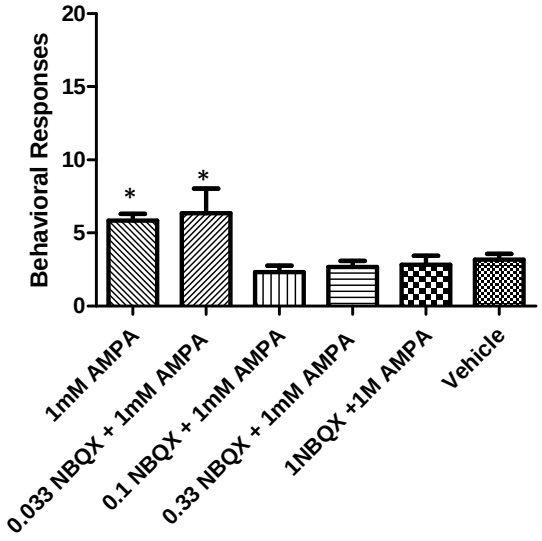


Fig. 4.3.2 Kainate Inhibition with CNQX

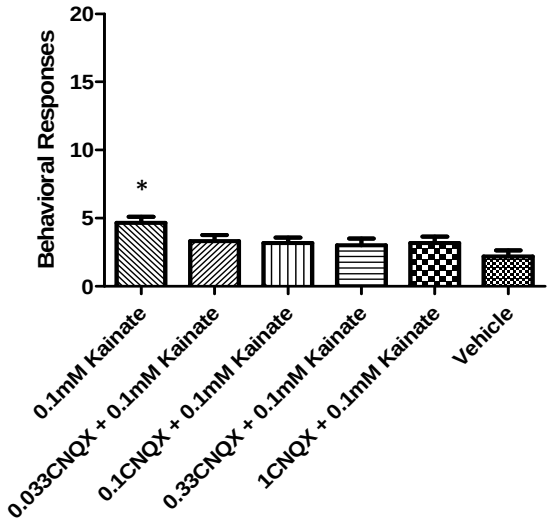


Fig. 4.3.3 NMDA Inhibition with D-AP5

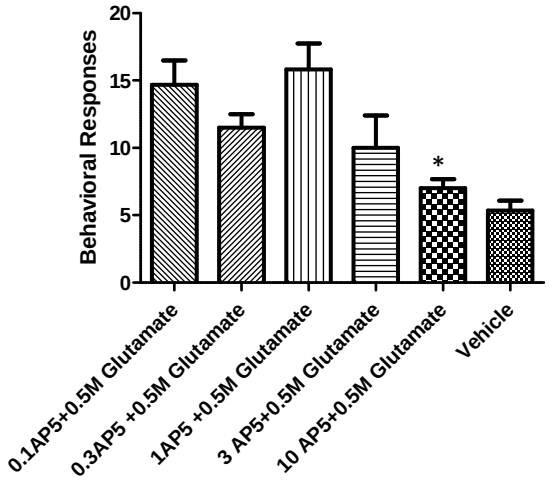


Figure4.3.**Nocifensive behavioral assay – EAAR antagonists**. NBQX, AMPA antagonist, significantly blocked responses induced by AMPA in the cornea (Fig. 4.3.1). In Fig 4.3.2, CNQX, kainate/AMPA antagonist, did not decrease responses significantly from 0.1mMkainate. D-AP5, NMDA antagonist, significantly blocked glutamate-induced nocifensive responses in a dose dependent fashion (Fig. 4.3.3). (* Indicates a significant difference, one way ANOVA, $P < 0.05$. n = 3-6 for each treatment group.)

