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A POSSIBLE POSITIVE FEEDBACK MODULATION OF ACETYLCHOLINE RELEASE THROUGH THE STIMULATION OF ALPHA-7 NICOTINIC ACETYLCHOLINE RECEPTORS ON BIPOLAR NEURONS IN PIG RETINA

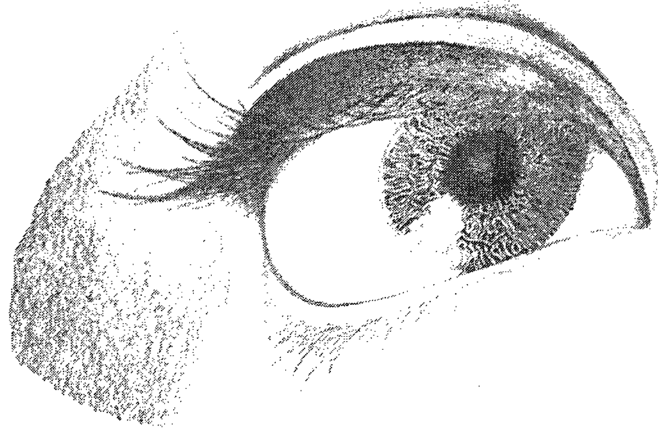
A thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science

By

Viralkumar M. Patel

To

Cell and Molecular Biology Department
Grand Valley State University
Allendale, Michigan
July, 2009



(Modified from Atul Sharma, Atul Art Gallery 2004)

This is dedicated to 'MA GAYATRI',
she helped me through the journey.

ACKNOWLEDGEMENTS

I thank the members of my graduate committee for their support and guidance. I also thank the Radiation Safety Committee and Dr. David Lutkenhoff for their support and radiation safety training. I thank DeVries Meats Co. for providing pig eyes for experiments. I thank Dr. Cindy Linn for her support. I thank Mr. Joshua Stickney for his support in ordering supplies. Above all, I thank John Lelli for his valuable ideas for troubleshooting.

ABSTRACT

A POSSIBLE POSITIVE FEEDBACK MODULATION OF ACETYLCHOLINE RELEASE THROUGH THE STIMULATION OF ALPHA-7 NICOTINIC ACETYLCHOLINE RECEPTORS ON BIPOLAR NEURONS IN PIG RETINA

by Viralkumar Patel

Glaucoma is associated with excitotoxicity in which increased glutamate release leads to apoptotic death of retinal ganglion cells (RGCs). Acetylcholine (ACh) has shown neuroprotection of RGCs through the stimulation of RGCs' nicotinic acetylcholine receptors (nAChRs). The cholinergic amacrine cells are the only cells in retina which synthesize and release ACh. They get excitatory inputs from bipolar cells. The presence of α7nicotinic acetylcholine receptors (α7nAChRs) on the cholinergic amacrines, bipolar cells and RGCs is documented. Recently, stimulation of presynaptic nAChRs of cholinergic cells was shown to enhance ACh release in the rat superior cervical ganglion. Therefore, we hypothesized that α7nAChRs stimulation by tropisetron and PNU282987 (α7nAChR agonists) could induce ACh release through either direct stimulation of cholinergic amacrine α7nAChRs or indirect stimulation of bipolar α7nAChRs. For ACh release studies, pig eyes were dissected and cholinergic amacrine cells were labeled with 40μCi of ^{3}H-choline in which the retina was flashed with light (3Hz) for 30 minutes to maximize ^{3}H-choline uptake. Then, the eyecup was transferred to a perfusion chamber, washed for 20 minutes. 1 minute output fractions were collected into vials and prepared for liquid scintillation counting. To assess the viability of the preparation, light and kainate were applied. Light (2-3 fold increase), kainate (3-4 fold; 10-100μM), α7nAChR agonists (2-4 fold; 0.01-100nM) evoked ACh release greater than the baseline in the absence of DNQX (a glutamate receptor antagonist). In the presence of DNQX, which blocked bipolar input to cholinergic cells, α7nAChRs stimulation did not increase ACh release from baseline. Hence, the possibility of indirect input of bipolar α7nAChRs for ACh release was supported. Our results indicate that ACh release through α7nAChRs stimulation is possible and specifically the bipolar α7nAChRs release of ACh via an indirect positive feedback mechanism. During excitotoxicity, ACh released by amacrine cells, might feedback on bipolar nAChRs to increase ACh release. The neuroprotective effect of tropisetron on α7nAChRs on isolated RGCs is documented. Our study suggests that tropisetron might also protect RGCs through increased ACh release by possible indirect modulation. This study indicates the possibility of dual therapeutic targets of α7nAChRs in the retina for neuroprotection against RGC excitotoxicity.

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CHAPTER I

INTRODUCTION

Neurodegenerative diseases are the result of cell death in associated organs like the brain, spinal cord and retina. Major causes of neurodegenerative diseases are excitotoxicity, inflammation, genetics, aging, injury and free radical generation (Beal et al., 2005). In excitotoxicity, an excitatory neurotransmitter, such as glutamate is increased in an associated organ, which causes neuronal cell death (neurodegeneration) by allowing an excess of calcium into the cell (in addition to other proposed mechanisms). Excitotoxicity is an important factor associated with many neuronal diseases in the central nervous system, including the retina (Choi et al., 1988; Romano et al., 1998; Slemmer et al., 2005). Neurodegenerative diseases caused by excitotoxicity in the brain include multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease (Kim et al., 2002; Hughes, 2009). Some of the diseases proposed to be caused by excitotoxicity in the retina are diabetic retinopathy, glaucoma and retinal ischemia (D'Amico 1994; Brooks et al., 1997; Levin, 2001).

Glaucoma is one of the major diseases causing blindness in the world. The exact mechanisms causing glaucoma have not been fully discovered, but glaucoma is usually associated with increased intraocular pressure which is believed to lead to excitotoxicity (Vickers et al., 1995; Brooks et al., 1997; Dkhissi et al., 1999). In this disease condition, an excess of non-specific cations enter the retinal ganglion cells (RGCs) of the retina

which trigger an intracellular signaling cascade, causing apoptotic death (Quigley et al., 1995; Lam et al., 1999).

In a pig model for glaucoma, excess glutamate can induce blindness. Glutamate excites pig RGCs through both NMDA (N-methyl-D-aspartic acid) and non NMDA receptors (Wehrwein et al., 2004). NMDA receptors are restricted to the inner retina, and the influx of calcium through NMDA receptors in RGCs is believed to trigger an apoptotic cascade. The loss of RGCs interrupts the normal visual pathway of the retina, eventually leading to blindness.

Visual Pathway

In the visual pathway (Brindley 1970), light entering the eye causes a series of events and eventually passes electrical and chemical signals to the brain (Figure 1). The vertical signal transmission is conducted by an excitatory neurotransmitter (glutamate) from photoreceptors to bipolar cells and horizontal cells (in the outer plexiform layer) and from bipolar cells to ganglion cells (in the inner plexiform layer) (Massey, 1990). Horizontal cells excited by the photoreceptor neurotransmitter glutamate provide lateral complex feedback to bipolar cells and photoreceptors in the outer plexiform layer through the release of the inhibitory neurotransmitter GABA. Bipolar cells, which receive signals from photoreceptors, pass signals to ganglion cells by making synapses in the inner plexiform layer where amacrine cells form a much more complex lateral feedback network. Amacrine cells make synapses with other amacrine cells, bipolar cells and RGCs (Dowling and Boycott, 1966). The amacrine cells use mainly two inhibitory neurotransmitters, GABA (gamma-aminobutyric acid) and glycine (Marc, 1995), and a

subset also use the excitatory neurotransmitter acetylcholine (Famiglietti, 1983) as a co-transmitter to pass the visual signal.

