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Systems Pharmacology Links GPCRs with Retinal Degenerative Disorders

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Abstract

In most biological systems, second messengers and their key regulatory and effector proteins form links between multiple cellular signaling pathways. Such signaling nodes can integrate the deleterious effects of genetic aberrations, environmental stressors, or both in complex diseases, leading to cell death by various mechanisms. Here we present a systems (network) pharmacology approach that, together with transcriptomics analyses, was used to identify different G protein–coupled receptors that experimentally protected against cellular stress and death caused by linked signaling mechanisms. We describe the application of this concept to degenerative and diabetic retinopathies in appropriate mouse models as an example. Systems pharmacology also provides an attractive framework for devising strategies to combat complex diseases by using (repurposing) US Food and Drug Administration–approved pharmacological agents.

Keywords

network; systems biology; pharmacological agents; polypharmacology; retina; blindness

INTRODUCTION

Systems pharmacology can be defined in various ways. Here we use the term to describe the use of a combination of two or more drugs to achieve a positive therapeutic effect that neither could invoke alone (without risking dose-limiting adverse drug reactions) by affecting downstream common effector(s) or signaling pathways. Thus, any two or more drugs selected must possess different toxicity profiles in addition to distinct pharmacodynamic properties to maximize the probability of success. Moreover, an optimal drug combination would provide a therapeutic effect with doses of each component that alone would be subtherapeutic, thereby minimizing the chances of dose-related toxicity.

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Regardless of the initial genetic and/or environmental cause, researchers have increasingly recognized that disease onset and progression involve a complex interaction of seemingly unrelated and unappreciated signaling events. Findings from systems biology studies could be explored fully to guide the selection of previously unevaluated pharmacological interventions under conditions of a given disease and used to probe the disease mechanisms and identify novel molecular targets to enhance future therapeutic design. Moreover, in contrast to single-drug approaches, systems (network) pharmacology employs more than one drug to affect different cellular signal transduction pathways that ultimately impinge upon a common effector. Such an effector could be an enzyme, a second messenger, a transporter, or any other biologically active molecule. Multiple pathways with common effectors can exist in single cells or more globally in groups of cells, organs, and tissues (1). By providing a combination of therapeutics directed toward the unique complement of ligands and signaling pathways that are active in an organ or tissue of interest, a systems pharmacology approach holds promise to provide a more rational and effective means of achieving selectivity compared to that offered by modulating a single target. Because each subset of cell types has its own unique set of networks, a combination of drugs acting on different molecular targets at low doses could amplify a desired response in a given tissue or organ, restoring homeostasis to genetically or environmentally perturbed loci within organisms. Hence, in this period of rapid growth of genetic information, precise identification of disease-causing lesions and the availability of a large number of safe, potent, US Food and Drug Administration (FDA)-approved drugs could allow systems pharmacology to become a novel and effective way of providing safe treatment for complex diseases.

The above strategy requires detailed functional and structural knowledge of the various cellular pathways involved in the pathology being treated. Although researchers have made much progress in achieving this objective, modern genetics can offer even more. Here we emphasize that a combination of genetic profiling, together with systems pharmacology, can offer a powerful approach for identifying combination therapies for retinal diseases and diabetes, as exemplified by mouse models. A similar approach could be tailored to identify novel, effective, combinatorial therapies for a multitude of etiologically complex diseases.

POLYPHARMACOLOGY VERSUS SYSTEMS PHARMACOLOGY

Polypharmacology is the term used to convey the idea that a single drug can exert effects on many cellular targets; it contrasts with the term polypharmacy, which connotes the administration of multiple drugs (often used to treat a combination of clinical disorders) (2). Thus, multiple pathways can be affected by a single drug, especially when the target is at the focal point of a biological activity node such as an enzyme, receptor, or second messenger (3–10). Unwanted consequences of polypharmacology can lead to drug withdrawal from the market owing to adverse side effects (9). The self-reporting FDA Adverse Event Reporting System provides empirical and potentially useful information about polypharmacology that may help researchers generate hypotheses of optimal therapies for further experimental validation (11). One way to study polypharmacology combines proteomics-based identification of targets with theoretical considerations (1, 12, 13). Further development of computational methods also holds substantial promise for predicting polypharmacology (14–16). Recently, investigators successfully employed an approach for automated profiling of

ligands against multiple drug targets (17). Structural analyses (e.g., using X-ray crystallography, nuclear magnetic resonance, or cryo-electron microscopy) can also produce molecular insights into possible mechanisms of polypharmacology.

In contrast, when multiple drugs modify one or several networks, the term systems pharmacology applies (18, 19). Combining drugs that act on different targets within the same network or a combination of related or nonrelated networks can be more efficacious than treating a pathological condition with a single drug because the flexibility and redundancy of biological systems allows them to compensate when just a single element is perturbed (19). For example, dorzolamide/timolol (Cosopt), an eye drop used clinically for treating glaucoma, consists of two components, namely dorzolamide and timolol. Dorzolamide decreases the production of aqueous humor by inhibiting carbonic anhydrase. Timolol is a nonselective β-adrenergic receptor antagonist that reduces the production of aqueous humor by blocking β_1- and β_2-adrenergic receptors of the ciliary epithelium. The combined effect of these two agents results in an additional intraocular pressure reduction compared to either component administered alone by the same regimen. Indeed, this practice of combining therapeutics is a long-established hallmark of therapy for cancer, with certain antimicrobial agents (e.g., trimethoprim/sulfamethoxazole), and in the treatment of HIV. The broader systems pharmacology approach is based on defining interactive networks of signaling pathways and targeting multiple common nodes in those pathways to achieve more effective and safer therapy. Systems pharmacology is an innovative extension of a time-honored concept. It is important to distinguish the new concept of systems pharmacology from the traditional practice of combining drugs that act via different mechanisms to achieve a synergistic action while diminishing side effects.

Improving our understanding of drug effects is also key to managing undesirable side effects within their targeted cellular networks (20). This complexity is particularly true when investigations move from the cellular to the whole organism level. However, if specific networks are identified, cellular homeostasis could be restored by combinations of drugs, each administered at reduced doses.

As a disease state often causes multiple abnormalities, promiscuity of a drug's action can be advantageous, as demonstrated by cancer chemotherapy and agents controlling mood and neurological disorders (5, 21, 22). For example, antidepressants with multiple molecular targets proved to have efficacy superior to those with a single mode of action (23). But even more desirable would be the identification of optimal therapeutic regimens with high selectivity and affinity but minimal off-target effects and toxicity. The biological complexity of diseases makes pharmacological approaches less predictable and more dependent on experimentation. A database of publically available resources to integrate drug actions with systems biology would help solve this problem (16).

Genes can be pleiotropic and in some cases have distinct functions in different tissues or organs (gene sharing) (24). The same gene may have enzymatic or signaling functions in one context and a structural role in others. For example, crystallins are expressed at high levels and are essential structural proteins in the lens, exerting a key role in maintaining its refractive index. These proteins are also present at lower levels in other cell types, including

those of the retina and brain. But in tissues outside the lens, crystallins can serve other functions, such as by acting as molecular chaperones to help maintain homeostasis, protecting cells from the accumulated insults of stress or aging (25). Moreover, a recent study also revealed that α-crystallin regulates breast cancer cell metastasis (26). Systems pharmacology could take advantage of these cell-specific functions, employing multiple drugs at low doses that target multiple regulatory sites peculiar to a specific diseased cell type and that could circumvent unwanted dysregulation of the same gene in other uninvolved tissues.

FUNCTIONAL GENOMICS AND THE POWER OF BIOLOGICAL COMBINATIONS

Efforts to establish the biological functions of genes and pathways (functional genomics) are continuously evolving and will accelerate as researchers develop more innovative and rigorous technologies. As more details emerge regarding different aspects of cellular function, we can begin to understand how different pathways interact. The human body has about 100 trillion cells, yet the number of protein-encoding genes is far less, totaling only approximately 21,000. Many are derived by gene duplication and encode proteins with similar modes of ligand binding and regulation. The relatively low number of protein-encoding genes and their different splice forms is markedly expanded by regulatory elements such as genetic mutations, epigenetic modifications, noncoding RNAs (including micro RNAs), metabolites, and modifications of proteins, which essentially constitute the diversity of an integrated biological system. The pattern of these elements also changes during the 24-h day-night cycle owing to the body's circadian rhythm, adding yet another layer of complexity. Functional diversity of different cell types can also result from the interplay among the unique set of signaling pathways operating within each cell type. Such intricacies of biological systems have profound implications for drug development, including drug selectivity.