Fig 4.4.1 Antagonist inhibition curve of AMPA with NBQX

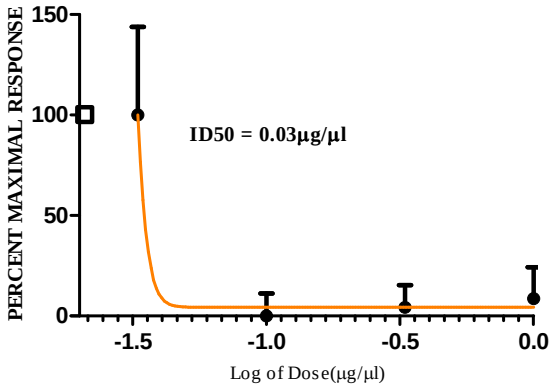


Fig 4.1.2 Antagonist inhibition curve of glutamate with D-AP5

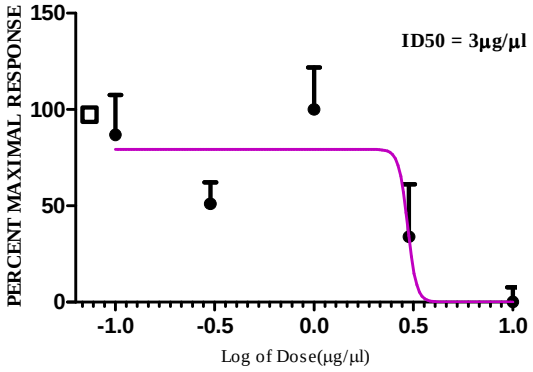


Figure 4.4 **Antagonist dose response curves**. Fig. 4.4.1 is antagonist inhibition curve for inhibition of AMPA-induced nocifensive behavior. Fig. 4.4.2 is the antagonist inhibition curve for D-AP5, NMDA antagonist, exhibiting inhibition of glutamate-induced behavior. NBQX and AP5 significantly blocked AMPA and glutamate induced responses (ID_{50} = 0.03µg/µl and 3µg/µl, respectively).

Fig 4.5 Comparison of responses between EAAR in rat cornea

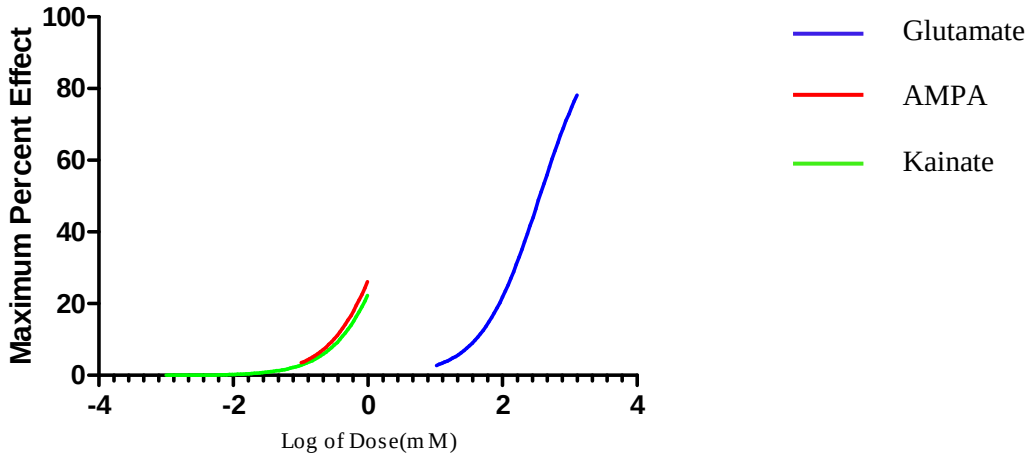


Figure 4.5; **Comparison of responses to AMPA, kainate and glutamate in the cornea**. AMPA and kainate have higher potency when compared with glutamate. Glutamate has a higher efficacy than AMPA and kainate in the cornea.

4.4 DISCUSSION

Glutamate is the excitatory neurotransmitter used by primary sensory neurons. In addition to release in the central nervous system, nociceptive afferents release glutamate from peripheral terminals in response to noxious or tissue damaging stimuli (Julius and Basbaum, 2001; Woolf and Ma, 2007; Miller et al., 2011a). Glutamate interacts with EAARs on peripheral terminals and excites the nociceptive afferents (Carlton et al., 1995; Du et al., 2001; Woolf and Ma, 2007; Miller et al., 2011a). This produces nocifensive responses and is perceived clinically as pain (Carlton et al., 1995; Du et al., 2001; Julius and Basbaum, 2001; Miller et al., 2011a). The present study demonstrated that topical instillation of glutamate, AMPA, and kainate into the cornea induces corneal irritation and nocifensive responses in a dose dependent manner. Findings in this study suggest that corneal glutamate receptors can play an important role in corneal nociception. These results are consistent with studies done in skin and muscle, where injection of glutamate and glutamate receptor agonists cause peripheral sensitization of primary afferents (Carlton et al., 1995; Cairns et al., 2001; Du et al., 2003; Du and Carlton, 2006; Quinta˜o et al., 2010; Miller et al., 2011a).

