PROTEIN KINASE C (PKC) BETA1 AND GAMMA ISOFORMS ARE ESSENTIAL FOR ROD PHOTORECEPTOR DIFFERENTIATION

A Dissertation in
Neuroscience

by

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Mammalian retinal differentiation is a tightly regulated process that requires the appropriate integration of intrinsic and extrinsic factors to regulate neuronal cell production from a single pool of proliferating progenitor cells. Using a model of post natal day 1 (P1) retinal explants we study the molecular mechanisms utilized by retinal progenitor cells to differentiate into rod photoreceptors. We found that both PMA and IGF1 treatments increased photoreceptor differentiation in a PKC dependent manner. Of the ten PKC isoforms we only detected expression of PKCβ1 and PKCγ in the rod progenitor population. Inhibition of either isoform resulted in partial inhibition of rod differentiation, whereas inhibition of both isoforms resulted in complete absence of rod photoreceptors in the retinal explants. We identified two potential mechanisms by which activation of PKC results in rod differentiation. First, activation of PKC with either IGF1 or PMA significantly reduced tyrosine phosphorylation of STAT3, a molecule that has been shown to promote progenitor cells proliferation and self renewal. In contrast, inhibition of PKC resulted in higher levels of tyrosine phosphorylated STAT3 accompanied by delayed cell cycle exit of progenitor (proliferating cell nuclear antigen or PCNA positive) cells. This suggests that activation of PKC promotes the transition of progenitor cells from proliferation to cell specification. Second, activation of PKC causes modification of epigenetic markers that may result in changes of gene expression necessary for rod photoreceptor differentiation.

Finally, we identified IGF1 as one physiological signal that during photoreceptor development triggers activation of PKC. We found that contrary to adult retinas, in neonatal retinas, when IGF1 binds to its receptor, the tyrosine kinase of the IGF1 receptor does not activate PI3K, and the absence of PI3K activity results in activation of PKC and rod development.
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CHAPTER 1

INTRODUCTION

1.1 RETINAL DISEASE

Monogenic diseases of the retina and vitreous affect approximately 1 in 2,000 individuals, or more than 2 million people worldwide (Berger W et al., 2010). The outcomes in patients suffering from these diseases vary greatly with some declared legally blind, and some with only nocturnal vision mildly affected. There is currently little treatment offered for these patients, and it mainly focuses on nutritional supplements that may prevent more cell death. An alternative approach is to restore vision by replacing lost cells. However, regeneration of lost retinal tissue has not yet been successful. There are many studies underway testing the ability of stem cells to regenerate injured neural tissue including the retina (Little CW et al., 1989, Barsch U et al., 2008, MacLaren RE et al., 2006). Significant problems may arise if transplantation of the correct cell does not take place. For example, if the stem cell preserves proliferation activity at the time of transplantation, tumorigenesis becomes a big issue rendering the patient to a life threatening condition. On the other hand, if the cell does not differentiate correctly, transplantation of the wrong cell type, or a cell type that was not intended to be transplanted, may occur. Therefore, in order for regenerative medicine to be successful in treatment of retinal diseases, a complete understanding of retinal cell development is necessary to accurately program the stem cells to become a specialized retinal cell before transplantation.

There has been substantial research in the molecular bases for retinal diseases that has elucidated a variety of genetic factors as well as some environmental factors that contribute to retinal
degeneration. Retinal disease can be divided into two mayor categories: Syndromic and nonsyndromic. Within these two categories, diseases have also been divided into four subcategories: Diseases that affect rods only, cones only, rods and cones, and vitreoretinopathies. In addition another factor that is taken into account is whether the disease is stationary or progressive.

In the nonsyndromic categories, the most prominent diseases that affect rods include congenital stationary night blindness, and progressive rod diseases with retinitis pigmentosa (RP) as the most common in this category. Rods comprise the majority of cells in the retina. They are specialized for low light intensities and are responsible for visual perception under dim light conditions; therefore symptoms of diseases that affect rods are characterized by some type of impairment in night vision. However, in the progressive diseases such as RP, rod photoreceptor cell death is commonly followed by cone cell death rendering the patient with severe vision impairment.

Congenital stationary night blindness (CSNB) is a non-progressive visual impairment present at birth. However, it is also one of the first symptoms present in other progressive retinal diseases including RP. Therefore, a complete genetic screening is necessary for the appropriate diagnosis and prognosis of patients and families, since most diseases are genetic. Currently 11 genes have been shown to be associated with CSNB (Audo et al., 2009, Dryja et al., 1993, 1996, 2005, Fuchs et al., 1995, Gal et al., 1994, van Genderen et al., 2009, Wycisk et al., 2006, Yamamoto et al., 1997, Zeitz et al., 2005, 2006) Mutations in these genes can be recessive, dominant or x-linked. The functional spectrum of genes includes calcium channel subunits (CACNA1F,
CACNA2D, TRPPM1), calcium binding protein (CABP4), metabotropic glutamate receptor (GRM6), phototransduction molecules (GNAT1, GRK1, PDE6B, SAG, RHO). Mutations in some of these genes such as the rhodopsin (RHO) and phosphodiesterase beta (PDE6B) can lead to stationary as well as progressive rod degeneration.

Retinitis pigmentosa (RP) comprises a group of clinically similar phenotypes associated with a different spectrum of genetic mutations. The onset and disease progression varies significantly among patients, even within the same family. The disease progresses from night blindness in the early phase to loss of vision that starts in the mid periphery and moves to the center of the retina resulting in tunnel vision (Hamel 2006). The visual impairment is caused by loss of photoreceptors due to apoptosis, however, the definite reason why photoreceptors undergo apoptosis is not clear. Because of the progressive loss of rods, patients with RP have reduced or absent a and b waves in ERG. Mutations in over 40 genes have been associated with RP, and these mutations can be inherited in autosomal recessive, dominant or x-linked mode. In addition, mutations in several genes can be either dominant or recessive (Abd El-Aziz et al., 2008; Banerjee et al., 1998; Bareil et al., 2001; Collin et al., 2008; denHollander et al., 2008; Dryja et al., 1995; Gal et al., 2000; Zhang et al., 2007a; Zhao et al., 2009).

The functions of genes associated with RP range from phototransduction (rhodopsin, phosphodiesterase A and B), retinal metabolism (Guanylate cyclase activator 1B, Ceramide kinase-like), tissue development and maintenance (Neural retina leucine zipper NRL, cone rod homeobox protein CRX), and cellular structure (Retinal outer segment membrane protein 1ROM1).
Although the functional properties of several RP associated genes have been studied extensively, genotype-phenotype correlations are not well understood. Around 10% of genes associated with RP are involved with splicing including PRPF3, PRPF8, PRPF31 and PAP1, perhaps due to the fact that photoreceptors have specialized splicing machinery that make them vulnerable to changes in splicing patterns. Other genes classically mutated in RP include Rhodopsin and RPGR (RP GTPase regulator) which usually compiles a more severe phenotype.

The other class of photoreceptors in the retina is the cone. Cone photoreceptors are responsible for daylight vision and are important for high visual acuity and color discrimination. In the nonsyndromic categories, the most prominent diseases that affect cones include complete and incomplete achromatopsia, cone dystrophies and macular degeneration (MD) monogenic and age related.

Leber congenital amaurosis (LCA) is the most severe of the retinal dystrophies with onset as early as one year of age. The frequency of the disease is 1 in 50,000. The most common clinical features include congenital or early onset of vision loss, sensory nystagmus and no light response upon electrophysiology. The most common mode of inheritance is autosomal recessive with more than 14 genes identified to date. However, mutations in CRX and IMPDH1 have been shown to cause LCA with dominant inheritance. One of the most common genes mutated in patients with LCA is the RPE65 (Marlhens et al., 1997) protein found in the RPE layer, an isomerase involved in the conversion of all trans retinol to 11 cis retinal.
Age related macular degeneration (AMD) has a prevalence of 0.05% in population younger than 50 years, and increases to about 12% in population older than 80 years of age (Augood CA et al., 2006, Javitt JC et al., 2003). Based on studies with twins and families with high prevalence of AMD, researchers have concluded that both genetic and environmental factors such as smoking, diet, obesity and chronic inflammation contribute to the pathophysiology of AMD (reviewed by Chamberlain et al., 2006). Patients with AMD present with abnormalities in the RPE layer as well as formation of protein and lipid deposits in the retina called drusen. Two types of AMD can be distinguished: (i) an exudative or wet and (ii) a non-exudative or dry. The dry form with time has ten percent probability of transforming into a wet one, with characteristic choroidal neovascularization which tends to cause vessel leakage and bleeding.

Some genome wide association studies have described a role of the complement pathway of the immune system in AMD. Studies have shown that uncontrolled activation of the system may lead to the formation of drusen (Sivaprasad and Chong 2006). Histological studies of retinas from AMD patients found many components of the complement system within these drusen (Hageman et al., 2001). Genetic modifications in many components of the complement pathway (complement factor H, complement factor B, C2, and C3) have been associated with increased risk of developing AMD (reviewed by Berger et al., 2010). In addition, several studies have linked the G allele of the toll-like receptor 4 (TRL4), a bacterial endotoxin receptor, to higher risk of developing either dry or wet AMD (Zareparsi et al., 2005).

More generalized retinal diseases include choroideremia and vitreoretinopathies that affect photoreceptors, RPE layer, choriocapillaris, choroid and vitreous that eventually result in complete blindness. In addition there are many other syndromic diseases in which the retina is
affected as well as other tissues in the body. The most common syndromic retinal diseases are Usher syndrome that also involves the inner ear as a consequence of mutations in myosin 7A or usherin (Bolz 2009, Saihan et al., 2009). Mutations in these proteins affect intracellular transport of proteins through the connecting cilium of photoreceptor and hair cells in the inner ear. Refsum disease which belongs to the peroxisome biogenesis disorder with a variety of organs involved in the disease. Bardet Diedl syndrome, Joubert syndrome and Senior Loken syndrome are other examples of complex syndromes in which the retina is also involved but the mechanism is not very well understood.

The retina can be also affected by common pathologies including high blood pressure, high cholesterol and diabetes that can cause severe neurological as well as vascular dysfunctions in the retina resulting in vision impairment or even blindness. It is the ultimate goal to better understand the molecular etiology of these diseases in order to develop better approaches for therapeutic interventions.

1.2 CURRENT AND POTENTIAL TREATMENTS FOR RETINAL DISEASES

The eye is an ideal organ for many different therapeutic approaches because its accessibility that enables easy application as well as easier monitoring of the delivered treatment. In addition the blood retinal barrier successfully reduces or even prevents exchange of therapeutics with other organs in addition to minimizing therapy provoked immune responses. However, currently, there is limited treatment to minimize symptoms and no treatment to revert cell damage.
Current treatments of retinal diseases may be divided into three categories: (i) Prevention of cell death, (ii) Halting the progression of cell loss, and (iii) regeneration of lost cells and vision. In the first category several compounds are currently being studied and they include:

* Vitamin A/beta-carotene, vitamin C, vitamin E and Zinc whose antioxidant effects may be useful in preventing oxidative damage and subsequent cell loss in diseased retina, but no clear prospective evidence in favor of vitamin supplementation yet exists. A recent epidemiologic study concluded that very high daily doses of vitamin A palmitate (15,000 U/d) slow the progress of RP by about 2% per year (Berson EL et al., 1993) Since the effects are quite modest this treatment must be weighed against the uncertain risk of long-term adverse effects from large chronic doses of vitamin

* Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid and antioxidant. Some studies reported fewer changes in ERG studies in patients that had higher levels of erythrocyte DHA (Berson EL et al., 2004). Further clinical trials must be carried out to determine DHA benefit

* Acetazolamide (Fishman GA et al., 1989) is a carbonic anhydrase inhibitor that has shown the most encouraging results with some improvement in visual acuity by decreasing macular edema.

* Lutein/zeaxanthin (Aleman TS et al., 2001) are macular pigments that cannot be produced by the body but can only be obtained from dietary sources. They are thought to protect the macula from oxidative damage, and oral supplementation has been shown to increase the macular pigment. The Age-Related Eye Disease Study II (AREDS II), a NIH clinical trial, is testing the
effectiveness of lutein and zeaxanthin to slow age-related macular degeneration. Their ability to prevent cone photoreceptor cell death has not been shown.

*Growth factors such as Ciliary neurotrophic factor (CNTF) has been shown to slow retinal degeneration in a number of animal models (Emerich DF an Thanos CG 2008, Thanos et al., 2004). Phase II clinical trials are underway using a surgically placed encapsulated form of RPE cells producing CNTF (Neurotech) for patients with Usher syndrome and RP. Results released from Neurotech’s study in May 2009, showed evidence of retinal thickening after 1 year of treatment. However, visual improvement was not seen at this time point and further studies are needed to evaluate long term effects of the treatment. Interestingly, patients with atrophic age-related macular degeneration treated with the same growth factor did have a measurable visual improvement after 1 year.

Therapies intended to halt the progression of cell death must in some way solve the problem that is causing cell damage and death. In many of the congenital retinal degenerative diseases, absence of an important functional protein, or accumulation of miss-folded proteins in the cytoplasm may be leading to retinal cell death. Gene therapy that restores function to mutated genes may be one possibility to stop progression of disease.

*Gene therapy focuses on replacing the defective protein by using DNA vectors (eg, adenovirus, lentivirus). Gene replacement will be more successful in diseases where the mutation results in loss of function of the protein. Gene therapy was successful in providing the missing protein to a naturally occurring dog model of RPE65 mutation that resembles human Leber congenital amaurosis (Acland GM et al., 2001). Transducing the RPE65 gene using an adeno-associated virus (AAV) to the dog allowed expression of the functional protein in about 20% of its RPE
cells, thereby allowing the dog to see. Phase I clinical trials are underway in human patients with early encouraging results in patients who noted vision improvements in the first 6 months (Bainbridge JW et al., 2008, Hauswirth WW et al., 2008, Maguire et al., 2008). No significant adverse effects have been noted.

Because of the wide heterogeneity of defects in RP, gene therapy must be targeted specifically to each mutation. Gain of function mutations will require an extra step in which the gene would have to be silenced, and then replaced with a wild type copy. Advances in RNA silencing would provide a suitable tool to accomplish this task.

In addition, it is not known which, if any, of the retinal degeneration phenotypes will show reversibility even with a nondestructive reinsertion of the appropriate gene in the appropriate locus with appropriate regulation. Therefore different approaches need to be investigated to regenerate lost vision either with cell transplantation or retinal prosthesis.

*Retinal prosthesis or phototransducing chip placed on the retinal surface has been under investigation for several years. Most retinas with photoreceptor degeneration contain healthy ganglion cells that can be stimulated, and implants in animal models have long-term stability. Chow One study placed subretinal microphotodiodes prosthesis in patients with severe RP (Chow AY et al., 2002). Some patients had minimal subjective improvement; however, the improvement occurred in retinal areas outside of where the chip was placed. Therefore, the effect was thought to be an indirect benefit to adjacent cells. Currently, no retinal prosthesis is available for clinical use, but this technology remains investigational holding a huge potential for future therapies of blindness.
Transplantation of cells into injured retina continues to be an exciting potential approach to treat patients with retinal degeneration. Small patches of retinal or RPE tissue have been transplanted into the subretinal space to rescue photoreceptors in animal models of RP. One approach that may prove useful is ex vivo modification of these cells to provide trophic factors. Stem cells are being actively investigated as a potential source to replace damaged RPE or photoreceptor cells.

Stem cell transplantation is the only current potential treatment that will regenerate lost cells due to disease or injury with the possibility of restoring lost vision. The ability to produce stem cells from patients’ own differentiated cells such as skin fibroblasts (Hirami Y et al., Neurosci Letters 2009) has opened the field to ideal conditions for transplantation. For example, it eliminates the necessity of obtaining stem cells from embryonic tissue, and it also reduces immunological rejection after transplantation. However, complete understanding of the physiological process of cell differentiation from stem cells to specific neuron or retinal cells is pivotal for successful transplant. Stem cells have the potential to form teratomas if not programmed correctly, and in a confined place such as the retina, this could be even more devastating than the disease itself. In addition, transplantation of the non-intended cell type or a nonfunctional cell would definitely not benefit the patient either. Therefore, proper programming of stem cells is necessary for stem cell transplantation to be successful in treating retinal diseases.

Furthermore, proper programming of stem cells can only be carried out with proper understanding of the molecular and genetic mechanisms that stem cells use during normal development to make the precise decisions that allows them to become a specific neuron/glia.
1.3 ROD PHOTORECEPTOR DIFFERENTIATION

The coordination of proliferation and cell differentiation in any higher level organism is a fundamental step in their normal development. In the case of genesis of most neural tissues, embryonic proliferation provides the majority of cells needed to populate the mature tissue for the rest of the animal life. During neurogenesis, cells must exit the cell cycle and undergo a complex program of gene expression that would lead to the necessary morphological changes to differentiate into functional neurons. It has been thought that these processes require the action of multiple secreted ligands that control the course of neuronal cell fate in a spatiotemporal manner upon binding to their respective receptors (Rapaport D.H et al., 2004). The mature retina contains a diverse but limited repertoire of intrinsic cell types, including sensory neurons (rod and cone photoreceptors), interneurons (horizontal, bipolar and amacrine cells), projection neurons (ganglion cells) and glia (Müller cells). In addition the retina contains astrocytes and microglia that migrate in during development (Dowling JE 1970). The intrinsic cell types are generated in a sequential yet overlapping order with ganglion cells differentiating first followed by amacrine cells and cones. In the rodent retina, the majority of these three cell types have exited mitosis and differentiated by embryonic day 18 (E18)(Rapaport D.H. et al., 2004, Young RW1985). Most populations of amacrine cells exit mitosis before birth, but a minority finish their differentiation by a couple of days after birth. Bipolar and Muller glia are the last cells to differentiate beginning their process soon after birth with 95% of the cells out of mitosis by post natal day 6 (P6). The majority of rod photoreceptors are born soon after birth, however, about 20% of rods exit mitosis between E16 and E20. 95% of rods are formed by P5.
Studies have shown that the seven types of intrinsic retinal cells are derived from the same progenitor pool (Turner DL and Cepko CL 1987, Holt CE et al., 1988) suggesting these progenitors are multipotent. The molecular and genetic mechanisms that these multipotent progenitors utilize to begin the process of commitment and differentiation have been an important topic of discussion in the last decades. It has been postulated that both intrinsic and extrinsic cues are important for this process. A current model of retinal differentiation suggests that retinal progenitor cells progress through a series of competent states during development, and each competent state favors specification of one or more cell fates. Cell intrinsic properties such as nuclear transcription factors, cell surface receptors and intracellular signal components determine the competent state of the progenitors responsiveness of the progenitor cell to extrinsic cues that signal differentiation (Cepko Cl 1996, Livesey FJ and Cepko CL 2001). After a progenitor divides, each daughter may independently assess whether to divide again, probably based on input from its environment. As additional postmitotic cells form, they alter the retinal environment as well, and consequently affect the types and quantity of cells produced later. Ablation experiments in the frog retina suggest that both the total number of retinal cells and the number of retinal cells of a specific type are regulated by negative feedback (Reh TA and Tully T, 1986; Reh TA, 1987).