Investigators have not generally solved the problem of drug selectivity satisfactorily, and many reasons explain why this is so. First, the number of signaling pathways that function in our bodies is limited, and traditional pharmacological strategies allow only the subset present in a particular cell type to be modified (27). Therefore, a clearer picture of how cells perform different tasks and communicate with each other can expand the spectrum of viable pharmacological targets only to a limited extent. Second, most known biochemical transformations occur in diverse cell types and unrelated tissues. This phenomenon, along with the similarity of ligand-binding properties among subsets of targets, presents a serious challenge for achieving drug selectivity without adverse drug reactions (28). To overcome this problem, researchers are paying greater attention to the development of local drug delivery. However, the complexity and expense of most such delivery methods, along with problems with patient acceptance, leaves oral administration as the preferred method for drug administration. Nevertheless, drug targets are the key to achieving selective efficacy and minimizing toxicity.

Among many possible signaling molecules, G protein–coupled receptors (GPCRs), such as the adrenergic, adenosine, and muscarinic receptors or any of 800 others in the human

genome, play essential roles in fundamental cellular processes. Moreover, GPCRs are also the most common types of receptors targeted by medications for disease treatment (29). They are master regulators of virtually all physiological processes, accessible to active compounds that do not need to enter the cell, and are critically connected to other signaling pathways such as those involving growth factor receptors (30). Allosteric regulators and drugs targeting heterodimerization of GPCRs could interact with specific pairs of receptors via unique allosteric binding sites linking highly selective ligands to further increase response selectivity (31–33). Expression profiling on a genomic scale can be developed to guide the rational targeting of specific pathways, not only by novel therapeutics but also by existing FDA-approved drugs. Because each subset of cell types has its own unique set of networks, a combination of drugs acting on different molecular targets at low doses could achieve a desired response in a given tissue or organ, restoring homeostasis affected by loci that have genetically or acquired perturbations within a tissue of interest. Moreover, identification of diurnal changes in gene expression could allow these therapeutic agents to be delivered at an optimal time. Hence, in this period of rapidly growing genetic information, by combining precise identification of disease-causing lesions and the vast array of safe and efficacious FDA-approved drugs, systems pharmacology could be an—perhaps the most—effective way of providing optimal treatments for complex diseases.

MOTIVATION FOR NEW GLOBAL APPROACHES

The idea of one drug, one target, and one disease was developed from the notion of a single disease-causing gene. The decline in new drugs introduced into the market (perhaps with the exception of inhibitors of protein tyrosine kinases in cancer), along with the multifactorial nature of many common diseases, necessitates a new concept for drug design, one that embraces both multidimensionality and interconnection (34). Thus, as elegantly summarized by Ravi Iyengar, “complex diseases require complex therapies” (35, p. 1039).

Any perturbation of a native state, even as a result of a single mutation, can result in a complex adjustment of many networks to produce a new steady state associated with a disease phenotype. For example, removal of the neural retina-specific leucine zipper gene *Nrl* alters a few dozen transcripts in the retina that completely change the retinal photoreceptor population (36). Similarly, A/J mice exhibit variations in hundreds of transcripts, driving more complex alterations associated with cone photoreceptor cell degeneration (37). The interconnectedness of various intra- and intercellular networks must be considered when evaluating drug safety and efficacy. Thus, the functional specificity of a drug does not guarantee its effectiveness as a treatment for a disease state. For example, gefitinib (Iressa), an epidermal growth factor inhibitor, failed to exhibit the expected efficacy in prolonging the survival of patients with adenocarcinoma of the lung (38). Recent progress in the omics disciplines, along with more detailed understanding of disease progression in animal models, enables the design and testing of more sophisticated therapeutic approaches. As exemplified in this review, retinal degenerative diseases leading to blindness, including diabetic retinopathy, manifest complex phenotypes and can serve as prime candidates for systems pharmacology approaches to develop new treatment options.

INTEGRATION OF OMICS DISCIPLINES INTO DRUG DISCOVERY

The shortcomings of current approaches in drug development could benefit from the expanded perspective afforded by omics technologies. Developing a new drug is an expensive and lengthy (approximately 120 months) proposition, with only about 30 drugs receiving FDA approval each year. Another problem is the paucity of pharmacologically validated protein targets for drug action. Only about 400–3,000 out of roughly 25,000 proteins (expanded 4-fold by different splice variants and another 4-fold by posttranslational modifications) are currently targetable (39–41). Thus, to date, less than 1% of potential protein targets have been druggable. In addition, genetic polymorphisms can alter the activity of functional proteins. For example, two cytochrome P450 2C9 (CYP2C9) polymorphisms, CYP2C9*2 and *3, slowed the metabolism of the anticoagulant warfarin, resulting in reduced dose requirements and hemorrhagic complications for individuals carrying these alleles who were treated with standard dosing protocols (42, 43). Genetic differences that preferentially occur in members of certain ethnic groups can further complicate this scenario. For instance, discrepancies among responses to the same drug treatment are often noted in patients of different racial origins. African Americans responded poorly to angiotensin-converting enzyme inhibitors compared to white patients with chronic heart failure (44) and left ventricular dysfunction (45). Similarly, hypertensive African Americans responded less favorably to treatment with the β_1-selective-adrenergic receptor antagonist atenolol compared with hypertensive Caucasians, an effect partly attributed to atenolol-induced metabolic changes that are dependent on race and genotype (46).

The advent of high-throughput omics technologies for sequencing genomes and transcriptomes, measuring global protein levels, and identifying protein modifications and fluctuations of metabolic products (i.e., genomics, transcriptomics, proteomics, lipidomics, glycomics, metabolomics, and so on) promises to provide a more complete picture of major determinants affecting organ and tissue homeostasis. Researchers previously thought that pathological alterations could thus be more readily identified and pharmacologically corrected. However, this relationship has turned out to be much more complex than anticipated (47, 48), as only a few new drug targets are still discovered each year (49). So what's wrong? Too many of these approaches relied on data derived from studies with transformed and immortalized cultured cells rather than normal cells in their native environment. Hence, they could not fully recapitulate more complex biological systems (50). A classic example is the bacteriostatic prodrug prontosil, which would be missed by high-throughput modern screening. Ineffective in tissue cultures, this compound must be converted to the active sulfanilamide by the host organism (51). The genetic homogeneity of cultured cells, compared to the expansive genetic diversity of the human population, provides yet another limitation. Moreover, it is difficult to discern which specific pathway would serve as the ideal target. The promise of these modern techniques can, however, still be fully realized if all the data are appropriately obtained and integrated. More highly developed genomics and automated drug discovery technologies (a combination of omics technologies) offer great promise (52), but the cumbersome nature and technical skills involved require that these technologies be further developed for broader and more robust use. Researchers also hope that the use of computational biology to assess complex systems

can contribute significantly. Computer simulations that integrate omics data are promising, but independent experimental validation of simulation results is critical (53).

Our approach involves initial expression profiling on a genomic scale that then is used not only to guide rational targeting of novel therapeutics to specific pathways but also to test existing FDA-approved drugs. Indeed, >900 active substances are FDA approved for use in about 100,000 different products (http://www.accessdata.fda.gov/Scripts/cder/drugsatfda/index.cfm), and about half of these active substances target human proteins (the other half being directed against pathogens). In our case, GPCRs have been the primary targets, but this approach could also be applied to enzymes, transcription factors, or other cellular components. GPCRs are affected by selective ligands. Drugs modulating GPCRs account for 30–50% of all pharmaceutical agents in clinical use, and a quarter of the 200 best-selling drugs target these receptors. However, only about half of all GPCRs, including olfactory receptors, have identified ligands. A multiplexed pharmacological approach could also be effective for drug discovery. For example, Roth and colleagues (54) have demonstrated that new molecular targets (receptors) can be identified by targeting multiple pathways. A similar strategy successfully revealed protein kinases as targets for cancer-targeted chemotherapies (5). The approach described here that combines cell- and tissue-specific transcriptomics analyses should also be of interest to those pursuing mechanism-based pharmacological development and identifying more comprehensive systems approaches to treat multifactorial clinical disorders.

