Loss of daylight vision in retinal degeneration: are oxidative stress and metabolic dysregulation to blame?

Claudio Punzo\textsuperscript{1}, Wenjun Xiong\textsuperscript{2}, Constance L. Cepko\textsuperscript{2}

\textsuperscript{1}Department of Ophthalmology & Gene Therapy Center
University of Massachusetts Medical School, Worcester, MA 01606
\textsuperscript{2}Department of Genetics and Department of Ophthalmology, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115

Running title: Oxidative stress and metabolic dysregulation in RP.

To whom correspondence should be addressed: Constance L. Cepko, Department of Genetics and Department of Ophthalmology, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115

Keywords: Retinitis Pigmentosa, oxidative stress, rod, cone, photoreceptor, retinal degeneration

SUMMARY

Retinitis pigmentosa (RP) is characterized by loss of night vision followed by complete blindness. Over 40 genetic loci for RP have been identified in humans, primarily affecting photoreceptor structure and function. The availability of excellent animal models allows for a mechanistic characterization of the disease. Metabolic dysregulation and oxidative stress have been found to correlate with the loss of vision, particularly in cones, the type of photoreceptors that mediate daylight and color vision. The evidence that these problems actually cause loss of vision, as well as potential therapeutic approaches targeting them, is discussed.

Introduction

There are more identified genes that cause blindness than there are for any other disease (retnet:www.sph.uth.tmc.edu/Retnet). In part, this is due to our ability to self-report any abnormality in vision. In addition, it may be due to a relatively large target size comprising the genes that are dedicated to vision. These genes, when mutant, do not impact reproductive fitness to the extent of e.g. genes that cause heart disease.

Vision begins with the process of phototransduction, an elaborate biochemical cascade carried out by the photoreceptor cells, the rods and cones, located in the neural retina, which lines the back of the eye (1). Rod photoreceptors initiate our night vision, and are able to recognize a single photon as a specific signal, a remarkable ability that has resulted from years of selective pressure applied to a critical behavioral node. This high degree of sensitivity is achieved by cells that have unusual and vulnerable structural features, are demanding in terms of their energy requirements, and exist in a fairly threatening environment. Cone photoreceptors carry out color and high acuity vision, providing our daylight vision, and have many of the same features and vulnerabilities as rod photoreceptors. In our modern world with electricity, low light vision is no longer critical, while cone-mediated vision is still essential for our quality of life. This review will consider a disease, retinitis pigmentosa (RP), which leads to loss of both rod and cone vision due to genetic lesions (2). In addition to its intrinsic importance, RP is an excellent model for other diseases that lead to loss of vision. It has defined genetic causes and there are several animal models with mutations in the same genes as in human RP (3).

Many of the RP genes are expressed only in rods, yet cones still malfunction and die. The non-autonomous death of cones is likely due to a common problem(s), as it is seen in all organisms where there is a rod-specific gene defect, and where rods are the most abundant photoreceptor type. Oxidative stress and metabolic dysregulation are two causes that may be common across RP disorders. As is becoming increasingly appreciated in many diseases, these two causes are likely intertwined. In RP, they are relatively new targets for therapy. The evidence for these mechanisms of cone death will be considered here, as well as some possible points of intervention based upon these mechanisms.
Clinical Progression of RP

RP is characterized clinically as loss of rod (low light) vision, followed by loss of cone (day light) vision, and is often accompanied by the appearance of pigment within the retina, as well as attenuated vessels and optic disc pallor (4,5). The symptoms typically begin at birth, with reduced, or absent, night vision. Loss of cone vision can begin at different ages and in different regions of the retina, but generally the final loss is in the center, in the macula, giving rise to “tunnel” vision. The macula comprises only cones in its very center, and is the area of our highest acuity color vision. The animal models of RP have a retina with the same composition as the human retina, in the area outside of the macula, where rods are >90% of the photoreceptors. In several mouse models of RP, cone death begins when the majority of the rods have died (6,7). While the question regarding the causes of cone death are particularly important due to the role of cones in vision, it is also an interesting basic science question. The synaptic partners of rods, the horizontal and bipolar cells, do not die until much later in the disease process (4), raising the question as to why cones are preferentially susceptible.