In most mammals photoreceptors comprise more than 70% of retinal cells (Young RW 1985) but rods outnumber cones by 30:1 in mice and 18-20:1 in humans (Carter-Dawson LD and LaVail MM 1979, Roorda A and Williams DR 1999). Rod and cone photoreceptors have been shown to be derived from the same type of committed post mitotic photoreceptor progenitor and a complicated interaction of several transcription factors determine whether the progenitor
becomes a rod or a cone (Cepko 1999, Swaroop et al., 2010). The process of development of photoreceptors can be divided into five major steps: First, proliferation of multipotent retinal progenitor cells (RPC); second restriction of the competence of RPCs; third, cell fate commitment and specification to photoreceptor precursors during final mitosis; fourth, expression of photoreceptor genes including genes specific for phototransduction and morphogenesis; and fifth, photoreceptor maturation that includes axonal growth, synapse formation and outer segment generation (Swaroop Anand, Douglas Kim and Douglas Forrest 2010).

The transition between cell proliferation to post-mitotic commitment is a very important step into terminal differentiation of retinal cells. STAT3 is a transcription factor that has been shown to regulate this transition in rod photoreceptors. Activation of STAT3 stimulates progenitor cell expansion and the cell is not allowed to proceed to differentiation until STAT3 is inhibited (Zhang et al., 2004, Rhee KD et al., 2004). Signal Transducer and Activator of Transcription (STAT) proteins were originally described as latent cytoplasmic transcription factors that required phosphorylation for nuclear retention (Fu X.Y et al., 1992). Unphosphorylated STAT proteins shuttle between the nucleus and cytoplasm waiting for the activation signal. Once STAT3 is activated by phosphorylation on tyrosine 705, it dimerizes and translocates to the nucleus. Once in the nucleus STAT3 dimers bind to a consensus DNA-recognition motif called gamma activated sites (GAS) in the promoter region of cytokine inducible genes and activates transcription of these genes. (Fu X.Y 1992, Schindler C et al., 1992). The STAT protein can be dephosphorylated by nuclear phosphatases which lead to inactivation of STAT and the transcription factor becomes transported out of the nucleus by exportin crm1/RanGTP. STATs
were originally identified using cell culture systems that responded to cytokines such as interferons (Schindler et al., 1992)(Darnell et al., 1997). Since then, it has been demonstrated an important role of the STAT family in innate and adaptive immune response from lower to higher organisms. Some evidence, including the fact that STAT3 KO are embryonic lethal, suggests that STAT family, specifically STAT3 may also be involved in other aspects of vertebrate development. Zhang et al., 2005 demonstrated that STAT3 activation by extrinsic factors such as ciliary neurotrophic factor (CNTF), interferon alpha (INF-α) and EGF, resulted in retinal progenitor cell proliferation during retinal development. Persistent activation of STAT3 correlated with expression of HES1 and OTX2 suggesting an important function of STAT3 in stimulating mitotic precursors. Furthermore, CNTF and leukemia inhibitory factor (LIF) specific activation of STAT3 blocked the production of rod photoreceptors in both mice and chicks by promoting retinal progenitor cell proliferation (Ezzeddine ZD et al., 1997, Kirsch M et al., 1998, Neophytou C et al., 1997, Schulz-Key S et al., 2002)

The extracellular signals that indicate the progression from a proliferating retinal progenitor cell to a committed post mitotic photoreceptor progenitor are far from being completely elucidated. However, one signal pathway that has been shown to be important in this process is the Notch signaling pathway (Yaron O et al., 2006). High levels of Notch maintain progenitor multipotency as well as proliferation, and inhibition of Notch drives progenitors to commit to the cone and rod photoreceptor fate. Notch signals through the bHLH transcription factors HES1 and HES5 responsible for downregulating the expression of pro-neural bHLH proteins in the developing retina thus maintaining the progenitor pool (Hatakeyama J, and Kageyama 2004).
Kamakura et al., 2004 demonstrated in cortical neuroepithelial cells that STAT3 and Hes1 were activated in the presence of active Notch. Activated Hes proteins, in turn, associated with STAT3 facilitating complex formation between JAK2 and STAT3 thus promoting STAT3 phosphorylation and activation. Although these results have not been confirmed in the retina progenitor cells, the interactions between STAT3 and Notch signaling may be one mechanism responsible for maintaining the retinal progenitor cell pool.

There has been extensive work on the genetic mechanisms that drive photoreceptor differentiation and six transcription factors have been shown to be responsible for dictating the key steps required for photoreceptor development namely, homeobox protein OTX2, cone-rod homeobox protein CRX, neural retina leucine zipper NRL, photoreceptor-specific nuclear receptor NR2E3, nuclear receptor RORβ, and thyroid hormone receptor β2 (Trβ2).

The paired-type homeodomain transcription factor OTX2 has been implicated in cell fate commitment and specification to photoreceptor precursors as it is expressed during final mitosis in retinal progenitor and in early precursors that become committed to the photoreceptor cell fate (Nishida A et al., 2003, Koike C et al., 2007). Mice that lack the expression of OTX2 in the immature retina develop a retina with very few photoreceptors. In addition, over expression of OTX2 during retinal development drives progenitor cells into a photoreceptor fate. These data suggest OTX2 is an essential gene that compels multipotent progenitors into a committed photoreceptor lineage. However, OTX2 has been shown to be expressed in other cell types including bipolar and Muller glia, making OTX2 not a specific signal for photoreceptor development.
The transcriptional regulator CRX acts downstream of OTX2 (Nishida A et al., 2003) and it 
exerts an important role on photoreceptor development and maturation since CRX KO mice 
produce abnormal photoreceptors that eventually degenerate (Furukawa T et al., 1999). CRX 
enhances expression of rod specific genes by binding to their promoter including the rhodopsin 
promoter. However, it has been shown that for proper rhodopsin expression, CRX requires NRL 
co-activation of promoter transcription (Furakawa et al., 1997, Chen S et al., 1997). Furthermore, 
CRX alone does not determine specific photoreceptor cell fate.

The neural retina leucine zipper protein NRL is an important transcription factor shown to 
activate expression of photoreceptor genes. It is a basic-leucine zipper transcription factor that 
has been shown to bind and regulate rhodopsin promoter via NRE, an AP-1 like element located 
in the proximal rhodopsin promoter region (Kumar R et al., 1996) NRL interacts with CRX and 
many other transcription factors to induce expression of rod specific genes (Mitton KP et al., 
2000, Yoshida S et 2004) Loss of NRL in retinal progenitors results in absence of mature rods 
with an excess of S opsin expressing cones, suggesting a conversion of fate from rods to s-cones. 
In addition, ectopic expression of NRL in developing retina results in transformation of s cones 
into rods. Studies have shown that NRL can directly bind to cone specific gene promoters 
including S and M opsin, cone arrestin and Trβ2, and in the presence of retinoid X receptor 
gamma (Rxrγ) and Nr2E3 repress expression of cone specific genes (Peng GH and Chen 2005, 
Peng GH et al., 2005)
The nuclear orphan receptor Nr2E3 is a direct transcriptional target of NRL, and it has been shown to play a major role in rod differentiation and maintenance (Chen J et al., 2005, Peng et al., 2005, Bumsted O’brien et al., 2004). In the presence of CRX and NRL, Nr2E3 potentiates CRX/NRL activation of transcription of rhodopsin gene, but represses their activity on the cone opsin promoters (Cheng J et al., 2005, Peng et al., 2005). However, Nr2E3 alone possesses limited regulatory activity on opsin gene promoters.

Orphan nuclear receptor RORβ is expressed in all cell layers of neural retina, and it has been shown to be important in photoreceptor differentiation as mice lacking RORβ do not express rods and have an excess of nonfunctional S cones. These mice do not express NRL, suggesting RORβ is upstream of NRL in the photoreceptor differentiation cascade (Jia L et al., 2009). Srinivas et al., 2006 showed that RORβ acts synergistically with CRX to initiate S opsin transcription in cones. This data together with RORβ’s patter of expression suggests it may be implicated in the process of differentiation of not only rod photoreceptors but other retinal cells as well.

The nuclear receptor Trβ2 is a ligand-regulated transcription factor expressed in the newly differentiated cones in the developing retina (Ng L et al., 2001). Mice deficient in Trβ2 contain a retina with cones that express only M opsin and lack S opsin. Transient transfection with Trβ2 in developing retinas in the presence of thyroid hormone, activated M opsin transcription and inhibited CRX mediated transcription of S opsin. (Yahagi Y et al., 2002). These data suggest a crucial role for Trβ2 signaling in opsin patterning perhaps by establishing the M/S opsin gradient in the mammalian retina.
Complex interactions between these six previous transcription factors regulate differentiation of photoreceptors in the developing retina from a pool of retinal progenitor cells. Studies have emerged showing each of these transcription factors regulates its own expression, regulates expression of the other factors by binding to their promoters, regulate the actions of the other transcription factors by acting in parallel or downstream, and feedback regulatory information to the promoters of the upstream factors that induce them (Henning AK et al., 2008). More research is necessary to completely understand all the components required for determination of a specific retinal cell type.

1.4 INDUCTION OF ROD FORMATION

As mentioned before, currently there are minimal possibilities for treating retinal degeneration. Repair of damage to photoreceptor cells by cell transplantation is one of the most feasible types of central nervous system repair as photoreceptor degeneration initially leaves the inner retinal circuitry intact and transplanted photoreceptors need to make single, short synaptic connections. Most importantly, a number of groups have demonstrated successful transplantation of rod photoreceptors into retinas of mouse models of retinal degeneration with transplanted cells making synaptic connections with host cells (del Cerro M et al., 1989, Bartsch U et al., 2008, MacLaren RE et al., 2006). Therefore, restoring visual activity in patients with retinal degeneration is a possibility if the proper number of cells at the proper developmental stage is obtained.
There is a variety of compounds that can positively or negatively regulate rod photoreceptor differentiation. One of the compounds that can increase the number of rods in developing retinas, both in vitro and in vivo is retinoic acid (RA). RA is a metabolite of vitamin A (retinol) that has been shown to have essential functions in growth and development. It acts through Hox genes, which ultimately control anterior/posterior patterning in early developmental stages. Retinoic acid acts by binding to the retinoic acid receptor (RAR) which binds to DNA as a heterodimer with the retinoid X receptor (RXR) in regions called retinoic acid response elements (RAREs). Binding of RA to RAR alters the conformation of the RAR affecting the binding of other proteins that either induce or repress transcription of nearby genes including Hox genes (Holland LZ. 2007). Kelly et al., in 1994 showed that RA promotes differentiation of rod photoreceptors in cultures of embryonic rat retinal cells. The effects of RA on rods were confirmed in vivo by Hyatt et al., who showed that RA given to zebra fish embryos, produced retinas with higher number or rods. In addition, Kelly et al., in 1999 validated the in vitro results by providing RA to developing rat retinas injecting pregnant rats at E18 and E20 with RA and looking at the effects in the retinas of the new born pups. Retinas of the treated newborn pups contained substantially more rods than untreated pups. These studies provided support for the importance of RA in rod development. However retinas developed normally in the RARβ2/RARγ2 double-null mutant mice, (Kastner P et al., 1995) suggesting RA is not necessary for rod development, perhaps due to compensation by another signaling cascade. Whether RA is necessary or not for rod development still leaves an important function for RA for in vitro differentiation of rods as it may be an important tool for obtaining large quantities of rods for transplantation.
Hedgehog is another signaling mechanism utilized by the CNS to establish the dorsal/ventral pattern (Ericson J et al., 1997). Hedgehog (Hh) proteins are secreted ligands whose receptor complex is composed of two multipass transmembrane proteins, Patched and Smoothened. Several different hh genes have been identified in vertebrates (Hammerschmidt M et al., 1997). Levine et al., 1997 found that addition of Hh to a culture of E18 retinal cells resulted in early increase in mitotic events with eventually ten times more rhodopsin expressing cells at the end of a fourteen day culture. Recent studies have shown Hedgehog signaling reduces the length of G1 and G2 phases producing a shorter cell cycle by modulating cyclin D1, cyclin A2, cyclin B1, and cdc25C activation. (Locker M et al., 2006). In addition, Yang et al., showed Sonic hedgehog expressed by daughter cells that have just exited cell cycle initiates maturation of progenitor cells by down-regulating the Notch ligand Delta1 expression (Yang HJ et al., 2009). These findings support a role for Hedgehog in transient amplification of progenitors that are closer to cell cycle exit, and therefore if activated in the right rod developmental stage, it will induce cell division of rod progenitors and eventually produce more rods.

Taurine has also been shown to promote rod photoreceptor differentiation in rat retina. Using low-density cell cultures of P0 rat retina, Altshuler et al., 1993 demonstrated that addition of taurine to the retinal disassociated culture caused an increase in the number of rhodopsin-expressing cells. Subsequently, Young and Cepko 2004 showed that taurine acts via GlyRα2 subunit-containing glycine receptors expressed by retinal progenitor cells at birth. The binding of taurine to the glycine receptor caused depolarization of the precursor cells and an increase of intracellular calcium thought to be responsible for activating rod differentiation in these cells. The downstream mechanism by which taurine activates rod development remains unclear.
Finally, Hunter et al., found that plating retinal progenitor cells on lamininβ2 rich matrices caused an increase in the number of rhodopsin positive cells in the cultures although the precise mechanism has not been elucidated (Hunter D. D et al., 1992)

On the other hand there have been studies where some factors that inhibit rod development have been elucidated. Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) were the first factors reported to inhibit rod photoreceptor (Lillien L. and Cepko C 1992). When the EGFR is activated by adding EGF or TGFα to a culture of retinal disassociated cells, the progenitor cells increase their rate of proliferation. Over-expression of EGFR on in vitro culture of RPCs decreased the number of rods present at the end of the culture while increasing the number of Muller glia cells when compared to control cultures. The number of other cell types present in the culture did not change significantly between controls and treated, therefore, the effects of EGFR activation are specific to rod differentiation (Anchan R. M et al., 1991, Lillien L. 1995).

Other critical negative regulators of rod photoreceptor are ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) signaling. Several groups have reported that CNTF/LIF specifically blocked the production of rod photoreceptors in both mice and chicks (Ezzeddine ZD et al., 1997, Neophytou C et al., 1997). Zhang et al., 2004 demonstrated that the effects of CNTF and LIF on rod photoreceptor differentiation were modulated specifically by the actions of STAT3. When the activity of STAT3 was inhibited using lentivirus encoding a dominant negative STAT3 protein, CNTF and LIF did not inhibit rod differentiation. Using a lentivirus
coding for dominant negative STAT1 did not abolish LIF/CNTF effects on rod development. In addition, inhibition of the MAPK/ERK pathway did not have any effects on the CNTF/LIF mediated inhibition of rods. They concluded that in addition to natural stimulatory factors, the STAT3 signal transduction pathway acts as a brake to regulate rod photoreceptor formation. They postulated the actions of STAT3 were occurring in a rod precursor cell or some earlier progenitor and that inhibition of STAT3 activity was linked to rod differentiation.

1.5 IGF1 AND NERVOUS SYSTEM DEVELOPMENT

Insulin is best known for its role in glucose uptake and metabolism, whereas the insulin-like growth factors are well characterized as growth promoting peptides. Both of these peptides signal through receptor tyrosine kinases (RTK) that are expressed in the nervous system (Bondy CA et al., 1990, Havrankova J et al., 1978), therefore, both may have an important role in cell proliferation and neurogenesis. Characterization of their RTK function with KO mice have provided evidence that in mice, IGF may be essential for proper central nervous system development, while the IR may either be redundant or play a more subtle role. For example, null mice develop normally but due to severe diabetic ketoacidosis they die shortly after birth (Accili, D et al., 1996) suggesting that the IR is not required for neuronal development. In addition, Bruning et al., 2000 generated a mouse strain with neuron-specific disruption of the Ir gene and the animals developed normal brains and the survival of neurons was not affected.

In contrast, Igflr−/− mice developed by Liu et al., 1993, are born with smaller brains that have altered brain structures, and a significant increase in the density of neural cells in the spinal cord and brainstem (Liu, J. P et al., 1993). Furthermore, detailed examination of cochlear
development showed that differentiation of the neurons in the inner ear is delayed, with significant decrease in the number of auditory neurons together with aberrant expression of early neural markers (Camarero, G et al., 2001). Moreover, evidence of IGF1R/IGF1 signaling involvement in nervous system development was presented by Vicario-Abejon et al., 2003, who demonstrated that locally born olfactory bulb stem cells proliferate in response to insulin-related factors and require endogenousIGF1 for differentiation into neurons and glia (Vicario-Abejon, C et al., 2003). Furthermore, ectopic expression of IGF in dorsal cells of xenopous embryos led to the induction of ectopic eyes and eye expansion and, when expressed in ventral cells, induced secondary head-like structures (Pera et al., 2001; Richard-Parpaillon et al., 2002).