NETWORK DYNAMICS

Network pathways are not only interconnected but also highly dynamic, as exemplified by diurnal changes. Dynamic changes in networks also occur in response to drugs, making it possible to identify homeostatic compensatory pathways that could also be pharmacologically targeted. Finally, network biology remains dynamic throughout life, and the onset and clinical feature of many diseases are age-related. Age-dependent physiological changes can have a genetic basis or result from changes in the state of an organ or cellular function (influencing pharmacodynamics) or alterations in drug metabolism (influencing pharmacokinetics). DNA undergoes continual changes, such as shortening of telomeres and chemical modifications. Alterations in cell biology throughout a lifetime can be significant and include the accumulation of intracellular and extra-cellular materials that cannot be effectively cleared (55–57). Genetic methods, such as analyses of polymorphisms and mutations of disease-causing genes, do not reveal the complex timing and regulation of gene expression, protein stability, or altered posttranslational modifications of proteins (58). No overtly abnormal phenotypes are identified in many gene-knockout mouse models, and the frequency of this phenomenon is difficult to generalize; many factors are involved, including compensatory changes during development. Nevertheless, an estimated 10–15% of generated mouse knockouts do not exhibit detectable pathophysiological changes (59). In addition, ablation of genes associated with human aging cannot be well recapitulated in mouse models (60). Even in the case of monogenic diseases, a new homeostatic state is established that causes alteration of numerous signaling pathways. This adjustment is a basic imperative of evolution. Thus, the notion of gene therapy—that restoring function of an inactivated protein will consequently restore tissues to their native state—is likely initial

simplification. It fails to consider that permanent changes have already occurred in the mutant cells and that a new, unstable homeostatic state has resulted, as was recently observed for the *Retinal Pigment Epithelium 65* (*RPE65*) gene transfer in Leber's congenital amaurosis (61). Systems pharmacology could apparently augment gene transfer approaches by restoring a more native state along with the function of an affected protein.

Networks of a given organ are also regulated by physiological functions of other organs and tissues within the same organism. Sears and colleagues (62) recently identified a novel interplay between peripheral and visceral organs. These authors showed that treatments targeting the liver in mice can also prevent oxygen-induced retinopathy by altering the peripheral capillary bed of the retina. Such examples of communication between different organs demonstrate the need to develop even more complex, whole-organism, systems biology approaches. In a short timeframe after drug administration, biochemical changes occur within cells, including changes that involve the metabolism of drugs and their metabolites. Drugs are actively eliminated from cells by multidrug-resistant transport pumps with different efficacies for each drug (63), making changes that are difficult to predict, especially in the context of multiple drug therapy (64). Thus, multiple therapies targeting specific, key signaling pathways based on rigorous transcriptomics and other omics analyses can provide important insights into the dynamics of network biology and its potential response to systems pharmacology. Also, an iterative approach could coordinate the advancement of computational models and therapeutic development. Testing multiple drug regimens to verify the models and rigorous omics analyses to define additional regulatory nodes in interactive signaling pathways would enable refinement of the computational models and identify additional drug targets.

SYSTEMS PHARMACOLOGY STRATEGIES USED TO TREAT COMPLEX DISORDERS

Just as different cells contribute specifically to particular functions of our organs and tissues, different cellular components (proteins, genetic material, membranous structures, metabolites, and so on) provide various functions to a broad spectrum of cell types with diverse roles. Cellular components are also influenced by factors such as age, localization, and temporal changes, including their rates of formation and degradation. At steady state, a homeostasis is established between the nucleic acid, protein, and metabolic components of a cell (Figure 1***a***). But germ-line, somatic, or environmentally induced modifications can perturb this balance. In many cases, cells can initially cope with these changes but later become more prone to premature senescence. Systems pharmacology can be used to introduce multiple modifications in signaling pathways that restore more stable, native-like homeostatic conditions (Figure 1***a***).

GPCRs provide an excellent example for the application of systems pharmacology (65). Multiple different signal transduction pathways can converge on a common effector molecule or second messenger (Figure 1***b***). Next-generation sequencing (NGS) initially provided a comprehensive view of transcripts expressed in the mouse retina (37, 66). Detailed data analyses then further suggested interconnected signaling components whose interactions might contribute to retinal physiology, such as several GPCRs, major GPCR

effector enzymes, and NADPH oxidase (Nox) subunits. Researchers therefore designed combination pharmacological interventions to address the possible implications of these interconnected signaling mechanisms in a mouse model of a retinal degenerative disorder. The results provided proof-of-concept evidence supporting the mechanistic implications of these signaling components in retinal degeneration; more importantly, they also laid the groundwork for therapeutic development, in part by optimizing the choice of pharmacological agents to be tested (Figure 1***c***) (66, 67).

GPCRS AND GPCR SIGNALING

Cells respond to various types of environmental cues through signaling mechanisms. Cell signaling plays an essential role in governing and coordinating a vast array of fundamental cellular activities, including cell proliferation, differentiation, and apoptosis, required for normal tissue homeostasis and physiology. Aberrant cell signaling perturbs normal cellular functions and contributes mechanistically to the pathogenesis of various disorders, including cancer, inflammatory diseases, and neurodegenerative disorders, among others. Cells are equipped with a rich variety of signaling mechanisms to carry out their basic functions through multiple events ranging from receptor-mediated perception of a signal to intracellular second messenger–mediated signaling transduction and targeted action that modify cellular function and gene expression. Extracellular signaling molecules and changes in the cellular environment are generally detected by cell surface receptors, leading to signaling initiation.

This connectivity partly explains why GPCRs play fundamental roles in a large array of physiological processes (68). These receptors recognize a wide diversity of extracellular physical and chemical signals, such as nucleotides, peptides, amines, Ca^{2+}, and photons, modulating vital physiological functions including sensory perception, chemotaxis, neurotransmission, and intercellular communication. As shown in Figure 2, GPCRs transduce extracellular stimuli to initiate various intracellular signaling responses through interaction of their intracellular domains with respective heterotrimeric G proteins (69–71). This can result in modulation of the adenylyl cyclase (AC)-mediated cyclic adenosine monophosphate (cAMP)-dependent pathway, small GTPase Ras homolog gene family, member A (RhoA) activation through rhodopsin (Rho) guanine nucleotide exchange factor (RhoGEF), phospholipase C (PLC)-mediated intracellular Ca^{2+} mobilization, and regulation of ion channels in the plasma membrane (30, 72). Because of their structure, localization, and mechanism of action, GPCRs are accessible to circulating ligands, chemical compounds, or antibodies that stabilize the receptor in an agonist-like or antagonist-like conformation. Nanoantibodies, small 15-kDa fragments, have great potential as attractive ligands to modulate GPCR action (73), representing one aspect of the multifaceted approach to the systems pharmacology of GPCR networks.

Knowledge of a candidate gene's expression in a particular tissue or cell type often provides important clues to its functional relevance in pathophysiological conditions. The design and interpretation of functional studies covering a wide range of gene products, including GPCRs, nuclear receptors, single transmembrane receptors, enzymes, and transcription factors, can be informed by genome-wide expression analyses, especially when such

analyses identify the expression of a large number of genes from the same family. Genes encoding GPCRs are found in the genomes of many species, with more than 800 members identified in humans (74). Based on their sequences as well as their known or speculated functions, human GPCRs are commonly divided into five major classes: the *Rhodopsin*, *Secretin*, *Adhesion*, *Glutamate*, and *Frizzled*/*taste receptor 2* (*TAS2*) families (74) (Figure 3).

COMPLEXITY OF GPCR NETWORKS

The tissue-specific expression and functional relevance of many GPCRs remain unknown. A variety of challenges, either intrinsic to the molecule of interest or resulting from limitations of conventional methods, can hinder the discerning of the tissue- and cell-specific localization of membrane proteins, including some GPCRs and their respective signaling partners (see sidebar, Transcriptomics Information Revealed by NGS). NGS of transcripts [RNA sequencing (RNA-seq)] offers new insights into both the presence and abundance of gene products in a high-throughput, quantitatively precise manner with an unlimited dynamic range. RNA-seq has revealed transcripts of many GPCRs in the retina distributed among the five major classes of the GPCR superfamily (Figure 3). The presence in the retina of several members of the same GPCR subfamily suggests that they contribute to retinal pathophysiology. For example, several receptors from the *Rhodopsin* GPCR class are expressed, including those encoding the 5-hydroxytryptamine (serotonin) receptor (HTR) family, such as *HTR1A*, *HTR2A*, *HTR2B*, *HTR4*, *HTR5*, *HTR6*, and *HTR7*. Interestingly, these HTRs function through different G_{α} proteins. HTR1A is a $G_{\alpha i}$-coupled GPCR, HTR2A is a $G_{\alpha q}$-coupled GPCR, and HTR6 and HTR7 are $G_{\alpha s}$-coupled GPCRs. Similarly, adrenergic receptor genes, including several forms of α *adrenergic receptor 1* (*ADRA1*) and *ADRA2*, are also found in the retina. ADRA1 is a $G_{\alpha q}$-coupled GPCR, whereas ADRA2 functions through $G_{\alpha i}$-mediated signaling. Moreover, transcripts of enzymes mediating the intracellular functions of GPCRs are also expressed in the retina, including many *PLC* isoforms (Table 1) and *AC* isoforms (66). These findings provide a rationale for further functional evaluation of these receptors and their respective intracellular signaling partners in retinal pathophysiology.