A simplified version of the progression of cell death, and examples of retinal tissue morphology in RP, are shown in Figure 1. Several points are highlighted as potential points for therapeutic intervention, and both specific and generic types of strategies can be envisioned. For example, a specific, recessive genetic defect in rods might be remedied by delivery of a normal allele of the disease gene, i.e. by specific gene therapy, prior to loss of the majority of the rods (8-10). This type of specific therapy can also be targeted to a dominant allele, using a ribozyme or shRNA for knock-down, as is being developed for dominant alleles of rhodopsin (11-13). Alternatively, a generic therapy aimed at slowing or preventing rod death, even in the absence of correcting rod dysfunction, could be envisioned. In such a case, night vision would likely not be achieved, but cone death should be delayed or prevented if rods are preserved. Here, an intervention in the rod death pathway might be successful Similarly, addition of a gene such as histone deacetylase 4 (HDAC4), which has been shown to prolong rod survival in what is likely a non-specific manner (14), can be carried out. Unfortunately, little is known about the rod death pathway(s), other than the fact that the rods die of apoptosis in those cases that have been examined (15). A second point of intervention is when the majority of rods have died, but cones would still be functional. For this, a greater understanding of the mechanisms of cone death is needed.

Several models for cone death in RP have been proposed. One class of models concerns the loss of trophic support (16). Rods may supply a factor or factors required for cone survival. Even if this is not the underlying cause, delivery of a growth factor might delay death, and is an approach that is being taken (15-17). Another class of models concerns toxicity due to rod death. The release of a toxic factor by dying rods might kill the nearby cones (18). We believe that the kinetics of rod and cone death make this latter model unlikely. If dying rods released a toxin, one would predict that there would be a close temporal and spatial association of rod and cone death. However, cone death often does not occur until many months after rod death (4-7,19). Another model holds that there is an increase in oxidative damage to cones once the rods have died (20-22). Finally, we recently proposed that the cones have a nutrient shortage and/or imbalance in metabolism due to a change in retinal architecture, brought on by the loss of the rods (7). These models are not mutually exclusive and will be explored further below.

Oxidative Stress in RP

Oxidative stress has been suggested to be one of the causes of cone dysfunction and death in RP (20-22). Photoreceptor cells are under constant environmental and intrinsic challenges that make them highly susceptible to oxidative stress. Their function as light sensors places them in an area where they are exposed to the ultraviolet radiation in sunlight, which induces free radical formation (23,24). The isomerization of the chromophore, 11-cis-retinal, by light as part of the normal visual cycle, can lead to the formation of compounds that are reactive with short wavelength light. Such reactions can lead to free radical generation (25). To make matters worse, the choroidal blood vessels expose photoreceptor cells to near-arterial levels of oxygen (26), and high oxygen tension induces the production of reactive oxygen species (ROS). ROS cause oxidative damage to proteins,
Oxidative stress and metabolic dysregulation in RP

lipids and DNA, all of which have been demonstrated to increase during the course of RP (20). Besides the environmental risks, the high metabolic rate of photoreceptor cells is an intrinsic risk factor for oxidative damage, as ROS form as a natural byproduct of mitochondrial metabolism. Given that cones contain twice as many mitochondria as rods in murine retinas, and ten times as many in primate retinas (27,28), vulnerability to oxidative stress is likely heightened in cones. Lastly, NADPH oxidase (Nox), an enzyme complex that deliberately produces ROS for host defense and cellular signaling, has also been shown to contribute to cone cell death in RP and in light-induced retinal degeneration (29,30).

If photoreceptors, especially cones, are naturally under a considerable level of oxidative stress, how do healthy retinas cope with oxidative stress for many decades? Photoreceptor inner segments, which are packed with mitochondria, rely on endogenous antioxidant pathways (31) (Figure 2). Natural antioxidant enzymes in mitochondria include superoxide dismutases (SODs), which convert superoxide radicals (O$_2^-$), the major reactive species produced by mitochondria, to H$_2$O$_2$, and glutathione peroxidases (GPXs) and catalase, which further metabolize H$_2$O$_2$ to H$_2$O. In contrast, the OS of photoreceptors appear to lack such enzymatic detoxifying agents. The strategy that has been proposed for removal of oxidized products in the OS is one where oxidized proteins and lipids are cleared by daily OS disc shedding and renewal (32). The shed OS are phagocytosed by cells of the retinal pigmented epithelium (RPE), which also provide other support functions for photoreceptors (33).