The findings in the current behavioral study corroborate data from our immunohistochemical study demonstrating that EAARs are present on corneal afferent nerves and epithelial cells. We also previously reported that glutamate and VGLUT2 is present in the rat corneal afferent nerves (Miller et al., 2011b; Ibitokun – Chapter 2). Additionally this is supported by studies localizing glutamate in superficial corneal epithelial cells and stroma (Langford et al., 2010). These observations from the current study further indicate that glutamate interacts with its receptors in the cornea to produce nociception. Furthermore, blockade of EAARs in rat cornea reduced EAAR-induced corneal irritation and nocifensive responses. This observation is consistent with

studies reporting that EAAR antagonists attenuate glutamate induced excitation in peripheral tissues (Carlton et al., 1995; Lin and Kinnamon, 1999; Peng et al., 2011; Miller et al., 2011a).

4.4.1 Glutamate. A number of studies have evaluated the nocifensive responses to glutamate, EAAR agonists, and EAAR antagonists from the skin of rats and mice. Injection of glutamate into the rat hindpaw or tail produces thermal hyperalgesia in the thermal plantar, hot water bath, and tailflick tests (Jackson et al., 1995; Carlton et al., 1998; Jin et al., 2009). These responses are enantiomer selective, dose dependent, occur within minutes of injection, and last up to 4 hr (Jackson et al., 1995; Jin et al., 2009; Lin et al., 2009; Carlton et al., 1998). Injection of intraplantar (i.pl.) glutamate into the rat hindpaw or subcutaneous (s.c.) injection into the rat tail produces mechanical hyperalgesia and allodynia in several behavioral tests, e.g., Randall-Selitto, plantar anesthesiometer, and von Frey filaments (Follenfant and Nakamura-Craig, 1992; Carlton et al., 1995; Coggeshall et al., 1997; Leem et al., 2001; Zanchet and Cury, 2003). L-glutamate, but not D-glutamate, produces a dose dependent mechanical hyperalgesia (Follenfant and Nakamura-Craig, 1992; Carlton et al., 1995; Coggeshall et al., 1997; Walker et al., 2001). The mechanical-evoked responses occur within minutes and can last up to 2 hr (Zanchet and Cury, 2003; Leem et al., 2001; Carlton et al., 1995; Coggeshall et al., 1997). Glutamate (i.pl. 0.01-0.3mM) causes dose dependent mechanical hyperalgesia, but little to no response occurs at a higher dose of 1mM. This has been interpreted to be due to desensitization of EAARs (Carlton et al., 1995; Coggeshall et al., 1997). In the present study, glutamate caused dose-dependent behavioral responses, but we did not observe decrease in response at higher concentrations, up to 1.3M glutamate.

Mechanical and thermal tests evoked nocifensive responses from subcutaneous (s.c.) injections, whereas our study observed spontaneous irritative behaviors in response to topical application of glutamate. In this regard, s.c. injection of glutamate evokes nocifensive responses or spontaneous

pain in rodents (Carlton et al., 1998; Lin et al., 2009; Beirith et al., 2002, 2003; Quintão et al., 2010). Spontaneous tail flicks occur when 5.0mM glutamate is co-administered s.c. with 0.01mM Substance P (SP), but no response occurs with glutamate alone (Carlton et al., 1998). In mice, i.pl. injection of glutamate produces dose dependent licking of the injected foot (Beirith et al., 2002; Lin et al., 2009). Licking behavior occurs within 15 min and lasts 30 min (Beirith et al., 2002). Also, there is evidence that injection (s.c.) of glutamate into the forehead of men and women elicits dose dependent pain and skin sensitization (Gazerani et al., 2006). The pain is reported as moderate in intensity and lasts 5-10 min. In addition, at high concentration (100mM, 100µl), glutamate produces secondary hyperalgesia in humans (Gazerani et al., 2006). Glutamate injection into the human masseter muscle causes dose dependent muscle pain perceptions for 5-10 min that spreads to other areas, e.g., temporomandibular joint (TMJ), teeth, and temple (Cairns et al., 2001a,b, 2003; Wang et al., 2004; Svensson et al., 2003, 2005, 2008; Arendt-Nielsen et al., 2008). The present experimental design was constructed to evaluate the stimulation of EAARs in the corneal epithelium by topical instillation. Most behavioral responses for glutamate and other EAAR agonists occurred within 15 sec following topical application, although nocifensive behavior was observed up to 30 sec. Activation of EAARs on stromal afferents cannot be excluded in this design, but EAARs in the corneal epithelium most likely were the first to be activated. Compared to other reports, response times in our study occurred quickly, but were of short duration. S.c. injection of glutamate and EAAR agonists most likely produce a depot effect (Moll et al., 2004)causing longer responses than topical delivery. Alternatively, the EAARs on corneal afferents or cells could desensitize quickly and behavioral responses, thereby, promptly curtailed. Future studies should explore this issue, possibly with longer acting EAAR agonists, as well as determining if the corneal afferents have been sensitized with EAAR agonists. This could be examined by using electrophysiological recording of ophthalmic afferents or with the use of evoked mechanical and thermal responses (Belmonte et al., 1997).