NADPH OXIDASES AND REACTIVE OXYGEN SPECIES GENERATION

As important signaling molecules, reactive oxygen species (ROS) and their mechanisms of generation remain an active research area for understanding the pathogenesis of retinal diseases and are often considered important therapeutic targets. ROS embody a variety of free radical and reactive molecules as well as nonradicals that can act as oxidizing agents, become easily converted into radicals, or both. ROS are formed through a reaction cascade that begins with superoxide production and can be generated as byproducts of functioning mitochondria (75), peroxisomes, cytochrome P450 (76), and other entities. However, phagocytic Nox was the first enzyme identified with the primary function of generating ROS. Nox is an enzymatic complex consisting of several membrane and cytosolic subunits that, upon activation, catalyzes the production of superoxide from oxygen and NADPH. This Nox function is not limited to phagocytes but is present in virtually all cell types. Investigators increasingly recognize that nonphagocytic cells such as fibroblasts, endothelial

cells, and vascular smooth muscle cells also express superoxide-producing enzymes analogous to the prototypical phagocytic Nox, namely Nox2 (77). Although they share structural similarities, Nox isoforms activate the Nox complex in different ways (Figure 4) (78, 79).

Researchers have also noted several major functional differences between phagocytic and nonphagocytic Nox enzymes (80, 81). Firstly, nonphagocytic Nox appears to generate constitutively low levels of superoxide in the unstimulated state. The extent of superoxide production by an activated Nox complex expressed in nonphagocytic cells is also much lower than that found in neutrophils. Lastly, nonphagocytic Nox produces mainly intracellular ROS, whereas neutrophil superoxide production is thought to occur in extracellular or phagosomal compartments. ROS react with many molecules, including proteins, lipids, carbohydrates, and nucleic acids. Thus, when overproduced, ROS may irreversibly destroy or alter the function of a target molecule, resulting in cellular damage through oxidative stress. This seemingly harmful effect of ROS is indispensable for normal host defenses, as a deficiency in ROS generation impairs the killing ability of neutrophils. In addition to cellular damage and pathogen killing, low levels of ROS produced by nonphagocytic Nox could function as important second messengers that regulate redox-sensitive signal transduction pathways (82). Therefore, dysregulation of Nox-mediated ROS generation is increasingly recognized as one of the central mechanisms contributing to the pathogenesis of various disorders (83). RNA-seq has revealed the basal expression of Nox family members and associated enzymatic partners in mouse and human retinas (Table 1). Transcripts of several Nox enzymatic complex components, particularly *p22^{phox}* and *Rac1*, were readily detected in both mouse and human retinas (Figure 4 and Table 1).

CROSS-REGULATION OF THE GPCR AND NADPH OXIDASE SIGNALING PATHWAYS

Many molecular components bridge the cell surface and the nucleus, forming various types of signaling pathways that carry out essential biological functions. A signaling pathway mediated by a particular receptor, regardless of its nature, appears to be a linear chain in itself, but this apparently unidirectional flow of information often interlinks with other pathways at various levels of the signaling cascade. Cross talk is the term used to define such interactions. Thus, each component or step of a signaling pathway could become a potential regulatory point or intersection with other signaling pathways. This common theme in signal transduction highlights the complexity of regulation in determining the fate of a cell under any given pathophysiological condition. For instance, cross talk is often noted among GPCR signaling pathways. Stimulation of a particular GPCR may not only result in activation of a single signaling pathway but also bring about changes in signaling mediated by other GPCRs. A scenario of synergistic interactions could cause amplification of a certain signal, whereas another would be involved in fine-tuning multiple signaling pathways. Notably, signal transduction from one receptor could also be negatively regulated by another receptor through feedback effects or by activation of an inhibitory pathway (84–86). Such cross talk could be further complicated by the combined actions of other independent signaling mechanisms. Thus, researchers have noted cross-regulatory

mechanisms among different GPCR signal transduction pathways and between GPCR signaling and other intracellular regulatory mechanisms, including Nox activation. As shown in Figure 5, the complex interaction of GPCR signaling and Nox activation modulates the levels of important second messengers, namely Ca^{2+} and ROS, which can participate in many cellular functions as well as in cell death. Indeed, an interlinked pattern of cross-regulatory mechanisms is often responsible for cell death. Therapeutic interventions directed at any critical component or multiple components of this cross-regulatory machinery could cause a significant change and thus protect against cell death. Systems pharmacology approaches focusing on the visual system applied to retinal degeneration and diabetic retinopathy illustrate this principle, as described below.

TWO APPLICATIONS OF SYSTEMS PHARMACOLOGY: LIGHT-INDUCED RETINOPATHY AND DIABETIC RETINOPATHY

Normal vision results from efficient detection of light, which requires rapid restoration of the preillumination physiological state. This continuous process depends on the retinoid or visual cycle; it is accomplished by photoreceptor cells and the neighboring RPE. The cycle involves the regeneration of 11-*cis*-retinal, a light-sensitive chromophore derived from vitamin A that is converted to all-*trans*-retinal upon absorption of a photon of light. The retinoid cycle is composed of a series of enzymatic events dependent on the regulated expression and function of many gene products that ensure the sustained conversion of all-*trans*-retinal back to its 11-*cis* isomer (see sidebar, Retinoid Cycle). Deficiencies in individual components of this enzymatic cycle contribute to a variety of retinal disorders (87) (see sidebar, Human Ocular Disorders Associated with Aberrant Retinoid Cycle). For example, the gene for *ATP-binding cassette transporter 4* (*ABCA4*) encodes a product that transports all-*trans*-retinal from the inside to the outside of photoreceptor disc membranes. Mutations in *ABCA4* cause Stargardt disease (SGD) and are associated with an increased risk of developing age-related macular degeneration (AMD) (27, 28).

Investigators have used animal models with genetic modifications of components of the retinoid cycle not only to reveal mechanisms contributing to the pathogenesis of retinal degenerative disorders but also to identify possible therapeutic strategies that are lacking for many retinal degenerative disorders. For instance, targeted deletion of *Abca4* and *retinol dehydrogenase 8* (*Rdh8*) (*Abca4*$^{-/-}$*Rdh8*$^{-/-}$) in mice results in an increased susceptibility to light-induced retinopathy that pathologically mimics many features of human retinal degenerative disorders such as SGD (88). Optical coherence tomography imaging and immunohistochemistry reveal that *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice exposed to bright light exhibit a severe loss of the outer nuclear layer and damage to photoreceptor inner and outer segments of the retina compared to illuminated wild-type controls or *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice unexposed to light (Figure 6***a***). Moreover, two-photon microscopy demonstrates enlargement of photoreceptor cells in the outer segments prior to significant clearance of photoreceptor cell debris (Figure 6***b***), suggesting that rod photoreceptor cells, where toxic retinoids accumulate, are the primary sites of light-induced retinal damage. Thus, this model can be used to select various systems pharmacology strategies that protect photoreceptor

cells against this insult. It also can be employed to screen and develop therapeutic compounds that exert protective effects against bright light–induced retinal damage.

Systems pharmacological strategies have revealed that a complex set of cross-regulatory mechanisms are involved in determining the fate of photoreceptor cells after light exposure in this mouse model (see sidebar, Systems Pharmacology Directed at Interconnected Signaling Pathways). Thus, increased activity of G_s-coupled GPCRs and decreased functionality of G_i-coupled GPCRs with subsequent activation of AC causes photoreceptor cell death (66). Additionally, increased functionality of G_q-coupled GPCRs and activation of their intracellular functional pathway [PLC/inositol 1,4,5-trisphosphate (IP_3)/Ca^{2+} signaling] communicates with Nox-mediated ROS production in promoting photoreceptor cell death upon bright light exposure (67). *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice were protected from acute light–induced photoreceptor degeneration by pharmacological interventions partly guided by results from transcriptomics analyses (Figure 7). These results indicate that a complex mechanism implicating network interactions of different GPCR signaling and Nox pathways could be involved in the pathogenesis of related retinal disorders.