Normally in the dark, rods and cones (photoreceptors) are in the depolarized state and release (Trifonov, 1968) glutamate as a neurotransmitter (Figure 2; Dowling, 1987 and Massey, 1990). When light is transduced by the photoreceptors, they become hyperpolarized and stop releasing glutamate. Due to this effect, one group of bipolar cells (known as OFF- bipolar cells) decreases conductance (Dacheux and Miller, 1976) and becomes hyperpolarized via metabotropic glutamate receptors, specifically mGluR6, which uses a G protein cascade (Slaughter and Miller, 1983; Nawy and Jahr, 1990; Dhingra et al. 2001). OFF bipolar cells glutamate receptors are also known as APB (2-amino-4-phosphonobutyrate) sensitive glutamate receptors (Slaughter and Miller, 1981). When light is transmitted, the other group of bipolar cells (known as ON-bipolar cells) depolarizes and increases conductance (Nelson, 1973; Toyoda, 1973) through ionotropic glutamate receptors i.e. (2-(aminomethyl)phenylacetic acid (AMPA) and kainate glutamate receptors; Slaughter and Miller, 1983). Finally, ON bipolar cells (in the dark) and OFF bipolar cells (in the light) release glutamate within the inner plexiform layer (Werblin and Dowling, 1969; Werblin, 1991), which excites postsynaptic RGCs (Raviola and Raviola, 1982) and amacrine cells (Cunningham and Neal, 1985).

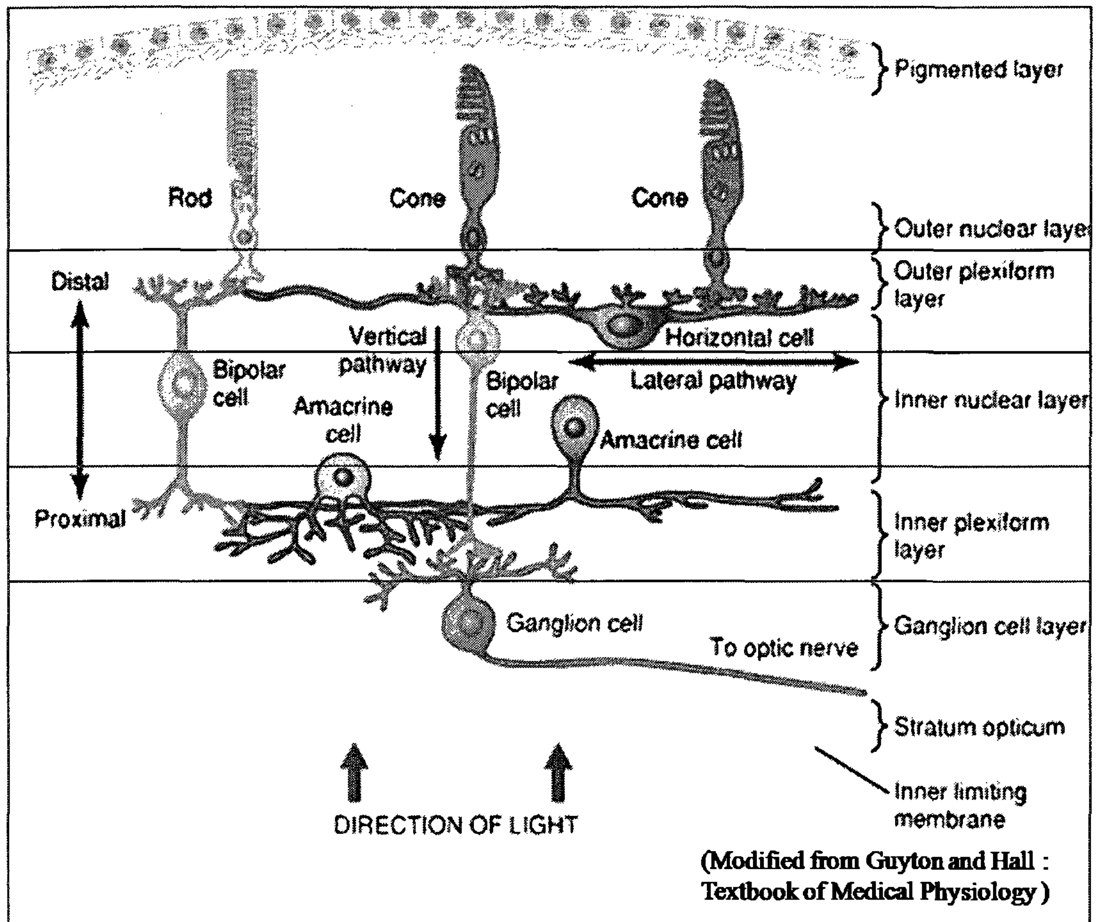

Figure 1. Visual pathway of the retina. Entered light excites photoreceptors to pass signals to bipolar cells (in the outer plexiform layer). Bipolar cells pass the signal to ganglion cells (in the inner plexiform layer) which send signals to brain. Horizontal cells and amacrine cells provide lateral feedback to the outer and inner plexiform layer respectively.