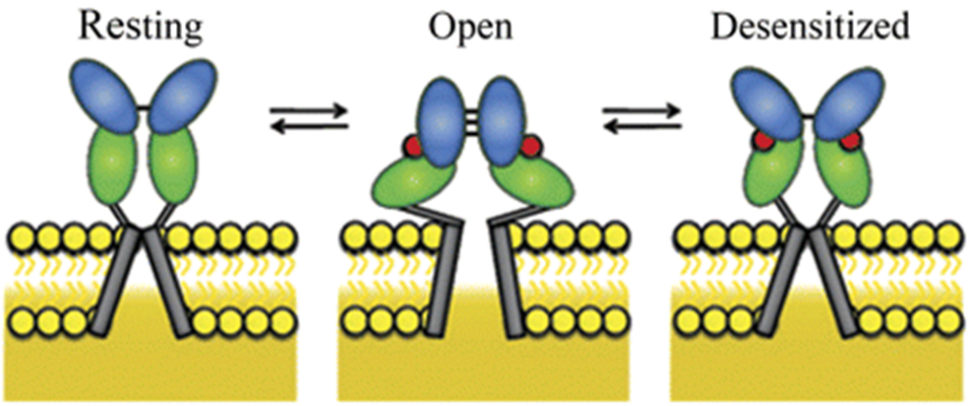
4.4.2 AMPA. I.pl. application of AMPA in rats dose-dependently produces thermal hyperalgesia within 15 min lasting for 3-4 hr (Jin et al., 2009). I.pl. AMPA-induced mechanical hyperalgesia occurs within 20 min and persists for 1hr (Zhou et al., 1996). In mice, i.pl. injection of AMPA produces mechanical hyperalgesia dose dependently, but with lower potency compared to glutamate (Walker et al., 2001). Potency is the amount of drug that is required to produce an effect while efficacy is the maximal response that can be produced by a drug. In our study, AMPA was only 30% effective in eliciting nocifensive behavior compared to glutamate (Fig. 4.5), although the concentration for AMPA's response was 1.0mM compared to 1.0M for glutamate. Thus in the cornea, AMPA has a lower efficacy but higher potency when compared with glutamate.

As with i.pl. injection of AMPA (Zhou et al., 1996), the maximal effect to i.pl. AMPA occurs at 1mM with a sharp decrease in response at 3.0 and 10.0mM. This has been interpreted as desensitization of EAARs (Carlton et al., 1995; Zhou et al., 1996; Coggeshall et al., 1997; Gonzalez et al., 2010). Desenstization of AMPA and kainate receptors has best been studied *in vitro* from dissociated CNS neurons or expressed in immortalized cells (Dingledine et al., 1999; Gonzalez et al., 2010). It has been hypothesized that the AMPA receptor dimer interphase is not coupled together at rest. When AMPA binds to its receptor, a dimer interphase is formed and it stabilizes the open channel form of the receptor, i.e., the receptor is activated (Fig. 4.5). With continued exposure to AMPA, the dimer interphase decouples and the channel closes causing desensitization (Chaudhry et al., 2009; Gonzalez et al., 2010). The termination of ionotropic glutamate receptor response has also been explained in terms of ‘‘deactivation (loss of bound agonist) and desensitization (conversion into an unresponsive state)’’ (Mcfeeters and Oswald, 2004). Desensitization of AMPA receptors is due to subunit composition and RNA splicing of AMPA receptor, e.g., the“flip/flop” region (Mcfeeters and Oswald, 2004). In the kainate receptor,

desensitization is due to differences in the amino acid residues of the subunits (Swanson et al., 1997).

Model of AMPA or kainate receptor channel activation and desensitization

in the presence of agonist



Agonist

Diagram IX. **Model of channel activation and desensitization in the presence of agonist in kainate and AMPA receptors.** In the resting state, no agonist is bound and the channel is closed. When agonist binds to the ligand binding domain, a cleft in the ligand binding domain closes and a conformational change occurs leading to channel activation, i.e., channel opening. In the desensitized state, there is decoupling of the ligand binding domain dimer interface due to mechanical stress (Swanson et al., 1997; Mcfeeters and Oswald, 2004; Gonzalez et al., 2010) and even though the agonist is bound, the channel remains closed. The desensitized state is a lower energy state (Chaudhry et al., 2009).