To further test the possibility that retinal protective effects may be enhanced by combined treatments that target different mechanisms, researchers administered drugs targeting G_q-coupled and G_i-coupled GPCRs simultaneously. A much greater protection against retinal degeneration was observed when light-stressed *Abca4*$^{-/-}$Rdh8$^{-/-}$ mice received a combination of guanabenz and doxazosin, agents that activate ADRA2 and antagonize ADRA1, respectively, than when such mice were pretreated with either of these drugs at the same dose (66). These findings were based on a transcriptome-aided design of pharmacological interventions and the ensuing selection of a combined treatment, serving as an example for treating retinal degenerative disorders. It is worth noting that several of the tested pharmacological agents are FDA-approved drugs (Table 2), which could markedly facilitate their evaluation for clinical applications (66, 67).

In addition to retinal degenerative disorders such as SGD and AMD, efforts to combat diabetic retinopathy could also benefit from a systems pharmacology approach. As noted above, we had previously identified a series of intrinsically linked events involving G_s-, G_i-, and G_q-coupled GPCR signaling pathways that contribute to the pathogenesis of light-induced photoreceptor degeneration. Recently, we found that these same pathways also participate in hyperglycemia-induced generation of superoxide, in large part by photoreceptor cells (89). Thus, links between different GPCR pathways related to superoxide generation by Nox could result from hyperglycemia-induced increases in cytosolic Ca^{2+} concentration. This appears reasonable because elevated Ca^{2+} levels are well known to induce superoxide generation (90). Specifically, pharmacological activation of ADRA2 (acting via a G_i signaling pathway) or inhibition of ADRA1 or serotonin (5-HT_2, 5-HT_4, 5-HT_6, or 5-HT_7) receptors (G_q- and G_s-coupled receptors, respectively) were the most beneficial in preventing early symptoms of diabetic retinopathy (91). These exciting findings suggest that the action of several GPCRs individually or in combination could attenuate retinal oxidative stress and prevent the degeneration of retinal capillaries caused by diabetes. The specific drug combinations and their dosage optimization will require extensive clinical evaluation. Additionally, researchers have reported that Nox-mediated

oxidative stress also plays an important role in the pathogenesis of retinopathy of prematurity (92). Thus, evaluating the impact of targeting related GPCRs and their intracellular signaling using a systems pharmacology approach could enrich the understanding of the mechanisms and therapeutic strategies of other ischemic retinopathies. Another common eye disease is glaucoma. A new treatment for glaucoma, brimonidine/timolol (Combigan), which combines an ADRA2 agonist (brimonidine) with a β-adrenergic receptor blocker (timolol), could be considered ocular systems pharmacology.

CONCLUSIONS

A practical systems pharmacology approach should be considered by those interested in using basic knowledge of signaling pathways to discover how more comprehensive systems approaches can enhance rational therapeutic strategies for treating complex disorders and diseases. Our proposal for systems pharmacology starts with a quantitative transcriptomics analysis not only of cells but also of tissues and organs of interest. Next, a reliable animal model that recapitulates the human condition(s) must be available to investigate combinations of drugs that act on one or several network pathways to select those most suited for human trials. Priority should be given to those with a minimal dose requirement under experimental conditions. At this stage, another set of transcriptomics analyses could help determine which drug actions affect the expression of which genes, propagating yet another round of drug discovery. This iterative approach could also expand the reach of current FDA-approved therapeutics, accelerating their clinical evaluation.

We believe that the future of drug development lies more in the modification of pathways and networks rather than in targeting single elements, making identification of combinations of drugs a more attractive option (93). Precursors of this approach have already been used for cancer chemotherapy (4, 5, 94), infectious diseases (7–9), neurological diseases (e.g., epilepsy) (48), hypertension (95), and glaucoma (96). A combination of drugs is an attractive option, as lower doses that target multiple elements can decrease the risk of off-target action and toxicity associated with higher doses of individual drugs. Finally, this approach uses the power of genetics to select appropriate pathways for systems pharmacological intervention. However, the information collected at the genome level only provides one fundamental layer and is subject to posttranscriptional and posttranslational modifications.

The cell biology and physiology of an organism could well be the key to more rapid progress in modern pharmacology. As the omics technologies continue to evolve and deliver vast amounts of information, the integration and synthesis of this knowledge into drug discovery efforts will considerably extend the power of systems pharmacology (97). Systems pharmacology, together with expanded knowledge of cell biological processes (49) in the whole organism, can promote further drug discovery. This approach has already shown promise in mouse models that recapitulate features of AMD and diabetic retinopathy. Because it is amenable to a wide variety of imaging, genomic, biochemical, and structural biology approaches, the visual system could provide the principles needed to develop systems pharmacology into a general paradigm for treating complex disorders.

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Glossary

FDA	US Food and Drug Administration
GPCR	G protein–coupled receptor
RPE	retinal pigment epithelium; RPE65 is the RPE-specific 65-kDa protein
NGS	next-generation sequencing
Nox	NADPH oxidase
AC	adenylyl cyclase
Rho	rhodopsin
PLC	phospholipase C
HTR	5-hydroxytryptamine (serotonin) receptor
ADRA	α adrenergic receptor
ROS	reactive oxygen species
ABCA4	ATP-binding cassette transporter 4
SGD	Stargardt disease
RDH	retinol dehydrogenase
IP_3	inositol 1,4,5-trisphosphate

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TRANSCRIPTOMICS INFORMATION REVEALED BY NGS

Transcriptomics information revealed by NGS could lead to novel insights into the potential participation of GPCRs and associated signaling molecules in retinal pathophysiology. Immunolocalization of membrane proteins such as GPCRs and their respective signaling partner molecules could be adversely affected by their low expression levels, antibodies lacking the required specificity and sensitivity, and other factors. In situ hybridization also can be problematic when transcripts are unstable or poorly expressed. Intercellular communication could modulate the activities of GPCRs in neighboring cells in addition to, and independent of, their functions in cells where they are expressed. RNA-seq not only overcomes the technical obstacles encountered by conventional methods but also detects all known and novel RNAs in a biological sample in an unbiased manner with high efficiency, thereby facilitating the identification of transcriptomic changes associated with various pathophysiological conditions including retinal degeneration.

RETINOID CYCLE

The retinoid cycle is a complex enzymatic pathway consisting of multiple gene products. Coordinated flow of retinoids through this cycle is essential for the continuous regeneration of 11-*cis*-retinal from all-*trans*-retinal, which is required for maintenance of normal vision. ATP-binding cassette transporter 4 (ABCA4) transports the fraction of dissociated all-*trans*-retinal from disc lumens back into the cytoplasm prior to its reduction to all-*trans*-retinol by all-*trans*-retinol dehydrogenase (atRDH). Interphotoreceptor retinoid-binding protein (IRBP) mediates the transport of retinoids between photoreceptor and RPE cells. In the RPE, lecithin retinol acyltransferase (LRAT) catalyzes the transformation of all-*trans*-retinol into all-*trans*-retinyl esters, whereas RPE65 converts all-*trans*-retinyl esters into 11-*cis*-retinol, which is then oxidized to 11-*cis*-retinal by 11-*cis*-retinol dehydrogenase (11cRDH). Cellular retinaldehyde-binding protein (CRALBP) is a soluble retinoid carrier that protects 11-*cis*-retinal from premature photoisomerization and enzymatic reverse isomerization in the RPE. Stimulated by retinoic acid 6 (STRA6) is the receptor for retinol-binding protein (RBP) involved in transporting retinol into the eye.

HUMAN OCULAR DISORDERS ASSOCIATED WITH ABERRANT RETINOID CYCLE

Mutations in the *ABCA4* gene cause SGD and are associated with an increased risk of developing AMD. Mutations in the *RDH12* gene, which encodes one of the atRDHs, can cause either Leber's congenital amaurosis (LCA) or retinitis pigmentosa (RP), whereas mutations in the *IRBP* gene are associated with autosomal recessive RP. Mutations in the *LRAT* gene can cause either LCA or RP. *RPE65* mutations are associated with both LCA and RP. Gene mutations in *RDH5*, an 11cRDH, are causative for fundus albipunctatus and are also associated with cone-rod dystrophy. *CRALBP* gene mutations are associated with both Bothnia dystrophy and retinitis punctata albescens. The *STRA6* mutation can produce Matthew-Wood syndrome with an ocular phenotype of either anophthalmia or severe microphthalmia.

