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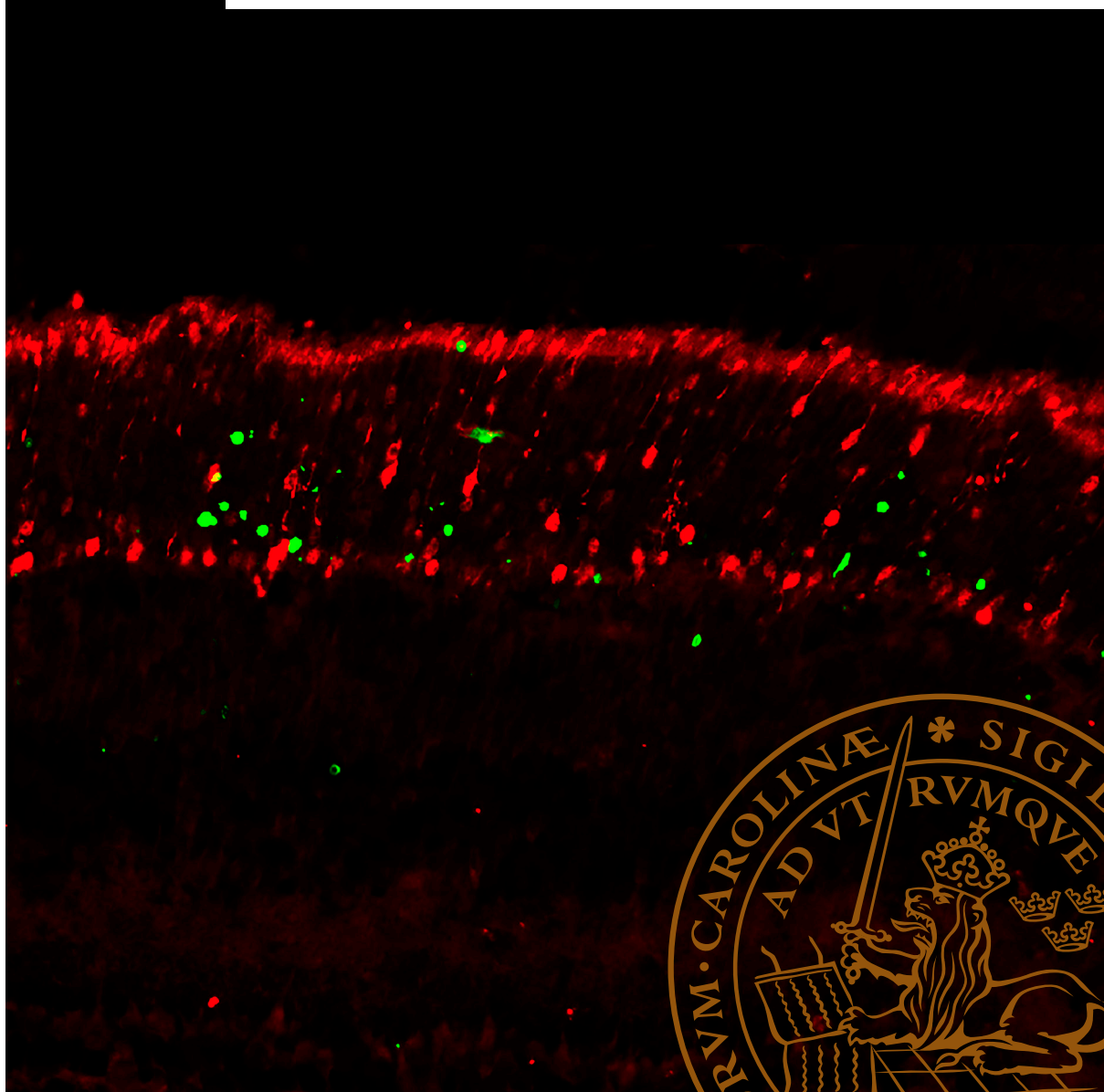
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DOCTORAL DISSERTATION

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Abstract

The eye disease Retinitis Pigmentosa (RP) represents a heterogeneous group of inherited retinal dystrophies, and it has been estimated that 1 in 3000 to 7000 people worldwide has the disease. The symptoms of RP are photoreceptor degeneration that leads to night blindness, followed by a decline in peripheral and central vision, and potential blindness. However, the mechanism(s) behind the photoreceptor degeneration is still not fully understood, although a high photoreceptor cyclic GMP (cGMP) level exists in several mouse models having RP (*i.e., rd1, rd2,* and *rd10* mouse). It is thus very likely that cGMP, as well as its targets, may play a key role in the degeneration. Currently, numerous cGMP targets are known, and one of them is the cGMP-dependent protein kinase (PKG). cGMP can extensively govern PKG activity, and during RP, it over-activates PKG, which may propel the disease progression. However, it is possible that also other targets exist, and exposing those might help comprehend the RP mechanism(s).

Therefore, this thesis studied the current cGMP targets and potential new targets. This was done by applying a proteomics approach utilizing affinity chromatography and various cGMP-analogs linked to agaroses to enrich and select for the proteins of interest. In Paper I, five potential new cGMP-interactors showed to be in proximity with cGMP in the photoreceptor layer, supporting that other cGMP-interactors may exist in the retina. Paper II showed that cGMP-interactors have various stereospecific requirements and that cGMP-analogs possess target specificity. Such selectivity could aid in studying functional and physical associations for a given cGMP interactor and provide valid information on the target specificity and drug design in the field of cGMP-analog-based therapies.

Paper III investigated the promising selectively modified cGMP-analog (*i.e.,* CN03), which has PKG inhibiting actions and can reduce degeneration. Paper III studied CN03's target-specificity by applying the newly generated CN03-agarose and found that CN03 is more target-specific when compared to regular cGMP studied in Paper I and thus CN03 is a promising RP therapy.

In Paper IV, *rd* and wt retinal cross-sections and organotypic retinal cultures were used. First, it was seen that some *rd* models might temporally increase the expression of the newly found retinal cGMP-interactor EPAC2 in the photoreceptors. Furthermore, some *rd* models revealed increased interaction between cGMP and EPAC2 in the photoreceptors over time, whereas such augmentation was not observed between EPAC2 and cAMP. The discrepancy in interactions may be explained by the co-localization of highly expressed EPAC2 and accumulated cGMP and the lack of such between EPAC2 and accumulated cAMP in diseased photoreceptors. Lastly, through pharmacological manipulation in *rd10* retinal cultures, it was seen that EPAC2 activity may have neuroprotective abilities.

Taken together, this thesis adds to the understanding of the cGMP-system by suggesting potential new cGMP-associated proteins, which may help pave the way for comprehending the cell death mechanism(s) within the photoreceptors as well as reveal new potential therapeutic targets.

Keywords: Retinitis Pigmentosa, Photoreceptors, cGMP, cGMP-interactors, retinal degeneration, neuroprotection

Classification system and/or index terms (if any)

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Cover illustration: Cross-sectional immunofluorescence staining of cGMP and TUNEL positive cells within the *rd1* retina
Cover photo by Michel Rasmussen

To the People living with Retinitis Pigmentosa

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ABSTRACT

The eye disease Retinitis Pigmentosa (RP) represents a heterogeneous group of inherited retinal dystrophies, and it has been estimated that 1 in 3000 to 7000 people worldwide has the disease. The symptoms of RP are photoreceptor degeneration that leads to night blindness, followed by a decline in peripheral and central vision, and potential blindness. However, the mechanism(s) behind the photoreceptor degeneration is still not fully understood, although a high photoreceptor cyclic GMP (cGMP) level exists in several mouse models having RP (*i.e., rd1, rd2,* and *rd10* mouse). It is thus very likely that cGMP, as well as its targets, may play a key role in the degeneration. Currently, numerous cGMP targets are known, and one of them is the cGMP-dependent protein kinase (PKG). cGMP can extensively govern PKG activity, and during RP, it over-activates PKG, which may propel the disease progression. However, it is possible that also other targets exist, and exposing those might help comprehend the RP mechanism(s).

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LIST OF PAPERS AND MANUSCRIPTS

The content of this thesis is based on the following papers and manuscripts referred to in the text by their roman numerals. To keep a uniform and simple reference, manuscripts III and IV will be referred to as Paper III and IV within the main content of the thesis. The original formats and several of their supplementary data are added as appendices in the book version of this thesis.

I. **Rasmussen, M**.; Welinder, C.; Schwede, F.; Ekström, P. The cGMP System in Normal and Degenerating Mouse Neuroretina: New Proteins with CGMP Interaction Potential Identified by a Proteomics Approach. *J. Neurochem.* **2020**. https://doi.org/10.1111/jnc.15251.

II. **Rasmussen, M**.; Welinder, C.; Schwede, F.; Ekström, P. The Stereospecific Interaction Sites and Target Specificity of cGMP Analogs in Mouse Cortex. *Chem. Biol. Drug Des.* **2021**. https://doi.org/10.1111/CBDD.13976.

III. Manuscript: **Rasmussen, M**.; Tolone, A.; Paquet-Durand, F.; Welinder, C.; Schwede, F.; Ekström, P. The photoreceptor protective cGMP-analog Rp-8-Br-PET-cGMPS interacts with cGMP-interactors PKGI, PDE1, PDE6, and PKAI in the degenerating mouse retina

IV. Manuscript: **Rasmussen, M**.; Zhou, J.; Schwede, F.; Ekström, P. Enhanced cGMP interactor rap guanine exchange factor 4 (EPAC2) expression and activity in degenerating photoreceptors - A neuroprotective response?

ABBREVIATIONS

AC	Affinity chromatography
ADP	Adenosine diphosphate
AIPL1	Aryl hydrocarbon receptor-interacting protein like-1
arRP	Autosomal recessive RP
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
cAMP	Cyclic adenosine-3,5'-monophosphate or cyclic AMP
cGMP	Cyclic guanosine-3',5'-monophosphate or cyclic GMP
cGMPAAs	Agaroses carrying different cGMP-analogs
cGMPS	Sp- and Rp-guanosine3',5'-cyclic monophosphorothioate
CNGC	cGMP-gated channel
CN03	Rp-8-Br-PET-cGMPS
C-subunit	Catalytic subunit
ERG	Electroretinographic response
EPAC2	Rap guanine exchange factor 4
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Ganglion cell layer
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GRK1	G-protein receptor kinase or rhodopsin kinase
GTP	Guanosine triphosphate
GUCA1B	Guanylyl cyclase-activating protein 1B
GUCY2D	Guanylyl cyclase 1
IF	Immunofluorescence
IMPDH1	Inosine-5´-monophosphate dehydrogenase 1
INL	Inner nuclear layer
IPL	Inner plexiform layer

LCA	Leber's Congenital Amaurosis
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LDH	Lactate dehydrogenase
MAPK1	Mitogen-activated protein kinase 1
MAPK3	Mitogen-activated protein kinase 3
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
ONL	Outer nuclear layer
OPL	Outer plexiform layer
p-CaMKIIα	Phosphorylated multifunctional serine/threonine-protein Kinase α
PDE6	Phosphodiesterase 6
PDE6β	Phosphodiesterase beta-subunit
pGC	Particulate or membrane-bound guanylyl cyclase
p-GSK3βSer9	Glycogen synthase kinase-3 phosphorylated at serine 9
PKA	cAMP-dependent kinase
PKG	cGMP-dependent kinase
PLA	Proximity ligation assay
PN	Postnatal day
PRPH2	Peripherin 2
RalGDS	Ral Guanine Nucleotide Dissociation Stimulator
RBD	Rap-binding domain
Rh*	Activated rhodopsin
RP	Retinitis Pigmentosa
RPE	Retinal pigment epithelium
R-subunit	Regulatory subunit
Tα•GTP	GTP-bound alpha-subunit
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick and labeling

INTRODUCTION

The human visual system is impressive and can provide valuable information about the world. It can quickly describe size, location, shape, color, and if objects are moving[1]. Equally amazingly, the information can be distinguished in light on a bright day or in faint light in the dark[1]. A reduced or absent vision can significantly impact people's lives. Here, daily activities, work opportunities, community interaction, and access to the public service may be challenging[2]. Financially, a considerable economic burden may also come to the patients and society[2]. The prevalence of people having blindness or vision impairment is around 2.2 billion worldwide[3]. Here, most people with vision impairments are at the age of 50 years[3]. However, a decline in vision may affect people of all ages[3]. Vision impairments can be caused by different factors, including trauma to the eye or by a disease, like Retinitis Pigmentosa (RP), where the latter will be emphasized here.