How might oxidative stress affect cones in RP? One hypothesis is that the redox balance in cones is disturbed by the loss of rods, and oxidative stress elevates beyond the antioxidant capacity of cones. Supporting this idea, studies have found that, after the death of rods, which comprise >90% of the photoreceptor population and thus consume the majority of oxygen delivered to the outer retina, the oxygen level per cone increases sharply (22,34). This is likely due to the inability of choroidal vessels, which nourish the photoreceptors, to regulate blood flow in response to the environmental oxygen level (35).

Consequently, the overload of oxygen may be toxic to the residual cones. This “oxygen toxicity” hypothesis is consistent with the fact that relative cell density is a crucial determinant of cone death (36-38). This model can at least in part explain why cone death in RP is usually a slow process that takes years or decades, during which time oxidative damage may accumulate and eventually kill cones.

In recent years, mounting evidence supports the hypothesis that oxidative stress contributes to cone mortality in RP. Oxidative damage in cones was evident in a transgenic RP model in pig and in a mouse RP model (20,21). Importantly, treating several mouse models of RP with exogenous antioxidants slowed cone death (21,39). In addition, overexpression of the endogenous antioxidant enzymes, including SOD and GPX, in some RP mouse models, decreased oxidative damage and prolonged cone survival (40-42).

Oxidative stress is believed to play an important pathogenic role in many retinal and brain neurodegenerative diseases, including diabetic retinopathy, age-related macular degeneration, Parkinson’s, Huntington’s and Alzheimer’s diseases (43-45). The neurons with especially high vulnerability to oxidative stress possess two common properties: high oxygen consumption and great energy demand. While no suitable animal model has been developed for some of these neurodegenerative diseases, the well-characterized animal models of RP have been exploited in the studies cited above. They can also serve as test subjects for therapeutic approaches, such as viral delivery of anti-oxidant enzymes. Gene therapy directed to the photoreceptors will solve one problem posed by the delivery of chemical anti-oxidants through, e.g. the diet. The blood-retinal barrier, and the soluble nature of many of these compounds, does not enable a high, steady state level of the anti-oxidants in the retina following systemic delivery. Moreover, ROS are important signaling molecules and a wholesale decrease in ROS from systemic delivery might not be without side effects (46). Viral gene delivery, ideally coupled with a cone-specific promoter, might provide a more effective approach, one that might be especially beneficial if the promoter was also regulated by the oxidation level of the tissue. Such vectors are being developed for use in other
Oxidative stress and metabolic dysregulation in RP

Metabolic Changes in RP

Common changes in gene expression at the onset of cone death in four mouse models of RP led us to investigate the status of the mechanistic target of rapamycin (mTOR), a key regulator of cellular metabolism (7,50) (Figure 2). The activity of mTOR is regulated by phosphorylation, which is driven by nutrient availability, energy levels, and growth factor signaling. When active, mTOR phosphorylates a number of targets that regulate translation, macroautophagy, and metabolic pathways. We found that the phosphorylation of mTOR was reduced in the dorsal cones in all four RP mouse models examined, as the earliest sign of pathology among cones. In addition, there was a significant reduction in the level of the red/green opsin protein in the ventral cones, without a concomitant decrease in the RNA for this gene. This may reflect a reduction in translation, which is controlled by mTOR, or enhanced degradation of this opsin. These changes suggested that the cones might be under metabolic stress. Indeed, the chaperone-mediated autophagy pathway was found to be activated in the RP cones, but not in other retinal cell types. This prompted us to hypothesize that the mTOR phosphorylation status might be low due to the cells suffering from some type of nutrient deprivation and metabolic dysregulation. As insulin signaling can promote mTOR activity, we attempted to decrease or increase mTOR activity, by reducing or augmenting insulin signaling, respectively. Cone survival was indeed improved upon insulin injection, while cone death was accelerated upon insulin depletion, in a mouse model of RP. These data provide evidence that cone survival can be regulated by insulin signaling. These observations are in keeping with findings from the delivery of other growth factors to animal models of RP, which also led to increased cone survival (15,17). It is important to point out, however, that it is not clear for insulin, or other therapeutic growth factors, if the action is directly upon cones, and/or is mTOR-mediated. An understanding of the mechanism of these effects might enable the design of more specific therapies.