SYSTEMS PHARMACOLOGY DIRECTED AT INTERCONNECTED SIGNALING PATHWAYS

Systems pharmacology directed at interconnected signaling pathways underlying photoreceptor cell death could be employed to target multiple elements in different pathways for the treatment of SGD-associated retinal degeneration. Increased activity of G_s-coupled GPCRs and decreased functionality of G_i-coupled GPCRs with subsequent activation of AC could cause photoreceptor cell death. Additionally, increased functionality of G_q-coupled GPCRs and activation of their intracellular functional pathway, PLC/IP_3/Ca^{2+} signaling, could communicate with Nox-mediated ROS production in causing light-induced photoreceptor cell death. These examples demonstrate a complex set of cross-regulatory mechanisms involved in determining the fate of photoreceptor cells in response to intense light. Systems pharmacological strategies targeting multiple GPCRs, their respective signaling pathways, and Nox can prevent the development of bright light–induced photoreceptor degeneration in a mouse model recapitulating human SGD.

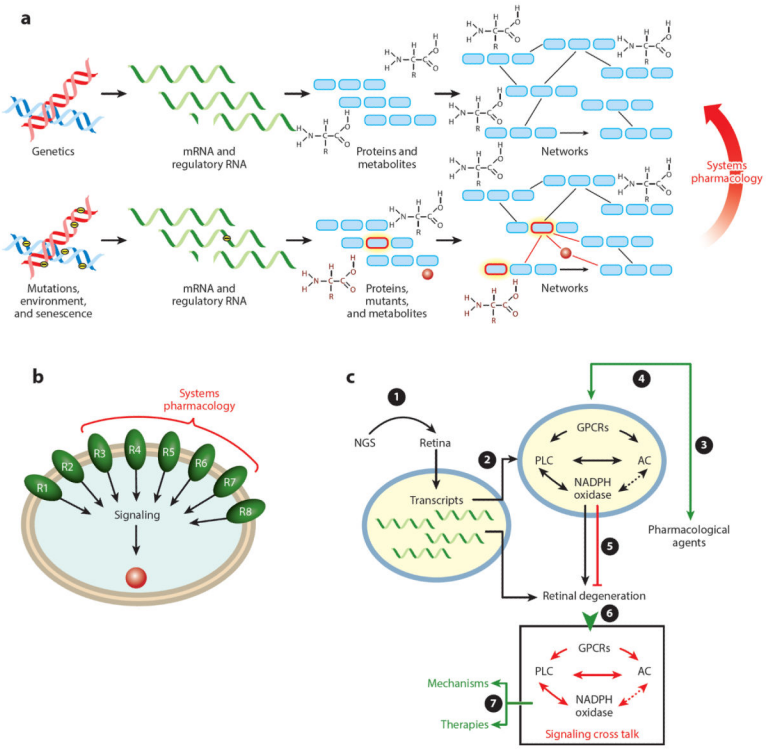

Figure 1.

Systems pharmacology strategies can successfully treat complex disorders. Here, this concept is applied to important eye diseases, providing a road map to analogous approaches for other biological systems. (*a*) Physiological phenotypes emerge from a tightly regulated sequence of events driven by genomic DNA sequences, mRNAs and regulatory RNAs, proteins, metabolites (exemplified by a generic amino acid), and interacting networks that contribute to the complexity of biological function. Complex disorders can emanate in part from genetic factors such as mutations, environmental factors that cause epigenetic changes, and senescence-associated genetic alterations. Such perturbations can lead to modifications in the flow of biological information, causing changes in transcripts and regulatory RNAs, production of mutant proteins, altered metabolite and second-messenger content, and disturbed network interactions. The blue ovals outlined in red and yellow and the balls represent modified proteins, metabolites, and second messengers, respectively. Systems pharmacology employs multiple targeting strategies that offer the potential of returning these pathological phenotypes to a more normal homeostatic state. Panel *a* modified with permission from Nature Publishing Group (98). (*b*) Functional alterations in various membrane receptors affect their respective intracellular signaling mechanisms, which can result in altered levels of a common second messenger. Therefore, a systems pharmacology approach could be employed to modulate the function of one or a combination of receptors to achieve a given therapeutic effect through that second messenger. (*c*) A stepwise systems pharmacology approach to a complex retinal degenerative disorder. ❶ NGS provided a comprehensive view of transcripts expressed in the mouse retina. ❷ Data analyses identified potentially interconnected signaling components. These analyses led to a rationale for selecting pharmacological interventions (❸) to elucidate the implication of these signaling

mechanisms in retinal degeneration by targeting these molecules (❹), which in turn was tested in mouse models of retinal degeneration (66). The retinal protection conferred by the selected pharmacological treatment (❺) provided proof-of-concept evidence that interconnected signaling events could mechanistically contribute to the pathogenesis of retinal degeneration (❻). ❼ This strategy helps to illustrate the disease mechanism and also provides a rationale for the mechanism-based therapeutic design. Dashed lines in the double-headed arrows indicate potential cross talk. Abbreviations: AC, adenylyl cyclase; GPCR, G protein–coupled receptor; mRNA, messenger RNA; NGS, next-generation sequencing; PLC, phospholipase C; R, receptor.

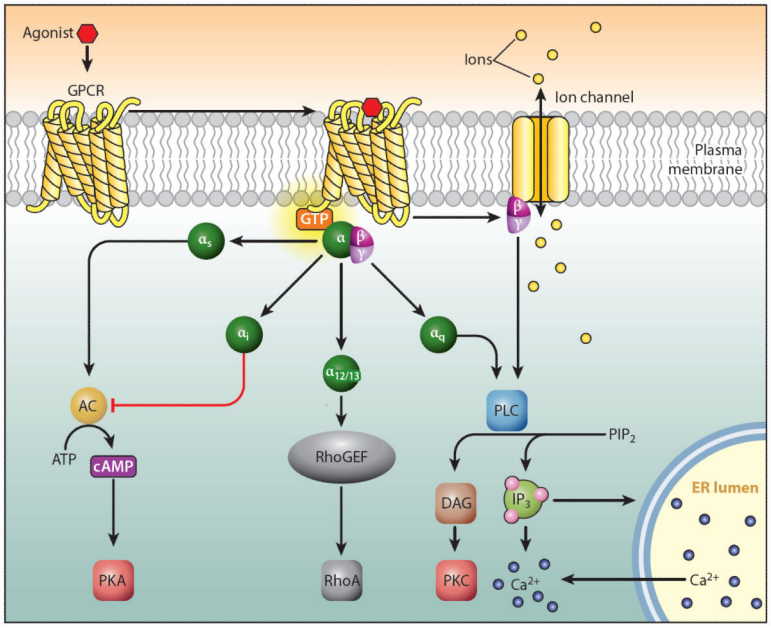

Figure 2.

GPCR signaling overview. GPCRs constitute a large family of transmembrane proteins that transduce extracellular stimuli to initiate intracellular signaling responses through an interaction of their intracellular domains with heterotrimeric G proteins. In GPCR signal transduction, binding of an agonist to the receptor induces a conformational change and activation of the GPCR. Exchange of GTP with GDP on the G_{α} subunit of the G protein then occurs, triggering dissociation of the G_{α} subunit from the $G_{\beta\gamma}$ dimer and receptor. Free G_{α} and $G_{\beta\gamma}$ then activate different signaling cascades and effector proteins. $G_{\alpha s}$ activates the cAMP-dependent pathway by stimulating the production of cAMP from ATP through direct activation of AC; cAMP then acts as a second messenger that activates PKA and other effectors, such as Epac and cyclic nucleotide-gated channels. $G_{\alpha i}$ inhibits the activity of AC and the production of cAMP. $G_{\alpha 12/13}$ is involved in RhoA activation through RhoGEF. $G_{\alpha q}$ stimulates its effector enzyme PLC, which then cleaves PIP_2 into two second messengers, IP_3 and DAG. IP_3 mobilizes the release of ER-stored Ca^{2+} into the cytosol, whereas DAG activates PKC. $G_{\beta\gamma}$ can act independently on effectors as well, including ion channels in the plasma membrane and PLC. Figure modified from Reference 72 with permission from Nature Publishing Group. Abbreviations: AC, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; ER, endoplasmic reticulum; GPCR, G protein–coupled receptor; GTP, guanosine triphosphate; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; RhoA, Ras homolog gene family, member A; RhoGEF, Rho guanine nucleotide exchange factor.