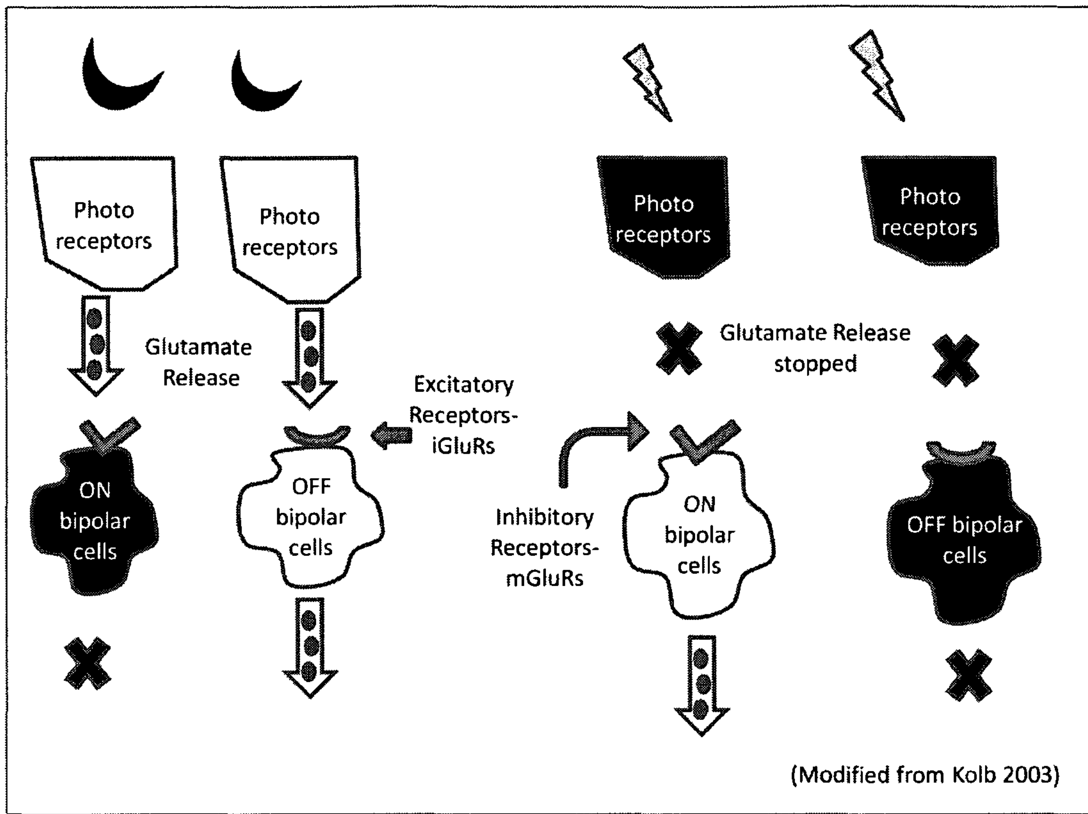

Figure 2. Visual pathway: Signal transmission from photoreceptors to bipolar cells. In the dark, photoreceptors (in depolarized state) release glutamate which depolarizes OFF bipolar cells (to release glutamate) and hyperpolarizes ON bipolar cells (to stop glutamate release). Vice versa happens in the presence of light.

Only cholinergic amacrine cells synthesize acetylcholine and release it as a neurotransmitter (Masland and Mills, 1979). Hence, cholinergic amacrine cells stimulated by bipolar glutamate input release ACh (Famiglietti, 1983; Masland 1988) and pass the signal to amacrine cells, bipolar cells and RGCs (Brandon, 1987; Ehinger et al., 1988; Marc et al., 1989). When excited, RGCs process and pass the signal received from amacrine and bipolar cells through the optic nerve to the brain.

ACh has shown neuroprotective effects on RGCs against excitotoxicity. ACh induced neuroprotection of various central nervous system cells (including RGCs) against excitotoxicity has been widely documented (Kaneko et al., 1997; Dajas-Bailador et al., 2000; 0'Neil et al., 2002; Nakamizo et al., 2005; Thompson et al., 2006; Wehrwein et al., 2004). As described above, in the normal state of retinal function, glutamate released by bipolar cells excites cholinergic amacrine cells which in turn release ACh, an excitatory neurotransmitter. ACh release is believed to increase during excess glutamate release as a natural defense mechanism against excitotoxicity (Wehrwein et al., 2004). ACh induces neuroprotection through activation of nAChRs. Various studies have shown nAChRs induced neuroprotection against excitotoxicity in the brain and retina (Marin et al., 1994; Dineley et al., 2001; Lafuente et al., 2001; Laudenbach et al., 2002; Wehrwein et al., 2004; Thompson et al., 2006). The nAChRs of the subtype α7 are suggested to be involved in the neuroprotection of the brain (Kaneko et al., 1997 and Dajas-Bailador et al., 2000). ACh neuroprotection against glutamate induced excitotoxicity was found to be partially mediated through activation of α7nAChRs (Wehrwein et al., 2004) and α4nAChRs (Thompson et al., 2006) in cultured pig RGCs. Furthermore, activation of

α7nAChRs through tropisetron (a partial α7nAChRs agonist) was found to be neuroprotective in pig cultured RGCs (Linn and Linn, 2003 and 2005).

Recently, α7nAChRs have been found on presynaptic neurons of bipolar cells and cholinergic amacrine cells (Dmitrieva NA et al., 2007). Moreover, the stimulation of presynaptic nicotinic acetylcholine receptors of cholinergic cells has shown to enhance ACh release in rat superior cervical ganglion (Liang et al., 1997). In that study, Liang et al. demonstrated that positive feedback of released ACh modulates ACh release through nAChRs at the presynaptic level of cholinergic cells in rat superior cervical ganglion.

Cholinergic and bipolar α7nAChRs might have physiological significant functional role in the retina which can be stated based on three results; the neuroprotective effect of α7nAChRs on RGCs against glutamate toxicity, the presynaptic presence of α7nAChRs on bipolar and amacrine cells, and a positive modulation of cholinergic cells through nAChRs in rat cervical ganglion.

Objectives

We hypothesized that the stimulation of cholinergic amarine α7nAChRs and/or bipolar α7nAChRs should affect the normal release of ACh. We predict that there might be the presence of a positive feedback modulation of ACh release via cholinergic amarine and/or bipolar α7nAChRs activation. If positive feedback modulation of ACh release occurs, there are at least two possible ways of positive feedback modulation. First, cholinergic α7nAChRs activation might directly modulate ACh release from thmselves to increase ACh release (direct feedback modulation). Second, if bipolar α7nAChRs are

activated, an increase in the release of the glutamate neurotransmitter would modulate glutamate receptors of the cholinergic amacrine cells in order to increase release of ACh (indirect feedback modulation). In the present study, we have stimulated the α7nAChR with the partial agonist tropisetron and specific agonist PNU282987.

CHAPTER II

MATERIALS AND METHODS

The approach in the study is same as previously used by Masland and Livingstone (1976) and Linn et al. (1991). Only a subset of the amacrine cells use ACh as a neurotransmitter and are commonly known as the 'starburst' amacrine cells based on their unique morphology. Only cholinergic amacrine cells synthesize and release ACh as a neurotransmitter when excited; because of this, the cholinergic amacrine cells of the retina can be selectively labeled with radio-labeled choline. ^{3}H-choline take up (via the choline transport system) is synthesized to ^{3}H-ACh by cholinergic amacrine cells (via choline acetyltransferase). Released labeled ACh is measured by scintillation counting. The materials and methods modified from Masland and Livingstone (1976) and Linn et al. (1991) are briefly described below.

Eyecup preparation

Porcine eyes were obtained from local abattoir (DeVries Meats). Eyes were obtained from freshly slaughtered animals and were kept on ice until used (usually within 3 hours). One eye was used for each experiment.

The eyeball was diagonally cut (hemi-sected) into two equal halves in such a way that the top portion of the eyeball, including the lens, was removed and the second half of the eye ball (posterior eyecup), including the optic nerve disc, remained intact. From the posterior eyecup, the vitreous humor was removed in order to empty the eyecup.

Dissection was conducted under a red dim light and the remainder of the study was conducted in the dark. Light affects ACh release (see introduction) and we used light as a stimulator; hence, the experiment was conducted in the dark or under red dim light whenever visual tasks needed to be performed.

Once the eyecup was dissected, it was labeled with ^{3}H-choline Ames solution using the steps shown in Figure 3. The eyecup was filled with ^{3}H-choline solution and the retina was stimulated with 3 Hz flashing bright white light (light Emitting Diode [LED], 1100 mcd) for 30 minutes (Masland and Livingstone, 1976). According to Masland, amacrine cells are thought to release maximum amounts of ACh under light stimulation at 3Hz. The application of light for 30 minutes causes amacrine cells to deplete all the unlabelled ACh stores with subsequent uptake of ^{3}H-Choline. During the labeling, the eyecup was kept on the small petri-dish containing Ames medium, which was in contact with 55°C water to keep the inner temperature of the eyecup at 37°C. After the labeling, the eyecup was transferred to the perfusion chamber.