This thesis will describe the following subjects: **1)** An overview of the eye disease RP and a statement of the need to understand RP's biological mechanism(s). **2)** An overall description of the cells that compose the retina and its cellular and molecular mechanisms, which allow us to see. **3)** A detailed description of the secondary messenger nucleotide cGMP's vital role within the visual system, and how mutations in proteins related to the cGMP cascade may negatively affect this system. **4)** How new cGMP targets can help elucidate the mechanism(s) leading to RP and the development of new therapeutic targets.

Retinitis Pigmentosa

Retinitis Pigmentosa (RP) is a common eye disease, and its prevalence is variably reported to affect 1 in 3000 to 7000 people worldwide[4–6]. It can be inherited in three ways: 1) Autosomal recessive inheritance, 2) Autosomal dominant inheritance, or 3) X-linked inheritance[7]. Furthermore, RP represents a heterogeneous group of inherited dystrophies that lead to remodeling of the retina and photoreceptor death[8].

However, the reason behind the photoreceptor degeneration is still not fully understood. Nonetheless, these dystrophies give rise to the symptoms of RP, which are nyctalopia (night blindness), followed by a decline in peripheral vision, then central vision, and, lastly, potential blindness[1]. At this point, mutations in over 60 genes have been identified to cause RP ("RetNet, https://sph.uth.edu/RetNet/")[9], albeit the knowledge of these mutations and their capability to cause RP is obscure. Due to the limited knowledge and the complexity of the RP mechanism(s), only a few gene therapy studies have entered the clinical stage. One of them is approved for a defect in the RPE65 gene, which concerns Leber's Congenital Amaurosis (LCA; an RP subtype)[10–12], but otherwise, imminent RP therapies are highly anticipated.

Therefore, there is a dire need for an improved understanding of RP mechanism(s) and the discovery of new therapeutic targets for treatments.

The Retina

The retina has an essential role in vision and is the light-sensitive tissue lining the back of the eye[13,14]. It receives the light and can convert it into an electrical signal through the cascade named phototransduction, a point we will return to later. This electrical signal then activates neurons, which conduct the message out of the eye and into the brain, creating conscious vision[15].

The retina (*i.e.,* human and mouse retina) is a complex nervous structure, which comprises four main distinct layers (Figure 1)[13,14]. The first layer **(1)** is the retinal pigment epithelium (RPE) layer. It is located outside of the choroid, a layer containing blood vessels that nourish the retina and the Bruch's membrane, which provides structural support and is essential in nutrition support[16]. The photoreceptor-supportive RPE layer holds numerous functions like [1] phagocytizing the shed outer segments, [2] detoxifying and degrading light-damaged components, [3] recycling of the usable fatty acids for energy consumption, and [4] recycling the restored retinal photosensitive pigments back to the photoreceptors[17]. In many ways, the photoreceptors' metabolic demand translates to the excitability of the RPEs[17]. Moreover, the RPE absorbs light that has not been absorbed by the photosensitive cells, which prevents light scattering. The second layer **(2)** after the RPE is the photoreceptor segments and the following outer nuclear layer (ONL) of the photoreceptors (*i.e.,* rods and cones)[1]. The changes induced in photoreceptors by light are transmitted to the **(3)** inner nuclear layer (INL)[1]. It includes bipolar, horizontal, and amacrine cells, where the two latter are responsible for the horizontal

transmission, thereby letting the activity in one area of the retina influence neighboring regions[18]. Between the two layers, ONL and INL, is the first plexiform layer (outer plexiform layer; OPL). It contains the photoreceptors projections, which terminate as either rod spherules or cone pedicles[19]. Upon illumination, photoreceptors become hyperpolarized and create a graded potential and a corresponding reduction in the rate of neurotransmitter (*i.e.,* glutamate) release[1]. The changes in glutamate and thus its inhibitory actions are noted by the dendritic processes of the bipolar cells[1], also located in the OPL. The bipolar cells then transmit the changes to their axons within the second plexiform layer (inner plexiform layer, IPL) and thus to the dendritic processes of the ganglion cells. The ganglion cells comprise the **(4)** fourth and last main distinct layer (GCL)[20]. The GCL then fires action potentials via the ganglion cell axons within the optic nerve[18,20], and together with the photoreceptors and bipolar cells, they provide lateral transmission.

As aforementioned, the photoreceptors are the ones that degenerate upon RP and, therefore, this thesis will emphasize photoreceptors further on.
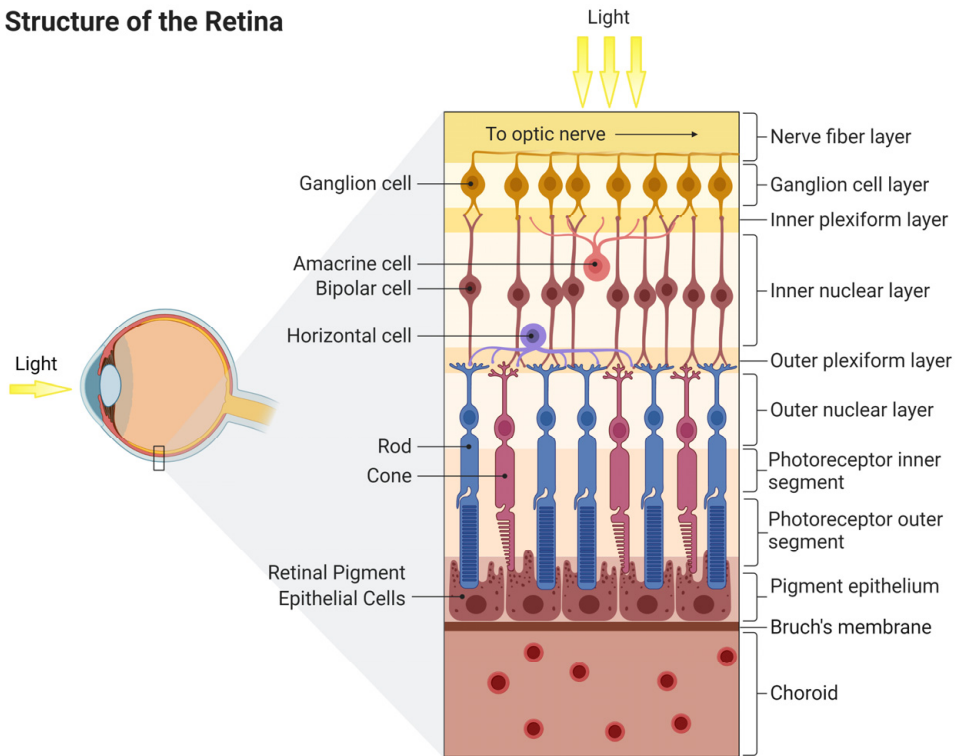
Figure 1 I Simplified illustration of the retinal layers. The light is refracted by the cornea and lens and focused onto the retina. The retina comprises four main distinct layers (from bottom to top); 1) Retinal pigment epithelium (RPE), 2) Outer nuclear layer (ONL), 3) Inner nuclear layer (INL), and 4) Ganglion cell layer (GCL). In addition, two plexiform layers exist. 1) Outer plexiform layer (OPL), which is located between ONL and INL, and 2) Inner plexiform layer (IPL), which is located between INL and GCL. Each layer consists of various cell types like retinal pigment epithelial cells, photoreceptors (rod and cone), horizontal, bipolar, and amacrine cells as well as ganglion cells. Created with BioRender.com.

The cGMP-signaling pathway in phototransduction

Regulation of the second messenger molecule cGMP (cyclic guanosine-3',5'-monophosphate) and its signaling pathway is crucial for the vision and the physiology of the photoreceptors and the phototransduction cascade[21]. Gene mutations related to the phototransduction[22,23] may lead to dysregulation of cGMP, which potentially affects cGMP targets as well as their signaling pathways and results in retinal degeneration[7,24].

Here the emphasis is on the cGMP synthesis and its involvement in the phototransduction cascade and thus how RP-related mutations may influence these systems.

cGMP cycle in the photoreceptor cells

The cGMP cycle within the photoreceptors' segments consists of six steps (Figure 2), and in Table 1 is given an overview of the proteins involved in the cGMP-synthesis and phototransduction. In the outer segments **[1]** cGMP is hydrolyzed by phosphodiesterase 6 (PDE6) to guanosine monophosphate (GMP; cGMP→GMP)[25]. PDE6 has four subunits, two inhibitory and two catalytic, where the latter is slightly different between rods and cones. Rod PDE6 has PDE6α (or PDE6A) and PDE6β (or PDE6B), where cone PDE6 have two of the same kind, named PDE6α' (or PDE6C)[26]. However, the inhibitory subunits vary in the PDE6γ sequences (rods: PDEG and cones: PDEH)[26]. The **[2]** GMP then diffuses from the outer segments to the inner segments through the space called connecting cilium[27]. Here GMP is either further degraded to **[3]** guanine (G) by 5'-nucleotidase (GMP→G) or recycled by guanylyl kinase, whose activity is mediated by the protein called RD3[27], to **[4]** guanosine diphosphate (GDP; GMP→GDP). GDP is then phosphorylated by nucleoside diphosphate kinase into **[5]** guanosine triphosphate (GTP; GDP→GTP)[27]. Finally, the GTP diffuses from the inner segments to the outer segments. Here it is used in various cascades[27], although a particulate enzyme called membrane-bound guanylyl cyclase (pGCs) will act upon stimulation from Ca^{2+}-binding and guanylyl cyclase-activating proteins (GCAPs; GCAP1 or GCAP2)[27–30] and convert **[6]** GTP into cGMP[31]. There exist two pGCs isoforms in the mouse photoreceptors retGC1 and retGC2, where cones only have retGC1[26]. The stimulation of pGCs can be affected by RD3, which can prevent the binding of GCAPs with pGCs and thus cGMP generation[28–30]. The newly generated cGMP can then either interact with various interactors or re-enter the phototransduction cascade (Figure 2).
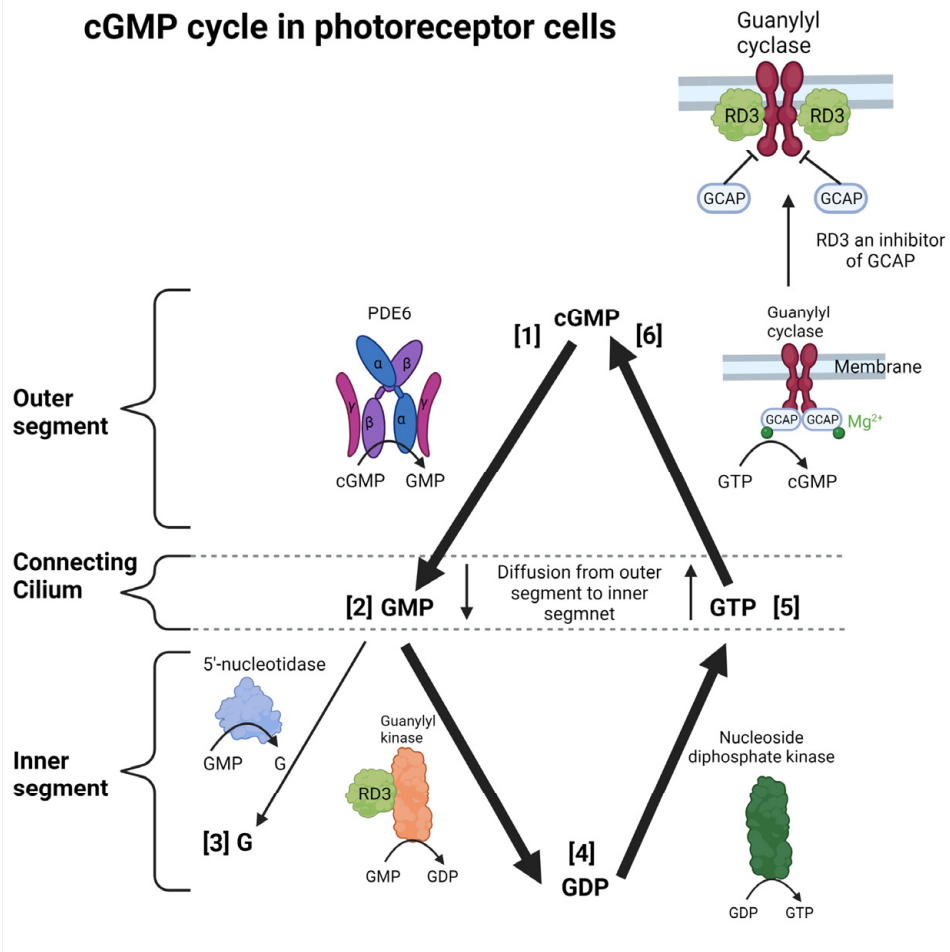
Figure 2 I Schematic illustration of the nucleotide cycle in photoreceptors. After illumination [1] cGMP is degraded to [2] GMP by PDE6 (cGMP→GMP). GMP diffuses through the connecting cilium to the inner segments where it is further degraded to [3] G or recycled by guanylyl kinase, which is regulated by RD3, to [4] GDP (GMP→GDP). The nucleoside diphosphate kinase generates [5] GTP (GDP→GTP). GTP then diffuses back through the cilium to the outer segments, where it can be synthesized to [6] cGMP by the guanylyl cyclase. RD3 can also mediate guanylyl cyclase by preventing GCAP binding thus no cGMP can be synthesized. This figure is inspired by Wimberg et al., 2018. Created with BioRender.com.