The insulin and IGF1 receptors are receptor tyrosine kinases (RTK) that undergo autophosphorylation upon ligand binding and subsequently associate and activate insulin receptor substrate (IRS) adapter proteins by inducing their phosphorylation. These IRS proteins are responsible for transducing insulin or IGF1 action by recruiting additional SH2 signaling proteins including phosphatidylinositol 3 kinase (PI3K), growth factor receptor bound protein 2 (GRB-2), SH2-containing phosphatase-2 (SHP-2), GTPase activating protein (GAP), and phospholipase C-γ (PLC-γ), among others (Russo VC et al., 2005, Li W and Miller WT 2006, Adams TE et al., 2000)

In mammalian systems, insulin stimulation has been shown to cause activation of the Ras/mitogen-activated protein kinase (MAPK) pathway which is one likely candidate pathway by which IR/IGF-IR signaling might control neurogenesis during development. Ligand binding to the IR results in tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins
and/or Shc, which through the adaptor protein Grb2, results in recruitment to the membrane of SOS for the activation of Ras. This MAPK activation has been shown to be required for nerve growth factor/epidermal growth factor-dependent differentiation of PC12 cells. (Tan and Kim 1999).

In addition, stimulation of the IR/IGFR also activates the phosphoinoside 3-Kinase (PI3K) pathway which has been shown to regulate cell growth by controlling ribosome biogenesis and protein synthesis. (Leeyers and Hafen 2004) Folli et al., reported IRS-1 and PI3K immunoreactivity in adult rat retina especially in the ganglion cell layer, but some amacrine and bipolar cells were also positive. Gosbell et al.,1 and Diaz et al., demonstrated that the photoreceptor layer also of adult rats expressed IRS-1, AKT and extracellular regulated kinase (ERK) 1 and 2 phosphorylation increases after supraphysiological insulin treatment for 24 hours in embryonic retinas, suggesting that these pathways may play an important role in neuroretina development. Activation of the Ser/Thr kinase AKT by PI3K is necessary for the pro-survival mechanism of insulin action because AKT inhibits apoptosis by phosphorylating a number of apoptosis regulators such as caspase 9, Bad, glycogen synthase kinase (GSK) among others. Barber et al., also demonstrated AKT dependent cell survival in culture retinal neurons in response to IGF-1.

1.6 EPIGENETICS AND DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM.

Epigenetics in general refer to stable and heritable modifications of DNA and its associated histone proteins (chromatin) that are independent of the underlying DNA sequence, that help to determine the phenotypic traits of cells during development (Bergel SL et al 2009). The
nucleosome is the core unit of chromatin, which consists of 147 bp of DNA wrapped around histone octamers formed by two of each of the histone proteins H2A, H2B, H3 and H4 (Bergel SL 2007). Proper regulation of gene expression during a particular stage of nervous system development is controlled by the transcriptional machinery as well as the modulation of epigenetic mechanisms including DNA methylation, nucleosome and chromatin remodeling, noncoding RNA-mediated posttranslational regulation, and histone modifications (Hsieh J, Gage FH 2005, Wu H, Sun YE 2006). Histones can be covalently modified at their N-terminal tails to regulate transcriptional activation and repression. Examples of these post-translational modifications include acetylation, methylation, phosphorylation, ADP-ribosylation, sumoylation, ubiquitylation and proline isomerization (Bergel SL 2007, Kouzarides, T 2007). Moreover, histones are often concurrently modified on several residues and these modifications generate distinct docking sites for the recruitment of multiprotein nuclear complexes that influence the structure and function of chromatin (Turner BM 2007).

DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmt) that include de novo (Dnmt 3a, Dnmt 3b) and maintenance methyltransferases (Dnmt1) (Bestor TH 2000, Robertson KD, Wolffe AP 2000). DNA methylation inhibits gene expression by either directly interfering with transcription factor binding to DNA or recruitment of methyl-CpG binding domain (MBD) proteins that forms a complex with histone deacetylases (HDAC) to transform chromatin to a repressive state (Watt F, Molloy PL 1988, Fan G, Hutnick L 2005). It was recently discovered that DNA methylation is reversible and subject to dynamic regulation throughout embryogenesis and possibly the postnatal CNS (Morgan HD et al 2004, Jost JP et al 2001, Feng J et al 2005). Consistent with its significant role during development, mutations of
Dnmts in mice all lead to embryonic or early postnatal lethality (Li E et al 1992, Okano M, et al 1999).

Whereas modification of DNA by methylation generally leads to gene silencing, posttranslational modifications of histone proteins can lead to gene activation or repression (Jenuwein T, Allis CD 2001). One of the best-studied modifications is the acetylation status of lysine residues, a reversible process that is catalyzed by either histone acetyl transferases (HAT) or histone deacetylases (HDAC). The addition of an acetyl group by a member of the HAT family decreases the interactions between the negatively charged DNA backbone and the positively charged histone tail. Decrease in interactions in turn results in a conformational change that may lead to a less compacted nucleosome which is more accessible to transcription factor complexes. Therefore, histone acetylation is associated with increased gene transcription. On the other hand, HDAC remove the acetyl group, potentially leading to a general repression of gene transcription. Methylation of histones is another form of epigenetic regulation that has heritable, long-term effects. Studies in the last few years have found that methylation on histone H3 at lysine 4 (K4) is associated with transcriptional activation whereas the di- and tri-methylation on histone H3 at lysine 9 (K9) is indicative of transcriptional inhibition (Martin C, Zhang Y 2005, Mosammaparast N, Shi Y 2010). These histone modifications can be regulated dynamically, and thus may contribute to fast adaptive transcriptional responses to extracellular cues. Even though the extracellular signals and intracellular pathways that lead to histone modification in neural progenitor cells remain poorly understood, we believe that chromatin-modifying enzymes are targeted by signaling pathways that directly link environmental cues to gene expression.
Histone lysine methylation had long been thought to be an irreversible chromatin mark. However, it was recently demonstrated that this process was reversible by the activity of histone demethylases (Martin C, Zhang Y 2005, Mosammaparast N, Shi Y 2010). Two classes of histone lysine demethylases were discovered, the amine oxidase-related enzymes and the Jumonji C-terminal domain (JmjC)-containing enzymes. Lysine-specific demethylase 1 (LSD1) was the first histone demethylase discovered and belongs to the superfamily of the flavin adenine dinucleotide (FAD)-dependent amine oxidases (Shi Y et al 2004). Subsequently, the first JmjC-containing histone demethylase (JHDM1a/FBXL11) was biochemically identified (Tsukada Y et al 2005). Therefore, like all other histone modifications, histone lysine methylation is dynamic and reversible. Its addition and removal are catalyzed by histonemethyltransferases and demethylases, respectively. Histone demethylases contribute to the regulation of the steady-state levels of histone methylation and are thus crucial for many chromatin-based cellular processes including cellular differentiation.

1.7 OVERALL GOAL

In this study I investigated the process of rod photoreceptor differentiation, specifically, I examined the signal pathways involved in converting the cell from retinal progenitor to rod photoreceptor. Many studies have reviewed the genetic processes that take place during rod photoreceptor development particularly transcription factors activation and inhibition. However, not many studies have addressed the question of how these transcription factors are regulated or what signal transduction pathways are utilized by progenitor cells to ultimately differentiate into rods.
These studies focus on the extrinsic signals that result in rod photoreceptor differentiation, their signal transduction pathways, and the activation of cytoplasmic molecules that would eventually lead to transcription modulation of rod specific gene.

I hypothesize that activation of PKC during retinal development, first allows the progenitor cells to exit mitosis by inhibiting STAT3 function, and second, it specifically activates transcription of rod specific genes, by manipulating epigenetic markers.
CHAPTER 2
MATERIALS AND METHODS

Animals
C57Bl/6J and PKC Gamma knockout (B6;129P2-Prkcc<sup>tm1Stl/J</sup>) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All experiments were approved by the Animal Care and Use Committee of Pennsylvania State University School of Medicine. Mice were housed on a 12-hr/12-hr light/dark cycle with ad libitum access to rodent chow. Embryonic day 17.5 (E17.5), postnatal day 1 (PN1), PN3, PN5, PN7 and PN13 were used for tissue sectioning and immunohistochemistry. PN1 retinas were used for retinal explant cultures. Most of the litters were born on E20, which was considered equivalent to postnatal day 1 (PN1). Embryos were dissected into cooled phosphate-buffered saline (PBS) for retinal explant isolation. Genotyping of the PKC Gamma KO animals was carried out using the following primers: Wild type oIMR0496: GCT CCG ACG AAC TCT ATG CCA, oIMR0497: GTG GAG TGA AGC TGC GTG AGA, Mutant generic neo primers oIMR6916: CTT GGG TGG AGA GGC TAT TC and oIMR6917: AGG TGA GAT GAC AGG AGA TC using a very specific protocol developed by Jackson Laboratory. This protocol amplifies two sets of primers at the same time with the annealing temperature being reduced by 5 degrees Celsius every cycle.

<table>
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<th>Reaction Component</th>
<th>Volume (µl)</th>
<th>Final Concentration</th>
<th>Total Volume (µl)</th>
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<tr>
<td>ddH2O</td>
<td>3.05</td>
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### 10 X AB PCR BufferII
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### 25 mM MgCl2
|            | 0.96 | 2.00 mM | 0.96 |

### 2.5 mM dNTP
|            | 0.96 | 0.20 mM | 0.96 |

### 20 uM oIMR6916
|            | 0.45 | 0.75 uM | 0.45 |

### 20 uM oIMR6917
|            | 0.45 | 0.75 uM | 0.45 |

### 20 uM oIMR0496
|            | 0.60 | 1.00 uM | 0.60 |

### 20 uM oIMR0497
|            | 0.60 | 1.00 uM | 0.60 |

### 5 mM DNA Loading Dye
|            | 1.66 | 0.69 mM | 1.66 |

### 5 U/ul Taq DNA Polymerase
|            | 0.07 | 0.03 U/ul | 0.07 |

### DNA
|            | 2.00 | - | 2.00 |

### PCR Cycle protocol

<table>
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<th>Time</th>
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<td>1.5 min</td>
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<td>2</td>
<td>94</td>
<td>35 sec</td>
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<td>3</td>
<td>64</td>
<td>45 sec*</td>
<td>*-0.5 C per cycle</td>
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<td>4</td>
<td>72</td>
<td>45 sec</td>
<td>repeat steps 2-4 for 12 cycles</td>
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<td>5</td>
<td>94</td>
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<td>7</td>
<td>72</td>
<td>45 sec</td>
<td>repeat steps 5-7 for 25 cycles</td>
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<td>9</td>
<td>10</td>
<td>-</td>
<td>Hold</td>
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Retina isolation and culture

Whole retinas were isolated from pups at post natal day 1 (PN1), by removing the sclera and most of the retinal pigmented epithelium (RPE) layer (Sparrow et al., 1990; Zhao and Barnstable, 1996; Zhang et al., 2002; Zhang et al., 2004) and cultured in UltraCulture™ (Cambrex Bio Science Rockland, ME, USA) serum-free medium supplemented with gentamycin antibiotic (10 µg/ml). Retinas were culture individually in 1ml of media in a 24-well culture dish at 37°C in a 5% CO2, balance air, atmosphere. Medium was changed every other day by replacing 0.5ml with fresh medium.

Dissociated retinal cells were obtained by incubating isolated retinas in 0.25% trypsin at 37°C for 15 min. Retinas were transferred to tubes containing DMEM medium with 10% FBS. A flame-polished glass Pasteur pipette was used to triturate the retinas to obtain single cells. Cells were then placed on a glass slide coated with poly-L-lysine (Sigma St Louis, Missouri), and fixed in 4% PFA for 30 min. Using immunohistochemistry, cells were subsequently double-labeled for PCNA (1:5000) and PKC βI (1:1000), PKC γ (1:1000) or STAT3 (1:1000).

Reagents

Polyclonal antibodies against PKCβ1, STAT3, PKC alpha, pSTAT3 Ser 737, pSTAT3 (Y705), pGSK3 β (Ser 9), GSK3 β, ppI3K p85 (Tyr 458)/p55 (Tyr 199), PI3K p85 (19H8), PI3K p110α, PI3K p110 β, PI3K p110 γ, PI3K p110 Class III, LSD1 (Cell Signaling Technology, Inc., Beverly, MA), pERK (Tyr 204), ERK , pAKT1/2/3 (Ser 473), total STAT3 C20 (Santa Cruz Biotechnology, Santa Cruz, CA) PCNA (Sigma St Louis, MO), H3K4Me2 (ChIP grade), anti H3K27Me3 (IP grade) (Upstate Charlottesvile, VA), SOCS3, H3K9Me2 (ChIP grade) and PKC
Gamma (Abcam, Cambridge, MA) were employed in these experiments. Antigen peptides for STAT3 were used for blocking reagents to check antibody specificity. Ret-P1 monoclonal antibody recognizes an epitope on the N-terminus of opsin of rod photoreceptors (Barnstable, 1980; Hicks and Barnstable, 1987). Recombinant LIF was purchased from Millipore (Billerica, MA), IGF1 mouse recombinant from Sigma (St Louis, MO), PMA from LC Laboratories (Woburn, MA), Go7874 and PKC β inhibitor, and AKT inhibitor VIII from Calbiochem (Darmstadt, Germany). PKC β inhibitor made by Eli Lilly and commercialized by Calbiochem has an IC50 of 12nM for PKC β1 and 5nM for PKC βII, 331nM for PKC alpha, and higher than 1uM for PKC Gamma and epsilon, PI3Kinase inhibitor (Ly294002) from Cell Signaling technology, Inc (Beverly, MA), FGF2, EGF, and BDNF from PeproTech (Rocky Hill, NJ).

**Western blot**

Whole cell extracts were prepared and Western blot assays performed as described previously (Asao and Fu, 2000; Zhang et al., 2004). Briefly, retinas from postnatal mice or explanted retina were suspended in a whole cell extract buffer. The tissues were frozen and thawed three times to lyse the cells. The supernatant was collected by micro-centrifugation, and protein concentrations were measured. 25 to 30 µg of lysate were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immun-BlotTM polyvinylidene difluoride membrane (Biorad Hercules, CA). After blocking with 5% non-fat milk in washing buffer, membranes were incubated with primary antibodies. Following washes, they were incubated in anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase. The immunoreactive bands were visualized using SuperSignalR Chemiluminescent Substrate (Thermo Scientific Rockford, IL). The quantitative image analysis was performed using Image J 1.29 (downloaded from [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)).
Histology and immunocytochemistry

Whole eyes and explanted retinas were fixed with 4% paraformaldehyde in PBS for 24 hr at 4°C. After three washes with PBS, fixed explants were dehydrated through a series of graded ethanols and embedded in paraffin. All samples for one experiment were placed in the same blocks and sectioned for immunohistochemistry. Antigen retrieval was performed using 6.5 mM Sodium Citrate pH 6 and boiling for 45 min. A standard immunohistochemistry protocol (Zhang et al., 2003, 2004) was employed for single or double labeling using fluorescent dye-conjugated secondary antibodies (Jackson Immuno-Research Laboratory, West Grove, PA). Sections were imaged using an Olympus Fluoview FV1000 confocal microscope (Olympus Center Valley, PA). For each set of experiments, acquisition parameters for each antibody were maintained constant.

Real-time RT-PCR

Total RNA was isolated by TRIzol (Invitrogen Carlsbad, California) and purified by RNeasy mini kits (Qiagen Hilden, Germany). Final concentrations were determined spectrophotometrically using a GeneSpect III (Hitachi Tokyo, Japan). For each sample, 50 μl RT reaction containing 2 μg RNA, 10 μl 5× first-strand buffer, 5 μl 10 mM deoxyribonucleotide triphosphates, 5 μl 0.1 mM dithiothreitol, 1 μlRNase-OUT, and 1μl SuperScript II reverse transcriptase. (Invitrogen Carlsbad, California) was incubated at 42°C for 50 min.

Real-time PCR used a 25-μl reaction volume containing 12.5 μl Sybr-green supermix (BioRad Hercules, CA) with primers and 12.5 μl diluted RT product. Annealing temperatures were set at
60°C for 40 cycles using an iCycler (BioRad Hercules, CA). Primer sequences were: opsin: fwd: 5′-TGC TGT TTT CCT TGG CCT TTG G-3′, rev: 5′-TCT CTT CAG CAT GCC AGG AAG T-3′; beta-actin: fwd: 5′-GTG GGG CGC CCC AGG CAC CA-3′, rev: 5′-CTCCTT ATT GTC ACG CAC GGA TTT C-3′; Crx fwd: 5′-TCA AGA ACC GGA GGG CT-3′, rev: 5′ATA GCT CTG GCC TGA TAG GGA

Chromatin Isolation for ChIP and Q-PCR.

Approximately 10 fresh or cultured mouse retinas were harvested and collected in PBS on ice. After washing retinas one time in PBS, cells were isolated in 1ml PBS by pipetting up and down and vortexing cell suspension. Cells were crosslinked in 1% formaldehyde and incubated on rotator at room temperature for 15 minutes. To quench crosslinking reaction 0.125ml 1M glycine was added and incubated on ice for 5 minutes. Cells were centrifuged for 7 minutes at 4000 rpm at 4°C and supernatant was discarded. Pellet was resuspended in 500µl L-CHIP buffer (+ 1mM PMSF + 5µl PI). After sonication to shear DNA to lengths of between 200 and 2,000 bp, protein concentration in lysate was measured and adjusted to 1mg/ml with L-CHIP buffer. This chromatin preparation was stored at 4°C until immunoprecipitation was performed. Before ChIP reaction retina chromatin was precleaned by centrifugation at maximum speed for 5 min.

Chromatin immunoprecipitation.

For ChIP reactions 350µl of chromatin was diluted 10 times in D-CHIP buffer and 5µg antibodies for specific histone modification were added. Reactions were incubated on a rotator overnight at 4°C. Simultaneously 30µl protein A or G beads (Sigma) slurry were washed 2 times
in washing buffer (9:1=D-CHIP: L-CHIP), resuspended in the same buffer with 500µg/ml salmon sperm DNA (Invitrogen) and 100µg/ml BSA (Invitrogen) for blocking and incubated on rotator overnight at 4°C. After washing 2 times with washing buffer beads were combined with the chromatin and antibody mix and rotated for 2 hours at 4°C. Beads were washed 4 times with 1 ml LS-CHIP buffer, 1 time with 1ml HS-CHIP buffer and were eluted with 350µl of E-CHIP buffer by rotating at room temp for 10 minutes. Immunoprecipitate (IP) and 50µl of input chromatin (Input) were treated with 0.5mg/ml of Proteinase K (Roche) and RNaseA (Roche) at 37°C for 30 min. IP and Input were incubated at 65°C overnight to reverse crosslinking. DNA was extracted twice with phenol/chlorophorm (Roche), once with chlorophorm and ethanol and then precipitated with glycogen (Roche) and sodium acetate. DNA was dissolved in 50µl water and subjected to Q-PCR.