Co-application of CNQX, AMPA/kainate antagonist, dose dependently blocks AMPA-induced mechanical hyperalgesia (Zhou et al., 1996). Spontaneous nocifensive responses caused by i.pl. glutamate can be blocked by i.pl. pretreatment (30 min) with NBQX (Beirith et al., 2002). In the current study, NBQX was used because it is specific for the AMPA receptor. Similar to effects

observed from s.c. injection of skin, pre- and co-treatment with NBQX dose dependently inhibits spontaneously evoked, corneal nocifensive responses.

4.4.3 Kainate. Kainate injected s.c. causes mechanical hyperalgesia in rats. Kainate s.c. causes a dose-dependent reduction in mechanical threshold within 20 min and lasting for 1 hr (Du et al., 2006; Zhou et al., 1996). 1.0mM kainate produces a maximal response with a sharp decrease in response at 5mM). Potency is the amount of drug that is required to produce an effect while efficacy is the maximal response that can be produced by a drug. Our data reveals that a kainate concentration of 1mM induced significant nocifensive response, but it is only 20% of behavior elicited by glutamate (1.3M), (Fig 4.5). Thus in the cornea, kainate has a lower efficacy but higher potency when compared with glutamate. As with mechanical hyperalgesia, corneal nocicfensive responses at higher concentrations (3.3- 10mM showed a decline in behavioral responses observed, probably due to desensitization, as described earlier (Carlton et al., 1995; Cairns et al., 1998; Cairns et al., 2001).

Co-application of CNQX with kainate dose dependently blocks the 1.0mM kainate induced mechanical hyperalgesia(Du et al., 2006; Zhou et al., 1996). I.pl. glutamate induces paw licking in mice (Beirith et al., 2002) and pretreatment (30 min) with γ-D-glutamylaminomethyl sulfonic acid (GAMS; kainate antagonist; 0.1–3.0 mmol/20µl) decreases the nofensive responses (Zhou et al., 1993).Glutamate injected into the rat tail produces mechanical hyperalgesiathat is blocked by co-injection of CNQX (Zanchet and Cury, 2003). Inhibition of glutamate responses by CNQX could occur by interaction of AMPA, kainate, or both receptors. In our study, we used CNQX (kainate/AMPA receptor antagonist) to block responses induced by kainate. We found that CNQX (0.033-1µg/µl) produced no significant responses. This most likely is due to the kainate concentration that was used as it did not produce significant responses on the dose response study.

4.4.4 NMDA. Injection of NMDA s.c. dose-dependently produces thermal hyperalgesia in the rat hindpaw within 15 min lasting for 3-4 hr (Jin et al., 2009) and in the rat tail within 5 min lasting for over 2 hr (Lin et al., 2009). I.pl. injection of NMDA produces mechanical hyperalgesia, butwith lower potency compared to glutamate (Walker et al., 2001). I.pl. administration of NMDA produces dose dependent mechanical hyperalgesia within 20 min lasting for more than 1 hr and mechanical allodynia for 30-40 min (Zhou et al., 1996). Following i.pl.injection of NMDA, mice undergo licking the injected foot (Lin et al., 2009). Unlike these previous studies with NMDA injection, NMDA did not produce any nocifensive behavior when instilled into the cornea. This may be due to the magnesium block of NMDA receptors, a lack of penetration of NMDA to access receptors, or the concentration used was too low for activation.

Mechanical hyperalgesia induced by NMDA i.pl. administration is blocked by MK-801, NMDA antagonist (Zhou et al., 1996). Since NMDA at the doses used in the current study did not produce behavior responses, D-AP5 did not show an effect, but we did see an effect with glutamate-induce behavior. Previous studies had demonstrated that glutamate induced thermal hyperalgesia is inhibited by MK-801 or AP-7 co-administration (Carlton et al., 1998; Follenfant and Nakamura-Craig, 1992). Glutamate i.pl. produces mechanical hyperalgesia and this effect is attenuated by concurrent administration of MK-801 and AP5 (Zanchet and Cury, 2003; Carlton et al., 1998). Furthermore, i.pl. glutamate-induced paw licking in mice is inhibited by pretreatment with MK 801(Beirith et al., 2002). When injected into the masseter muscle, 1.0M glutamate produces pain responses in human males, but co-injection of ketamine, NMDA antagonist, attenuates glutamate induced pain responses (Cairns et al., 2003). Ketamine reduces the peak response to glutamate as well as the overall amount of pain (Cairns et al., 2003).