Preparation of ^{3}H-choline solution

40μl of ^{3}H-choline (tritiated-choline, ^{3}H-Ch; 80 μCi/mmol; New England Nuclear) in ethanol was taken into a tube and evaporated to dryness by placing the tube in a beaker containing water at 100°C. ^{3}H-choline was redissolved in 1 ml of Ames media (Sigma; Ames and Nesbett, 1981) and held at 37°C until used for labeling.

Eyecup mounting and perfusion system

After labeling, the eyecup was tilted upside down and mounted on an eyecup support as shown in Figure 4. The eyecup was then held to the eyecup support with a ring. The perfusion solution (Ames media) was pumped using variable flow peristaltic pump (VWR International) over the eyecup at 37°C at 1.5 ml/minutes. Inflow temperature was maintained at 37°C by putting a long loop of tubing in water maintained at 40°C before the perfusion solution flowed over the retina (eyecup). The eyecup was washed for 20 minutes so that background activity of ACh reaches to steady state level. The output was collected in a waste beaker.

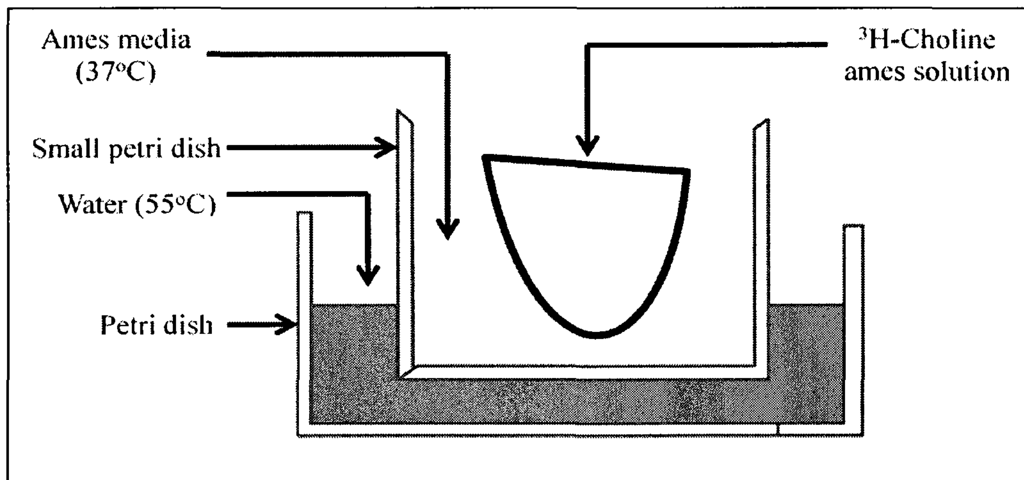

Figure 3. The eyecup set-up used during labeling. The eyecup, which was filled with ^{3}H-choline Ames solution and stimulated with 3Hz of bright flashing light, was kept in a small petri dish containing Ames media. The temperature of Ames media was maintained at approximately 45°C by keeping the small petri dish containing the eyecup in a large petri dish heated to 55°C.

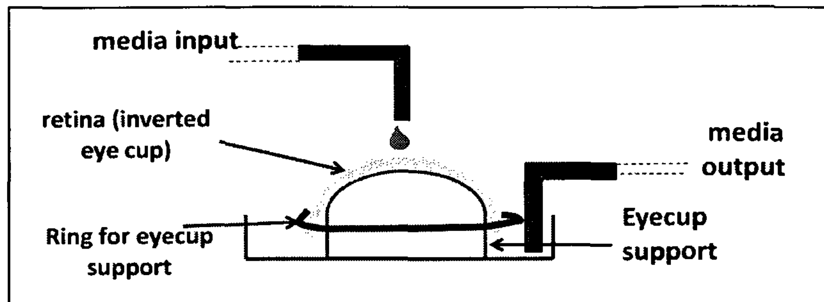

Figure 4. Perfusion system. The eyecup (inverted) was mounted on a cylindrical shaped eyecup support. A plastic ring was used to hold the eyecup to the eyecup support. Input media flow was over the top of the eyecup while output media flow was at the bottom of the petri dish.

After a 20 minute washing period, the experiment was started and output was collected into vials via a fraction collector. One minute outflow fractions were collected. The study was conducted using two types of stimuli, i.e., light and drugs.

A total of four different kinds of stimuli were used. Except for flashing light, each drug was applied for 1 minute through the input of perfusion solution. 1) 2 to 4 minutes of light stimulation (3 Hz of bright white light 1100 mcd LED) were applied to assess viability of the system and physiological input of the system. 2) Kainate (glutamate receptor agonist), which acts specifically on bipolar cells, was applied to validate direct selective pharmacological input and as a positive control (Linn et al., 1991). 3) Potassium chloride (KCl) was applied to check ACh release through direct depolarization of the membrane (direct, non selective stimulation; Friedman and Redburn, 1990). 4)

Tropisetron (partial α7nAChR agonist) and PNU282987 (α7nAChR agonist) were applied with or without DNQX (glutamate receptor antagonist) as an experimental variable to determine the presence of α7nAChRs stimulated ACh release.

Even after a prolonged wash (20 minutes), the baseline (background radiation) declined steadily throughout the experiment. And so, 5 minutes of fractions were collected as the basal efflux before the application of each stimulus. Tropisetron abruptly increased the basal efflux, so 10 to 15 minute washings were taken after its application.

Preparing vials for counting

1 minute fractions were subjected to scintillation counting. 2 ml of EconoScint (Packard Fisher) solution was added to each collected fraction vial. Vials were then counted in a standard scintillation counter (Packard CA2200).

Data analysis

Released labeled ACh was detected as Count per Minute (CPMs). The readings of CPM were plotted against the fraction number of vials. Each fraction number represents either baseline efflux or stimulation induced efflux. The peaks of agonist induced ACh release (labeled) were compared with baseline efflux of ACh. The average fold increase (response / preceding basal efflux) of the stimuli was plotted against respective stimuli. Statistical analysis was done using One way ANOVA followed by student-Newman-Keuls test of post-hoc analysis. P-values less than 0.05 were considered as significant.

CHAPTER III

RESULTS

Physiological and Pharmacological stimuli

We calibrated the responses of the pig retina *in vitro* system using physiological and pharmacological stimuli. After 20 minutes of washing, background radioactivity reached a steady state level. Light was used as a stimulus to release ACh (^{3}H labeled), to ensure that the physiological responsiveness of the retina was intact through photoreceptor activation. 3 Hz flashing light was applied for 2 to 4 minutes giving a 2 to 3 fold increase in ACh release (Figure 5 or 6). This is comparable to what Masland (1976) demonstrated and was used to indicate a functional retina.

We presumed that the released radioactivity by stimuli was in the form of ACh rather than another metabolite or choline. Previous work has indicated that essentially all the radioactivity released due to light was in the form of ACh (Masland and Livingstone, 1976; Massey and Neal, 1979; Massey and Redbum, 1982). Moreover, the analysis of released ACh by stimuli like kainate (KA), quisqualate (QQ) and NMDA has shown that more than 90% of radioactivity was ACh as opposed to choline (Linn et al., 1991).