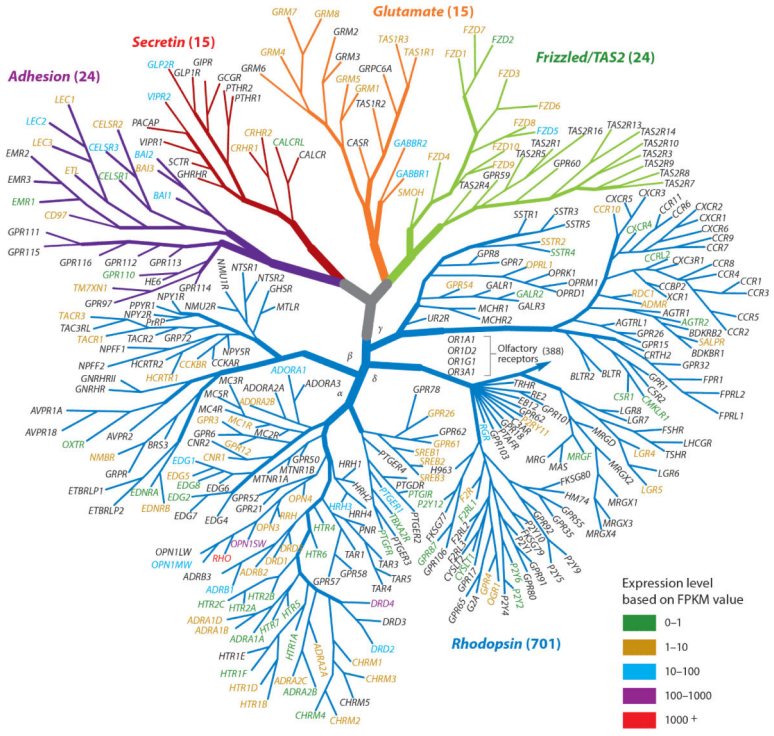

Figure 3.

The GPCR superfamily. A phylogenetic tree of human GPCR-encoding genes is shown. These genes are classified into five major families, namely *Rhodopsin*, *Secretin*, *Adhesion*, *Glutamate*, and *Frizzled/taste receptor 2* (*TAS2*). Expression of GPCR genes in the retina was documented by RNA sequencing. Each GPCR gene is color-coded based on the expression level indicated by its FPKM value. Figure modified from Katritch et al. (99) with permission from Elsevier. Abbreviations: FPKM, normalized fragments per kilobase of exon per million mapped reads; GPCR, G protein–coupled receptor.

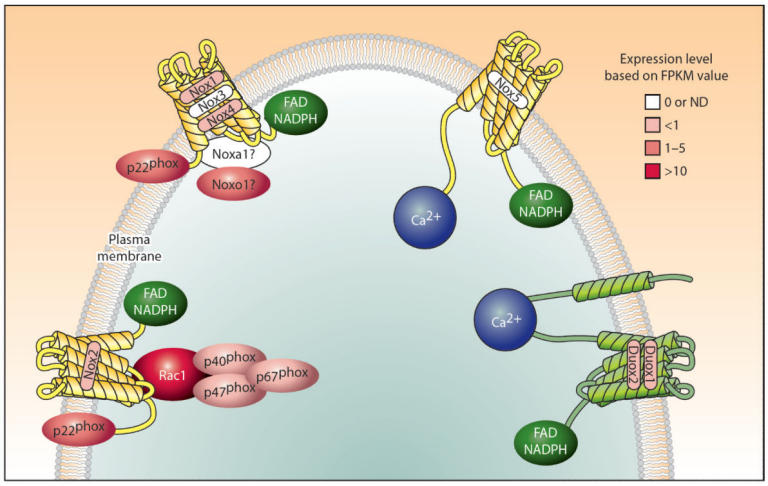

Figure 4.

The Nox family. Nox serves as a critical part of a transmembrane redox chain that transfers electrons in a stepwise manner from NADPH, the electron donor, to FAD across the cell membrane, where oxygen is eventually reduced to superoxide. Conserved structural features of Nox family homologs include C-terminal NADPH binding sites and FAD-binding regions, as well as six transmembrane domains. Nox isoforms activate the Nox complex in different ways. Nox2 is the prototypical Nox that often associates with its membrane partner, $p22^{phox}$. Activation of Nox2 occurs through enzymatic complex formation achieved by membrane translocation of its cytosolic subunits, including Rac1, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$. Nox1, 3, and 4 function in a similar fashion that not only depends on $p22^{phox}$ but also could require cytosolic subunits, including Noxa1 and Noxo1. Exerting its enzymatic function in the absence of $p22^{phox}$ and other cytosolic subunits, Nox5 is distinguished from Nox enzymes 1–4 by the presence of an intracellular N-terminal Ca^{2+}-binding EF hand. Duox1 and 2 have a Nox homology domain, an N-terminal functional Ca^{2+}-binding EF-hand domain, and a peroxidase homology domain but do not require the cytosolic subunits used by Nox2. RNA sequencing has revealed the expression levels of Nox family members and associated enzymatic components in mouse retina; these are color-coded based on their respective normalized FPKM values. Abbreviations: Duox, dual oxidase; EF hand, a Ca^{2+}-binding helix-loop-helix structure domain; FAD, flavin adenine dinucleotide; FPKM, normalized fragments per kilobase of exon per million mapped reads; ND, not determined; Nox, NADPH oxidase; Noxa1, Nox activator 1; Noxo1, Nox organizer 1.

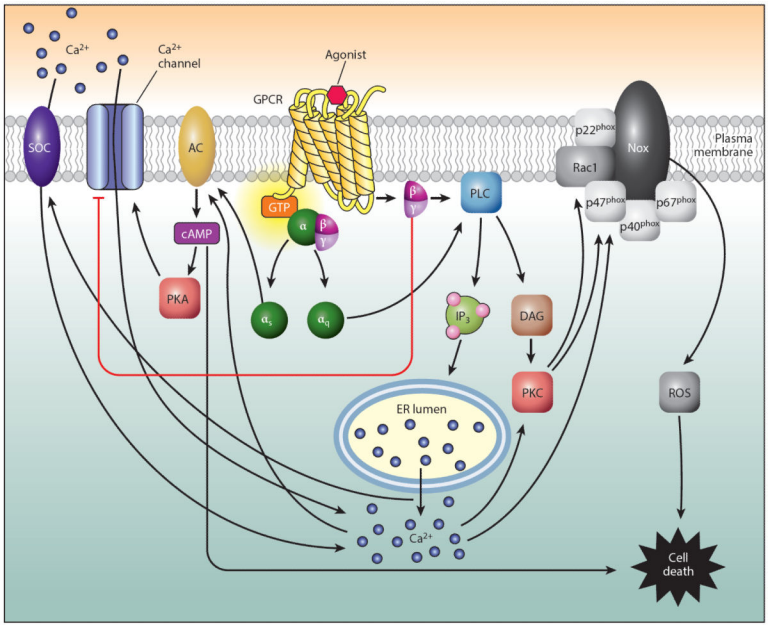

Figure 5.

Cross-regulation of GPCR and Nox signaling pathways can induce cell death by apoptosis. Cross-regulatory mechanisms contributing to cell death have been noted among different GPCR signal transduction pathways and between GPCR signaling and Nox activation. This simplified schematic illustrates an example of such complex interactions. Increased activity of a $G_{\alpha q}$-coupled GPCR activates a PLC effector pathway, resulting in production of the second messengers DAG and IP_3. DAG then stimulates PKC, whereas IP_3 causes mobilization of Ca^{2+} from the ER lumen into the cytoplasm. This elevation of PLC signaling can also induce Nox activation through a direct action of Ca^{2+}- and PKC-mediated phosphorylation of Rac1 and $p47^{phox}$. The two major cytosolic subunits of Nox then generate ROS. Ca^{2+} mobilization induced by IP_3 also activates AC, followed by opening of Ca^{2+} channels in the plasma membrane as well as SOCs. These regulatory mechanisms acting together could cause further elevation of Ca^{2+} in the cytosol. Whereas Ca^{2+} channel–mediated Ca^{2+} influx is facilitated by AC signaling, which could be a direct result of G_s activation and an indirect consequence of G_q signaling, $G_{\beta\gamma}$ could negatively regulate the action of this channel. Complex interactions of these signaling cascades alter the levels of the second messengers—Ca^{2+} and ROS—thereby playing an important role in promoting cell death. Abbreviations: AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; ER, endoplasmic reticulum; GPCR, G protein–coupled receptor; GTP, guanosine triphosphate; IP_3, inositol 1,4,5-trisphosphate; Nox, NADPH oxidase; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species; SOC, store-operated calcium channel.