Under resting membrane potential, the NMDA receptor is blocked by Mg^{2+} (Diagram VI), but depolarization causes phosphorylation of the NR1 subunit at the serine-896 site and release from the Mg^{2+}block (Chen and Huang, 1992; Xiong et al., 1998; Lan et al., 2001). We did not observe pNR1 in corneal afferents, but did observe pNR1 immunoreactivity in epithelial cells. This would indicate that the Mg^{2+} block in corneal afferents is intact (Mayer and Westbrook, 1987, Monyer et al., 1994) and may be the reason for a lack of response to NMDA in the present study. We tested if D-AP5, NMDA antagonist, could block glutamate induced responses in the current study. We observed that pre- and co-instillation with DAP-5 blocked glutamate-induced nocifensive responses in a dose dependent fashion. This may indicate that glutamate activation of AMPA and kainate receptors lead to membrane depolarization and removal of the magnesium block of the NMDA receptor, and activation of the NMDA receptor (Mayer and Westbrook, 1987). As a result in the current, D-AP5 was able to have an antinociceptive effect.

The observations from the current study indicate that EAARs can produce substantial irritative or nocifensive behaviors and inhibition of EAARs attenuates these responses. Future studies should be directed toward investigating EAARs under corneal inflammation or damage. The results from the current and future studies may aid in the development of topical corneal pain relief by addressing EAAR activation and inhibition.

CHAPTER 5

CONCLUSIONS

Corneal nerves are often damaged and/or inflamed during accidental injuries, abrasions and penetration by foreign objects leading to ocular pain and discomfort. Ocular surgical procedures for correction of refractive errors, such as photorefractive keratectomy (PRK) and Laser in-situ Keratomileusis (LASIK) also have been implicated(Belmonte et al., 2003; Rosenthal et al., 2009). Likewise, infections such as herpes simplex keratitis or autoimmune disease like Sjrogren syndrome (Rosenthal et al., 2009) are known causes of ocular discomfort and pain. Uncontrolled corneal inflammation can impair corneal transparency and eventually lead to loss of vision.

Steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are analgesics of choice, but their use often is limited due to unwanted side effects like desquamation of corneal epithelial cells and corneal perforation (Kimura et al., 1962; Guidera et al., 2001). Local anesthetics are limited because of their short duration of action(Borsook and Rosenthal, 2011) and direct toxic effect on the cornea (Boljka et al., 1994). Opioids sometimes are used for ocular pain, but use is limited due to their addictive properties and other side effects, such as constipation and respiratory depression. Thus, long lasting management of corneal pain is a clinical need.

In this study, the focus was on neurotransmitters, receptors and channels in nociceptors within the primary sensory neuron family. Since glutamate previously was demonstrated in superficial corneal epithelial cells and stroma (Langford et al., 2010) and glutamate is released peripherally from nociceptors in other tissues, the focus was on the mechanism of release of glutamate in the

cornea, the presence of corneal excitatory amino acid receptors (EAARs, i.e., glutamate receptors) and the role of EAARs in the cornea. At the cellular level, synaptic vesicle proteins, vesicular glutamate transporters, and EAAR subunits were evaluated with immunolocalization techniques. Behavioral studies were performed to determine the role of specific EAARs in the cornea under basal conditions.