Phototransduction cascade

Both rods and cones detect light according to the same basic system, namely phototransduction. During dark conditions, the phototransduction cascade allows for high levels of cGMP[32] in the photoreceptor's outer segments. It results in sensitization of photoreceptors down to the level of a single-photon sensitivity[33], where rods are generally more sensitive to light stimuli and thus become more hyperpolarized than cones, which are less sensitive to light stimuli, despite similar light exposure[34,35]. Anyhow, during these conditions, cGMP binds its phototransduction target cGMP-gated channel (CNGC), which is located in the outer membrane of the photoreceptors' outer segments[36]. These channels are tetrameric and consist of different subunits named CNGA1 and CNGB1 in rods and CNGA3 and CNGB3 in cones[26]. This interaction affects the intracellular Ca^{2+} concentration, and the homeostasis then regulates the dynamic equilibrium between the influx of Na^+/Ca^{2+} and efflux of Na^+/Ca^{2+} via the exchanger $Na^+/Ca^{2+}/K^+$ (rods: NCKX1; cones: NCKX2 and NCKX4)[37] in the outer segments. When the intracellular Ca^{2+} is high, as it is under dark conditions, more of the Ca^{2+} binds GCAPs and inhibits pGCs, preventing cGMP synthesis[28] (Figure 3).

Upon illumination, the photoreceptors respond to captured photons by photoreceptive pigments called opsins (rhodopsin in rods, and S, M, L-opsin in cones). These opsins are located in the photoreceptors receptor disks[38] (see Figure 3). The light stimulation of opsins (*e.g.,* Rhodopsin; Rh*) then activates the G-protein cascade, which involves a heterotrimeric G protein, named transducin (rods express α1, β1, and γ1, whereas cones express α2, β3, and γ8 subunits)[26]. Transducin binds Rh* and an exchange of GDP to GTP produces activated transducin (GTP-bound alpha-subunit; Tα•GTP). Tα•GTP binds PDE6γ, which then relieves its restrain on the PDE6α and PDE6β subunits. This activates the membrane-bound PDE6, which hydrolyses cGMP and thus decreasing the intracellular cGMP level[25]. This decrease hampers cGMP binding to CNGC, leading to a decline of the Ca^{2+} concentration by the closure of CNGCs[37]. In addition, the closure of the channels creates membrane hyperpolarization of the outer segment due to continuous efflux from the NCKX exchangers[37]. As previously mentioned, this promotes the generation of signals transmitted to the downstream cells in the INL and GCL. With the reduced intracellular Ca^{2+} levels, Mg^{2+} replaces the Ca^{2+} bound to GCAPs, activating pGCs and promoting cGMP synthesis[28].

The inactivation of the cascade is regulated by the rates at which Rh*, transducin, and PDE6 return to their basal conformations, and the cGMP level return to its default dark condition state[26]. Rh* is silenced by multiple phosphorylations by rhodopsin kinase (also called G-protein receptor kinase; GRK1) and arrestin binding

(rods: arrestin-1 and cones: arrestin-1 and arrestin-4)[39]. Activated transducin and PDE6 are abolished by hydrolysis of Tα•GTP to Tα•GDP by the aid of PDE6γ and the three GTPase-accelerating proteins (GAPs; RGS9, Gβ5, and R9AP)[40], which then prepare the transduction proteins for new light stimulation.

Table 1: Photoreceptor transduction protein isoforms in mouse rod and cone

	Rod	Cone
Photopigment	Rhodopsin	Opsin (S, M, L-opsin)
G protein (transducin)	α1, β1, and γ1	α2, β3, and γ8
Phoshodiesterase 6	PDE6A, PDE6B, and PDE6G	PDE6C and PDE6H
cGMP-gated channels	CNGA1 and CNGB1	CNGA and CNGB3
G-protein receptor kinase	GRK1	GRK1
Arrestin	Arrestin-1	Arrestin-1 and arrestin-4
GAPs	RSG, Gβ5, and R9AP	RSG, Gβ5, and R9AP
Guanylyl cyclase	retGC1 and retGC2	retGC1
GCAPs	GCAP1 and GCAP2	GCAP1
Na$^+$/Ca^{2+}/K$^+$ exchanger	NCKX1	NCKX2 and NCKX4

This table is adapted from N.T. Ingram et al., 2016.

Figure 3 I Phototransduction in rod photoreceptors. Absorption of light activates the visual pigment (Rhodopsin, Rh*), which then catalyzes the exchange of GDP for GTP on the α subunit of transducin (Tα) activating the transducin (Tα*). Tα* binds to the cGMP heterotetrameric protein phosphodiesterase 6 (PDE6) inhibitory PDEγ subunit, where it relieves its restrain to the catalytic subunit (PDEα and PDEβ). It activates PDE6, which then hydrolyze cGMP to GMP. The hydrolysis of cGMP leads to the closure of cGMP-gated channels located in the plasma membrane of the outer segment and induces hyperpolarization of the cell. On the contrary, under the dark condition, the cGMP interacts with the cGMP-gated channels which cause an opening of the channels. To inactivate the cascade Rh* is silenced by multiple phosphorylations by rhodopsin kinase and the binding of arrestin (not shown in the figure). Activated tarnsducin and PDE6 are abolished by hydrolysis of Tα•GTP to Tα•GDP by the aid of PDE6γ and the three GTPase-accelerating proteins (GAPs; RGS9, Gβ5, and R9AP), which then prepare the transduction proteins for new light stimulation. Created with BioRender.com.
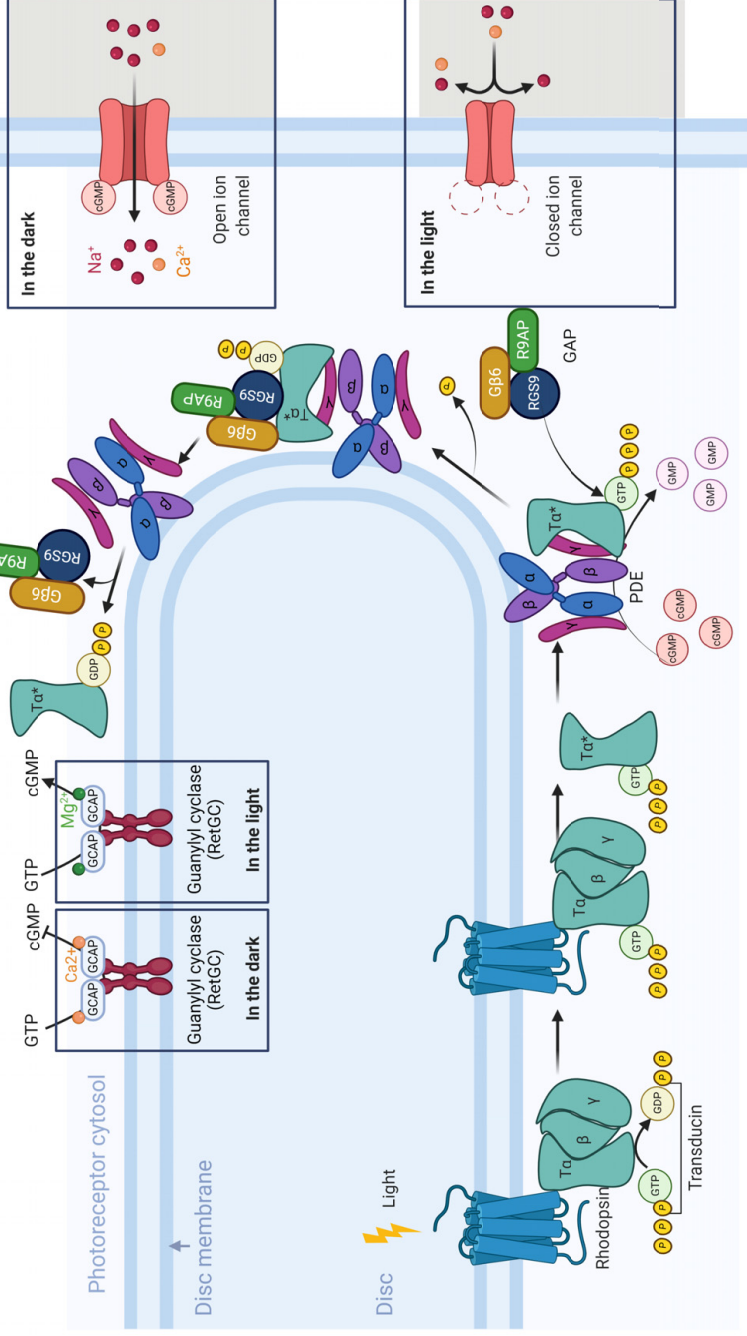
Phototransduction in rod photoreceptors

RP disease-linked cGMP regulators relevant to phototransduction

Disease-causing mutations in enzymes relevant to the above-mentioned systems can lead to dysregulations within the phototransduction[41] and give rise to disruption in the cGMP synthesis leading to toxic cGMP accumulation[42–44]. Moreover, these mutations may give rise to the phenomenon observed in different animal models for RP[24] (*i.e., rd* models) and the RP subtype LCA[24].

Some gene mutations in members relevant to the phototransduction cascade (some mentioned in Table 1)[24,41] are likely to affect the cGMP levels more strongly and cause a more severe early onset and rapid degeneration than other mutations[24]. These are, for example, found in aryl hydrocarbon receptor-interacting protein like-1 (AIPL1; a protein which stabilizes PDE6)[45,46], photoreceptor guanylyl cyclase 1 (GUCY2D; synthesizes cGMP)[47,48], and RD3 (an inhibitor of GCAP, governs pGCs and guanylyl kinase activity, and trafficking between segments)[27,49,50], which cause the phenotype for LCA[51]. Other disease-causing mutations that may reduce cGMP hydrolysis, and result in slower progressive degeneration and a milder phenotype of RP[7], are those found in the PDE6α/β protein[52–54] and guanylyl cyclase-activating protein 1B (GUCA1B)[55,56]. Alternatively, mutations within rhodopsin[44] and CNGC[57–59] instead impact the cGMP synthesis indirectly, where dysregulation of inosine-5′-monophosphate dehydrogenase 1 (IMPDH1) disrupts the cellular guanine nucleotide homeostasis[60].