Photoreceptor Metabolism

Photoreceptors have evolved an elaborate structure, the OS, where photons are captured and phototransduction is carried out. To accomplish this, the OS is densely packed with membranes and opsin proteins (51,52). In fact, lipids comprise 15% of the mass of a photoreceptor, compared to 1% for “average” cells (52). Each photoreceptor contains roughly 60 pg of protein (52). Since photoreceptors shed 10% of their OS daily, they need to synthesize the membrane and protein equivalent of a proliferating cell each day (53,54). Additionally, photoreceptors are neurons, and thus, as is typical for a neuron, they need large amounts of ATP in order to maintain membrane potential. It is thus not surprising that photoreceptors are rated as the highest energy consuming cells in the human body (55). The high-energy requirements of photoreceptors make them especially vulnerable to any imbalances. This is exemplified by the fact that mutations in a gene that is broadly expressed and affects general cellular metabolism (e.g. isocitrate dehydrogenase 3 beta) is primarily associated with photoreceptor degeneration resulting in RP (56). One might also predict that photoreceptor metabolic activity displays signs of both a postmitotic neuron and of a proliferating cell. These dual demands likely require robust regulatory mechanisms that apportion the sources of energy and anabolic materials accordingly.

Postmitotic neurons synthesize their large quantities of ATP by complete catabolism of glucose or lactate. In culture, photoreceptors can take up lactate released by Müller glia (57), which have extensive contacts with photoreceptors in vivo, and can release lactate as a by-product of their own metabolism. As has been proposed for other CNS neurons (58), lactate from glia might provide the majority of the acetyl CoA that enters the mitochondria for energy generation (59,60). Proliferating cells need the building blocks derived from glucose for anabolic purposes. One study of photoreceptors has led to the suggestion that, as in proliferating cells, most of the glucose taken up by photoreceptors never enters the Krebs cycle, and that it fuels membrane and protein biosynthesis (57). Lactate and glucose may thus be utilized differentially within photoreceptors for ATP synthesis and anabolic processes (57,59-61), respectively.
Metabolic Model of Rod-Dependent Cone Death

Based upon the observations cited above concerning mTOR phosphorylation and chaperone-mediated autophagy, we suggested that a metabolic problem contributes to cone death in RP (7). The model is based upon the idea that glucose uptake is more affected than lactate uptake in cones in RP. This may be due to the collapse of contacts between the photoreceptor OS and the RPE (Figure 3). The choroidal blood supply fuels photoreceptors through the RPE cells. The flow of nutrients, such as glucose, from the RPE to the remaining cones may be disrupted following the collapse of the interface between the RPE and the cones, which occurs when the rods degenerate. Lactate uptake likely occurs independently of the RPE, from the Müller glia, whose processes surround the photoreceptor cell bodies, and whose processes also form the outer limiting membrane between the photoreceptor cell bodies and the IS. This area appears to not be as impacted when the rods die, making it likely that lactate uptake is not as disrupted as glucose uptake. Consistent with the idea of reduced glucose in cones is reduced OS length. A reduction in anabolic processes, which depend upon the glycolytic products of glucose, without an equal reduction in OS catabolism, would lead to a reduction in OS length. Following the reduction in OS length is an overall change in the structure of the cone plasma membrane, which appears very disorganized (62). An overall reduction in membrane surface area might create a downward spiral, as it may lead to less surface area over which transporters could operate to bring in more nutrients. However, if lactate uptake can still occur at a level that is less reduced than that of glucose uptake, it might provide an explanation for why cones survive for extended periods of time in RP, even in the absence of any OS. Using lactate as an energy source, cones may still produce enough ATP through oxidative phosphorylation to at least survive. Consistent with this, a recent study showed that mitochondrial fuel, such as pyruvate, was sufficient to prevent the photoreceptor death caused by depletion of glucose in a retinal explant culture system (63).