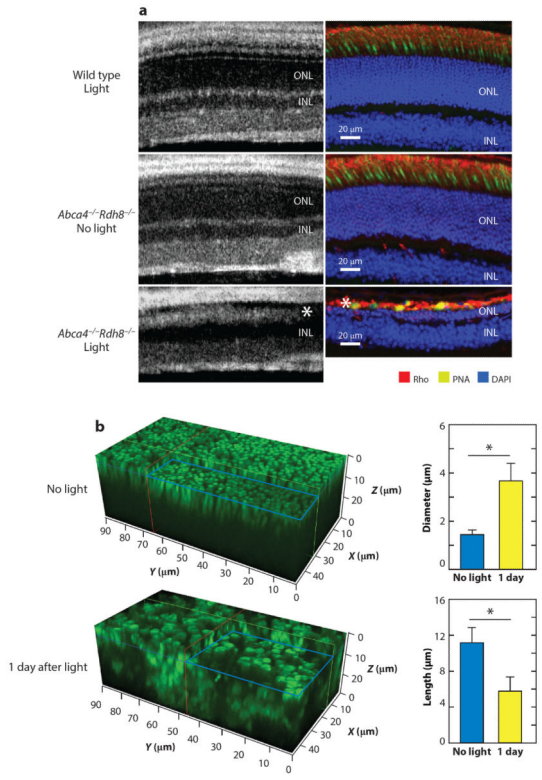

Figure 6.

Light-induced retinopathy in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice. Genetic ablation of *Abca4* and *Rdh8* in mice causes increased susceptibility to bright light–induced retinopathy. (*a*) As revealed by OCT imaging (*left*) and immunohistochemical examination (*right*) 7 days after exposure to bright light, *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice exhibited a severe thinning of the ONL and OS/IS layers, compared with little damage shown by light-stressed wild-type controls and *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice not exposed to light (asterisks indicate disrupted photoreceptors in the retinal structure). (*b*) TPM examination further demonstrated enlargement of photoreceptor cell OS prior to significant clearance of photoreceptor cell debris. TPM imaging was performed 1 day after albino *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice were exposed to intense light. (*Top*) A 3-D TPM section reveals regularly arranged photoreceptor cells in the retina from an *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mouse unexposed to intense light. (*Bottom*) A 3-D TPM section reveals photoreceptor cells with reduced lengths, enlarged diameters, and darker centers in a mouse retina 1 day after bright light exposure (asterisk indicates 1 day versus no light, $p < 0.05$). Panel *b* modified from Maeda et al. (100) published by *PNAS*. Abbreviations: 3-D, three-dimensional; Abca4, ATP-binding cassette transporter 4; DAPI, 4′,6-diamidino-2-phenylindole; INL, inner nuclear layer; IS, inner segment; OCT, optical coherent tomography; ONL, outer nuclear layer; OS, outer segment; PNA, peanut agglutinin; Rdh8, retinol dehydrogenase 8; Rho, rhodopsin; TPM, two-photon microscopy.

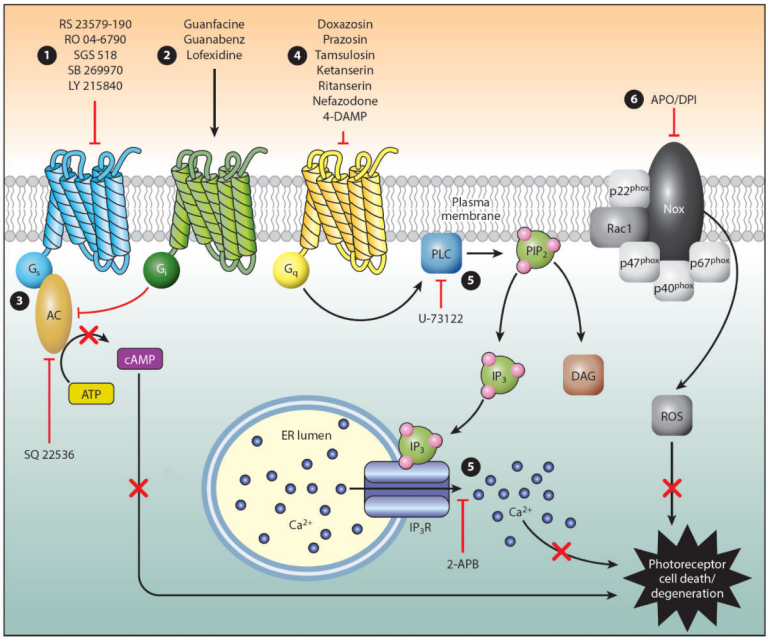

Figure 7.

Protection against acute light–induced photoreceptor degeneration can be achieved by pharmacological interventions, including ❶ antagonists blocking the activation of G_s-coupled GPCRs, RS 23579-190 (5-HT4 receptor), RO 04-6790 and SGS 518 oxalate (5-HT6 receptors), and SB 269970 and LY 215840 (5-HT7 receptors); ❷ agonists activating G_i-coupled ADRA2, including guanfacine, guanabenz, and lofexidine; ❸ the AC inhibitor SQ 22536; ❹ antagonists of G_q-coupled GPCRs, including doxazosin, prazosin, and tamsulosin (ADRA1), ketanserin, ritanserin, and nefazodone (5-HT2 receptors), and 4-DAMP (muscarinic 3 receptor); ❺ inhibitors targeting PLC/IP_3/Ca^{2+} signaling, including U-73122 and 2-APB; and ❻ Nox inhibitors, including APO and DPI. Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; 4-DAMP, 4-diphenylacetoxy-*N*-methyl-piperidine methiodide; AC, adenylyl cyclase; ADRA, α adrenergic receptor; APO, apocynin; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; DPI, diphenylene iodinium; ER, endoplasmic reticulum; GPCR, G protein–coupled receptor; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; Nox, NADPH oxidase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; ROS, reactive oxygen species.

Table 1

Expression of signaling molecules detected by NGS analyses as FPKM[a]

Gene	C57BL/6 mouse eye	C57BL/6 mouse retina	Rho$^{-/-}$ mouse eye	Human retina
PLCB1	1.38	1.72	1.31	1.19
PLCB2	0.12	0.11	0.32	1.32
PLCB3	8.73	3.51	16.88	2.17
PLCB4	13.33	16.89	14.44	5.97
PLCD1	12.03	6.48	26.11	5.70
PLCD3	20.72	45.07	16.37	49.10
PLCD4	0.98	1.34	1.82	125.53
PLCE1	2.80	1.70	3.57	3.44
PLCG1	6.76	8.89	10.46	9.60
PLCG2	0.89	0.35	1.94	0.58
PLCH1	9.96	14.20	9.35	7.18
PLCH2	50.14	161.94	39.16	208.38
Rac1	101.14	42.46	49.20	25.03
Rac2	0.83	0.12	0.19	0.52
Rac3	10.08	13.51	15.88	10.86
Nox1	0.02	0.03	0.01	0.08
Nox2	1.14	0.06	0.10	0.81
Nox3	0.00	0.00	0.00	0.00
Nox4	0.81	0.02	0.06	2.13
Nox5	ND	ND	ND	0.12
Noxa1	0.00	0.00	0.01	32.13
Noxo1	1.23	1.81	1.59	1.15
Duox1	0.36	0.02	0.02	0.32
Duox2	0.31	0.01	0.00	0.00
p22^{phox}	15.57	4.31	5.60	5.20
p40^{phox}	0.62	0.13	0.18	0.51
p47^{phox}	1.45	0.57	0.71	0.41
p67^{phox}	0.96	0.24	0.22	0.19

Abbreviations: FPKM, normalized fragments per kilobase of exon per million mapped reads; ND, not determined; NGS, next-generation signaling; PLC, phospholipase C; Rho, rhodopsin.

[a] Limited analyses of these results have been published previously (36, 37, 66, 101). NGS revealed basal expression of genes encoding various forms of PLC and NADPH oxidase subunits in mouse and human retinas, respectively.

Table 2

Examples of FDA-approved drugs targeting GPCRs shown to be effective in protecting mouse retinas against light-induced degeneration[a]

Agent	Trade name(s)	Major action	Indication(s)
Nefazodone	Serzone	5-HT2R antagonist	Depression
Doxazosin	Cardura	ADRA1 antagonist	Hypertension, benign prostatic hyperplasia
Prazosin	Minipress	ADRA1 antagonist	Hypertension
Tamsulosin	Flomax	ADRA1 antagonist	Benign prostatic hyperplasia
Guanabenz	Wytensin	ADRA2 agonist	Hypertension
Guanfacine	Intuniv, Tenex	ADRA2 agonist	Attention deficit/hyperactivity disorder, hypertension

Abbreviations: FDA, US Food and Drug Administration; GPCR, G protein–coupled receptor.

[a]Table modified with permission from results previously published (66, 67).