Buffers for ChIP

L-CHIP (lysis): 1% SDS, 10mM EDTA, 50mM Tris-HCl pH8.0

D-CHIP (dilution): 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH8.0, 167mM NaCl

LS-CHIP (low salt): 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8.0, 150mM NaCl

HS-CHIP (high salt): 0.1% SDS, 1% Triton X-100, 2mMEDTA, 20mM Tris-HCl pH8.0, 500mM NaCl

E-CHIP (elution): 1% SDS, 0.1M NaHCO3
**Quantitative-PCR.**

For quantitative real-time PCR we used 2x iQ-SYBR Green PCR supermix from Bio-Rad. Samples in the triplets were run on iQ5 Multicolor Real Time PCR Detection System (Bio-Rad). Each run included suitable standard curve samples using wide range of concentration for genomic DNA prepared similar to inputs.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Reverse</th>
<th>location</th>
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<td>rhodopsin 1000</td>
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<td>gac tag tgc gag acc tga g</td>
<td>chr7:16458611+16458786</td>
</tr>
</tbody>
</table>

**Table 1.** Primers for qPCR used for ChIP analysis with chromosomal location.

**Statistical analysis**

Statistical analyses were performed using the GraphPad Prism software. Student’s t test (two-tailed, unpaired) was used to compare two groups and one-way ANOVA (with Newman–Keuls post test) was used to compare more than two groups.
Chapter 3 evaluates the effects of some growth factors known to promote cell differentiation, on rod photoreceptor development using retinal explants from newborn mice. Integration of diverse intrinsic and extrinsic factors is responsible for regulating fate choices of neural progenitors. Like many regions of the CNS, the retina develops from a multipotential set of neuroblasts that give rise to specific cell types in a stereotypic sequence (Turner and Cepko 1987, Holt et al., 1988). Rod photoreceptors are among the last cell types to differentiate from this progenitor cell pool. Explants of embryonic retinas are capable of forming all the major classes of cells suggesting much of the information necessary to generate retinal neurons, including rod photoreceptors, is intrinsic to the retinal epithelium (Sparrow et al., 1990). Within the retinal epithelium, however, photoreceptor differentiation is influenced by a number of cell interactions mediated by either direct cell-cell contacts or through soluble factors released from cells (Watanabe and Raff, 1990, 1992; Reh, 1992; Altshuler and Cepko, 1992). Several factors have been shown to promote the formation of rods but the mechanisms by which these signals regulate the transition from progenitor to postmitotic neuron are only partially understood (Altshuler et al., 1993; Levine et al., 2000; Yang, 2004). Therefore the microenvironment generated during the differentiation process of specific neurons determines important modifications in genetic expression that would give rise to a specific cellular phenotype. For example, specific soluble factors may be responsible for the activation of a network of protein kinases, followed by phosphorylation of specific transcription factors and could represent a common pathway of gene regulation and cell differentiation. Lacovitti et al., 2001 proposed
protein kinase C (PKC) as a likely candidate involved in the regulation of cell growth and differentiation. They showed that phorbol esters, known to be specific activators of PKC, together with FGF-1, and dopamine induced differentiation of an embryonic carcinomal stem cell line into dopamine neurons. Furthermore, Pillai et al., 2006 demonstrated that activation of PKC through PMA, induced mature human astrocytes to modify their morphology and transform into cells with neuronal phenotype that expresses neuronal markers. Finally, Mangoura et al., 1997 demonstrated that in vitro activation of PKC by phorbol esters determined the expression of an immature phenotype in astrocytes.

As discussed in chapter 1, IGF1 signaling through the IGF1R has a pivotal role in development of the CNS illustrated by the profound defects on brain and spinal cord development (Liu et al., 1993). In addition, Zygar et al., 2004 showed that in the teleost retina where rod photoreceptors are produced continually, IGF1 produced by cones photoreceptors mediates at least in part the production of new rod photoreceptors in the adult retina.

In this chapter we test the hypothesis that activation of PKC during retinal development induces rod photoreceptor differentiation. We show that IGF1 treatment of P1 WT retinal explants increased the number of rods at the end of a four day culture, and that this effect was mediated by PKC activity. As the first step to study the pathways by which rod differentiation is controlled, we decided to compare the actions of some growth factors that have been shown to modulate CNS development. We utilize retinal explants cultures from postnatal day 1 WT mice cultured for a period of up to eight days and we used rhodopsin expression as a marker of differentiated rods.
3.1 Retinas explanted from P1 WT animals develop a higher number of rods at the end of a four day culture when the media was supplemented with IGF1 or PMA.

To test whether extrinsic signals from peptide growth factors or neurotrophins could stimulate the number of rod photoreceptors generated in explant cultures we added 50ng/mL IGF1, 100nM FGF2, 100nM EGF, or 100nM BDNF for four days to P1 retinal explants. We also tested the action of a phorbol ester, PMA, a molecule known to stimulate differentiation in many cell types. After four days in culture, retinas were fixed overnight on 4% PFA, paraffin embed, 5 micron sections were cut and labeled with an opsin antibody to detect the presence of rod photoreceptors. Both EGF and FGF2 increased the number of rods present at the end of the culture (p < 0.05), but IGF1 (4.92 ± 0.56 N=6 < 0.0001) and PMA (4.60 ± 0.50, n=6, P<0.0001) gave a significantly greater increase (Fig 1A,B).

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**Figure 1.** Effects of stimulation of WT P1 retinal explants with IGF1, FGF, EGF, BDNF and PMA for four days. A). Immunofluorescent detection of opsin (green) overlaid with nuclear
counterstain (Hoechst, blue) of four day P1 retinal explants. P1 retinas were cultured from WT mice for four days in the presence of 50ng/mL IGF1, 100nM FGF2, 100nM EGF, 100nM BDNF or 100nM PMA. OL, Outer retinal layer; IL, Inner retinal layer. B). Quantitative graph of the number of rods present on P1 retinas at the end of a four day cultured in the presence of 50ng/mL IGF1, 100nM FGF2, 100nM EGF, 100nM BDNF or 100nM PMA, obtained by averaging the number of rods present in three individual histological cross section of a single retina. At least three different retinas were studied per treatment focusing on the central areas of the retina. ***P<0.005, *<0.05 vs control.

We decided to continue to investigate the effects of PMA and IGF1 only, and since the effects were very similar, we hypothesized that IGF1 and PMA might induce rod photoreceptor differentiation utilizing similar molecular mechanisms. A major effect of phorbol esters is the activation of PKC. We therefore tested whether the increase in opsin-positive cells induced by PMA and IGF-1 might be due to activation of PKC. To do so, we repeated the retinal explants experiments in the presence Go 7874, a pan inhibitor of PKC. Retinas cultured in the presence of IGF1 and Go7874 or PMA and Go7874 did not show any significant increase in opsin-positive cells over control (Fig 2 A, B). These results suggest activation of PKC, either chemically or by the growth factor IGF-1 can increase the production of opsin-positive rod photoreceptors in the neonatal mouse retina.
Figure 2. Inhibition of PKC abolishes the effects of PMA and IGF1 on rod photoreceptor differentiation. (A). Immunofluorescent detection of opsin (green) overlaid with nuclear counterstain (Hoechst, blue) of four day P1 retinal explants. P1 retinas were cultured from WT mice for four days in the presence of 50ng/mL IGF1, 100nM PMA or IGF1 plus 100nM Go7874 and PMA plus 100nM Go7874. OL, Outer retinal layer; IL, Inner retinal layer. (B) Quantitative graph of the number of rods present at the end of a four day culture of P1 WT retinas in the presence of 50ng/mL IGF1, 100nM PMA, or 100nM Go7874 together with PMA or IGF1. Numbers were obtained by averaging the number of rods present in the in three individual histological cross sections of a single retina. At least three different retinas were studied per treatment focusing on the central areas of the retina. Numbers were normalized to control values. ***P<0.005 vs control. Scale bar, 40µm

To ensure that the changes brought about by PKC activation were on rod photoreceptor differentiation rather than just on opsin expression, we examined the expression of the transcription factor Crx, another early marker of rods. After four days in culture we found that activation of PKC by PMA or IGF1 increased expression of Crx RNA over eight-fold (8.58±5.30, N=4 P<0.02) and (9.831 ± 2.185 N=4 P<0.007) respectively, an increase
comparable to that of opsin itself (5.20±2.42, N=4, P<0.02) and (7.205 ± 1.214 N=4 P<0.005) (Fig 3 A and B). Similarly, treatment with either IGF-1 or PMA increased Crx protein levels by approximately 2-fold (1.580 ± 0.1706 N=3 P<0.05 and 1.887 ± 0.04854 N=3 P<0.05 respectively) (Fig 3 D). These results suggest that activation of one or more isoforms of PKC is sufficient to trigger expression of multiple genes that are characteristic of the early stages of rod photoreceptor differentiation.

**Figure 3** IGF1 and PMA treatment of WT P1 retinal explants increase expression of rod specific genes. (A), (B) Quantitative RT-PCR assay showing expression of opsin and Crx in P1 retinal explants cultured for four days in the presence of PMA (A) or IGF1 (B). **P<0.005, *P<0.05. Beta-actin and GAPDH expression was used to normalize individual mRNA levels. Values in graph are normalized to control levels. (C), (D) The amount of opsin (C) and Crx (D) protein found in four day retinal explants of P1 retinas using Western blot analysis. Beta-actin expression was used to normalize total protein levels. Values in the graphs are normalized to
control levels. *P<0.05, **P<0.01 vs control. At least three retinas were studied per treatment.

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### 3.2 PKCβI and PKCγ are expressed in dividing progenitors at the time of rod photoreceptor differentiation.

PKC exists as a series of eight classical (α, βI, βII, and γ) and novel (δ, ε, η, and θ) isoforms that can be activated by phorbol esters, as well as atypical forms that are not sensitive to these agents. To define which PKC isoforms might be responsible for inducing rod formation we first examined the temporo-spatial pattern of classical and novel PKC isoform expression during retinal development. No expression of βII, δ, ε, η, and θ isoforms was detected by either immunohistochemistry or western blotting. On the other hand, both PKCβI (Fig 4 E-H) and PKCγ (Fig 4 A-D) were expressed in the retina during the late embryonic and early postnatal stages when rod photoreceptors are forming.

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**Figure 4** PKCβI and PKCγ isoforms are expressed in the retina during retinal development. Immunofluorescent detection of PKC Alpha (I-L red), Beta1 (E-H red) and Gamma (A-D red) from embryonic day 17.5 (A,E,I), P1 (B,F,J), P5 (C,G,K) and co-localization with opsin (green) expression at P5 (D,H,L).
Both PKCβ1 and PKCγ were expressed strongly at the outer margin of the retina from embryonic stages until the end of retinogenesis in the first postnatal week. In addition, both PKC isoforms were expressed by cells in the inner retina. On the other hand, PKCα was detected only after P5 when the differentiation of most retinal cells is substantially advanced (Fig 4 I-L) and it is expressed mainly in the bipolar cells of the inner nuclear layers, suggesting that it has a limited involvement, if any, in rod development.

At early postnatal stages the outer layer of the retina contains both proliferating progenitors and post-mitotic neurons. To test which cell population in this region expressed PKCβ1 and PKCγ we co-stained the sections with proliferating cell nuclear antigen (PCNA) antibody to label the dividing cells. We found both PKCβ1 and PKCγ co-localized with PCNA positive cells in the outer retina during late embryonic and early postnatal stages of retinal development (Fig 5). By PN3 it became harder to detect dividing cells in central retina, although many were still present in more peripheral regions.
**Figure 5** PKCβ1 and PKCγ isoforms are expressed in dividing cells during early retinal development. Expression of PKC Beta1 and Gamma (red) and co-localization with PCNA (green) in the central and peripheral retina of E15.5, E17.5, P1 and P3 retinas. OL, Outer retinal layer; IL, Inner retinal layer. Scale bar, 40µm.

Because much of the PKCβ1 and PKCγ labeling was in the cell processes, particularly the endings at the outer surface, it was difficult to get an accurate estimate of the extent of overlap in labeling. To overcome this difficulty we stained in a similar manner cells dissociated from E17.5 retinas. At this stage 95% of the cells were PCNA-positive, and of these 83% were also PKCβ1 positive and 85% were also PKCγ positive. This result confirms the expression of PKCβ1 and PKCγ isoforms in progenitor cells and indicates that most of the cells expressed both isoforms.
3.3 The effects of IGF1 and PMA on rod photoreceptor differentiation are abolished if the activities of PKCβI and PKCγ are inhibited.

To examine whether IGF-1 or PMA effects on rod photoreceptor differentiation were specific to PKC we tested the effects of these agents in the absence of the activity of PKCβI and/or PKCγ on retinal explants. We first obtained a PKC β inhibitor developed by Eli Lilly and commercialized by Calbiochem. This PKC inhibitor has an IC50 of 12nM for PKC β1, 5nM for PKC βII, 331nM for PKC alpha, and higher than 1uM for PKC Gamma and epsilon. Since this inhibitor is 10 to 100 fold selective for PKC β1 and βII respectively, and PKC βII is not expressed in retinas during rod development, we used it as a specific inhibitor of PKC β1 in our retinal explants. Addition of PKC β inhibitor to PMA treated retinas reduced the number of rods present at the end of a four day culture compared to PMA alone (Fig 6 A, B), although the total number of rods present in the retinas was still two fold greater than untreated controls (2.1 ± 0.21 N=3 P<0.005). Even ten-fold higher concentrations of PKCβ inhibitor did not further reduce the number of rods, suggesting that the partial reduction was not due to sub-optimal concentrations of the inhibitor. As in previous experiments, however, addition of the pan-PKC inhibitor Go 7874 reduced the number of detectable rods to control levels.

We repeated these experiments using IGF1 to activate PKC and found that IGF1 treatments increased the number of rods present by fivefold (5.733 ± 0.3712 N=3 P<0.005). Addition of the PKCβ inhibitor substantially reduced the increase, although it continued to be two fold greater than untreated retinas (2.733 ± 0.1453 N=3 P<0.005). As in the previous experiments, ten-fold higher concentrations of PKCβ inhibitor did not change the magnitude of this increase whereas the pan-PKC inhibitor Go 7874 reduced the number of rods to control levels (Fig 6 C, D)
**Figure 6.** PKC β inhibitor reduces IGF1 and PMA effects on rod development but does not completely inhibit it. (A) and (C) Immunofluorescent detection of opsin (green) overlaid with nuclear counterstain (Hoechst, blue) of four day P1 retinal explants. P1 retinas were cultured from WT mice for four days in the presence of 100nM PMA (A), 50ng/mL IGF1 (C) or PMA plus 30nM PKCβ inhibitor and IGF1 plus 300nM PKCβ inhibitor. (B) and (D) Quantitative graph of the number of rods present at the end of a four day culture of P1 WT retinas in the presence of 100nM PMA (B), 50ng/mL IGF1 (D) or 30nM and 300nM PKCβ inhibitor together with PMA or IGF1. Numbers were obtained by averaging the number of rods present in the in three individual histological cross section of a single retina. At least three different retinas were studied per treatment focusing on the central areas of the retina. Numbers were normalized to control values. ***P<0.0005, **P<.005 vs control.

We then tested the effects of absence of PKCγ activity in the retina using PKCγ KO animals developed by Dr. Susumu Tonegawa at the Center for Cancer Research at the Massachusetts Institute of Technology.

As shown in Fig 7, when PKCγ KO retinas were cultured in the presence of PMA for four days they contained over seven times the number of rods compared with PKCγ KO control retinas cultured in medium alone (7.5 ± 0.52 N=4 P<0.0001). However, when the PKCγ KO retinas
were cultured in the presence of the PKCβ inhibitor the PMA stimulation of rod generation was abolished, and the total number of rods was reduced to below the levels in untreated cultures (0.57 ± 0.11 N=4 P=.09). Similarly, when the retinas from PKCγ KO animals were cultured in the presence of IGF1, we found that there were about nine times more rods than in untreated retinas (9.21 ± 0.55 N=4 P<0.0001), but retinas cultured in the presence of IGF1 plus the inhibitor of PKCβ had numbers of rods not significantly different from untreated retinas. This result confirms that the effects of PMA and IGF1 on rod development are mediated by either PKCγ or PKCβ1, however, in the absence of both isoforms, neither PMA nor IGF1 can induce rod formation.

**Figure 7.** In the absence of PKCβ1 or PKCγ, neither PMA nor IGF1 can induce rod photoreceptor formation. (A) and (C) Immunofluorescent detection of opsin (green) overlaid with nuclear counterstain (Hoechst, blue) of four day P1 retinal explants. P1 retinas were cultured from PKCγ KO mice for four days in the presence of 100nM PMA (A), 50ng/mL IGF1(C) or PMA plus 30nM PKCβ inhibitor and IGF1 plus 30nM PKCβ inhibitor. (B) The number of rods present at the end of a four day culture of P1 PKCγ KO retinas in the presence of 100nM of PMA, and PMA plus 30nM PKCβ inhibitor. (D) The number of rods present at the
end of a four day culture of P1 WT and PKCγ KO retinas in the presence of 50ng/ml IGF1 or IGF1 and 30nM PKCβ inhibitor. Total number of rods was counted from at least three cross sections of a single treated retina. At least three retinas were studied and the average was plotted. ***P<0.0005 vs control.

3.4. IGF1 receptor is expressed during rod photoreceptor differentiation

To this date, the expression of IGF1 receptor during retinal differentiation has not been studied. Our previous experiments indicated that IGF1 may be contributing to rod photoreceptor differentiation. Therefore, we investigated whether the IGF1 receptor was present during the time of rod development, or whether IGF1 may be exerting this effect by binding to another receptor. Using an antibody specific for IGF1R, we investigated its temporo-spatial pattern of expression from embryonic stage 17.5 (E17.5) to post natal day 7 (P7). At E17.5 and P1, we found a strong signal in all cells of the retina, including cells in the outer layers or the retina, a region where rod photoreceptor progenitors develop. As the retina matured, the signal became weaker, and localized to the inner layers of the retina (Fig 8).