Kainate (KA) is a selective KA receptor agonist, since KA receptors have been demonstrated to be the physiologically activated receptor on cholinergic amacrine cells (Linn et al., 1991) and bipolar cells. We used kainite as a selective pharmacological input to kainite receptors of bipolar and cholinergic amacrine cells. Our results showed that the

average fold increase of ACh release compared to respective basal efflux was 2.06 fold by light, 1.81 by 10 μM kainate and 2.45 by 100 μM kainate (Figure 6).

We used KCl to insure that ACh release was working through direct chemical stimulation. Elevated KCl is often used as a, non-selective input to depolarize the plasma membrane of cholinergic amacrine cells (Friedman & Redburn, 1990). Our results indicated that elevated potassium (100 mM KCl) caused up to a 7 fold increase in basal efflux (Figures 5 and 6).

In this study, direct pharmacological (kainate) and physiological (light) input had increased ACh release compared to base line, and essentially functioned as our positive controls to validate the system.

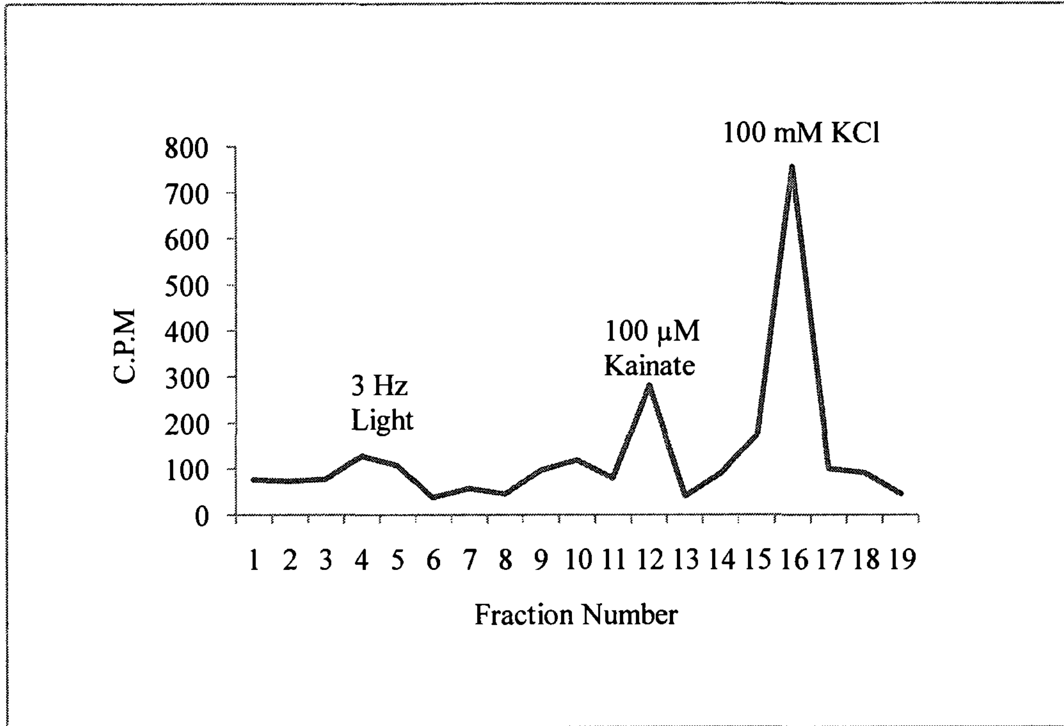

Figure 5. ACh release (CPMs) changes in the presence of light, kainate and KCl (control stimulators). Release of ACh from pig retina (*in vitro*) in response to stimulation by light, 100 μM kainate and 100 mM KCl. Release data in CPMs were plotted against the fraction number. One-minute fractions were collected, and kainate (glutamate receptor agonist) and KCl (non selective input, membrane depolarizer) were applied for 1 minute. Light stimulation at 3 Hz for 2 minutes.

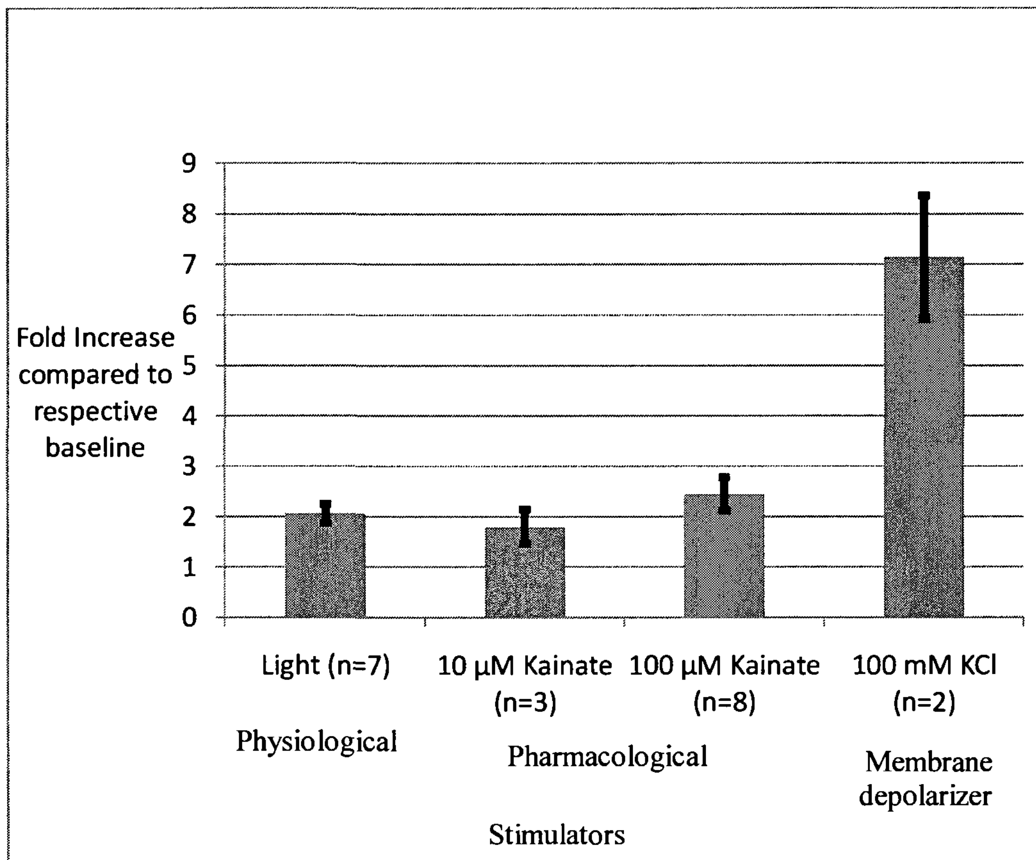

Figure 6. Average fold increase of ACh release due to light, kainate and KCl (control stimulators). The summary of data (ACh release in pig retina *in vitro*) collected for different positive controls used in the study to validate the system, light (flashing white -3 Hz) functions as a physiological stimuli, kainate as pharmacological stimuli and potassium (KCl) as membrane cell depolarizer. The average fold increase in ACh release compared to the respective baseline verses control stimulators was plotted in the graph.