Other proteins not having an obvious relation to the phototransduction can also elevate the cGMP level to some extent[24]. An example of such protein is the peripherin 2 (PRPH2), which is an outer segment structural protein[44,61]. In addition to the above-mentioned mutations, abnormalities in the retina's energy production can also cause RP since the retina and, more specifically, the photoreceptors have a high metabolic demand[41,62,63]. Numerous studies show that alterations in the blood floow[64–66] or disruption of glycolysis through mutations in enzymes essential for energy production can result in RP[41]. Furthermore, a connection between the cGMP-system (*i.e.,* cGMP-PKG-system) and, *e.g.,* oxidative phosphorylation[67] and the insulin/mTOR-signaling[68,69] have been suggested.

While this thesis will not discuss the above-mentioned proteins further, it is clear that dysregulation within the cGMP-system is involved in the retinal degenerative disease RP[24,70]. Moreover it supports the interest in cGMP, its interactors, and their likelihood to become targets for therapeutic interventions.

Targeting the cGMP-signaling pathway

As described above, both intracellular cGMP and Ca^{2+} concentrations in the photoreceptors are closely connected by a feedback loop that under normal conditions control their physiological ranges[71]. However, this feedback loop becomes disrupted due to diseased-related mutations in cGMP's effector proteins, of which numerous have been identified and elicit a wide variety of cellular functions[72], like maintaining the cGMP-signaling pathway[71]. These proteins comprise the already mentioned regulatory PDE6 (and other PDEs)[73], pGC[31,74], and CNGC[75]. Targeting some of these cGMP-interactors by, *e.g.,* attenuating CNGC[57] and pGC[50] activity reduces retinal degeneration, and therefore these proteins constitute potential targets to prevent photoreceptor cell death. However, studies investigating the CNGC show contradictive results, since some suggest that excessive activation of CNGC and high Ca^{2+} levels may propel photoreceptor degeneration[76–78], whereas other studies lack in finding such association[79–82]. This reinforces the complexity and the challenges of investigating retinal degeneration.

In addition to the cGMP targets within the phototransduction cascade, cGMP also has targets outside the cascade. Here the seemingly unrelated protein mitogen-activated protein kinase 1 (MAPK1)[83] and mitogen-activated protein kinase 3 (MAPK3)[68] have likewise been associated with the cGMP-PKG system[84,85], and knock-out of both MAPK1/3 kinases in the RPE cells leads to photoreceptor degeneration[86]. However manipulation of the cGMP-PKG system to see whether it would directly alter the MAPK1/3 activity and thus elucidate if the cGMP-PKG-MAPK1/3 cascade plays an essential role in the degeneration mechanism(s), has not been investigated within the photoreceptors until now (*this will be discussed further in the Results and Discussion sections, Paper III*).

Compared to MAPK1/3, the serine/threonine-specific protein kinases cGMP-dependent kinase and cAMP-dependent kinase (PKG and PKA) are well-established cGMP-interactors[87–90]. PKG is a homodimer of two identical subunits. Each subunit comprises an N-terminal regulatory domain, two cyclic nucleotide-binding (CNB-A/B) sites, an autoinhibitory sequence, and a C-terminal catalytic domain[90] (Figure 4). Furthermore, PKG is expressed as two different genes in mammals: the *PRKG1* gene, which encodes PKGIα and PKGIβ isoforms, whereas the *PRKG2* gene encodes the membrane-bound PKGII. It is known that PKGI and II phosphorylate several biological targets and can regulate various biological systems[91,92]. For example, PKGI mediates numerous NO/cGMP effects, including vasodilation, proliferation, and apoptosis[91,93]. In addition, PKGII regulates bone growth, homeostasis of Na^+, Cl^-, and functions within the nervous system[91,93]. However, PKG's role in the photoreceptors is obscure. Within the retina, PKGI is predominantly localized in the inner segments, ONL, and GCL, whereas PKGII is mainly expressed in the inner segments, INL, and GCL[94–96]. Here cGMP and the similar second messenger cyclic AMP (cAMP) may govern its activity.

Furthermore, several retinal targets of PKG are suggested, *e.g.,* PKA[92] and vasodilator-stimulated phosphoprotein[92]. In contrast to PKG, limited is known about PKA's role in RP. However, the few studies[112] (one unpublished[97]) that have been investigating PKA have so far not shown that PKA occupies a key position in retinal degeneration.

Overall, the evidence indicates that other cGMP-interacting proteins not obviously relevant to the cGMP system may exist (*this will be discussed further in the Results and Discussion sections, Paper I*), and these may, like PKG[98], be potential targets for neuroprotective RP therapy.
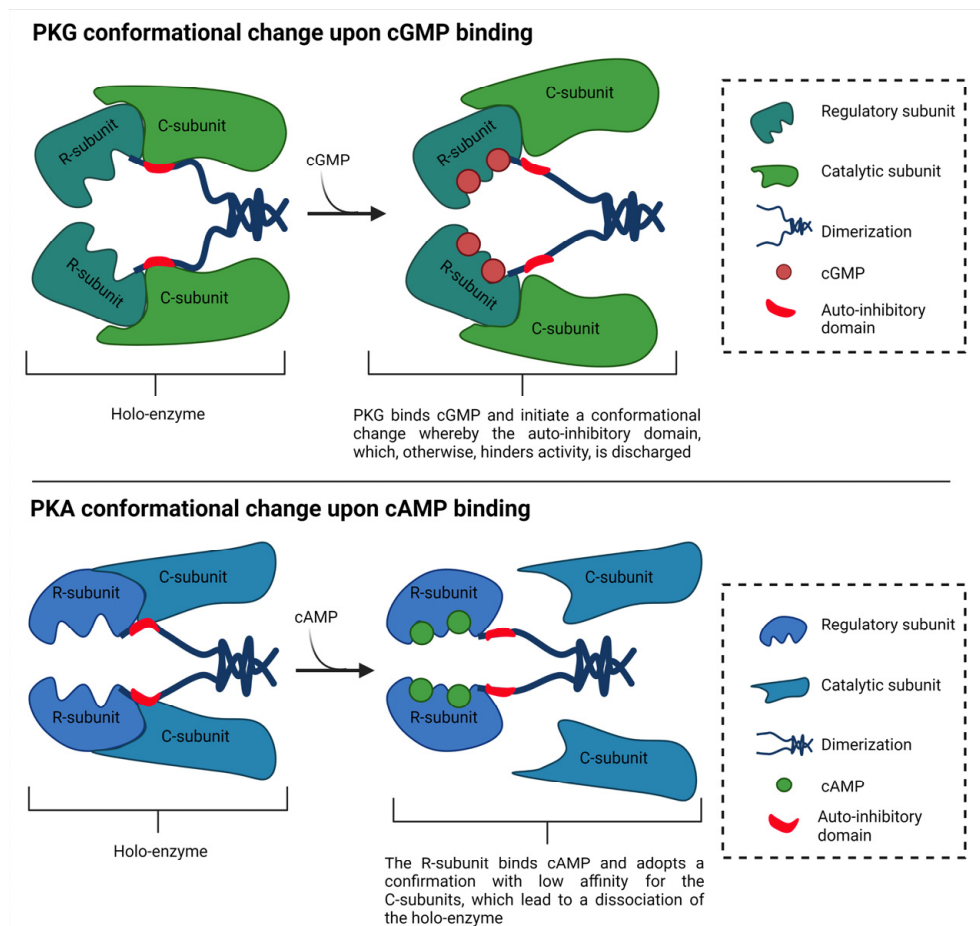
Figure 4 I Schematic illustration of conformational dynamics of the PKG and PKA. *Upper panel*) The holo-enzyme PKG is activated when four cGMP or cAMP molecules bind and initiate a conformational change whereby the auto-inhibitory domain which hinders activity is discharged. *Bottom panel*) The holo-enzyme PKA becomes activated by binding of four cAMP or cGMP to the regulatory subunit (R-subunit), which adopts a confirmation with low affinity for the catalytic subunit (C-subunits), which lead to a dissociation of the holo-enzyme. Created with BioRender.com.

Crosstalk by cGMP and cAMP

Several lines of evidence indicate that cGMP and cAMP pathways not only function as separate mechanisms but may also interfere with each other's target proteins like PDEs, PKA, PKG, and the rap guanine exchange factor 4 (EPAC2, see Figure 5)[99–102]. However, even though EPAC2 can interact with cAMP and cGMP[101,102], its potential role in RP has not been investigated until now (*this will be discussed further in the Results and Discussion sections, Paper IV*).

Nonetheless, several studies have investigated PKG's selectivity for the above-mentioned nucleotides. PKG is the primary receptor of cGMP, though PKGI and PKGII have different selectivities towards cGMP as well as cAMP. For PKGI, the CNB-B sites result in a 240-fold increased selectivity for cGMP compared to the CNB-A site[87–89,103]. The high affinity is due to the CNB-B site's structure which creates contact to the guanine moiety of the cGMP[104]. Although similar to PKGII, the CNB-B site also has a higher selectivity for cGMP[105], though its CNB-A site has a higher selectivity for cAMP[105]. For example, for PKGI to become activated, four cGMP molecules have to bind and initiate a conformational change whereby the auto-inhibitory domain, which otherwise hinders activity, is discharged (Figure 4)[87,90,92]. In addition, cGMP does not solely interact with PKGI. It may also crosstalk with other proteins like PKA[92,103], though cAMP is the primary activator of PKA and thus binds with higher affinity[106]. When PKA becomes activated by binding four cAMP to the regulatory subunit (R-subunit), it undergoes a distinct conformational change compared to PKGI[107,108]. The R-subunit adopts a confirmation with low affinity for the catalytic subunit (C-subunits), which leads to a dissociation of the holo-enzyme (Figure 4)[107,108]. This dissociation may give rise to further challenges to illuminate the pathways that cGMP may influence since dissociated C-subunits may re-target, while the R-subunits do not[109]. In turn, this suggests that PKA or parts of the PKA could be involved in multiple interaction profiles[109,110].

cGMP and cAMP interactors and cross-talks
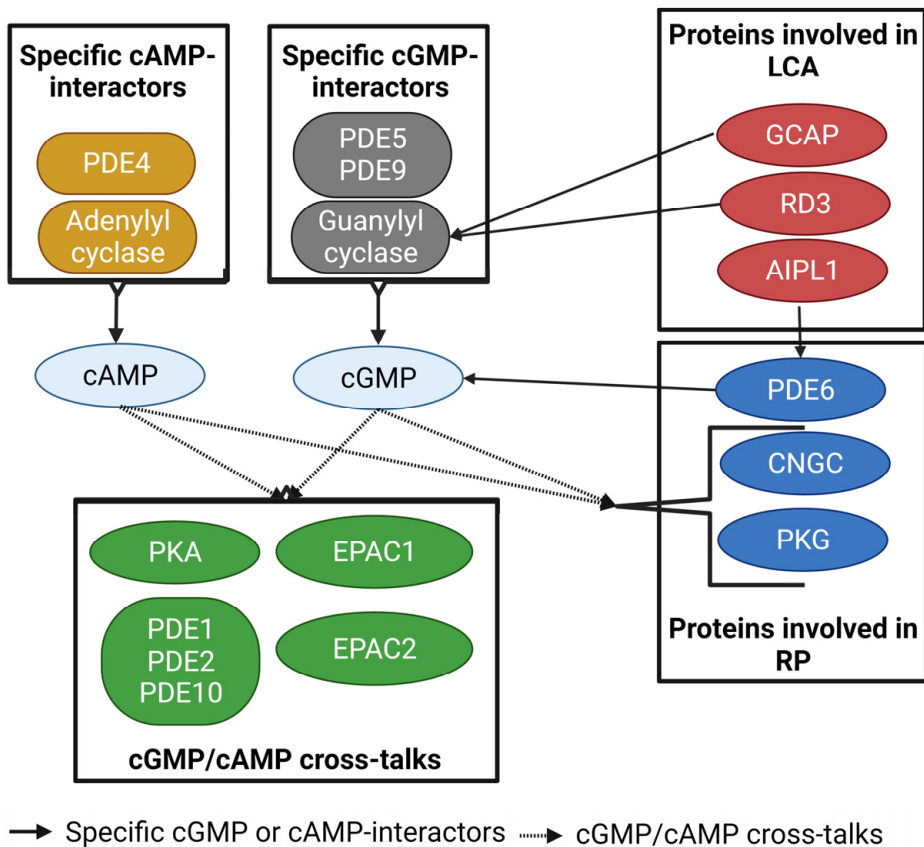
Figure 5 I Schematic illustration of potential cross-talks between cAMP, cGMP, and their interactors in the retina. Various PDEs are known to directly interact with either cAMP (*i.e.,* PDE4) or cGMP (*i.e.,* PDE5 and 9), whereas other PDEs (*i.e.,* PDE1, 2, and 10) have shown to have dual-specificity and can interact with both cAMP and cGMP. Proteins that are specific for the cGMP-system are the PDE6, guanylyl cyclase, AIPL1, RD3, GCAP, whereas adenylyl cyclase is specific for cAMP. However, cGMP and cAMP may cross-talk with several downstream targets like PKA, EPAC1, EPAC2, PKG, and CNGC. The proteins involved in LCA or RP are designated to their respective boxes. Created with BioRender.com.