Figure 8. IGF1 receptor is expressed in the retina during rod photoreceptor differentiation.
Immunofluorescent detection of IGF1R (red) from embryonic day 17.5 to post natal day 7 overlaid with nuclear counterstain (Hoechst, blue).

3.5 Summary of results

In this chapter we tested the hypothesis that activation of PKC during retinal development resulted in increased number of rod photoreceptors at the end of a four day culture. We investigated the effects of some growth factors and compounds that modulate cell differentiation, on rod photoreceptor differentiation in retinal explants. We wanted to identify mechanisms that retinal progenitor cells utilize during rod development to induce their differentiation. We found that of the five compounds tested; PMA, IGF1, EGF, FGF2, and BDNF, IGF1 and PMA had the highest effect on rod development measured as rhodopsin expression in P1 retinas cultured for four days. We confirmed that the IGF1 and PMA effects were not limited to rhodopsin expression, but that the effects were rod specific by measuring CRX protein and mRNA levels, another rod photoreceptor specific gene.

Since PMA has been shown to activate PKC, we examine whether the PMA and IGF1 induced effects on rod development were modulated by PKC. We demonstrated that the induction of rod development by IGF1 and PMA was abolished by Go7874, a pan inhibitor of PKC.

We therefore, searched for the specific PKC isoforms expressed in retina during development and found that only PKCβ1 and PKCγ were present during retinal development and that their expression co-localized with retinal progenitor-PCNA positive cells.

When PKCβ1 activity was inhibited using a specific inhibitor, the effects of PMA and IGF1 on rod differentiation were markedly reduced but not completely abolished. Furthermore, when both isoforms were inhibited, neither PMA, nor IGF1 increased the number of rods present at the end of a four day culture of P1 retinas.
These experiments confirmed our hypothesis, demonstrating that activation of PKC, specifically PKCβ1 and PKCγ, using PMA or IGF1 induced rod photoreceptor differentiation in retinal progenitor cells.
CHAPTER 4

FUNCTIONS OF PKCβ1 AND PKCγ IN ROD PHOTORECEPTOR DIFFERENTIATION DURING RETINOGENESIS

In the previous chapter we demonstrated that addition of PMA or IGF1 to a four day culture of WT P1 retinas substantially increased the number of rod photoreceptors present at the end of the culture, and that this effect was mediated by the activation of PKCγ and/or PKCβ1. In this chapter we investigated whether expression of PKCγ or PKCβ1 is necessary for rod photoreceptor differentiation during retinal development, and the mechanism that the two isoforms may utilize to influence rod differentiation.

The protein kinase C (PKC) family of serine/threonine phosphorylating enzymes is ubiquitously expressed and implicated in multiple cellular functions. These kinases transduce signals involved in many different processes including ion fluxes, neurotransmitter release, receptor modulation, cell proliferation, synaptic remodelling and gene expression. According to sequence homology and sensitivity to activators, at least 10 isoforms (encoded by nine different genes) have been described and they are grouped in three distinct classes: (1) calcium-dependent or classical cPKCs : α, βI, βII, γ (2) Ca Independent (Novel): δ, ε, η, θ, and (3) Atypical : λ, ζ, τ (Inoue et al., 1977) (Mellor et al., 1998). Conventional PKC isoymes are Ca2+-dependent, while novel and atypical isoymes do not require Ca2+ for their activation. All PKC isoymes, with the exception of the atypical group, are activated by diacylglycerol (DAG).
Most of the PKC resides in the cytosol in an inactive form. In this state, the pseudosubstrate sequence of the regulatory domain of PKC, found at the N terminus of the C1 domain, interacts with the catalytic domain and prevents access of the substrate to the catalytic site. The C1 domains in the regulatory domain of the classical and novel PKC isozymes bind the second messenger DAG as well as phorbol esters. Activation of PKC results from binding of a hormone or other molecules to a membrane receptor that activates phospholipase C (PLC) or phospholipase A2 (PLA2) via a G-protein-dependent phenomenon. The activated PLC hydrolyzes phosphatidylinositol-4, 5-bisphosphate (PIP2) to produce DAG and inositol-1,4,5-trisphosphate (IP3). The IP3 causes the release of endogenous Ca2+ that binds to the cytosolic PKC and exposes the phospholipid-binding site. The binding of Ca2+ to the C2 region of conventional and novel PKCs translocates PKC to the membrane and allows it to interact with DAG. These interactions of PKC with DAG transform PKC into a fully active enzyme because it
reduces the affinity of the pseudosubstrate domain for the catalytic site thus opening the folded conformation and allowing the activation of the enzyme. (Nishizuka et al., 1986) (Ng and Cantrell, 1997) (Eilers et al., 1995). The activity of PKC also depends on its localization within the cell. PKC interactions with carrier proteins such as receptors for activated C kinase (RACKs), proteins that interact with C kinase (PICKs), and substrates that interact with C kinase (STICKs) have been implicated in PKCs compartmentalization. Although through slightly different mechanisms, the common function of these anchoring proteins is to recruit the various PKCs into specific signaling networks where they can execute selected cellular functions. (Reviewed by Amadio M et al., 2006).

Anti PKC antibodies have been widely used as molecular marker for rod bipolar cells in the mature vertebral retina (Negishi et al., 1988) (Greferath et al., 1990). However, PKC expression during retinal development had not been investigated before. In the previous chapter we elucidated the expression of PKCγ and PKCβ1 during retinal development. In this chapter we investigated their function in rod photoreceptor differentiation. In 1998 Malek and Halvorsen demonstrated in neural crest-derived SH-SY5Y human neuroblastoma cells that specific activation of PKC with phorbol ester PMA targeted STAT3 to proteosome dependent removal of tyrosine phosphorylated STAT3. Moreover, administration of BIM a specific inhibitor of PKC prevented STAT3 degradation after administration of PMA. We hypothesized that activation of PKC in rod progenitor cells inhibits STAT3 activation, which has been linked to retinal progenitor cell proliferation, resulting in cell cycle exit and subsequent rod differentiation.
4.1 Activity of PKC β1 and PKCγ is essential for rod photoreceptor differentiation.

To examine the function of PKCγ on rod development we used PKCγ knock out (KO) mice. We crossed them back to C57BL6/6J WT animals to generate heterozygous littermates. These animals were crossed once more to generate in the same litter homozygous WT, KO, and heterozygous PKCγ animals to evaluate the effects of absence of PKCγ in retinal development using littermates with the same genetic background. Comparing rod photoreceptor development among littermates we found that at P5, the PKCγ KO animals did not contain any opsin-positive rod photoreceptors, whereas wild type littermates already had a substantial number of rods. At P7, the PKCγ KO animals appear to have fewer opsin-positive cells since the fluorescence intensity of equal areas of opsin-stained retinas was reduced by fifty percent in PKCγ KO retinas compared to wild type littermate retinas (0.43 ± 0.11 N=4 P<0.009). By P13, however, retinas from PKCγ KO animals had no detectable difference in the fluorescence intensity of opsin labeling photoreceptors compared to their wild type littermates (Fig 10 A, B). This suggests that the absence of PKCγ causes a substantial delay of rod development, but that eventually a full complement of rods is formed. One mechanism by which absence of PKCγ could delay rod differentiation is by retarding the exit of cells from the proliferative pool. To test this idea, we investigated the expression of PCNA in the outer layer of the retina, where photoreceptor progenitors are thought to be located, using the outer plexiform layer as boundary (Fig 10 A, C). By measuring fluorescence intensity we found that at P5 PKCγ KO contain twice as much labeling in the outer layer of the retina as wild-type littermates (2.82 ± 0.69 N=4 P<0.007). The difference was even more striking in the outer layer of the retinas of P7 PKCγ KO animals where we found over twelve times more PCNA labeling than their wild type littermates (12.69 ± 0.6847...
N=4 P<0.005). This maintenance of the progenitor pool was transient because at P13 neither the PKCγ KO nor the WT littermates had any PCNA positive cells.

Figure 10. Rod photoreceptor differentiation is delayed in retinas of PKCγ KO animals. (A) Immunofluorescence detection of opsin and PCNA (green) of PKCγ KO and WT littermate retinas of ages P5, P7 and P13. Sections were overlaid with nuclear counterstain (Hoechst, blue). (B) Fluorescence intensity of opsin present in retinas at ages P5, P7 and P13 comparing PKCγ KO retinas to wild type littermates. (C) Fluorescence intensity of PCNA present in the outer layer of the retinas at ages P5, P7 and P13 comparing PKCγ KO retinas to wild type littermates. We used the outer plexiform layer as the limit between outer and inner layers of the retina. *P<0.05, **P<0.01, ***P<0.005 vs control. Values were obtained by averaging the fluorescence intensity of at least three representative areas of at least three different retinas. Scale bar, 40µm
We hypothesized that since the activity of PKC β1 is intact in these animals, PKC β1 may be compensating for the absence of PKCγ activity, although the compensation was not complete and a delay in rod differentiation was observed. To test this hypothesis we used the specific PKCβ inhibitor in retinal explants of PKCγ homozygous mutant and WT littermates to generate retinas that lack the activity of both PKC isoforms. To effectively distinguish between a delay in rod development seen in PKCγ KO animals, and a complete block in rod development, we cultured PKCγ KO and wild type littermate retinas for eight days, which is enough time to achieve rod differentiation. We found that rod development was almost completely inhibited when both PKCγ and PKCβ1 activities were absent, with only 8% of the opsin labeling seen in wild type cultures (0.08 ± 0.018 N=4 P<0.009) (Fig 11). These data suggest that the presence of either PKCγ or PKCβ1 activity allows rod formation but if the activity of both isoforms is absent normal rod development is halted.

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**Figure 11.** Absence of PKCβ1 or PKCγ isoforms during retinal development inhibits rod photoreceptor formation. (A-D) Immunofluorescence detection of rhodopsin (green) in P1 retinas after eight days culture of PKCγ KO and WT littermates in the presence of 30nM PKCβ inhibitor. Sections were overlaid with nuclear counterstain (A, B) (Hoechst, blue). (E) Fluorescence intensity of rhodopsin present in P1 retinas after eight days culture of PKCγ KO and WT littermates in the presence of PKCβ inhibitor. ***P<0.009 vs control. Values were obtained by averaging the fluorescence intensity of at least three representative areas of at least
three retinas. Scale bar, 40µm.

4.2 Activation of PKC β1 and PKCγ results in reduced tyrosine 705 phosphorylation of STAT3

Activation of STAT3 during retinal development inhibits differentiation of rod photoreceptors (Ezzeddine et al., 1997, Kirsch M et al., 1998, Neophytou C et al., 1997, Schulz-Key S et al., 2002, and Zhang et al., 2004). We hypothesized that IGF1 and PMA mediated activation of PKC resulted in higher number of rods generated at the end of a four day culture because it inhibited the activity of STAT3. The function of tyrosine phosphorylation has been extensively studied, and it has been demonstrated it promotes STAT3 dimerization, translocation, and DNA binding (Fu et al., 1992). The functions of serine phosphorylation of STAT3 however, are not well understood. In spite of this, recent data support an important role for serine 727 phosphorylation of STAT3 in cellular growth control and differentiation (Bromberg et al., 1998; Turkson et al., 1998, 1999, Turkson et al., 1999, Frank et al., 1997), and it was also demonstrated recently that constitutively serine phosphorylated STAT binds to DNA even in the absence of tyrosine phosphorylation (Hazan-Halevy et al., blood 2010). Therefore, we investigated serine and tyrosine phosphorylation of STAT3 changes after activation of PKC in developing retinas, as a possible mechanism utilized during retinal development to promote rod differentiation.

We also looked at the effects of activation of PKC on total protein of STAT3. In 1998 Malek and Halvorsen found that PMA activation of PKC in neural crest-derived SH-SY5Y human neuroblastoma cells resulted in protein degradation of STAT3 which may be another mechanism utilized in the developing retina to inhibit STAT3 activity.
Postnatal day 1 retinal explants were pre-incubated in medium for five hours to allow activated STAT3 levels to return to baseline (Zhang et al., 2005). The process of retinal extraction activates a stress response that involves STAT3 tyrosine phosphorylation. We found that five hours incubation allows phosphorylation of STAT3 to return to baseline. We then treated the explants with PMA or IGF1 for thirty minutes and analyzed the levels of serine and tyrosine phosphorylation of STAT3 in addition to total levels of STAT3 (Fig 12). Thirty minutes treatment with PMA or leukemia inhibitory factor (LIF) did not significantly change the levels of serine 737 phosphorylation or the total levels of STAT3 proteins.

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 12.** Retinas treated with PMA or LIF for five minutes do not change their levels of total STAT3 or serine phosphorylation of STAT3. (A) Quantitative graph of the amount of serine 737 phosphorylated STAT3 found in P1 WT retinal explants cultured for five hours in ultraculture medium followed by thirty minutes treatment with 100nM PMA or 20ng/mL LIF. (B) Quantitative graph of the total amount of STAT3 found in P1 WT retinal explants cultured for five hours in ultraculture medium followed by thirty minutes treatment with 100nM PMA or 20ng/mL LIF. At least three retinas were used per condition.

On the other hand, as shown in figure 13, WT P1 retinas treated with PMA or IGF1 decreased the levels of tyrosine phosphorylation of STAT3 relative to untreated controls by 70% and 80% respectively (0.31 ± 0.11 N=6 P<0.001), (0.26 ± 0.11 N=3 P<0.002). However, simultaneous
addition of Go7874 not only abolished the effects of IGF1 and PMA, but the levels of phosphorylation of STAT3 were higher than control levels with IGF1 and Go7874 (2.93 ± 0.41 N=3 P<0.002) and similar to control levels with the PMA and Go7874. On the other hand, phosphorylated levels of STAT3 obtained with inhibition of PKC were significantly lower than the levels obtained with addition of STAT3 stimulatory compounds such as LIF. In our experiments, thirty minutes treatment with 20ng/mL LIF resulted in an increase of four to six times in the levels of tyrosine phosphorylation of STAT3. These data suggest that IGF1 and PMA can regulate the tyrosine 705 phosphorylation levels of STAT3 through modulation of PKC activity, and that PKC activity also plays a role in determining the baseline level of STAT3 phosphorylation.

**Figure 13.** PMA and IGF1 treatment of WT P1 retinal explants reduced STAT3 Tyr 705 phosphorylation in a PKC dependent manner. Amount of tyrosine phosphorylated STAT3 found in P1 WT retinas after five hours explant culture followed by 30 minutes treatment with 100nM PMA, 50ng/mL IGF1, and 100nM Go7874. **P<0.001, **P<0.005 vs control. At least three retinas were used per treatment.
A prediction from these results is that the absence of PKCβ1 and PKCγ should lead to increased levels of tyrosine phosphorylated STAT3. To investigate this hypothesis we checked the levels of phosphorylated STAT3 in retinas of new born PKCγ KO pups in the presence or absence of PKCβ inhibitor. The retinas were isolated from P1 PKCγ KO and WT littermates, cultured for 5 hours and the protein extracted and analyzed for the levels of tyrosine phosphorylated STAT3. We found that the levels of phosphorylated STAT3 were twice as much in the PKCγ KO animals as in the WT littermates (2.71 ± 0.55 N=3 P<0.05) (Fig 14). In addition when PKCβ inhibitor was added to the culture for the entire 5 hours, phosphorylation of STAT3 was further increased to almost five times the levels of control WT untreated retinas (4.98 ± 0.98 N=3 P<0.005).

Since STAT3 can promote its own expression through a STAT3 binding site in its promoter (Narimatsu M et al., 2001, Zhang C et al., 2008), we examined the effects of inhibition of PKCγ on total STAT3 protein and found that PKCγ KO had twice as much STAT3 total protein as the WT littermate control (2.71 ± 0.32 N=3 P<0.027). When we added the PKCβ inhibitor to the PKCγ KO retinas, the total levels of STAT3 increased but the change did not reach statistical significance.
Figure 14. Retinas obtained from PKCγ KO animals contained higher levels of tyrosine phosphorylated STAT3. Amounts of tyrosine phosphorylated STAT3 and total STAT3 found in retinas of P1 PKCγ KO and WT littermates cultured for five hours in the presence or absence of 30nM PKC B inhibitor. **P<0.005, *P<0.05 vs control. At least three retinas were used per condition.

To test whether PKC activation could also prevent LIF induced STAT3 tyrosine phosphorylation we added IGF-1 or PMA to the cultures at the same time as LIF or we preincubated for 3 hr before adding LIF. Simultaneous addition of IGF-1 or PMA was not able to reduce the induction of STAT3 phosphorylation modulated by LIF, but 3 hr preincubation significantly reduced the effect of LIF without any significant effect on STAT3 protein levels (Fig 15). These data suggest that PMA and IGF1 can prevent the activation of STAT3 by LIF, which has been previously shown to inhibit rod differentiation.

Figure 15. Activation of PKC with IGF1 or PMA prevents LIF induced tyrosine phosphorylation of STAT3. Amounts of tyrosine phosphorylated STAT3 found in P1 WT retinas after five hours explants culture followed by 3 hours pre-incubation with either 100nM PMA or IGF1 with subsequent addition of LIF for 30 min. ***P<0.0005, **P<0.005 vs control.
We wanted to examine if activation of PKC would block the LIF induced inhibition of rod photoreceptor differentiation during retinal development. We repeated the previous experiments adding PMA and LIF at the same time or pre-incubating with PMA for six hours before adding LIF. The effects were apparent at the cellular level since pre-incubation with PMA for 6 hr blocked LIF induced inhibition of rod photoreceptor development (Fig 16) and increased the number of rods present at the end of the culture by fivefold when compare to untreated controls (5.17 ± 0.67 N=9 P<0.0001).

**Figure 16.** Activation of PKC with IGF1 or PMA rescues LIF induced rod photoreceptor development block. (A)Immunofluorescence detection of rhodopsin (green) in WT P1 retinas after four days of culture in the presence of 100nM PMA, 20ng/mL of LIF, PMA and LIF added at the same time, and six hours pre treatment with PMA followed by LIF. Sections were overlaid with nuclear counterstain (B) Number of rods present at the end of a four day culture of WT P1 retinas in the presence of PMA, LIF and combination. Total number of rods was counted from at
least three cross sections of a single treated retina. At least three retinas were studied and the average was plotted. ***P<0.005 vs control.