Finally, although cones are alive for a significant period of time after their OS have collapsed, they do not carry out phototransduction at a functional level (64). Several problems might lead to loss of phototransduction. One is the loss of the OS structure, as the OS is where the phototransduction process is carried out, within highly organized membranous discs. Another might be a reduction in opsin proteins, as there is a reduction in red/green opsin in ventral cones (7,64). Photoransduction may also be reduced due to insufficient 11-cis-retinal. The first step in vision is the photoisomerization of the opsin-bound 11-cis-retinal, to 11-trans-retinal (65). Within photoreceptor OS, 11-trans-retinal is reduced to 11-trans-retinol through a retinaldehyde dehydrogenase, with NADPH as the hydride donor (66,67). NADPH may be limiting for this reaction, as it is likely in high demand in RP cones. This is because NADPH also is used to reduce ROS, which, as described above, are increased in RP retinas. In addition, the fact that NADPH generation is dependent upon glucose, through the pentose phosphate pathway, might mean that NADPH generation is limited due to reduced glucose uptake. A paucity of 11-cis-retinal might also occur due to the disruption of the interactions between cone OS and the RPE. There is a shuttle of retinals between the RPE and photoreceptors, and as the RPE and cone OS interactions are disrupted in RP, this might reduce the availability of 11-cis-retinal to cones (68). Cones may also be able to acquire 11-cis-retinal from Müller glia, however, as there is evidence for this in chicks, ground squirrels (66), and zebrafish (69).

Insulin Signaling in Photoreceptors

Given the high energy demands of photoreceptors, it would not be surprising if the insulin/mTOR pathway, a key regulator of cell growth and homeostasis, played a central role in photoreceptor survival. In keeping with the aforementioned result of insulin on RP cone survival, loss of insulin receptor in rods, or one of its downstream targets, Akt2, increased susceptibility to light-induced retinal degeneration (70,71). Phosphorylation of the insulin receptor in rods appears to be light and opsin dependent (72) and dephosphorylation is mediated in the dark by protein-tyrosine phosphatase-1B (PTP1B) (73). Loss of PTP1B has a protective effect in a model of light-induced retinal degeneration (73) indicating that the increased level of phosphorylated insulin receptor is protective. However, there may be differential effects of
insulin signaling in rods and cones. ATP consumption is significantly reduced in rods during the day, while it is increased in cones (61). Therefore, the insulin/mTOR pathway might differentially regulate how much energy flows into the anabolic versus catabolic pathway in rods and cones under normal day/night conditions. In support of this idea is the light-dependent phosphorylation of the insulin receptor in rods (72,73). Additionally, loss of 3 of the 5 regulatory subunits of PI3K resulted in cone, but not rod, degeneration after 12 months, perhaps due to the difference in day and night activities between rods and cones (74,75). PI3K modulates the signal from different growth factor receptors and is downstream of the insulin receptor, but upstream of mTOR kinase activity.

The importance of proper regulation of metabolism in photoreceptor function and survival is evident from their energy demands. However, the regulatory pathways that control anabolic processes, oxidation, membrane synthesis, and, more generally, homeostasis, in photoreceptors are just being discovered. Greater understanding of the regulation of these processes within photoreceptors under normal and stress conditions may lead to new treatment approaches for photoreceptor degenerative diseases.

Future Prospects

As we learn more about the mechanisms that lead to photoreceptor death, different targets for therapeutics that combat oxidation, metabolic dysregulation, and as yet undiscovered mechanisms, will undoubtedly be developed.

There is great deal of excitement about the possibility of using gene therapy to this end. Vectors derived form adeno-associated virus (AAV) have proven successful in the clinic, to treat people with Leber’s congenital amaurosis 2, a disease that leads to photoreceptor dysfunction (76,77), where the RPE is the site of the gene defect (78,79). Multiple groups are developing AAV vectors encoding photoreceptor genes for complementation of recessive diseases (80-84), as well as AAV vectors encoding growth factors (85,86) or anti-oxidant enzymes (47-49). The emerging field of optogenetics is also being brought to bear on diseases of the eye. Light activated channels and pumps are being delivered to the eye, either to augment light responses in ailing photoreceptors (64), or to convert non-photoreceptor cells into photosensitive cells (87-91). Stem cell approaches are under development, with more efficient protocols for generating photoreceptors from stem cells being reported (92-94). Nanoparticles are being tested for gene delivery to photoreceptors (95,96), and protein transduction methods have been shown to work in the eye (97). The longevity of these latter approaches will likely need to be extended for diseases such as RP, where the time line is likely decades. Importantly, combinations of approaches are likely to be more powerful than any individual approach (98). Having a number of approaches for gene and protein delivery, and a number of different targets, makes one hopeful that there will be some therapeutic benefits in the coming years for a group of diseases that greatly diminish the quality of life for a growing number of people.