Design of cGMP analogs to regulate PKG activity

Various PKG activators and inhibitors exist[99], although PKG inhibitors will be emphasized here. Numerous inhibitors of PKG's functional regions have been developed to potentially create therapeutic drugs based on PKG intervention[99]. Some of these are *Cyclic nucleotide* analogs (binds the cGMP binding domain), *K-Series-* (binds ATP-binding domain), *H-Series-* (binds ATP-binding domain), *W-Series-* (binds the substrate domain), *DT* (binds the substrate domain), and *Coccidian PKG-inhibitors* (blocks the ATP-binding domain)[111]. These inhibitors have different specificities and permeabilities which may affect their applications *in vitro* as well as *in vivo* and thus their capability as therapeutic drugs[84,111]. An overview of the different inhibitor's inhibitory effects on PKG and PKA is provided in Table 2[111]. Some of these inhibitors may also impede other proteins' activity besides PKG and PKA, and such kind lack of specificity is not preferred for therapeutic drugs. For example, the *K-Series* may also hinder protein kinase C activity, whereas the *H-Series* can impede both protein kinase C and myosin light chain kinase action[111]. In addition to specificity, membrane-permeability is likewise crucial for the drug. Thus it can enter the cells, and here the *W-Series* seems to fail[111].

However, some cGMP analogs have overcome several of these hurdles with tissue specificity and insufficient cell penetration and were synthesized and screened for therapeutic potential, especially against retinal degeneration in *rd* models[112].

This thesis will focus on the newer generation of hydrolysis-resistant Sp- and Rp-guanosine 3',5'-cyclic monophosphorothioate (cGMPS) analogs which comprises agonistic and antagonistic abilities and thus are more potently than cAMP and cGMP[106]. Several derivatives of these cGMPS are synthesized, and one of them is the Rp-8-bromo-β-phenyl-1,N²-etheno-cGMPS (Rp-8-Br-PET-cGMPS, CN03), which will be further emphasized here. CN03 is relatively more lipophilic and can cross membranes compared to regular cGMP, which is more or less unable to penetrate intact cellular membranes due to its cyclic phosphate moieties' ability to create polar interactions[99]. In addition, the analog has a higher affinity for PKGIα and PKGIβ compared to PKGII, combined with high discrimination for PKAI and II[106]. Moreover, it demonstrates resistance to hydrolysis by various PDEs[106,113]. Furthermore, CN03 indeed acquires great properties in studies, and for instance it preserves the phototransduction cascade and light-induced cone response by counteracting primary rod cell death and thus photoreceptor loss in an *rd* mouse model[98,112].

However, it is most likely that cGMP-analogs, like CN03, have other targets besides PKG and PKA, which it may cross-talk with[92,114]. So far, only a few studies indicating the specificity of cGMP-analogs have been published[83,115] and investigations elucidating cGMPS-analogs and CN03's known and potential new interactors have, until now, not been presented (*this will be discussed further in the Results and Discussion sections, Paper II and III*).

Table 2: Overview of PKG inhibitors

PKG Inhibitors	PKGIα K_i (µM)	PKGIβ K_i (µM)	PKGII K_i (µM)	PKAII K_i (µM)
Rp-cGMPS	20	15	0.5	20
Rp-8-Br-cGMPS	3.7	15	-	20
Rp-8-Br-PET-cGMPS	0.035	0.03	0.45-.9	11
Rp-8-pCPT-cGMPS	0.5	0.45-0.6	0.29-0.7	8.3
KT-5823	0.23	-	-	>10
H-7	5.8	-	-	3
H-8	0.5	-	-	1.2
H-9	0.9	-	-	1.9
H-89	0.48-0.5	-	-	0.05
W45	0.49-1.15	-	-	559
DT-2	0.012	-	-	12.7-20.3
DT-3	0.025	-	-	493
D-DT-2	0.0008	-	-	8.7-15.3

This table is adapted from Wolfertstetter et al., 2013 and represents the inhibition constants (Ki) for PKG and PKA isoforms.

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AIMS OF THE STUDY

There is an emerging need to elucidate the mechanism(s) behind the photoreceptor death, which occurs during RP. The comprehension of proteins that may play a key role within the cGMP system may help explain this degeneration mechanism. Furthermore, such cGMP-relevant proteins that can govern cell survival or cell death may potentially also function as therapeutic targets for RP.

Paper I

To find and make a tentative list of potential new cGMP-interacting proteins in the retina from a healthy and three well-established retinal degeneration mouse models by applying an optimized proteomic approach.

Paper II

To disclose the stereospecificity of cGMP-interactors and the target specificity of general cGMP and cGMPS-analog agaroses (cGMPAAs) that are selectivity-modified to obtain, *e.g.,* inhibitory actions on a specific target, like the PKG.

Manuscript III

To elucidate the modified cGMP-analog CN03's target-selectively and potential new associations. In addition, based on these proteins, suggest which pathways may be affected by pharmacological manipulation with CN03.

Manuscript IV

To elaborate on Paper I's findings. More specifically, to investigate the newly found retinal cGMP-interacting protein EPAC2 and its connection to the cGMP-system and its potential role in degenerating photoreceptors.

METHODOLOGY

Immunological and analytical techniques as well as the proteomics approach, including the separation method affinity chromatography (AC), were conducted by Michel Rasmussen at the Faculty of Medicine, Department of Clinical Sciences Lund, Lund University, Ophthalmology, Lund, Sweden. The Mass spectrometry (MS) method to identify proteins was performed by Charlotte Welinder at the Faculty of Medicine, Department of Clinical Sciences Lund, Mass Spectrometry, Lund University, Lund, Sweden. Organotypic retinal cultures were mainly performed by Jiaming Zhou or Hodan Abdshill with help from Michel Rasmussen within the Ophthalmology group at Lund University or by Arianna Tolone at the University of Tübinge, Institute for Ophthalmic Research, Germany.

This section presents a short description of the main methods applied within this thesis. For further detailed information about, *e.g.,* compounds, antibodies, and dilutions, please see the original formats (Paper I, II, III, IV), which are added as appendices in the book version of this thesis.

Ethical considerations

In this thesis, all efforts were made to comply with the 3 R's: Replacement, Reduction, and Refinement. The retina consists of a complex composition of various cells, as previously described. It is therefore challenging to replace retinas since replacements may not reflect the whole picture of what occurs in the retina as well as the protein/gene composition, which may diverge from the retina. However, an option to replace retinas from mice is to use photoreceptor-like cells originating from retinoblastoma tumors[116–118] or the cell line 661W[119,120], which is easily accessible and is thus widely used. 661W is an immortalized cone photoreceptor cell line that originated from the retinal tumor of a mouse expressing SV40 T antigen-photoreceptor cells[119]. In addition to studying cones, it is also possible to differentiate the cell line into rod-like cells[120]. However, it has been indicated that the 661W cell line may be the same as the commonly used RGC-5 line, which otherwise exhibits a phenotypic expression of many retinal ganglion cell markers[121]. Therefore, additional studies are necessary to clarify how well the 661W cells can replicate retinal photoreceptors[120]. However, photoreceptor-like cells like those

mentioned above were not used within this thesis and will therefore not be discussed further.

A great benefit of using mouse models is that they, to some extent, can recapitulate the human disease at the protein/gene level[122–124]. In addition, explanted retinas retain much of the cytoarchitecture of the retina, thus pharmacological manipulation of such organotypic retinal cultures may provide an insight into how a drug may impact different cells, proteins, and signaling pathways. However, to reduce the number of animals in Paper I, II, and III, a small pilot experiment using brain tissue as a replacement for retinas was applied to optimize the proteomics approach. To refine animal suffering, animals were housed under standard white cyclic lighting and had free access to food and water. Further, we applied all the procedures issued by the Malmö/Lunds Djurförsöksetiska Nämnd animal ethics committee (permit #M92-15, #02124/2020, and AK02/19M), including the law on animal protection issued by the German Federal Government (Tierschutzgesetz) and authorized by the institutional animal welfare office of the University of Tübingen, as well as the ARVO statement for the usage of animals in ophthalmic and visual research.

Retinal degeneration mouse models

As an aid to better understand the mechanism(s) behind the degenerating photoreceptors within the RP disease, there are several human homolog animal models (*i.e., rd* models) available that carry similar mutations as certain RP patients[44]. The models used in Paper I, II, III, and IV were the so-called C3H *rd1/rd1* (*rd1*), C57BL/6J *rd10/rd10* (*rd10*), C3H *rd2/rd2* (*rd2*), and the healthy counterpart (wt) mouse with either the C3H or C57BL/6J background (samples from C57BL/6J were provided from the University of Tübingen and were only used in Paper III)[44,125,126]. It should be noted that the genetic background and the mutation which the mouse model comprises may provide variations within epigenetics and protein composition, which could affect the experimental outcome[125–128], and therefore comparisons and thus conclusions based on such differences should be drawn with caution. Therefore, within Paper I, II, III, and IV, the development and of the retina as well as the synthesis of cGMP within the cortex were considered. In addition, the main emphasis was given to the animals' genetic background and their spontaneous gene mutations, which are associated with human RP. Depending on the mutations, the temporal progression of retinal degeneration is very variable in the different models (*i.e., rd1, rd10,* and *rd2*)[44]. Therefore, to avoid bias due to asymmetry within the ONL, the retinas were taken at PN11 and pulled, respectively, in Paper I and III. The selected time point, PN11, was based on the *rd1* model since, at this point, the degeneration is well underway, but the ONL thickness has not been reduced yet[129,130].

rd1

The rodless retina (C3H *rd1/rd1*) was discovered in wild mice first by Keeler in 1924[131,132] and later by Brückner in 1951[133] as an autosomal recessive mutation leading to retinal abnormality (*i.e.,* loss of photoreceptors). In 1993, Pitler et al. demonstrated that the line of *rd1* mice, reported by Keeler and Brückner, contained a homozygous nonsense point mutation in exon 7 (codon 347) and intronic polymorphisms in the rod phosphodiesterase beta-subunit gene (Pde6β)[22]. The mutation causes dysfunction in the PDE6β protein[44,54,134] and prevents the development of outer and inner segments of the photoreceptors[54,135]. Further, the *rd1* retina is characterized by no electroretinographic response (ERG)[136–138] and with a severe and early onset of retinal degeneration, which peaks at around postnatal day 13 (PN13) within the ONL[44]. Here rapid rod degeneration precedes cone degeneration, and by 4 weeks, no photoreceptors are left (Figure 6), whereas the INL and GCL are not affected[44,134]. The lack of cGMP-PDE activity has shown to result in an accumulation of cGMP (peaking at PN14)[44,137], which may affect its downstream signaling[98,139] and most likely negatively impact the photoreceptors' viability and promote cell death[98].