In chapter 2, synaptophysin I (Fig. 2.3-2.4), VGLUT2 (Fig. 2.1 and 2.2) and SNAP25(Fig. 2.5) were evaluated with immunohistochemistry and Western blotting in rat cornea. Retrograde tracing from the cornea demonstrated that these proteins were from trigeminal ganglion neurons. Their presence in corneal sensory afferents, therefore, provides a cellular mechanism for release into the cornea via synaptic vesicles. In chapter 3, ionotropic EAAR subunits were detected with immunohistochemistry in the cornea. EAARs are expressed on corneal epithelial cells (pNR1; fig. 3.1, GluR2/3; fig 3.4) and afferent nerve fibers in the cornea (NR1; fig 3.2, GluR1; fig 3.3, KA2; fig 3.5). Retrograde tracing from the cornea demonstrated that EAARs are expressed by corneal trigeminal ganglion neurons. Since studies in skin, muscle and joint show that glutamate interacts with its receptors to cause sensitization (Carlton et al., 1995; Cairns et al., 2001; Du et al., 2001; Du et al., 2003; Du and Carlton, 2006; Miller et al., 2011a), we hypothesized that glutamate in the cornea interacts with EAARs to cause irritation/nocifensive behavior. This issue was addressed in chapter 4. Glutamate (Fig. 4.1.1 and 4.2.1), AMPA (Fig 4.1.2 and 4.2.2) and kainate (Fig 4.1.3 and 4.2.3) produced significant corneal irritative and nocifensive behavior in a dose dependent fashion. NMDA, however, did not elicit nocifensive behavior (Fig. 4.1.4 and 4.2.4) possibly due to the magnesium block in the NMDA channel while cellular membranes are hyperpolarized. My study demonstrated that glutamate interacts with EAARs in the cornea to produce irritative and nocifensive behavior. Another important finding was that EAAR antagonists, NBQX (Fig. 4.3.1 and 4.4.1) and AP5 (Fig. 4.3.3 and 4.4.3), were observed to attenuate corneal irritation caused by glutamate or respective glutamate receptor agonists. If corneal EAARs contribute to corneal

nociception and their antagonists can control glutamate induced pain, then EAARS may serve as an important pharmacological target for controlling corneal pain. Topical EAAR antagonists may help to control corneal irritation/pain, thereby circumventing possible CNS side effects, lessening systemic concentrations, and minimizing systemic side effects.

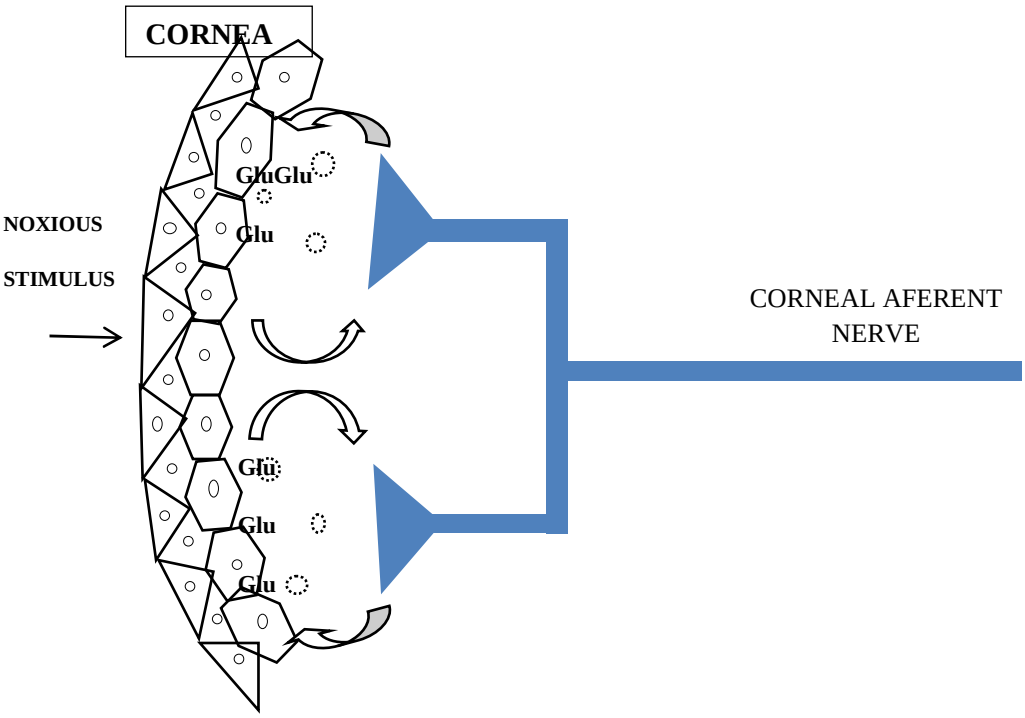


Diagram X. **Summary of study**. This is an illustration of an afferent nerve terminal in the cornea. On application of noxious stimulus, glutamate is released into the cornea by synaptic vesicle exocytosis (Chapter 2). A stimulus at one nerve terminal may cause glutamate release from a collateral axonal terminal by way of the axonal reflex (Yaprak, 2008). Released glutamate acts via its receptors on corneal epithelial cells and afferent nerve fibers in the cornea (Chapter 3) to sensitize resident corneal cells, the afferent nerve from where it is released, and surrounding afferent nerves (Chapter 4). Glu in the figure represent glutamate and dotted arrows represent the direction of glutamate action.