References

Oxidative stress and metabolic dysregulation in RP

Oxidative stress and metabolic dysregulation in RP

Oxidative stress and metabolic dysregulation in RP


Acknowledgement

CLC would like to thank the Foundation for Retinal Research, Thome Foundation, HHMI, and Foundation Fighting Blindness for their critical support over the years. CP thanks the University of Massachusetts for their support. WX is a postdoctoral fellow of HHMI.

Figure Legends

**Figure 1.** Photoreceptor death in RP. (A) Simplified time course of rod and cone death kinetics. Time points for possible therapeutic interventions are indicated. Rods can be targeted using an approach specific to a particular disease gene, e.g. by AAV-mediated gene therapy replacing a recessive gene
(80,81,83) or via knock-down of a dominant gene (13). Alternatively, rod survival can be prolonged by non-specific therapies, e.g. delivery of growth factors (85,86), or HDAC4 (14), aimed at a wider group of RP diseases. Cones can be targeted using anti-oxidant therapy (20,99) or gene manipulations that might alter metabolism. Once cones have become unable to carry out normal phototransduction, they can be transduced with halorhodopsin, a light activated chloride pump (64). After the loss of cones, non-photoreceptor cells, such as bipolar cells and retinal ganglion cells, can be made to respond to light following delivery of channel rhodopsin2 or melanopsin (87,88). (B). Retinal cross-sections of a mouse model for RP (100), at 8 weeks (wk) and 17 wk of age. The photoreceptors are located in the outer nuclear layer (ONL) which can be seen to degenerate to one or two rows of cells, primarily cones, by 17 wk. Note the collapse of the cone OSs during this time, revealed by the binding of the lectin, peanut agglutinin (PNA, red). Accompanying degeneration is the upregulation of the glial fibrillary acidic protein (GFAP, green). ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer, DAPI: nuclear stain.

**Figure 2.** Metabolism in cones. Photoreceptors are highly active metabolically and require substantial glucose and oxygen, which are supplied by the choroidal vessels via the RPE cells. Lactate may also be released by retinal Müller glia, and taken up by photoreceptor cells. Mitochondrial oxidative phosphorylation provides cells with large amounts of ATP for neuronal functions, but also can cause an excess of ROS. Removal of ROS requires the actions of the endogenous antioxidative enzymes (SOD, GPX, Catalase) and the natural antioxidant, glutathione. The pentose phosphate pathway (PPP) generates NADPH, which is important for glutathione recycling and lipid synthesis. Sufficient supplies of NADPH, ATP and the metabolic intermediates ensure rapid macromolecular synthesis, underlying the continuous self-renewal of cone OS. Growth factor signaling, including the activation of the insulin receptor, which stimulates mTOR phosphorylation, can positively regulate the key steps of glycolysis. Cones also need NADPH for an early step of the visual cycle, reduction of 11-trans-retinal, which ultimately results in regeneration of 11-cis-retinal. The RPE participates in this cycle, with intermediates cycling between cone OS and the RPE. The major metabolic pathways are highlighted in blue, with the key proteins and molecules of therapeutic interest shown in red.

**Figure 3.** Schematic representation of rod photoreceptor loss leading to changes in retinal architecture. Before the onset of rod photoreceptor death (A), the interactions between photoreceptor OS and RPE cells are important for photoreceptor nutrient uptake, the visual cycle, and maintenance of photoreceptor structure. When rods first start to die (B), the RPE-OS interactions are not greatly perturbed. However, as the disease progresses (C), the loss of rods becomes very extensive, and the collapse of the remaining cone photoreceptor OS’s becomes evident. Loss of rods also leads to an elevated oxygen level. (D) Eventually, the RPE-OS interactions become completely disrupted, which may cause a reduction in nutrient flow, particularly glucose, into remaining photoreceptors. Cones in blue or red/green; Rod in grey; RPE in pink; Choroid in red.
Oxidative stress and metabolic dysregulation in RP

Figure 1.

Figure 2.
Figure 3.