Overall, the *rd1* mouse is a well-established animal model whose mutation in the Pde6β gene phenotypically resembles the mutation which is found in a cohort of human patients with autosomal recessive RP (arRP; OMIM 180072)[52,140].

rd10

The *rd10* (C57BL/6J *rd10/rd10*) mouse model was first described by Chang et al. in 2002[125] and showed to harbor a missense mutation in exon 13 of the Pde6β gene. This mutation reduces the cGMP-PDE activity, although opposed to *rd1,* the PDE6β can be detected early in the *rd10* retina (PN10)[137]. However, the expression of PDE6β is reduced compared to its age-matched wt model[137]. The *rd10* mouse is characterized by an ERG, which is only recordable until the age of 3 weeks[137,141]. Further, the *rd10* has a relative later-onset starting at PN14 and a slower retinal degeneration that peaks shortly after PN18[44,125]. As the *rd1,* the INL and GCL are not affected[138] (Figure 6). The partial loss of function and reduced PDE6β enzymatic activity[137] in the *rd10* model increases the cGMP levels in the photoreceptors (Figure 6)[44], which, like in the *rd1,* may propel cell death[137]. Although, due to its slow progression, the *rd10* may be a more suitable model than the *rd1* for experimental-based therapy for RP (OMIM 180072)[138].

rd2

The *rd2* (C3H *rd2/rd2*) mouse model was identified in 1978[142] and showed to contain a mutation in the peripherin 2 protein (PRPH2), an integral membrane glycoprotein that is essential in photoreceptor disc membrane morphogenesis in the outer segments of photoreceptors[61]. The *rd2* harbors a mutation consisting of an insertion mutation of a 9.2 kb repetitive genomic element into exon 2 of the Prph2 gene causing transcription of an atypically large mRNA[143] and abnormal development of photoreceptor's outer segments in the retina[61] that, otherwise should begin to develop on PN7[44,125]. The *rd2* has a slow progression of retinal degeneration with a relatively early onset, at around PN14 with a peak a few days after PN17 (Figure 6). By 9 months of age, the ONL has completely degenerated in the peripheral retina, and by 12 months in the central retina[144,145]. This is supported by ERG, which is progressively diminishing with time and gone by 12 months[146]. Opposite to *rd1* and *rd10,* the accumulation of cGMP is not a hallmark in *rd2*, even though an elevated cGMP level is indicated[44,147]. The mutation within the *rd2* model has a common feature (*i.e.,* loss of photoreceptor function and photoreceptor cell death) with the peripherin-related RP (OMIM 179605)[148].

Cortex

In Paper II, we were interested in looking into various cGMP-nucleotide-agaroses available and their targets stereospecificity as well as target-specificity. Therefore, to reduce the number of animals and their suffering, we used the cortex from the C3H wt mouse[149]. Furthermore, the cortex was isolated at the age of PN11 since at this time point, cGMP is synthesized (initiated at embryonic day 15[150]), and the cortex expresses cGMP interactors like PKGI[95].
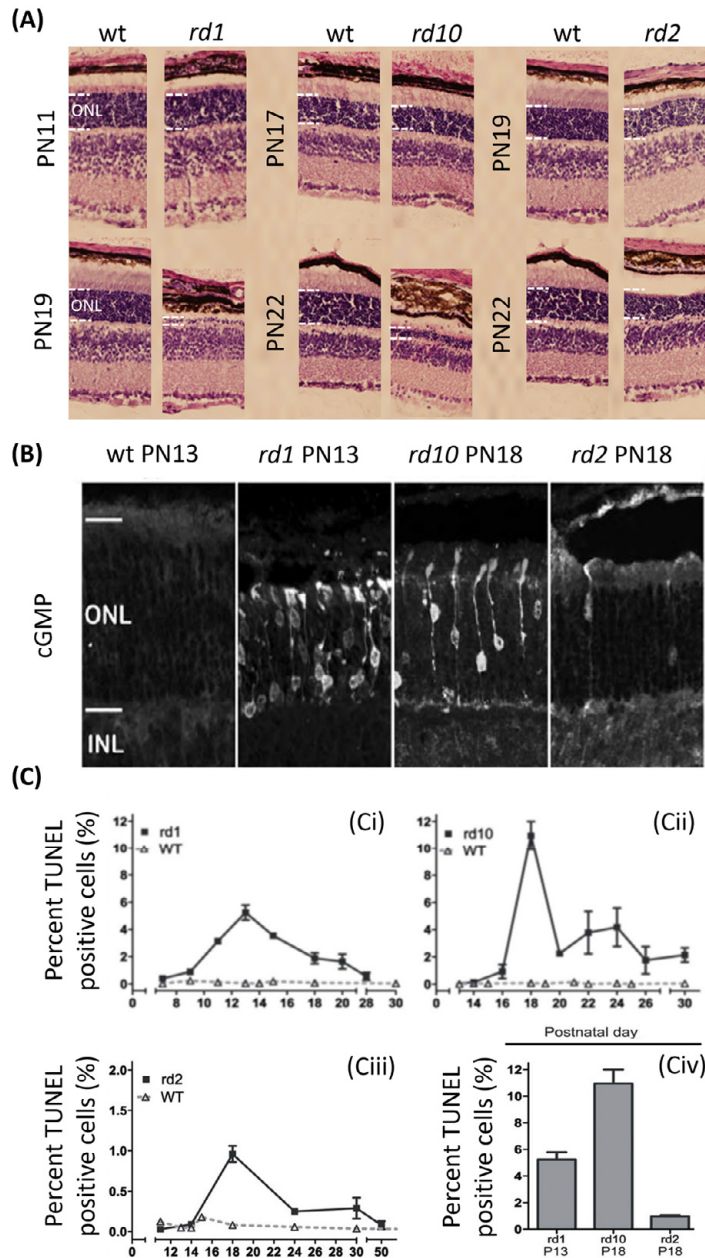
Figure 6 I Progression of photoreceptor degeneration in *rd* mouse models. Depending on the mutations, the temporal progression of retinal degeneration is very variable in the different models (*i.e., rd1*, *rd10*, and *rd2*). (A) Hematoxylin and eosin staining of wt, *rd1*, *rd10*, and *rd2* retinas at different time points. White dashed line indicates ONL. (B-C) Arango-Gonzalez et al. 2014 stained for cGMP accumulation in the ONL and quantified dying photoreceptors by using TUNEL assay. Ci-iii) TUNEL-positive photoreceptor cells in the ONL allowed determination of the evolution and the peak of photoreceptor death for each of these animal models. Civ) The bar graph shows a comparison of maximum peak heights for all the models. Note the different scales in line graphs showing less TUNEL positive cells for the *rd2* model. Data from (B-C) are adapted from the paper Arango-Gonzalez et al. 2014.

Proteomics approach

In Paper I, II, and III, the proteomics approach included two main steps: Affinity Chromatography (AC) and Mass Spectrometry (MS). For Paper I and III, retinal tissue was applied, and in Paper II mouse cortex was chosen as a biological sample. The amount of protein used [1.5 – 2 mg] was optimized in pre-experimental set-ups and inspired by previous studies[83,115,151]. The same optimized AC protocol was applied for all the proteomics approaches.

Affinity Chromatography

Agaroses with, *e.g.,* cGMP chemically linked were applied to catch and isolate cGMP-interacting proteins. The various agaroses had cGMP linked at different positions. Thus, it would theoretically be possible to pull down various cGMP-interacting proteins without the linker at one position, potentially preventing a protein from binding. This was especially important in Paper I, where we looked for potential new cGMP-interacting proteins. In Paper II, the general cGMP and modified cGMPS-analog agaroses aided in elucidating the cGMP-binding proteins indicated stereospecific interaction sites and the cGMP- and cGMPS-nucleotides (cGMP(S)) target-specificity. To avoid unspecific binding (*i.e.,* proteins that most likely do not interact with cGMP), we used EtOH-NH-agaroses (not having cGMP bound) together with elutions with the free nucleotides guanosine diphosphate (GDP) and adenosine diphosphate (ADP), as previously optimized[115,151]. Although, there may still be a high amount of non-specific binding, resulting from a combination of the property of agarose matrices[152], and proteins not binding cGMP-directly but that rather exist in protein complexes. Furthermore, we did not add nicotinamide adenine dinucleotide (NADH) to the elution sequence, which could be a limitation. NADH could potentially have removed lactate dehydrogenase (LDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and thus possibly have improved the enrichment procedure and subsequent MS analysis[151].

After removing unspecific proteins, the remaining proteins were eluted away with cAMP followed by cGMP. Finally, the successful pull-down of proteins was verified using an electrophoretic system including SDS-PAGE combined with silver staining, which provided more detection sensitivity to the system. As aforementioned, cGMP and cAMP may cross-talk with each other's targets. Therefore, it may be difficult to isolate pure cGMP-interactors using cGMP-nucleotide-agaroses. Thus, to overcome this hurdle, both the cAMP and cGMP eluates were subjected to MS and analyzed, respectively. The preparation methods in-solution or in-gel digestion were used to prepare the eluates before the samples were injected into the LC-MS/MS (Liquid Chromatography with tandem mass spectrometry) for identification (Figure 7).

Mass Spectrometry

In Paper I, II, and III, the isolated and enriched proteins derived from, *e.g.,* the cGMP and cAMP eluates (*i.e.,* either retinal or cortex proteins) were identified by LC-MS/MS. In Paper I, the methods to prepare the samples for LC-MS/MS were in-gel or in-solution digestion. However, it was observed that the in-solution method was not suitable when using agaroses since these clogged the LC-MS/MS system. Therefore, to avoid losing valuable proteins due to the small protein amount, the preparation method in-gel digestion was chosen as the primary preparation step for Paper II and III. Shortly, in the in-gel method, the eluates were loaded to a gel and run briefly, where after the protein bands were cut out. This removed the otherwise remaining agaroses. The proteins within the bands were then trypsinized, followed by extraction of the peptides. The peptide solution was then injected into the LC-MS, where they were concentrated on a pre-column (C18) and eluted depending on their hydrophobicity. The initial MS survey scan was performed using the Orbitrap Fusion (operated in the positive data-dependent acquisition), and based on the full scan MS, the most intense ions were applied for fragmentation in the MS2. Here the ions were run through the quadrupole before being detected in the Orbitrap. The data from the MS2 were analyzed with Proteome Discovery™ Software (Figure 7).
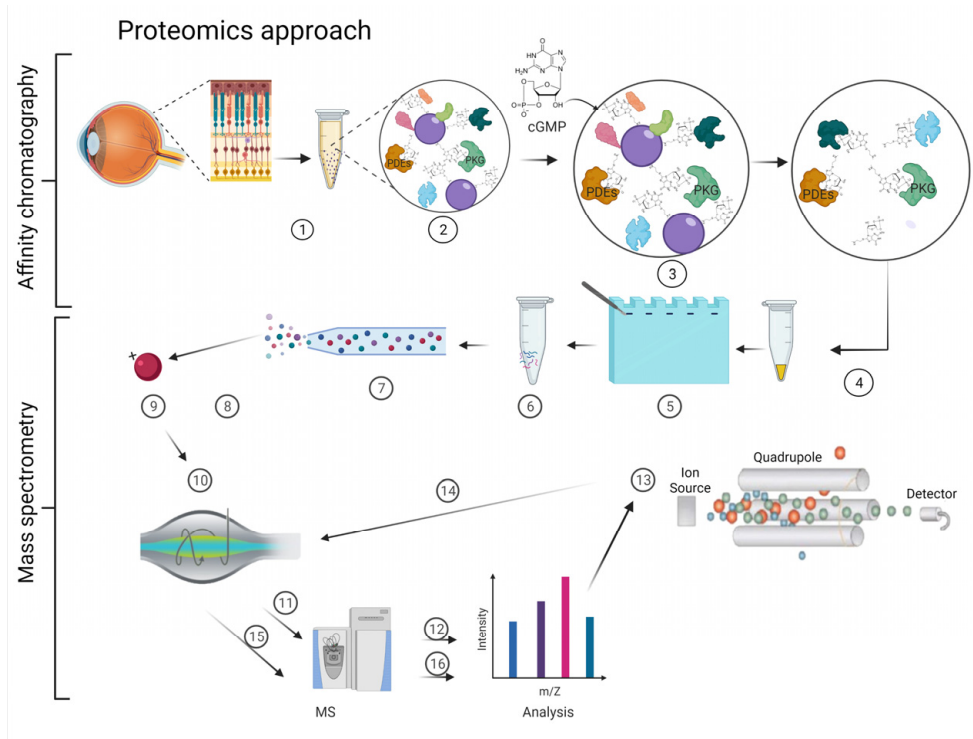
Figure 7 I Simplified overview of the proteomics approach. 1) The biological sample is collected, *e.g.,* retina. 2) The homogenized retina is then subjected to affinity chromatography, where, *e.g.,* cGMP-agaroses are mixed with the retinal supernatant. Subsequent elutions to remove unspecific proteins are performed by eluting with ADP and GDP. 3) cGMP-binding proteins are then eluted by the addition of cAMP followed by cGMP. 4) The cGMP-associated proteins are now in free solution and subjected to SDS-PAGE. 5) The proteins are loaded to the gel and run briefly before the bands are cut out. 6) These proteins are then trypsinized and 7) added to the liquid chromatography separation, where 8-9) the peptides are positively ionized. 10-12) An MS survey scan is performed using the Orbitrap Fusion and based on the full scan MS, the most intense ions are applied for fragmentation in the MS2. 13-16) Here the ions are run through the quadrupole before the Orbitrap and finally analyzed. Created with BioRender.com