Together these experiments suggest that activation of PKCγ and PKCβ1 by growth factors such as IGF1, or agents such as PMA can release the block of rod photoreceptor differentiation caused by activated STAT3.

**4.3 Activation of PKCβ1 and PKCγ inhibits tyrosine phosphorylation of STAT3 by recruiting a phosphatase that removes the tyrosine phosphate group of STAT3.**

Given that we demonstrated that PKCγ and PKCβ1 are responsible for reducing STAT3 tyrosine phosphorylation during retinal development, we investigated potential mechanisms by which activation of PKC inhibited STAT3 tyrosine phosphorylation.

Suppressors or cytokine signaling (SOCS) comprise a family of intracellular proteins which have been shown to regulate the responses of immune cells to cytokines. SOCS family proteins, as well as other SOCS-box-containing molecules, have been shown to function as E3 ubiquitin ligases and mediate the degradation of proteins that are associated with these proteins through their N-terminal regions (Kubo M et al., 2003, Naka T et al., 2005). In addition to suppressing signaling by ubiquitin-mediated degradation of the signaling complex, both SOCS1 and SOCS3 can suppress cytokines signaling by inhibiting JAK tyrosine kinase activity directly through their kinase inhibitory region (KIR), which has been proposed to function as a pseudosubstrate (Yasukawa H et al., 1999). Finally, in 2007 Ozawa et al., demonstrated that SOCS3 was required during retinal development to shut down the residual STAT3 activation necessary for proper rod differentiation.
To test whether activation of PKC that resulted in reduction of STAT3 tyrosine phosphorylation was due to an increase SOCS3 signaling, we tested the levels of SOCS3 found in retina during development after activation of PKC. However, the levels of SOCS3 protein remain unchanged in explants where PKC was activated with PMA or IGF1, conditions that reduce the levels of tyrosine phosphorylated STAT3 (Fig 17). In addition, levels of SOCS3 did not vary in retinas extracted from P1 PKCγKO compared to WT littermates. This data suggest PKC does not regulate activation of STAT3 through modulation of SOCS3 protein levels.

**Figure 17.** PKC activity in retinal development does not regulate SOCS3 levels. (A) Amount of total SOCS3 protein found in WT P1 retinas after thirty minute treatment with 100nM PMA, 20ng/mL LIF or 50ng/mL IGF1. (B) Amount of total SOCS3 protein found in PKCγ KO retinas and WT littermates cultured for five hours in the presence or absence of PKC β inhibitor. At least three retinas were studied and the average was plotted.

An alternative pathway that would result in decreasing phosphorylation of STAT3 is the activation of a phosphatase that would remove the phosphate group of the STAT3 protein. To
test the potential role of a phosphatase we used the general phosphatase inhibitor sodium orthovanadate (NOV).

We pre-incubated P1 retinal explants for one hour with 100µM of NOV before adding PMA or IGF1 (Fig 18 A). In explants treated with PMA the tyrosine phosphorylated levels of STAT3 were reduced to one half compared to controls (0.42 ± 0.07 N=8 P<0.0001) and explants treated with IGF1 decreased pSTAT3 to almost undetectable levels (0.20 ± 0.12 N=6 P<0.0001). One hour of pre-incubation with NOV blocked the PMA inhibition of STAT3 phosphorylation as the levels remained similar to control levels (1.104 ± 0.1132 N=8 P=0.79). NOV also substantially increased the pSTAT3 levels in the presence of IGF1, although the level did not reach control values (0.6999 ± 0.1245 N=3 P<0.05).
Figure 18. Activation of PKC inhibits tyrosine phosphorylation of STAT3 through modulation of a phosphatase activity. (A) Amount of tyrosine phosphorylated STAT3 found in WT P1 retinas after 30 minutes treatment with 100nM PMA, 50ng/mL IGF1, or one hour pre-incubation with 100µM NOV (Sodium Orthovanadate) before addition of IGF1 or PMA. (B) Amount of tyrosine phosphorylated STAT3 found in WT P1 retinas after treatment with 100nM PMA, 20ng/mL LIF, three hours pre-incubation with PMA followed by thirty minutes LIF, or one hour pre-incubation with 100µM NOV, followed by three hour pre-incubation with PMA, and thirty minutes treatment with LIF. (C) Quantitative graph of the amount of tyrosine phosphorylated STAT3 found in WT P1 retinas after treatment with 50ng/mL IGF1, 20ng/mL LIF, three hours pre-incubation with IGF1 followed by thirty minutes LIF, or one hour pre-incubation with 100µM NOV, followed by three hour pre-incubation with IGF1, and thirty minutes treatment with LIF. ***P<0.0005, **P<0.005, *P<0.5 vs control.

The above experiments suggest that IGF1 and PMA regulate the resting levels of tyrosine phosphorylated STAT3 through the actions of a phosphatase. To test whether PKC regulation of cytokine induced STAT3 activation is also regulated by a phosphatase, we repeated the experiments in the presence of LIF. A three hour pre-incubation with PMA prevented LIF-induced activation of STAT3 as the tyrosine phosphorylation of STAT3 was the same as control (0.8658 ± 0.1179N=3 P=0.5220) (Fig 18 B). One hour pre-incubation with NOV abolished this effect and maintained the pSTAT3 levels similar to LIF treatment (1.525 ± 0.1338 N=3 P=0.1740). Similarly, three hours pre-incubation of IGF1 prevented LIF induced activation of STAT3 setting the tyrosine phosphorylation level to half of the control (0.4316 ± 0.1528 N=3 P<0.002) (Fig 18 C). One hour pre-incubation with NOV blocked the ability of IGF1 to inhibit LIF induced STAT3 phosphorylation setting the values close to LIF treated ones. These data suggest that activation of PKC with either PMA or IGF1 reduces STAT3 tyrosine phosphorylation by activating one or more phosphatases. STAT3 activation has been shown to be important for maintaining the proliferation and self renewal abilities of progenitor cells.
Therefore, PKC activation may be important in rod photoreceptor differentiation by stopping progenitor cell divisions and forcing cells towards a differentiation process.

4.4 Summary of results

In the previous chapter we demonstrated that IGF1 can modulate rod photoreceptor differentiation by activating PKCβ1 and γ isoforms. In this chapter we tested the hypothesis that PKC activation was necessary for rod photoreceptor differentiation through inhibition of STAT3 activity. We first confirmed that PKCβ1 and γ activity is necessary for rod photoreceptor development in the absence of other factors. When one isoform is missing, the other one is able to compensate for that absence, however, the compensation is not complete, since the PKCγ KO retinas presented with a delay in rod development due to sustained proliferation of progenitor cells. In addition, our experiments demonstrated that PKCβ1 and γ function is necessary for rod development because when the activity of both isoforms is inhibited, rods are not able to differentiate. However, further investigation into the specific functions of each isoform is needed.

We also investigated the mechanism that PKCβ1 and γ may utilize to modulate rod differentiation. Our lab previously demonstrated that continuous activation of STAT3 prevented rod differentiation by constantly promoting cell proliferation. We therefore examined the effects of activation of PKC of P1 retinal explants on STAT3 activity. We showed that activation of PKC did not alter serine 737 phosphorylation or total protein levels of STAT3. However, activation PKCβ1 and γ isoforms significantly reduced the tyrosine 705 phosphorylation of STAT3 even in the presence of LIF, which has been shown to increase tyrosine 705 phosphorylation of STAT3. Conversely, inhibition of PKCβ1 and γ activity in retinas,
substantially increased the tyrosine phosphorylation of STAT3, confirming that PKC activity regulates tyrosine phosphorylation of STAT3 during retinal development.

We later investigated some possible mechanisms utilized by PKC to inhibit phosphorylation of STAT3. We first investigated whether SOCS3 a known regulator of STAT3 activity was being recruited by PKC activation. However, the total levels of SOCS3 protein did not change in any of our experiments involving activation or inhibition of PKC. We cannot disregard the possibility that some post-translational modification of SOCS3 may be regulated by PKC activity.

On the other hand, we demonstrated that the effects of PKC activation on tyrosine phosphorylation of STAT3 were completely abolished if sodium orthovanadate, a phosphatase inhibitor, was added to the culture. These results suggest that PKC recruits a phosphatase that in turn removes the tyrosine phosphate of STAT3 in order to inhibit its activity in retina during rod development.
CHAPTER 5

IGF1 ACTIVATES PKC DURING ROD PHOTORECEPTOR DEVELOPMENT BY INHIBITING PI3K ACTIVITY

Insulin and insulin growth factor 1 (IGF1) signal through the insulin receptor tyrosine kinase which autophosphorylates itself upon binding of ligand, and allows for downstream phosphorylation of other proteins. Ligand binding to the α-subunits of the IGF1R induces tyrosine kinase activity in the β-subunits by the phosphorylation of three tyrosine residues within the receptor autocatalytic domain. This leads to formation of the signaling complex by tyrosine phosphorylation of several docked intracellular proteins. (LeRoith D et al., 1995, Laviola L et al., 2007, Li W and Miller WT 2006, Adams TE et al., 2000)

Several labs have demonstrated that insulin and IGF-1 can activate the PI3K pathway in the retina (Reiter et al., 2003, Rajala et al., 2004). In most tissues the activated IR phosphorylates insulin receptor substrate (IRS)-1, a principal substrate of the IR, on multiple tyrosine residues, which, in turn, recognizes and binds to the SH2 domain of PI3K. However, it was shown that in the retina IR activation does not induce the activation of IRS-1 but rather induces the activation of IRS-2. They also demonstrated that this interaction results in activation of PI3K. Furthermore, Rajala et al., showed that the IR can directly interact with the p85 subunit of PI3K and activate it in the absence of IRS-2.

PI3Kinase belongs to the large family of PI3K-related kinases (PIKK) whose most recognized members include mammalian target of rapamycin (mTOR), ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia mutated and RAD3 related (ATR), and DNA-dependent protein
kinase. All possess a PI3K-homologous kinase domain and a highly conserved carboxy-terminal tail (Kuruvilla FG et al., 1995). However, PI3K is the only member known to have an endogenous lipid substrate. The PI3K enzymes are divided into three classes (I, II, and III) depending upon their substrate specificity (Hawkins PT et al., 2006, Stephens et al., 1994). The class I PI3K phosphorylates PI-4,5-P₂ to produce PI-3,4,5-P₃ and class III enzymes produce PI-3-P from PI (Martin TF 1998, Fruman DA 1998). The function of class II PI3K is not well established but it probably involves the production of both PI-3,4-P₂ and PI-3-P (Engelman JA et al., 2006). The class I PI3K is the most characterized group and it is composed of heterodimers of a catalytic subunit referred to as the p110 subunit (110KD) and an adaptor regulatory subunit known as the p85 subunit (85KD) (Carpenter CL et al., 1990). There are four class I PI3Kp110 genes known in mammals; Pik3ca, Pik3cb, Pik3cg, and Pik3cd and are referred to as PI3Kα, β, γ, and δ (Zhao L et al., 2008). These genes produce proteins with a high level of homology at the N terminal end which contains a GTPase Ras domain, a C2 lipid binding domain, the phosphatidylinositol kinase domain, and the catalytic domain (Hirsch EC et al., 2007). The p110 catalytic subunits bind to a regulatory subunit in order to be activated. In mammals there are five known regulatory subunits collectively known as the “p85s” (p85α, p85β, p55γ, p55α, and p50α) (Geering BP et al., 2007). These p85 are encoded by three genes: Pik3r1, Pik3r2, and Pik3r3, and the Pik3r1 can be expressed in splice variants that encode p85α, p55α, and p50α. All members of the p85 regulatory domain family contain a p110 catalytic domain-binding region that interacts with a specific domain present at the N-terminal ends of the class IA p110 catalytic domains (Hiles ID et al., 1992).
<table>
<thead>
<tr>
<th>Class</th>
<th>Catalytic Subunit</th>
<th>Regulatory Subunit</th>
<th>Activator</th>
<th>Phosphoinositide Product</th>
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<td>III</td>
<td>VSP34p</td>
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Table 2. Members of the PI3K family.

The regulatory p85 subunit contains a SH3 domain that allows the interaction with the p110 catalytic domain and two SH2 domains that allow interaction with the tyrosine kinase of the IR. The regulatory subunit maintains the catalytic subunit in a low activity state until the receptor tyrosine kinase gets phosphorylated upon ligand binding. The activated IR usually phosphorylates insulin receptor substrate (IRS)-1 or (IRS)-2 (in retina) on multiple tyrosine residues, which, in turn, recognizes and binds to the SH2 domain the p85 subunit allowing activation of the p110 catalytic subunit (Fruman DA et al., 1998, DeFronzo RA 1992).

Once the PI3K is activated, it generates 3’-phosphoinositides such as phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4,5-bisphosphate (PI3,4P2) and phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5P3) by transferring a phosphate from ATP to the D3 position of the inositol ring. This lipid second messenger recruits AKT, also known as PKB, to the cellular membrane by interacting with its pleckstrin homology domain (Andjelkovic MD 1997, Meier RD 1997, Wijkander J 1997). Once AKT localizes to the membrane it is phosphorylated on Thr
308 of its activating loop and in Ser473 of is hydrophobic motif (Manning B. D. and Cantley L. C. 2007). AKT is completely activated when it is phosphorylated in both sites a process that is modulated by mTOR (Thr308) and phosphoinositide dependent protein kinase 1(PDK1)(Ser473) (Alessi DR et al., 1998). AKT is a ser/thr kinase, and once it is activated, it phosphorylates several downstream proteins including glycogen synthase kinase 3 (GSK3), ribosomal protein S6 kinase (p70S6K), Bcl-2, IKK kinase, endothelial nitric oxide synthase, mTOR, eukaryotic translation initiation factor 4E binding protein (4E-BP), forkhead transcriptional factor, and caspase 9. By modulating these proteins AKT plays an important role in regulating metabolism, apoptosis, cell cycle, protein transcription, cell proliferation and survival (New DC et al., 2007, Parcellier AC et al., 2008, Frake TF et al., 1997).

In addition to the PI3K pathway, activation of the IGF1 receptor tyrosine kinase also results in activation of other intracellular proteins including the mitogen-activated protein kinase (MAPK). The MAPK pathway is activated when phosphorylated IGF1R stimulates Ras leading to activation of the Raf, MEK, MAPK, and ERK proteins. This pathway has regulatory effects on gene transcription and cellular metabolism, and is known to promote cell growth and survival (Tan and Kim 1999). Other second messengers that have been implicated in insulin/IGF1 action, include, growth factor receptor bound protein 2 (GRB-2), SH2-containing phosphatases, GTPase activating protein (GAP), and phospholipase C-γ (PLC-γ), among others. (Reviewed by Carolyn A Bondy and Clara M Cheng 2004).
5.1 IGF1 effects on rod photoreceptors are not mediated by AKT activity.

To investigate the pathway by which IGF1 activates PKC during development, we looked at the role of the major enzymes regulated by IGF1 receptor activity, namely AKT and PI3Kinase (PI3K). Addition of the pan AKT inhibitor VIII to P1 retinal explants for four days did not result in any significant change in the number of rods in control conditions, or the increased number of rods seen following IGF1 treatment (Fig 19). This suggests that the effects of IGF1 on rod development are not dependent on AKT activity.

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Figure 19. AKT activity does not regulate IGF1 effects on rod photoreceptor development. (A) Immunofluorescence detection of rhodopsin (green) in WT P1 retinas after four days of culture in the presence of 50ng/mL IGF1 and 500nM AKT inhibitor VIII. Sections were overlaid with nuclear counterstain. (B) Number of rods found in P1 WT retinas at the end of a four day culture in the presence of 50ng/mL IGF1 and 500nM AKT inhibitor VIII. ***P<0.0001 vs control. Three retinas were used per condition.

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5.2 IGF1 effects on rod photoreceptor development are mediated by the inactivation of PI3K activity.

When cultures were treated with the compound Ly294002 to inhibit the activity of PI3K the number of rods was almost ten times that of control cultures (9.40 ± 0.21 N=3 P<0.0001) (Fig 20
B). Adding IGF1 together with Ly294002 did not increase the number of rods over that induced by the inhibitor alone (10.12 ± 0.20 N=3 P<0.0001) (Fig 19 A, B).

Figure 20. Inhibition of PI3K results in increased expression of rod photoreceptors. (A) Immunofluorescence detection of rhodopsin (green) in WT P1 retinas after four days of culture in the presence of 50ng/mL IGF1, 50µM Ly294002 (PI3K Inhibitor) and combination. Sections were overlaid with nuclear counterstain. (B) Number of rods found in P1 WT retinas at the end of a four day culture in the presence of IGF1, 50µM Ly294002 and combination. ***P<0.0001 vs control.

Subsequently, we tested whether the effects of PI3K inhibitor were PKC activity dependent. To do so, we repeated the experiments on retinas extracted from PKC γ KO animals in the presence of PKCβ inhibitor. As shown in figure 21 the effects of PI3K inhibitor on rod development were PKC-dependent since the stimulatory effect of adding Ly294002 to the PKC γ KO retinal cultures was blocked by the PKCβ inhibitor.
Figure 21. Inhibition of PI3K results in increased expression of rod photoreceptors in a PKC dependent manner. (A) Immunofluorescence detection of opsin (green) in PKCγ KO P1 retinas after four days of culture in the presence of 50µM Ly294002, 30nM PKCb inhibitor and combination. Scale bar, 40µm (B) Number of rods found in PKCγ KO P1 retinas at the end of a four day culture in the presence of Ly294002, PKCb inhibitor, and combination. Total number of rods was counted from at least three cross sections of a single treated retina. At least three retinas were studied and the average was plotted. **P<0.01 vs control.