Partial agonist of α7nAChRs – Tropisetron

Tropisetron ((3-endo)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl 1H-indole-3-carboxylic acid ester monohydrochloride) has potent 5-HT_3 (serotonin, 5-hydroxytryptamine) receptor antagonistic & anti-emetic activity (Seynaeve et al., 1991; Middlemiss and Tricklebank, 1992; Mhatre et al., 2004). It has partial α7nAChRs activity (Papke et al., 2005) and has shown neuroprotection of RGCs against glutamate toxicity in RGCs culture (Linn and Linn, 2003 and 2005). Moreover, it has passed clinical trials and is used as an antiemetic (anti-vomiting) outside of the US. Hence, its effect on α7nAChRs mediated ACh release might be valuable.

Tropisetron increased ACh release compared to basal efflux from 1.13 fold to 2.79 fold in a dose dependent manner. Figure 7 shows the result of one experiment. Peaks of increased ACh release are seen with three different tropisetron concentrations. The data suggests concentration of tropisetron in 0.01-100 nM tropisetron had 1.13 fold-2.79 fold increases in ACh release compared to basal efflux.

Figure 8 shows summary of tropisetron experiments. ACh release induced by 1 nM and 100 nM tropisetron concentrations is significantly different from the ACh release induced by 0.01 nM tropisetron concentration ($P<0.05$). However, ACh release induced by 1 nM and 100 nM tropisetron is not significantly different from each other (Figure 8).

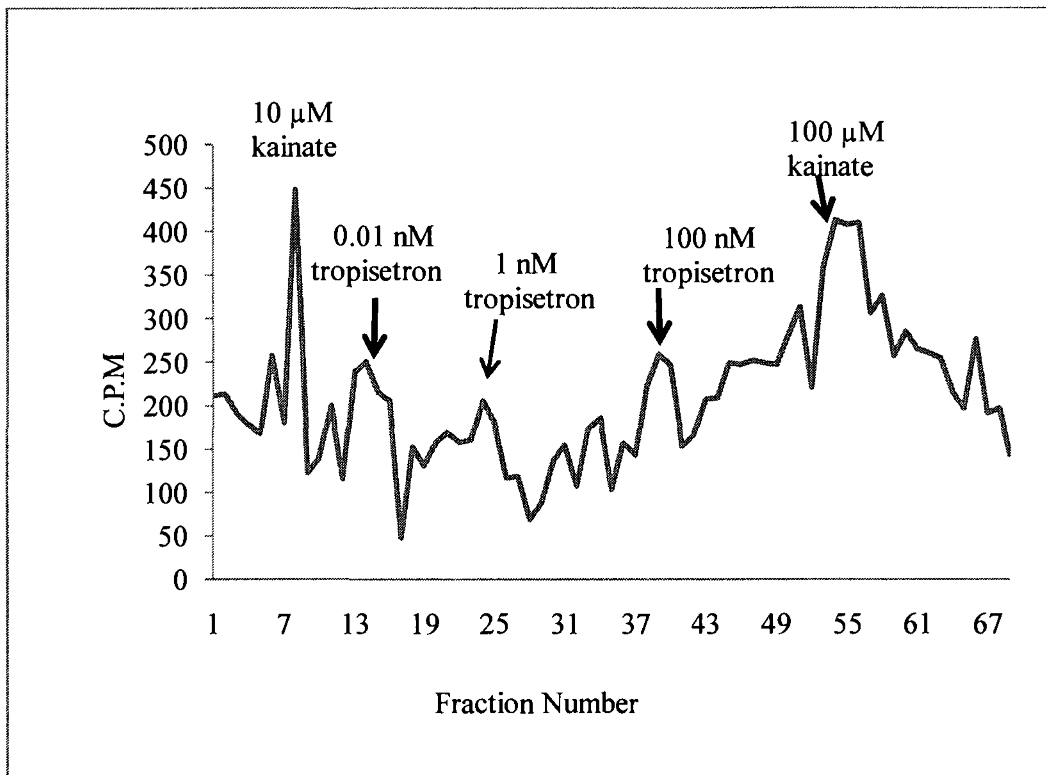

Figure 7. ACh release (CPMs) changes in the presence of different concentrations of tropisetron. Release data in CPMs were plotted against the fraction number. One-minute fractions were collected, and different concentrations of tropisetron were applied for 1 minute. The washtime between two applications of tropisetron were more than 10 minutes. Normally a steady decline in radioactivity is observed, due to the finite pool of releasable ACh.

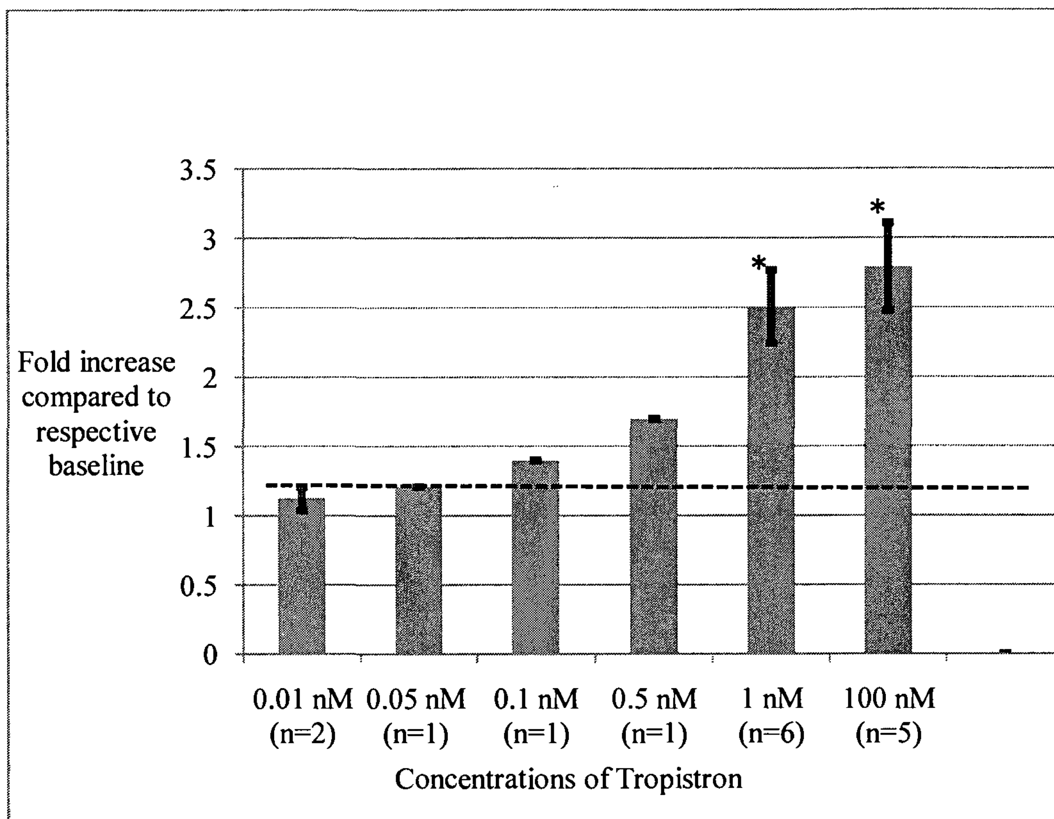

Figure 8. Average fold increase in ACh release for different concentrations of tropisetron. The average fold increase in ACh release compared to the respective preceding baseline verses different concentrations of tropisetron was plotted in the graph. * P<0.05 from 0.01 nM tropisetron (One way ANOVA, Student-Newman-Keuls test of post-hoc analysis).