Future directions

Although this study localized VGLUT2 and EAARs in the cornea (Chapter 2 and 3), it did not establish if they are up regulated during corneal inflammation as has demonstrated by studies done in the skin. Studies should be done to determine if expression of VGLUT2 in the cornea is augmented in sensory afferents during corneal inflammation. Also, this current study localized ionotropic EAARs in the rat cornea, but did not determine presence of metabotropic EAARs. If these receptors are present in the cornea, they may also contribute to corneal irritation/pain. A future direction will be to investigate for the expression of metabotropic glutamate receptors in rat cornea which may reveal more effective glutamate receptors leading to the possible development of more potent topical ocular analgesics. This study demonstrated that EAAR antagonists block glutamate-induced corneal irritation/pain (Chapter 4) in experimental animals under basal conditions. However, it does not represent how the antagonist might be used clinically because, as in any basic science research, interpretation of data in rats is subject to limitations, i.e., behavioral responses in rats are interpreted based on our perception of sensations in rats. Nevertheless, behavioral responses observed in rats are comparable to responses due to corneal irritation/pain in man such that our data may be translatable to humans. Thus, a future direction will be to formulate EAAR antagonists in ophthalmic solutions that can be topically instilled into the cornea during inflammation or before ocular surgeries. Electrophysiological studies should be done to explore the possibility of the use of longer acting EAAR agonists to desensitize corneal nerves. This is based on the finding that corneal afferent can easily be desensitized (Chapter 4). Further studies also should be done to determine if there is hyperalgesia after EAAR agonists are applied to the cornea.

On antagonist studies, AMPA and kainate receptor antagonism of glutamate-induced nocifensive behavior should be further explored. Studies on corneal inflammation, quantitation of EAARs followed by behavioral responses to EAAR agonists and antagonists will generate useful

information on antagonist concentration that will be beneficial for pain relief. Ultrastructural studies will also give information on changes that occur to EAARs in the cornea during inflammation. Studies comparing varying concentrations of EAAR agonists with potency (IC_{50}) of their corresponding antagonists should be pursued.(Zhou et al., 1996)

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VITA

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Title of Study: THE ROLE OF GLUTAMATE IN CORNEAL NOCICEPTION

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Major Field: Biomedical Sciences

Scope and Method of Study: At sensory peripheral nerve terminals, glutamate is released into tissues, e.g., skin, where it sensitizes surrounding afferent nerves and resident cells. Sensitization of peripheral sensory afferents causes hyperalgesia and allodyna. Although neurotransmitters like glutamate are released into the cornea in response to triggering stimuli, neither the mechanism of release nor the presence of glutamate receptors in the cornea has been studied in detail. In this study the following projects were performed: (1) immunohistochemistry, Western blot, and retrograde tracing were used to demonstrate synaptophysin I and vesicular glutamate transporter 2 (VGLUT2) in rat cornea sensory nerve fibers and trigeminal neuronal cell bodies; (2) immuno-histochemistry and retrograde tracing were used to localize ionotropic glutamate receptor subunits in rat cornea sensory nerve fibers and trigeminal neuronal cell bodies; (3) nocifensive behavioral analysis was used to determine the role of glutamate receptors in the rat cornea.

Findings and Conclusions: VGLUT2, synaptophysin I, and ionotropic glutamate receptor subunits for NMDA (N-methyl-D-aspartate), AMPA (2-amino-3-(5-methyl-3-oxo-1, 2- oxazol-4-yl) propanoic acid) and kainate are present in intact rat cornea. Glutamate, AMPA, and kainate interact with corneal glutamate receptors to cause nociception/irritation. NMDA did not induce corneal irritation possibly due to magnesium block of the receptor. Ionotropic glutamate receptor antagonists: AP5, (D-(-)-2-amino-5-phosphonopentanoic acid), NBQX (2, 3-aioxo-6-nitro-1, 2, 3, 4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide disodium salt), and CNQX, (6-cyano-7-nitroquinoxaline-2, 3-dione disodium) blocked glutamate-, AMPA-, and kainate -induced corneal irritation, respectively, in a dose dependent fashion. This study suggests that: 1) there is a potential for vesicular glutamate release from corneal afferent nerves, 2) ionotropic glutamate receptors are expressed in corneal afferent nerves and epithelial cells, 3) glutamate interacts with its receptors in the cornea to cause nociception. This suggests that blockade of these receptors may help in controlling inflammatory or maladaptive pain from the cornea.

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