In many cell types IGF-1 stimulates the activity of PI3K and subsequent AKT phosphorylation. Since our results were more compatible with an inhibition of PI3K mediated by IGF-1, we compared the actions of IGF-1 on freshly isolated neonatal and adult retinas by measuring the phosphorylation of AKT five and thirty minutes after addition of IGF1. As shown in Figure 22, in adult retinas IGF-1 treatments resulted in increase phosphorylation of AKT with a maximum stimulation at five minutes that was about three times compared to control (3.31 ± 0.6, n=3, P<0.05). The stimulation gradually decreased after thirty minutes exposure to IGF1 (1.703 ± 0.1602, n=6, P<0.005), a result in accord with other studies (Barber et al., 2001 JBC, Biswass et al., IOVS 2008). In the neonatal retinas, however, IGF-1 treatment led to a significant reduction in phosphorylation of AKT to a maximum of eighty five percent reduction after five minutes treatment (0.15 ± 0.09, n=3, P<0.003). Thirty minutes treatment with IGF1 resulted in a partial recovery but the samples still showed a fifty percent decrease of phosphorylation of AKT compared to control (0.45 ± 0.03, n=7, P<0.001).
Figure 22. IGF1 treatment of P1 and adult retinas results in opposite effects on phosphorylation of AKT and GSK3β. (A) Amount of phosphorylated Ser 473 AKT found in adult and P1 WT retinas after 5 and 30 minutes treatment with 50ng/mL IGF1. (B) Amount of phosphorylated Ser 9 GSK3β found in adult and P1 WT retinas after 5 minutes treatment with 50ng/mL IGF1. (C) Amount of phosphorylated Tyr 204 ERK found in adult and P1 WT retinas after 5 minutes treatment with 50ng/mL IGF1. ***P<0.005, *P<0.05 vs control. Dotted line depicts the levels of untreated retinas. At least three retinas were studied per condition.

To confirm the effects of IGF1 on the PI3K-AKT pathway, we looked at the phosphorylation of GSK3β on Ser 9 which is mediated by AKT upon stimulation of PI3K. We found a reduction of about twenty percent on the phosphorylation of GSK3β after five minutes addition of IGF1 (0.79 ± 0.04 N=3 P<0.005) confirming the inhibitory effects of IGF1 on the PI3K/AKT pathway in the neonatal retina (Fig 22 B). On the other hand, five minutes treatment with IGF1 on adult retinas resulted in increased phosphorylation of GSK3β by about seventy five percent when compared to untreated retinas (1.73 ± 0.08 N=3 P<0.05).
To determine whether these effects were due to IGF receptor dysfunction or to a change at the post-receptor level, we examined whether tyrosine phosphorylation on residue 204 of ERK changed after addition of IGF1 into the cultures (Fig 22 C). In the adult retinas five minutes treatment with IGF1 resulted in seventy percent increase on the phosphorylation of ERK (1.73 ± 0.162 N=3 P<0.05). In the neonatal retinas, ERK phosphorylation increased by almost five fold (4.98 ± 0.95 N=3 P<0.05) suggesting the tyrosine kinase activity of the IGF1R is active throughout development in the retina and that the effects we observe are at the level of PI3K or its coupling to the receptor.

Thus, IGF-1 causes an inhibition of PI3K activity in neonatal retinas that leads to a PKC-dependent inhibition of STAT3 tyrosine phosphorylation that is important to trigger rod photoreceptor differentiation.

5.3 Addition of IGF1 to WT P1 retinas does not cause phosphorylation of the regulatory domain of PI3K.

In section 5.2 we demonstrated that IGF1 had opposite effects on activity of PI3K on neonatal retinas compared to adult retinas. We hypothesized the difference was due to a change in the regulation of the catalytic domain of PI3K. Therefore, we investigated the expression of the regulatory domain of PI3K in adult and P1 retinas.

We cultured retinas from WT P1 and two month old animals for 5 hours to allow activation of stress response pathways to return to base line. Subsequently we added IGF1 for five minutes at which time we collected cell lysates. We used an antibody specific for the p85 regulatory domain of PI3K and we tested its expression at a base line level or after IGF1 stimulation. As shown in
We subsequently used the same protein lysates to test the tyrosine phosphorylation of p85 regulatory protein of PI3K using an antibody specific for p85 tyrosine phosphorylation at residue 458 or p55 splice variant tyrosine phosphorylation at residue 199. We did not detect any phosphorylation of the p85 Tyr 485 in neither the P1 nor the adult retinas before or after treatment with IGF1.

One important difference between P1 and adults was that as shown in figure 23B we found significant amounts of phosphorylated p55 Tyr199 in adult retinas, while we did not detect any in the P1 retinas even after IGF1 treatment for five minutes. We identified an increase of about
fifty percent in phosphorylated p55 after five minutes of IGF1 treatment in adult retinas (1.5 ± 0.07450 N=3 P<0.05)

In addition we also tested for expression of the three p110 catalytic domain subunits of class I PI3K. We found that p110β or p110γ are not expressed in adult or neonatal retinas. On the other hand p110α is expressed in both adult and P1 retinas, and the amount of protein found in cytoplasm doubles after five minutes treatment with IGF1 in adult retinas, but it does not change in P1 retinas (Fig 24).

**Figure 24.** Expression of total p110α catalytic subunit of PI3K in P1 and adult retinas. Quantitative graph of the amount of total p110β protein found in adult and P1 WT retinas after 5 minutes treatment with 50ng/mL IGF1. **P<0.001 vs control.

This data suggest a significant difference between P1 and adult retinas in the expression of PI3K regulatory and catalytic domains that may be responsible for the differences in regulation of PI3K activity after IGF1 stimulation.
5.4 Summary of results

In this chapter we further investigated the mechanisms by which IGF1 resulted in an increase in the number of rods. We tested the hypothesis that IGF1 activates PKC through the PI3K/AKT pathway. The IGF1 receptor has been shown to signal through the receptor tyrosine kinase that eventually phosphorylates PI3K and subsequently AKT. Therefore, we looked whether IGF1 effects on rod development were mediated by PI3K or AKT. We found that inhibition of AKT did not have any effects on rod development. However, contrary to our hypothesis, inhibition of PI3K resulted in a higher number of rods at the end of a four day culture, even when compared to IGF1 alone. Consistent with our hypothesis that PKC activation is necessary for rod photoreceptor differentiation, the increase in the number of rods that resulted from PI3K inhibition was also PKC dependent, since inhibition of PKCβ1 and γ activity abolished PI3K inhibitor effects on rods.

These results were consistent with IGF1 mediating inhibition of PI3K activity that resulted in activation of PKC and increase of rod differentiation. To test this hypothesis we evaluated the effects of IGF1 treatment on phosphorylation of AKT in adult and P1 retinas. We found that treatment with IGF1 in P1 retinas resulted in reduction of phosphorylated AKT and GSK3β whereas treatment with IGF1 in adult retinas resulted in the opposite effects.

We hypothesized that the differences in the effects of IGF1 treatment between adult and P1 retinas were due to changes in the regulation of the catalytic domain of PI3K. Therefore, we examined the expression of the regulatory and catalytic domains of PI3K in adult and P1 retinas. We found that P1 and adult retinas do not express p110β or p110γ catalytic domains of PI3K, but they both expressed p110α catalytic domain. We also found that in both stages the p85
regulatory domain is expressed, however, it does not get phosphorylated upon IGF1 binding. On the other hand, the p55 regulatory domain gets phosphorylated upon IGF1 binding only in adult retinas. In fact, we did not detect any p55 phosphorylation in P1 retinas before or after IGF1 treatment. Therefore, we concluded that IGF1 binding to its receptor, triggers rod photoreceptor differentiation by activating PKC activity. However, IGF1- induced PKC activation is not mediated by activation of PI3K/AKT pathways, but it is the lack of activity of PI3K that results in PKC activation and subsequent rod development.
CHAPTER 6

PKC MAY CONTROL ROD PHOTORECEPTOR DIFFERENTIATION BY REGULATING EPIGENETIC MECHANISMS OF GENE EXPRESSION

In previous chapters we demonstrated that activation of PKC led to inhibition of STAT3 activity, which has been shown to be essential for maintaining progenitor cell proliferation. Therefore, we concluded that activation of PKC was an important step to trigger progenitor cells to exit mitosis and begin the process of cell differentiation. In this chapter we explore the idea that PKC is not only controlling STAT3 activity to terminate cell proliferation and begin differentiation, but is also establishing the fate of the progenitor cell by specifying gene expression of rod specific genes through modulation of histone modifications.

The development of retina requires accurate gene transcription control in response to proper environmental signals. Epigenetic mechanisms including histone modifications, DNA methylation, and other chromatin-remodeling events, are critically important in mediating precise gene regulation. During mammalian development, at the time of tissue specification two important processes occur simultaneously: 1) cells cease proliferation with an accompanying dramatic down-regulation of genes responsible for cell cycle progression and 2) in the resulting post-mitotic cells large numbers of tissue-specific genes are up-regulated to give the mature phenotype. In addition, genetic inactivation of many chromosomal loci at the end of tissue maturation correlates with formation of compact blocks of heterochromatin. The histone code hypothesis suggests that combinatory patterns of histone modifications play an important role in the regulation of gene expression and ultimately in the differentiated status of the cell (Strahl and Allis, 2000).
Lysine methylation of histone 3 (H3) has been associated with activation and repression of gene transcription because of its effects on regulating accessibility of other transcription factors and transcription regulators to a particular DNA sequence such as gene promoters or gene enhancers (Osipovich O et al., 2004). Histone lysine methylation has been well characterized at the lysine 4 (K4), Lysine 9 (K9), and lysine 27 (K27). These residues may contain one, two or three methyl groups and each of these modifications translates into specific regulation of gene expression. For example, generally trimethylation of K4 on H3 is associated with complete activation of gene transcription, whereas monomethylation of K4 occurs in both active and inactive promoters (Santos-Rosa H et al., 2002). On the other hand H3K9 methylation is correlated with silencing of gene expression. Dimethylation at K9 on H3 marks silent euchromatin important in proliferating cells, whereas trimethylation is enriched in centromeric regions of heterochromatin, usually poorly transcribed (Tachibana M et al., 2002, Rice JC et al., 2003, Peters AH et al., 2003). Methylation at K27 on H3 has been associated with repression of gene expression in several developmental processes (Lachner M et al., 2003, Tachibana M et al., 2001).

Pre-existing acetylation, phosphorylation, or methylation of histone tails affects subsequent histone modification. For example, several transcription-related HATs, including GCN5, PCAF, and p300, prefer S-10 induced phosphorylation of H3 as a substrate (Cheung et al., 2000; Lo et al., 2000). Furthermore, S10 phosphorylation negatively regulates K9 methylation by SUV39H1 (Rea et al., 2000). In addition, Hengbin et al demonstrated that previous K9 methylation inhibited subsequent K4 methylation and vice versa. Therefore, the gene expression pattern that gives rise to a specific cellular phenotype is regulated by interactions of chromatin markers that determine which group of genes becomes transcriptionally active or repressed. In the mature rod photoreceptors for example, a very characteristic architecture is distinguished in the nucleus with
a large central domain of condensed chromatin surrounded by a thin border of extended euchromatin. Kizilyaprak et al., showed that nucleosomes were hyperacetylated, contained higher levels of H3K4 methylation and were hypomethylated on H3K9, H3K27 and H4K20 in the euchromatin-rich peripheral regions. These characteristic regions were the only ones in the nucleus at which RNA polymerase II molecules were detected, and were frequently at a fixed distance from the euchromatine/heterochromatine interface playing a role in the organization of transcription units. In the central heterochromatin compartment, nucleosomes were generally hypoacetylated and hypermethylated on H3K9, H3K27 and H4K20. Other groups have demonstrated that when this characteristic chromatin organization is perturbed, such as in the case of spinocerebellar ataxia 7, normal development of rods is not achieved and retinal degeneration emerges (Helmlinger D et al., 2006).

Recently, it was discovered that methylation of histones is a highly dynamic process in which newly uncovered demethylating enzymes such as LSD1 can change the methylation state of H3 on a specific promoter and switch gene transcription activation to repression or vice versa and regulate physiological processes including cell differentiation (Bannister, A. J et al., 2002, Metzger, E et al., 2005, Shi, Y et al., 2004). LSD1 is an amine oxidase homologue that demethylates H3K9Me2 and Me1 as well as H3K4Me2 and Me1. It therefore has a dual effect on gene expression. It can remove mono and dimethyl groups from H3K4 thereby removing activation marks on promoter, but it can also remove the inhibitory mark of H3K9. Even though the mechanism by which LSD1 decides when to activate or repress gene expression on a specific gene promoter has not been completely elucidated, Metzger et al 2010 established that one mechanism used by androgen dependent signaling to remodel chromatin is the activation of
PKCβ1 which in turn phosphorylates H3 at threonine 6 preventing LSD1 from binding to H3K4 and demethylating the residue, keeping the promoter in an active state.

LSD1 is expressed in neural progenitor cells and plays an important role in their proliferation abilities. Sun et al 2010 demonstrated that inhibition of LSD1 activity markedly diminished neural stem cell proliferation both in culture and in adult mice dentate gyrus proliferating cells. The functions of LSD1 have not yet been studied during retinal development, but we hypothesize that PKC activation may be guiding expression of rod specific genes by modulating LSD1 activity on rod specific gene promoters.

6.1 P1 WT retinas cultured in the presence of LSD1 inhibitor failed to develop rod photoreceptors after eight days in culture.

Sun et al 2010 demonstrated an important effect of LSD1 during neural stem cell proliferation. We therefore, investigated the effects of inhibition of LSD1 during rod photoreceptor development. We used P1 WT retinas and we cultured them in the presence of LSD1 inhibitor (Pargyline hydrochloride, or trans-2-phenylclopropylamin hydrochloride) for four days and assessed the number of rods present at the end of the culture. We found that retinas cultured in the presence of LSD1 inhibitor did not contain any rods whereas controls already contained substantial number of rods (Fig 25 A). To investigate whether the reduction of rods present in the retinas was due to a developmental delay, or whether it was due to a block on rod differentiation we repeated the experiment and cultured the retinas for eight days. In our model of retinal explants, eight days in culture is enough time for rhodopsin positive cells to populate the
majority of the tissue. We also investigated the effects of PKC activation in these retinas by coculturing them with IGF1.

**Figure 25.** LSD1 inhibition of WT retinal explants results in absence of rods. (A) Rhodopsin (green) expression in WT P1 retinal explants cultured for four days in the presence of LSD1 inhibitor (Pargyline hydrochloride) colocalized with nuclear counterstain (Dapi blue). (B) Rhodopsin (green) expression in WT P1 retinal explants cultured for eight days in the presence of IGF1, LSD1 inhibitor or combination colocalized with nuclear counterstain (Dapi blue). (C) Fluorescence intensity of rhodopsin expression in WT P1 retinal explants cultured for eight days in the presence of IGF1, LSD1 inhibitor or combination. ***P<0.0001, *P<0.01 vs control.
Addition of the LSD1 inhibitor to the retinal explants almost completely inhibited rod differentiation with only nine percent of rhodopsin expressed after culturing them for eight days (0.09 ± 0.06 N=3 P<0.0001). Furthermore, LSD1 inhibitor prevented rod formation even in the presence of IGF1 with eighteen percent of rhodopsin expressed compared to control (0.17 ± 0.06 N=3 P<0.0001) suggesting LSD1 activation is necessary for rod formation, and PKC activation may be inducing rod differentiation by modulating LSD1 activity (Fig 25 B, C).

6.2 LSD1 is expressed during retinal development with high protein levels at the peak of rod differentiation and protein levels decreasing as the retina matures.

Based on the data from the previous section that showed a significant inhibition of rod development when LSD1 activity is inhibited, we wanted to investigate the expression of LSD1 protein in retina during development. To do so, we used cells lysates from freshly extracted retinas obtained from wild type animals at embryonic day 17.5, postnatal day 1, 7, 15 and 56. We used an antibody specific to LSD1 to test for the presence of the protein in the cell lysates and we found low levels of LSD1 at E17.5 that gradually increased as the animal developed, with highest levels of expression at P7. After P7, the levels of expression began to decrease with LSD1 levels at P56 lower than E17.5 (Fig 26 A).
Figure 26. LSD1 is expressed in retina, with highest levels expressed at P7. (A) Western Blot of LSD1 expression during retinal development measured. (B) LSD1(green) expression in WT P1 retinal explants cultured for four days co-stained with rhodopsin (RetP1 green) and nuclear counterstain (Dapi blue). (C) LSD1 expression in WT P1 retinal explants cultured for four days colocalized with PCNA (green). (D) 200X magnification of outer retina stained with PCNA (green), LSD1 (red), and nuclear counterstain (Dapi blue).

To determine the localized expression of LSD1 in retinas, we cultured P1 WT retinas for four days and using IHC stained sections with LSD1 specific antibody. We found LSD1 to be expressed in all layers of the retina (Fig 26 B). However, when we co-stained with PCNA as a marker of dividing cells, we found that in the outer layer of the retina where rods will develop, LSD1 was only present in non-dividing cells (26 C, D).

In addition, to investigate whether PKC modulates rod photoreceptor differentiation by modifying LSD, we tested whether activation of PKC during retinal development changed the expression of LSD1. We cultured WT P1 retinas for four days in the presence of PMA or IGF1
and using IHC we examined the expression of LSD1. As shown in figure 27 we found that treatment with either PMA or IGF1 caused a significant increase in LSD1 expression measured by fluorescence intensity.

![Figure 27](image)

**Figure 27.** LSD1 expression in retina increases with activation of PKC. (A) LSD1 (green) expression in WT P1 retinal explants cultured for four days in the presence of PMA or IGF1, colocalized with PCNA (green) and nuclear counterstain (Dapi blue). (B) Fluorescence intensity of LSD1 expression in WT P1 retinal explants cultured for four days in the presence of PMA or IGF1. *P<0.05

6.3 PKC activation leads to histone modification changes that promote expression of genes linked to rod maturation.

To investigate whether activation of PKC caused histone modification changes on rod specific genes that may account for the specific induction of rod formation, we first assessed the effects of IGF1, LSD1 inhibition and PKC inhibition on the total amount of several histone modification markers. We cultured the retinas for four or eight days in the presence of IGF1 or LSD1 inhibitor. In order to inhibit PKC activity we also cultured retinas from PKCγKO animals in the presence of PKCβ inhibitor for four or eight days. We collected protein at the end of the cultures to measure amount of histone 3 lysine 9 dimethylation (H3K9Me2), histone 3 lysine 4 dimethylation (H3K4Me2), and histone 3 lysine 27 trimethylation (H3K27Me3).
As shown in figure 28 all three treatments increased the levels of H3K4Me2 after four days in culture, with PKC and LSD1 inhibition causing an increase of more than two and half times above control. IGF1 treatment resulted in an increase of one and half times above control. However, after eight days in culture, inhibition of LSD1 or PKC activity but not IGF1 continue to show higher levels of H3K4Me2 compared to control.