Specific agonist of α7nAChRs – PNU 282987
(N-(3R)-1-Azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide; Tocris Bioscience)

PNU has shown neuroprotective activity on RGCs against glutamate toxicity. Here it was used as a selective agonist for α7nAChRs stimulation. We observed increased efflux after tropisetron application and PNU 282987 also increased ACh release compared to a basal efflux (Figure 9).

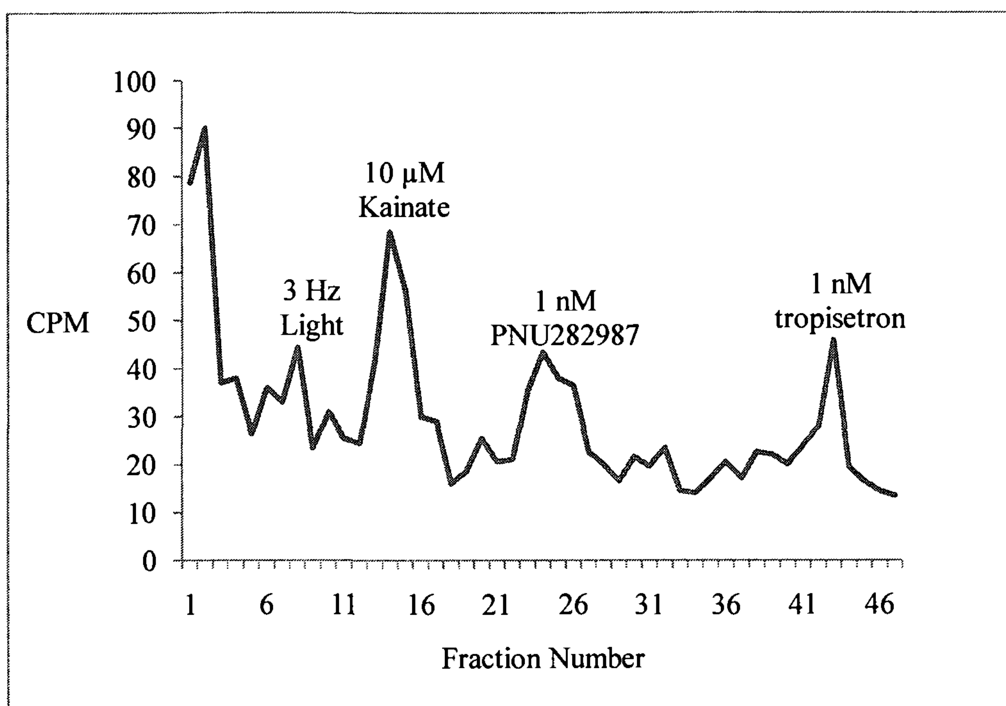

Figure 9. Release of ACh from pig retina (*in vitro*) in response to stimulation by light, kainate, PNU282987 and tropisetron. Release data in CPMs were plotted against the fraction number. One-minute fractions were collected, and different concentrations of tropisetron were applied for 1 minute. Washing timings between the application of PNU282987 and tropisetron were kept more than 10 minutes. Light stimulation at 3 Hz for 2 minutes.

KA antagonist - DNQX and α7nAChR agonist PNU-282987

Stimulation of α7nAChRs by tropisetron and PNU 282987 increased ACh release compared to basal efflux. 6,7-dinitroquinoxaline-2,3-dione (DNQX) is a glutamate receptor antagonist (a selective KA receptor antagonist, Linn et al. 1991). It is used to differentiate between a direct (cholinergic α7nAChRS mediated) or indirect (bipolar α7nAChRS mediated) effect of ACh release (Linn & Massey, 1992). 10μM DNQX blocks the effect of KA and physiological stimuli (light) on cholinergic amacrine cells completely (Linn et al., 1991). We blocked KA receptors with DNQX with a10 minute pre-treatment before PNU 282987 application. Light, 100 μM Kainate and 100 μM PNU 282987 were applied with and without DNQX application. There was an increase in ACh release compared to basal efflux with application of light, kainate and PNU 282987 in the absence of DNQX; while there was no change in ACh release compared to baseline in the presence of DNQX in response to stimulation (Figure 10).

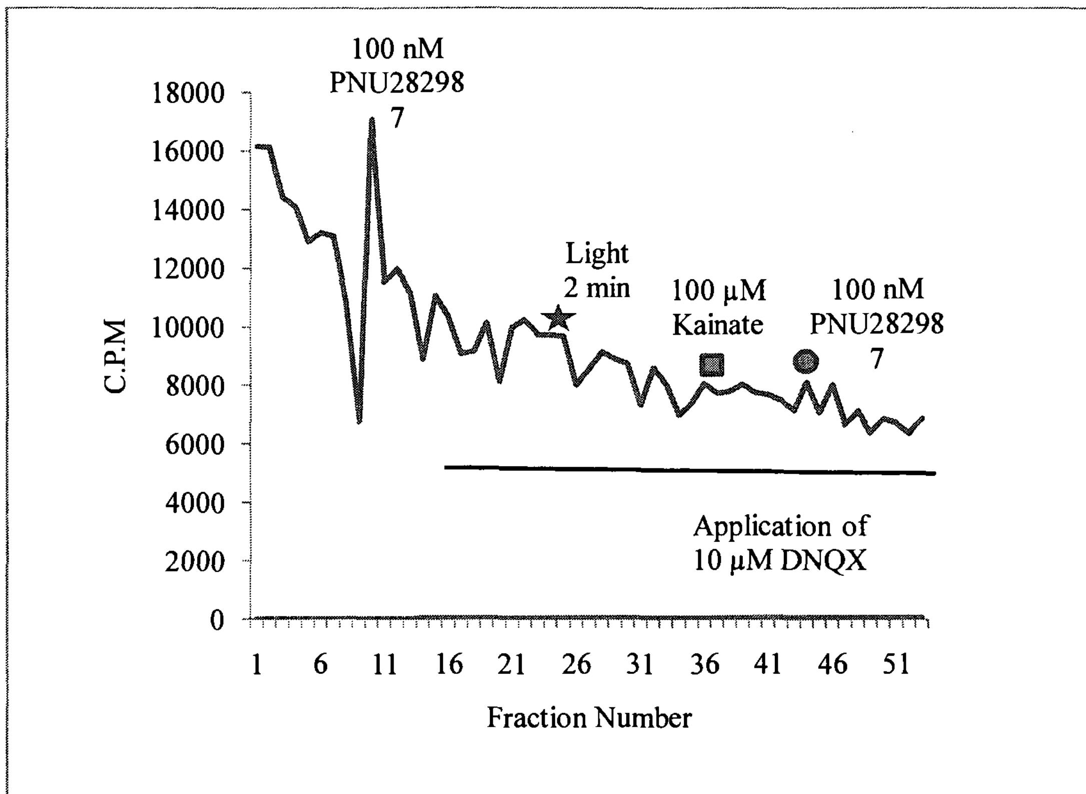

Figure 10. Release of ACh from pig retina (*in vitro*) in response to stimulation by light, kainate, PNU282987 in the presence and absence of DNQX. Release data in CPMs were plotted against the fraction number. One-minute fractions were collected, and different concentrations of tropisetron were applied for 1 minute.