Retinal Cultures

The organotypic retinal cultures derived from the *rd* models, *rd10* and *rd1,* and the wt retina were prepared as previously described[153]. In general, animals were sacrificed by decapitation, and eyes were enucleated and subjected to an R16 retinal medium. To aid in separating the retina from the sclera, the proteinase K enzyme was added to the R16 medium, where the enzyme activity later was blocked by the addition of 10 % fetal bovine serum. The retina with the RPE remaining was isolated from the rest of the eye and flat-mounted onto a culture membrane insert. The R16 medium used for culturing the explants was complemented with different supplements and free of serum and antibiotics[154]. Serum consists of various components, *i.e.,* antibodies and growth factors, albeit the specific concentration of the components may be elusive. Therefore, by excluding serum in the medium, the possibility of interference in drug effects by unknown factors is thus eliminated, and the conditioned culture medium can likewise be analyzed to investigate biomolecules released by the retina[154]. To replicate the physiological conditions, the explants were incubated in a humid incubator (5% CO_2) at 37°C. Prior to pharmacological manipulation, the explants were left undisturbed for 48 h to allow for adaption to the culture conditions. The culture paradigm for both Paper III (CN03 manipulation; C3Hwt and C3H*rd1:* PN5-11; C57wt and C57*rd10:* PN12-24) and IV (EPAC2 manipulation; C3Hwt and C3H*rd10:* PN12-24) were chosen depending on their disease background. The long-term cultures (PN12-24) allowed the systems under investigation to be exposed to the manipulation for a long time and adapt to the condition. All explant cultures were terminated by fixation with 4 % paraformaldehyde and cryoprotected with graded sucrose solutions before being embedded and sectioned.

TUNEL Assay

This assay was applied in Paper III and IV. The cleavage of the double-stranded genomic DNA by endonucleases generates small nucleosomal fragments showing free 3'-hydroxyl groups at their termini. These fragments are a biochemical hallmark of early and late-stage apoptosis[155]. The terminal deoxynucleotidyl transferase biotin-dUTP nick and labeling (TUNEL) allow the *in situ* detection of such cleavage fragments in tissue sections. The terminal deoxynucleotidyl transferase (TdT) is a template-independent DNA polymerase, which contains an amino sequence that inhibits its interaction with double-stranded DNA, directing its activity towards the addition of a properly labeled deoxynucleotide triphosphate (*e.g.,* dUTP nucleotides) to a single-stranded sequence with a free 3'-hydroxyl terminus[155]. Even though TUNEL assay is easy to use, it also has drawbacks. TUNEL assay may be nonspecific since the assay will label all free 3'-hydroxyl

termini irrespective of how these termini were generated. Thus, the assay will not only detect apoptotic cells, but it can also detect non-apoptotic cells like necrotic degenerating cells, cells damaged by mechanical forces, cells undergoing gene transcription, or cells undergoing DNA repair[156].

Immunofluorescence

Immunofluorescence (IF) was applied in Paper I, III, and IV, where they aided in detecting the localization and expression of the proteins of interest. To visualize these proteins using IF, paraformaldehyde-fixed retinal sections were incubated in a blocking buffer thus unspecific binding from the antibody would be avoided. The retinal cross-sections were then incubated with primary antibodies overnight, followed by rinse and incubation with secondary antibodies before mounting. For staining of cGMP binding proteins, fluorescent-labeled cGMP(S) was applied to unfixed retinal cross-sections for 45 min. IF outcomes were visualized in the Zeiss Axio Imager.M2 microscope. Any analysis of the pictures was performed with Zen (2.5) blue edition software (Zeiss Zen software).

Rap1-GTP Assay

To measure the EPAC2 activity in Paper IV, a Rap1-GTP assay was applied since EPAC induces the activation of the Ras-like GTPase family members Rap1 and Rap2[157]. Rap1 exists in an inactive GDP-bound form and an active GTP-bound form. When Rap1 is active (Rap1-GTP), it binds specifically to the Rap-binding domain (RBD) of Ral Guanine Nucleotide Dissociation Stimulator (RalGDS) to regulate downstream signaling cascades[158]. Therefore, this assay uses RalGDS-RBD agarose beads to selectively isolate and pull down Rap1-GTP. The Rap1-GTP is then subjected to a western blot where an antibody specific for Rap1 is used to detect the pulled-down Rap1-GTP and Rap1. However, since we could not isolate photoreceptors from the retina without the risk of losing such or contaminating the samples with other retinal cells, it is, therefore, notable that this assay reflects the total Rap1-GTP activity from the whole retina and not solely the photoreceptors.

Proximity Ligation Assay

In Paper I and IV, the method proximity ligation assay (PLA) was utilized. PLA is a method that can indicate if a protein and, *e.g.,* cGMP are in proximity and thus may interact in the tissue. However, the PLA outcome may give a false-positive

result (Figure 8) if the protein and cGMP are ≤ 40 nm apart. This gives rise to two scenarios: 1) Direct interaction; cGMP and the protein of interest interacting with each other directly, or 2) Indirect interaction, meaning that cGMP and the protein of interest may interact in a complex, but they do not interact directly. Still, the PLA may aid in selecting for proteins, which are more likely to be in proximity or even interacting directly with cGMP, by the number of positive PLA outcomes.
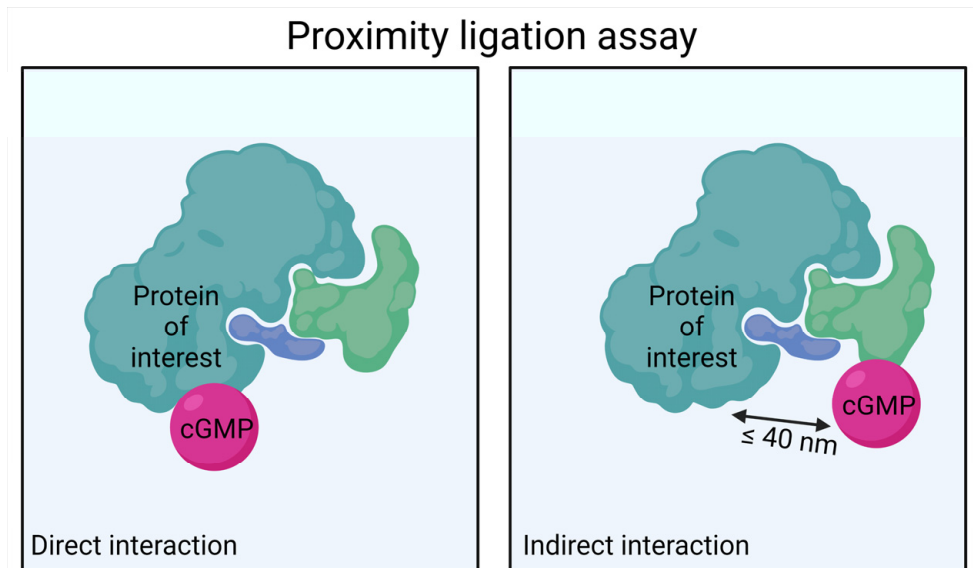
Figure 8 I Illustration of the direct and indirect binding upon proximity ligation assay. *Left panel*) Here the cGMP binds directly to the protein of interest. *Right panel*) cGMP and the protein of interest interact in a complex, but they do not interact directly. However, since the protein and cGMP are ≤ 40 nm apart the assay will provide false-positive results. Created in Biorender.com

RESULTS

In this section, I present a summary of the main findings of each paper/manuscript. The original formats and several of their supplementary data are added as appendices in the book version of this thesis. Here, the reader can find an in-depth description of the outcomes. For supplementary data only accessible in Excel I refer to the published version of Paper I-II where these data are available. For Paper III - IV these data can be provided by Michel Rasmussen

Paper I: The cGMP system in normal and degenerating mouse neuroretina: New proteins with cGMP interaction potential identified by a proteomics approach

Here we employed an optimized proteomics approach to study the cGMP-interactors in three well-established *rd* models (*i.e., rd1*, *rd10*, and *rd2*) as well as a healthy counterpart. In addition, several measures (1) using control agaroses with no cGMP attached (EtOH-NH), (2) elutions with the nucleotides GDP and ADP prior to cAMP and cGMP elutions, and finally, (3) only proteins labeled with HIGH, meaning the peptides were identified and associated with its corresponding protein, were applied to exclude non-specific proteins; thus, the outcome would most likely reveal proteins relevant to the cGMP-system. As a result, multiple regular known cGMP-interacting proteins, like PKGI, PKAI/II, and PDE1/2/5/6, were identified, which aided the validation of the proteomics approach. The Basic Local Alignment Search Tool (BLAST), with PKGI as query, was applied to reveal potential new interactors associated with cGMP in the retina. Hereof ten proteins with high sequence similarity to PKGI, and which thus might possess possible associations with cGMP, were identified. Five of these: CaMKIIα, GSK3β, MAPK1/3, and EPAC2, were designated based on their relevance to photoreceptors and the retinal degeneration seen in RP. Together with PKGI, they showed proximity with cGMP *in situ* in the photoreceptor layer, respectively. Furthermore, phosphorylated CaMKIIα (*p*-CaMKIIα) and EPAC2 showed more proximity with cGMP than GSK3β phosphorylated at serine 9 (*p*-GSK3β^{Ser9}) and MAPK1/3. With the potential for a false-positive result with the PLA an indirect binding of cGMP to some of these proteins (*e.g., p*-GSK3β^{Ser9} and MAPK1/3) could not be excluded. Although, the results indicated that these proteins might be cGMP-relevant and aid in deciphering the cGMP signaling pathway and thus the degeneration mechanisms as well as support the concept that cGMP-interactors could be used for RP therapies.

Paper II: The stereospecific interaction sites and target specificity of cGMP-analogs in mouse cortex

This study applied the proteomics approach and mouse cortex proteins to isolate cGMP-interactors and elucidate the general cGMP- or modified cGMPS-analogs' target specificity and their interactors' stereospecificity. The modified cGMPS-analogs included sulfur-containing cyclic phosphorothioates that gave activatory or inhibitory actions towards PKG (*i.e.,* Rp/Sp-8-AET-cGMPS, Rp/Sp-2'-AHC-cGMPS), whereas the cyclic phosphate cGMP-nucleotides (*i.e.,* 8-AET-cGMP, 2-AH-cGMP, 2'-AHC-cGMP) were not selectively modified. The seven nucleotides were chemically linked to agaroses (cGMPAAs) at different sites. In addition, the EtOH-NH agarose was also included since it functioned as a control.

This study disclosed that all cGMPAAs were able to capture some or several of the known cGMP-binding proteins like the PKAI/II regulatory and catalytic subunits, PDE1/2, PKGI, and EPAC2. The disparity in pull-down profiles and thus target-selectivity could be due to stereospecific requirements or differences in cGMPAA binding, or perhaps variations in affinities. The latter, however, was not studied here. Furthermore, 25 proteins that could be associated with the cGMP-system were found by applying the BLAST search with PKGI as a query, *e.g.,* MAPK1/3, GSK3β, CaMKII, and EPAC2. Furthermore, when analyzing all identified proteins and their involvement in KEGG pathways, we discovered that the cGMP-binding proteins were involved in 40 KEGG pathways. Here, *e.g.,* PKA regulatory and catalytic subunits were engaged in 30 of these pathways. Hence the cGMPAA's selectivity could aid in studying functional and physical associations for a given cGMP interactor and provide valid information on the target specificity and drug design in the field of cGMP-analog-based therapies.