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**Figure 28.** Modifying PKC activity during retinal development results in changes of histone modification markers. Quantitative graphs of total modification (A) H3K4Me2, (B) H3K9Me2, (C)H3K27Me3 found in WT P1 retinal explants after four or eight days of culture in the presence of IGF1, LSD1 inhibitor or PKCγ KO in the presence of PKCβ inhibitor.

On the other hand, when we looked at H3K9Me2 we found that only inhibition of PKC activity resulted in changes in this epitope, with fifty percent increase after eight day culture. Finally, we found that retinas cultured for four days with IGF1, LSD1 or PKC inhibitor total H3K27Me3 was reduced compared to control. After eight days in culture, only IGF1 and LSD1 treatments resulted in decreased levels of H3K27Me3 whereas, inhibition of PKC did not change the levels of H3K27Me3 when compared to control.
This data suggest that PKC may regulate the levels of methylation on lysine 4 of histone 3 during retinal development through modulation of LSD1.

Since the previous experiments showed that treatments with IGF1, LSD1 inhibitor, and blockade of PKC activity during retinal development caused general changes in the histone modification markers, we investigated where in the genome these changes occurred and whether they would affect rod differentiation. Thus, we investigated changes in the histone modification markers on rod specific gene promoters. We performed chromatin immunoprecipitation (ChIP) after eight day treatment of WT retinal explants with or without IGF1 or LSD1 inhibitor, and on PKCγKO retinas in the presence of PKCβ inhibitor. ChIP was done with antibodies specific for H3K9Me2, H3K4Me2 and H3K27Me3, and we designed specific primers for promoter regions of rod specific genes (opsin, Nrl and Crx) to calculate changes in the histone modifications on rod specific promoters using quantitative PCR.
Figure 29. Modifying PKC activity during retinal development results in changes of histone modification markers in the promoter of rod specific genes. Graphs of modifications found in the promoters of opsin, Nrl, and Crx after performing ChIP with H3K9Me2, H3K4Me2 and H3K27Me3 on WT retinal cultures treated with IGF1 and LSD1 inhibitor and PKCγKO retinas treated with PKCβ inhibitor.

As shown in figure 29 we found that compared to control, IGF1 treatments resulted in fewer number of inhibitory markers (H3K9Me2 and H3K27Me3) on the promoters of the rod specific genes opsin, Nrl, and Crx. Furthermore, IGF1 treatment decreased the number of the activation marker H3K4Me2 on the same gene promoters. On the other hand, inhibition of LSD1 resulted in increased number of the inhibitory marker H3K9Me2 and substantial decrease of the amount of the activation marker H3K4Me2. As expected, since LSD1 does not interact with lysine 27 of
histone 3, no changes in the amount of H3K27Me3 were detected. Finally inhibition of PKCβ1 and γ increased the amount of both inhibitory markers (H3K9Me2 and H3K27Me3) and substantially reduced the amount of the activation marker H3K4Me2 on rod specific promoters.

6.4 Summary of results
In this chapter we tested the hypothesis that PKCβ1 and γ activation influence rod differentiation by modulating epigenetic markers in promoters of rod specific genes. We first investigated the effects of inhibiting LSD1 during retinal development, since in other cellular models PKC has been shown to modulate the activity of LSD1. We found that inhibition of LSD1 during retinal development blocks rod photoreceptor differentiation, suggesting LSD1 activity during genesis of rods is necessary for normal development. In addition, activation of PKCβ1 and γ with IGF1 during retinal development did not result in increase in the number of rods at the end of an eight day culture if LSD1 activity was inhibited; suggesting one mechanism by which PKC modulates rod differentiation is by regulating LSD1 activity. Subsequently, we established that LSD1 expression peaks around the time of rod photoreceptor differentiation confirming its activity is important for rod photoreceptor differentiation. We also showed that during rod development LSD1 is only expressed in post-mitotic cells found in the outer layer of the retina, and its levels increased after four day cultures in which we activated PKC with PMA or IGF1. Furthermore, inhibition of LSD1 and PKCβ1 and γ activity during eight days of retinal explants resulted in higher levels of total H3K4Me2 expression measured by WB. Finally, ChIP using antibodies specific to H3K4Me2, H3K9Me2, and H3K27Me3 showed that inhibition of PKCβ1 and γ as well as LSD1 activity resulted in reduced expression of the activation marker H3K4Me2 in rod specific gene promoters accompanied by increased expression of the inhibitory marker.
H3K9Me2. On the other hand, activation of PKC with IGF1 resulted in decreased expression of both inhibitory and activation markers.
CHAPTER 7

GENERAL DISCUSSION

A fundamental step in the process of development is the coordination of proliferation and cell differentiation. In the case of genesis of most neural tissues, embryonic proliferation provides the majority of cells needed to populate the mature tissue for the rest of the animal’s life. During the process of rod photoreceptor differentiation, progenitor cells must exit the cell cycle and undergo a complex program of gene expression that would lead to the necessary morphological changes to differentiate into functional neurons. To achieve this, rod photoreceptors need to undergo five consecutive steps that include: First, proliferation of multipotent retinal progenitor cells (RPC); second restriction of the competence of RPCs; third, cell fate commitment and specification to photoreceptor precursors during final mitosis; fourth, expression of photoreceptor genes including genes specific for phototransduction and morphogenesis; and fifth, photoreceptor maturation that includes axonal growth, synapse formation and outer segment generation (Swaroop Anand et al., 2010). One key factor that regulates the first step of the process is STAT3. STAT3 serves as a key transcription factor regulating mouse embryonic stem cell self-renewal and in the embryonic neocortex STAT3 activity maintains neural precursor cells (Xie et al., 2009; Yoshimatsu et al., 2006). Our previous results had indicated that STAT3 is an important player in the process of rod photoreceptor differentiation since it serves as a gatekeeper between the transition from progenitor proliferation and commitment to rod differentiation. Therefore it serves a pivotal role in determining whether retinal progenitor cells remain in a proliferating pool or transition into a terminal differentiation pathway. Some evidence comes from our previous published data that demonstrated that sustained activation of STAT3 by virally encoded, constitutively-active STAT3 completely blocked rod photoreceptor
formation (Zhang et al. 2004). STAT3 can be activated by a variety of signals including Notch-Delta-like 1, FGF1, IFN-γ and LIF, and the maintenance of a progenitor state is presumably due to the sum of their activity (Yoshimatsu et al., 2006; Zhang et al., 2004; 2005).

In the present studies we have explored the other side of this signal mechanism, namely how is STAT3 signaling shut off to allow cells to transition into terminal differentiation pathways. Several growth factors had been shown to influence rod development, however, their mechanisms have not been completely elucidated. In our studies we demonstrated that key intermediates in this signal pathway responsible for rod differentiation are two of the many isoforms of PKC. Inhibition of either PKCβ1 or PKCγ reduced the number of rods formed following stimulation by either IGF-1 or direct chemical stimulation by PMA and completion blockade of rod formation was only achieved when the activity of both isoforms was blocked. Since both isoforms were expressed in many of the progenitor cells this suggests true redundancy of function rather than expression in separate cell populations. Several lines of evidence indicate that no other PKC isoforms regulate rod production. First, PKCβ1 and PKCγ were the only two isoforms expressed in the retina during the time when rods were forming. Second, when both isoforms were blocked no rods were produced even in the presence of IGF-1 or PMA. Although PKCβ1 and PKCγ show redundancy in this assay, it is possible that they have additional separate functions during retinal development.

One mechanism by which activation of PKC results in increase number of rods is by modulating the activity of STAT3 through reduction of its tyrosine phosphorylation by recruiting a phosphatase (Fig 30). Support of this statement comes from the substantial reduction of tyrosine phosphorylation after activation of PKC, with no changes in serine phosphorylation or total levels of STAT3. In addition the PKC induced reduction of tyrosine phosphorylation of STAT3
is completely abolished by pre-treatment with sodium orthovanadate, a known inhibitor of phosphatases. We also confirmed the effects of PKCβ1 and PKCγ activity on STAT3 in vivo since the retinas of PKCγ KO contained twice as much tyrosine phosphorylated STAT3 as control littermates, and four times as much as littermate controls if the activity of PKCβ1 was pharmacologically inhibited for five hours.

**Figure 30.** Proposed mechanism of action of PKC during rod development. Early in development, the GP130 receptor is active resulting in tyrosine phosphorylation of STAT3, which allows STAT3 to dimerize and translocate to the nucleus, where it acts as a transcription factor, promoting retinal progenitor proliferation and self renewal. Later at the peak of rod differentiation (P1) IGF1 binds to its receptor, PI3K is de-activated resulting in PKC activation. PKC in turn recruits a phosphatase that removes the tyrosine phosphate of STAT3, and renders it inactive. The inactivation of STAT3 results in photoreceptor progenitors exiting cell cycle and initiating their differentiation process.
In 2007 Ozawa et al demonstrated that SOCS3 was required during retinal development to shut down the residual STAT3 activation necessary for proper rod differentiation. However, in our model, activation or inhibition of PKCβ1 and PKCγ did not result in any changes of SOCS3 protein levels suggesting PKC induced inhibition of STAT3 activity is not modulated by changes in SOCS3 levels. However, we cannot rule out that PKC activation may induce post-translational modifications on SOCS3 that result in subsequent inhibition of STAT3 tyrosine phosphorylation.

Of the growth factors tested in our experiments, IGF-1 was the most effective at inducing rod formation in the neonatal explants. In 2006 Modanlou et al showed that in the retina, IGF-1 levels increase during rod photoreceptor development from below 200pg/mg at birth to 300pg/mg at P7, and above 400pg/mg at p21 consistent with our model in which IGF1 serves as an extracellular signal that triggers rod formation by activating PKC.

A number of studies have implicated IGF-1 in neural development although many of these studies have focused on the role of this factor in proliferation and survival of cells. One surprising result was that the action of IGF-1 could be mimicked by an inhibitor of PI3 kinase. In many cell types IGF-1 binds to its receptor and activates PI3 kinase, a result we confirmed in mature retinal tissue. The simplest explanation of our findings is that in neonatal tissue the coupling of the IGF-1 receptor to PI3 kinase is altered such that IGF-1 prevents activation of PI3 kinase (Fig 31). This effect was confirmed by looking at two downstream effectors of PI3 kinase activity, AKT and GSK-3beta. Both enzymes showed reduced phosphorylation after treatment of neonatal tissue with IGF-1 but enhanced phosphorylation when adult tissue was treated in this way. Treatment of both neonatal and adult retinas with IGF-1 led to an increase in ERK activation (phosphorylation). The IGF-1 receptor regulates ERK phosphorylation through a
ras/raf pathway (Gosbell et al 2000, Diaz et al 2003, Varela Nieto I et al 2003) therefore, our result indicates that the IGF1 receptor was activated in both neonatal and adult retinas.

Figure 31. Proposed mechanism of IGF1 effects PI3K activity during rod development. IGF1 binding its receptor results in activation of multiple pathways including the MAPK/ERK, the PI3K/AKT pathway, and simultaneous activation of phosphatases that feed back and balance the activity of the stimulated pathways. In adult retinas, binding of the IGF1 to its receptor results in phosphorylation of the p55 regulatory domain of PI3K, which in turn activates the PI3K catalytic domain p110 with a net effect of transient phosphorylation of AKT and GSK3β. In P1 retinas, IGF1 binding to its receptor does not phosphorylate the p55 regulatory domain of PI3K, thus, its p110 catalytic domain remains inactive. Therefore, AKT does not get phosphorylated, but the actions of phosphatases activated by IGF1R result in a reduction of AKT phosphorylation.

This suggests that the different response of PI3-kinase was due to a change at a later step. One important difference between the neonatal and adult retinal PI-3-kinase itself is that only the adult p55 regulatory subunit of the enzyme gets phosphorylated upon binding of IGF1 to its
receptor. We were not able to establish whether the lack of p55 phosphorylation in neonatal retinas was due to absence of expression of p55 protein since we did not obtain antibodies specific for this subunit. Therefore the absence of p55 phosphorylation in neonatal retinas after IGF1 treatment may be due to inhibitory mechanisms that prevents p55 binding to the receptor tyrosine kinase or to absence of the p55 subunit expression. More importantly this data demonstrates that during rod photoreceptor development, IGF1 does not induce phosphorylation/activation of the regulatory domain of PI3K, and therefore, the catalytic domain of PI3K may not be activated either. However, since IGF1 receptor tyrosine kinase remains active the reduction in phosphorylated AKT may be due to unbalanced activation of phosphatases in response to IGF1 binding to the receptor with a net result of decreased phosphorylated AKT. In addition, five minutes IGF1 treatment resulted in an increase of about four times in the levels of total regulatory p85 and catalytic subunit p110 of adult retinas, with no significant change in neonatal retinas. Such a rapid increase in total protein is more likely due to positional changes in the cellular compartments after IGF1 binding. As I centrifuge my protein samples before I run the Western Blot, tightly membrane bound PI3K subunits may have been lost in the pellet. A difference in translocation of PI3K subunits in response to IGF1 binding may also account for the lack of PI3K activity upon IGF1 binding in neonatal retinas. Further investigation of PI3K cellular localization is needed to clarify this matter.

However, the substantial increase in number of rods with the PI3K inhibitor that was even higher than IGF1, insinuate that PI3K activity during rod development is regulated through multiple pathways. We propose that other growth factor actions may result in activation of PI3K by binding to different receptors, and such signal cascade inhibits differentiation of rod development, by reducing PKC activity. Perhaps, partial inhibition of PKC and subsequent
inhibition of rod fate, allows for concomitant differentiation of other cell types. Binding of IGF1, results in reduced activity of PI3K with a net effect of some activation of PKC and induction of rod differentiation. On the other hand, complete inactivation of PI3K would result in even higher activity levels of PKC and subsequently more rods formed at the end of the culture. We have not investigated the fate of other cell types that are known to differentiate during the same period as rods, namely Muller glia and bipolar cells, but it is a question that needs to be addressed in future experiments.

Although we have described our results as a single pathway, it is clear that the key step, activation of PKCβ1 and PKCγ, can be regulated by multiple pathways (reviews in Ananthanarayanan B et al., 2003). It is possible that many of the factors that have been shown to influence rod formation in the developing retina converge at this step of PKC activation and that the level of activation is the sum of multiple pathways. Since many of these factors change their level of expression as part of the development of other retinal cells, or other systemic events, this provides a mechanism for the retina to generate rod photoreceptors in synchrony with development of the whole organism.

The finding of delayed rod development in the PKCγ KO animals supports a model in which PKC is involved in progenitor commitment to rod differentiation. However, it also provides some clues for PKC involvement in the second and third step of rod differentiation namely the restriction and commitment to rod photoreceptor phenotype. These steps may be regulated by changes in chromatin modification that allows for specific gene expression after proper signal transduction activation. We demonstrated that changes in PKC activity result in general changes of epigenetic markers. Specifically inhibition of PKCβ1 and PKCγ during eight days of retinal explants increased substantially the amount of the active marker H3K4Me2 assayed by western
blots. Investigation of epigenetic changes during retinal development performed in our laboratory has demonstrated that the activation marker H3K4Me2 is highly present in progenitor cells, and it is gradually reduced once the retina matures and cells differentiate suggesting transcription inhibition is an important process involved in rod development. Consistent with this idea, the chromatin organization of mature rods is very peculiar as it contains a large central area of heterochromatin surrounded by a thin area of euchromatin, also indicating the process of chromatin condensation is a very important process in rod differentiation (Carter-Dawson LD, La Vail MM (1979). In addition Helmlinger D et al 2006 demonstrated that rod nuclear organization normally compartmentalizes rod specific genes in peripheral euchromatin, and changes in this chromatin organization due to hyperacetylation, results in aberrant differentiation of rod development and eventually rod degeneration.

Therefore, our results suggest that PKC activation during retinal development is involved in organizing chromatin in rods so that non essential genes are silenced by removing the active mark H3K4Me2. This task may be accomplished at least in part by recruiting LSD1 which removes the methyl group of the lysine 4 of H3. Supporting this argument are the experiments that show that IGF1 cannot drive rod development if LSD1 activity is pharmacologically inhibited.

In addition, results from our ChIP assays indicate that IGF1 activation of PKC results in transcriptional activation of rod specific genes during retinogenesis, as retinas treated with IGF1 contained less inhibitory markers (H3K9me2 and H3K27Me3) on rod specific gene promoters. In addition, inhibition of PKCβ1 and γ resulted in higher number of inhibitory markers on the same promoter area. From these results we hypothesize that PKC induced reduction of the inhibitory markers on rod specific gene promoters is essential for the transcription activation of
rod specific genes, as it has been previously reported that inhibitory histone markers prevent binding of acetyl transferases, and RNA polymerases necessary to initiate transcription.

The ChIP results on the activation marker H3K4Me2 after activation or inhibition of PKC seem contradictory at first, since both treatments result in reduction of the histone modification on rod specific gene promoters. Even though at present we were unable to look at the changes on H3K4Me3, we predicted that IGF1 treatments resulted in trimethylation of H3K4, a histone modification that is not a substrate for LSD1, which would also result in activation of transcription. In addition, further investigation of these changes at earlier stages of differentiation, two days and four days in culture, would be necessary to better understand this process.

In summary, we identified a key intermediate of signal transduction pathways that regulates rod differentiation. Different growth factors can regulate PKC activity, in my experiments, PKC activity is modulated by IGF1, but most certainly, other signal pathways can modulate PKC in different ways. Activation of PKCβ1 and γ isoforms, allows the transition of progenitor cells from proliferative state to terminal differentiation through the inhibition of STAT3 activity.

In addition, we have evidence that PKC activation also results in changes of chromatin modifications that may be involved in subsequent steps of rod differentiation.
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