CHAPTER IV

DISCUSSION

Stimulation of α7nAChRs by agonists (e.g. tropisetron or PNU282987) increased the ACh release compared to basal efflux suggesting possible involvement of either cholinergic α7nAChRs and/or bipolar α7nAChRs (Figures 7, 8, and 9). Our results indicated that in the presence of DNQX (glutamate receptor blocker), the blockade of light and kainate induced ACh release is observed; which is consistent with the previous findings by Linn et al., 1991 (Figure 10). To find out which α7nAChRs (either cholinergic or bipolar α7nAChRs) mediated ACh modulation, we used DNQX. In the presence of DNQX, the KA receptors of cholinergic amacrine cells are blocked. 10μM DNQX blocks all the inputs from kainate and light. Hence, DNQX blocks glutamate input from bipolar cells to cholinergic amacrine cells (Linn et al., 1991). Basically, if the modulation is through direct effects on the cholinergic amacrine cell, this modulation should not be affected.

There are two possibilities for positive feedback modulation: 1) amacrine cell α7nAChRs mediated (direct positive feedback modulation) and 2) bipolar α7nAChR mediated (indirect positive feedback modulation). In the presence of DNQX, bipolar cell mediated ACh release should be blocked and not induce ACh release above baseline. This is based on the hypothesis that glutamate (released by bipolar α7nAChR activation) input to amacrine cells will be blocked in the presence of DNQX. Moreover, if ACh

release is mediated by α7nAChRs in the cholinergic amacrine cells, ACh release should increase compared to baseline even in the presence of DNQX.

Our study showed an increase in ACh release when α7nAChRs agonists (tropisetron and PNU282987) were applied (Figures 7, 8, and 9) in the absence of DNQX. However, in the presence of DNQX, there was no increase in ACh release compared to baseline (Figure 10). This does not support the possibility of a significant role of α7nAChRs in the cholinergic amacrine cells in the modulation of ACh release. Hence, bipolar α7nAChRs induced ACh release might be a possible mechanism (indirect positive feedback modulation). See figure 11 for a model explaining positive feedback modulation based on our results.

Figure 11. Model explaining positive feedback modulation. The figure shows neurotransmitter network of glutamate and ACh among bipolar cells, cholinergic cells and RGCs. Photoreceptors passes glutamate neurotransmitter signal to bipolar cells and horizontal cells. Bipolar cells passes glutamate neurotransmitter signal to cholinergic amacrine cells and RGCs. Cholinergic amacrine cells might feedback ACh to bipolar α7nAChRs as an indirect positive feedback modulation of ACh release.

This study supported the possibility of ACh release mediated by indirect positive feedback through the activation of bipolar α7nAChRs. As shown in figure in 7, ACh release through the stimulation of α7nAChRs is possible. Amacrine cells might get indirect positive feedback from ACh, inducing them to release more ACh (Figures 8 and 11). During excitotoxicity, ACh released by amacrine cells, might feedback on bipolar nAChRs to increase ACh release. This finding is significant. Normally, it would not be expected to see any increase if α7nAChRs were only physiologically important on RGCs. There is no known feedback from RGCs onto the cholinergic amacrine cells. So, this effect has to be direct or pre-synaptic (onto a cell that synapses onto the cholinergics).

Interestingly, activation of bipolar α7nAChRs modulated ACh release while cholinergic α7nAChRs did not. This raises questions regarding the physiological significance of the cholinergic α7nAChRs (Figure 11). We are hesitant to minimize the importance of the cholinergic α7nAChRs based on the findings of only one approach (ACh release), particularly since the effects of the bipolar synapse could be amplified through the high-gain, 'ribbon' synapse (Linn & Massey, 1992). Moreover, if α7nAChRs are only physiological significant, one would not expect any increase in ACh release through the stimulation of α7nAChRs.

Bipolar α7nAChRs in bipolar cell synaptic terminals receive GABAergic negative feedback from amacrine cells (Yazulla et al., 1987). At bipolar cell terminals, GABA inhibits Ca^{++} conductance by acting through metabotropic $GABA_B$ receptors. Decreased Ca^{++} conductance inhibits glutamate neurotransmitter release from bipolar synaptic terminals (Heidelberger and Matthews, 1991). Our experiments indicate a possible

enhancement of glutamate release through the stimulation of bipolar α7nAChRs. Opposing the negative GABAergic negative feedback, an increase in bipolar Ca^{++} levels might be central to α7nAChR mediated glutamate release (Rogers and Dani, 1995; Ragozzino et al., 1998).

Though we calibrated the *in-vitro* system of the pig retina, it should be kept in mind that the previous *in-vivo* studies of rabbit retina (Linn et al. 1991) induced significantly higher increases in ACh release when glutamate analogues were applied. This suggests a limitation of our model. However, our results are comparable to other *in-vitro* preparations examining ACh release from the rabbit retina (Masland & Livingstone, 1976; Friedman & Redburn, 1990) and rat nervous tissue (Liang & Vizi, 1997). This indicates that our results may underestimate the *in vivo* effect of glutamate analogue.

Previous research indicated that RGC neuroprotection from excitotoxicity through the stimulation of RGC α7nAChRs by tropisetron is possible (Linn and Linn, 2003). The present study indicates that the stimulation of bipolar α7nAChRs by tropisetron also caused ACh release. This raises the possibility of dual therapeutic targets for RGCs neuroprotection through direct action on RGC α7nAChRs & indirect action on bipolar α7nAChRs. Neuroprotection of RGCs is critically important in glaucoma and our study suggests that the stimulation of α7nAChRs using drugs like tropisetron, which is clinically approved, might be therapeutically targeted in dual way for protection of RGCs against excitotoxicity. However, the presence of α7nAChRs on bipolar cells, RGCs and various amacrine cells including cholinergic amacrine cells (Dmitrieva et al. 2007)

suggests a complex and 'layered' control of ACh release and requires further study to understand α7nAChR mediated neuroprotection.

Further exploration of this 'layered' structure would probably involve more direct monitoring of individual cells, such as with whole-cell recording. In their investigations of the GABA-B receptor on bipolar cells, Maguire et al (1989) used the retinal slice preparation to record from bipolar cells with and without intact axons. Responses to GABA-B agonists were only found on bipolars with intact axons indicating their localization to the axon terminal. One would assume that the alpha7 receptors are also restricted to the axon terminal since they appear to increase neurotransmitter release. The intracellular mechanisms of this increase also deserve further investigation. One would assume that they increase the permeability of specific calcium channels involved in neurotransmitter release. This mechanism would be in contrast to the neuroprotective mechanism invoked by alpha7 activation on RGCs. The mechanism of action of alpha7 receptors on amacrine (particularly the cholinergic) cells will probably be more elusive since currently there is not a way to selectively culture these cells (as there is with the RGCs) and they are more difficult to directly record from compared to bipolar cells.

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