Paper III: The photoreceptor protective cGMP-analog Rp-8-Br-PET-cGMPS interacts with cGMP-interactors PKGI, PDE1, PDE6, and PKAI in the degenerating mouse retina

The newly generated CN03-agarose was combined with the proteomic approach and retinal tissue from three retinal degeneration models (*i.e., rd1, rd10,* and *rd2*). The results revealed that CN03 could pull down seven known cGMP-binding proteins, like PKGIβ, PDE1β, PDE1c, PDE6α, PKA1α regulatory subunit, and PKAα catalytic subunit, whereas regular cGMP-agaroses could, in Paper I, pull down 12 known cGMP-binding proteins. Thus, this suggests that CN03 is likely more target-specific when compared to regular cGMP. Furthermore, when applying the BLAST search with PKGI as a query, 28 other PKGI-similar proteins (*e.g.,* MAPK1/3, CamKIIβ/δ/γ, and GSK3β) were found to potentially be associated with CN03. Moreover, we found that all the identified proteins, which were designated to be associated with CN03, were involved in numerous pathways like the cGMP and cAMP signaling, as well as pathways involving MAPK. It may thus be that CN03 can govern its effect through these pathways.

The connection between MAPK1/3 expression and activity with CN03 was studied via immunoflourescence and organotypic retinal cultures. Our results showed that the *rd2* segments express significantly higher MAPK1 as well as elevated activated MAPK1/3 when compared to its respective counterpart and the *rd2* ONL. On the contrary, the *rd1* and *rd10* models did not show significant differences, although a general higher expression was likewise seen in the segments. Nonetheless, CN03 has been shown to improve photoreceptor survival in all three *rd* models, likely through inhibition of PKG[98,112], and CN03 may regulate MAPK3 activity in the *rd1* retina[68]. We, therefore, employed retinal cultures of *rd1*, *rd10*, and their corresponding healthy counterparts to investigate what effect a short and long-term pharmacological manipulation with CN03 could have on MAPK1/3 within the ONL. The outcome did not show whether CN03 could govern MAPK1/3 expression or activity within the ONL.

Paper IV: Enhanced cGMP interactor rap guanine exchange factor 4 (EPAC2) expression and activity in degenerating photoreceptors - A neuroprotective response?

We employed cross-sectional retinal stainings and found EPAC2 localized in the photoreceptors and, more specifically, within the rods' inner segments in the mouse retina. The connection between EPAC2 and rods governed us to investigate EPAC2's potential association to the degeneration in retinal degeneration models (*i.e., rd1*, *rd10*, and *rd2*) as well as their healthy counterparts. Here *rd1* and *rd10* showed a significant temporal increase of EPAC2 expression in the photoreceptors, where *rd1* also had a significant increase in interaction between cGMP and EPAC2 in the ONL. However, the number of bindings between cAMP and EPAC2 was not significantly altered within the healthy retina or the *rd* models. Notably, the *rd2* model did not show significant outcomes in EPAC2 expression or interaction between EPAC2 and the nucleotides, respectively. Furthermore, stainings of cross-sectional *rd1* retinas indicated that accumulated cGMP overlapped with accumulating cAMP in some photoreceptors. However, accumulating cAMP was the only nucleotide that coincided with TUNEL[+] photoreceptors. In addition, photoreceptors having increased EPAC2 expression within their ONL also seemed to co-localize with accumulating cGMP. A similar outcome was not studied between EPAC2 and cAMP, nonetheless, it still suggests the following order of appearance by EPAC2 and accumulated nucleotide in the diseased photoreceptors: cGMP/EPAC2→cAMP→cell death. The *rd10* model was chosen for organotypic retinal cultures due to its slow cell death progression, which allowed pharmacological manipulation to be initiated before the major part of *rd10* cell death[44], and a long treatment paradigm could thus be performed (PN12 – PN24). A Rap1-GTP assay was applied to *rd10* retinas (PN 17) to test the effect of the activating and inhibiting components on EPAC2's activity. Firstly, the assay indicated that the EPAC2 activity was lower in the *rd10* retina compared to a healthy retina. Secondly, the compounds showed their expected effect in retinal cultures. Moreover, manipulation of EPAC2 activity by treating cultured *rd10* retina with an EPAC2 activating compound significantly impeded the reduction of the ONL thickness when compared to EPAC2 inhibiting conditions. Hence, EPAC2 activity could have a neuroprotective effect in some *rd* models like the *rd10*.

DISCUSSION

An optimized proteomics approach identified known retinal cGMP-interacting proteins as well as potential new cGMP-relevant associations (*i.e.,* MAPK1/3, *p*-CaMKIIα, *p*-GSK3β[ser9], and EPAC2, Paper I). For the latter protein, EPAC2, we revealed that EPAC2 might be relevant to the degeneration mechanism, and its activity may have neuroprotective abilities (Paper IV). Furthermore, the combination of the proteomics approach with various cGMP(S)-nucleotides showed that cGMP-interactors have stereospecific interaction sites but also that cGMP(S)-nucleotides have target-specificity (Paper II). Such target specificity was also seen for the CN03 analog (Paper III). Moreover, these studies led to the disclosure of various pathways with which the cGMP binding proteins and CN03 may interfere (Figure 11).

EPAC2 and PKG bind cGMP and cGMP(S)-nucleotides with their cyclic nucleotide-binding pockets through defined stereospecific binding-sites[87,102]. In Paper II, the proteomics approach showed that the cGMP(S)-nucleotides' attachment to the agarose is essential but also complex. It can be posited that the nucleotide's original affinities, the combination of modifications, *e.g.,* sulfur substitution and bromide addition, variations in linker composition and positionings, may affect the binding affinities for the various interactors by altering binding distances and bond energies. These variables should be noted when performing affinity chromatography and may thus have influenced the outcome in Paper III since this study only included one CN03-agarose. It may, therefore, be that Paper III does not include all proteins that potentially could have bound CN03 but were prevented by the linked agarose at the 2'-OH (2'-ribose hydroxyl) position. On the contrary, in Paper I, such a hurdle was avoided by incorporating three different cGMP-agaroses. However, common for Paper I, II, and III, these studies did not look into membrane-bound cGMP-interactors like the CNG-channels and PKGII since the proteomics approach applied was not optimized for such proteins. Firstly, no strong detergents like sodium dodecyl sulfate were utilized; thus, the extraction of membrane proteins may have been inadequate in the initial supernatant fractions[159]. Secondly, hydrophobic membrane proteins like CNG channels[160,161] are more difficult to analyze by MS than hydrophilic proteins due to the frequently used desalting steps since these are based on the hydrophobicity of peptides. This desalting step, which was used in Paper I, II, and III, could have discarded highly hydrophobic and hydrophilic peptides[162,163]. It is, therefore, possible that the results

from the proteomics approach in Paper I-III may have been restricted since membrane-bound cGMP-interactors or potentially other proteins could not have been detected.

Nonetheless, Paper I, II, and III all identified proteins of which several overlapped between the general cGMP targets and the cGMP-associated proteins (see Table 3), although EPAC2 was one of the exceptions.

Table 3: Overview of overlaps between known cGMP- and new potential cGMP-interactors from Paper I-III

Nucleotides bound to Agaroses	cGMP (retinal samples)	cGMP(S) (cortex samples)	CN03 (retinal samples)
Known cGMP interactors			
PKGI	X	X	X
PDE1α	X	X	
PDE1β		X	X
PDE1c	X		X
PDE2α	X	X	
PDE5α	X		
PDE6α	X		X
PDE10α		X	
PKAIα	X	X	X
PKAIIα	X	X	
PKAIβ	X	X	
PKAIIβ	X	X	
Total identified	**10**	**9**	**5**
Potential new cGMP interactors			
CaMKIIα	X		
CaMKIIβ	X		X
CaMKIIδ	X	X	X
CaMKIIγ	X	X	X
EPAC2	X	X	
GSK3α	X	X	
GSK3β	X	X	X
MAPK1	X	X	X
MAPK3	X	X	X
Total identified	**9**	**7**	**6**

Note, this is a general overview of pull downed proteins by the nucleotide-agaroses and thus do not reflect what the respective cGMP-, or analog-agarose pulled down. Moreover, catalytic PKA subunits are not included, since they do not directly bind cGMP, as previously described.

EPAC2, which is further described below, was pulled down by cGMP(S)-nucleotides. However, the cAMP/cGMP or CN03 nucleotides were not able to elute EPAC2 from the CN03-analog in Paper III. This discrepancy may be explained by EPAC2's stereospecific interactions sites. Rehmann et al., 2003[102] showed that EPAC2 interacts with the 2-NH_2 site of cGMP nucleotides. Further, modifications to the positions C8 (carbon 8) and possibly 2'-OH on cAMP may modulate general EPAC activity and binding[102]. It can thus be posited that these sites (C8, 2'-OH, and 2-NH_2), where the 2-NH_2 site seems to be the most important (Paper II), are needed

for EPAC2 to bind cGMP. This was further supported by the fact that EPAC2 interacted with a modified cGMPS (*i.e.,* Rp-2'-AHC-cGMPS) in Paper II. This cGMPS resembles CN03 with the substitutions of the exocyclic oxygen with sulfur in the cyclic phosphate moiety and a linker attached at the 2'-OH[110] site. This linker positioning prohibited EPAC2 from interacting with the 2'-OH site. However, Rp-2'-AHC-cGMPS was still able to pull down EPAC2 despite the lack of the available 2'-OH site (Paper II). Furthermore, a cGMP-agarose with a linker attached to the C8 site (*i.e.,* 8-AET-cGMP) likewise managed to pull down EPAC2, indicating that the C8 site, like the 2'-OH, is not essential for EPAC2 interaction (Paper II). Consequently, the prevention of EPAC2 binding CN03 may be explained by the polyethylene terephthalate addition and removal of the crucial 2-NH$_2$ site. It can, therefore, be indicated that EPAC2 most likely can be excluded as a target of the CN03 analog (Table 3 and Figure 9).
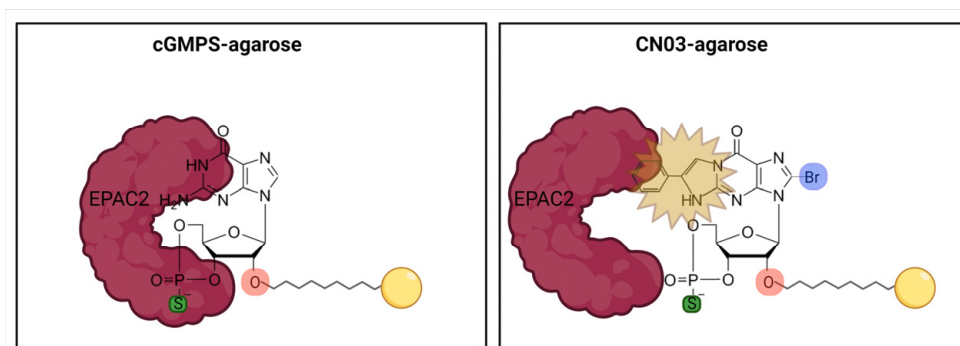
Figure 9 I A simple illustration of EPAC2's potential interaction with a cGMPS- and not the CN03-agarose. EPAC2 can bind a cGMPS (*i.e.,* Rp-2'-AHC-cGMPS) which contains a substitution of the exocyclic oxygen with sulfur in the cyclic phosphate moiety (highlighted green) and a linker attached to the 2'-OH site (highlighted red). However, CN03 has identical sulfur substitution and linker attachment, but it also has modifications, *e.g.,* bromide (highlighted blue) and polyethylene terephthalate addition (highlighted as a yellow star). These latter modifications, especially the polyethylene terephthalate, may prohibit EPAC2 interaction. Created with BioRender.com.

EPAC2 - General EPAC has potential therapeutic abilities within eye pathologies like glaucoma and macular edema[164]. Furthermore, the EPAC-Rap1 pathway may assist in maintaining the basal barrier properties in the retina, where activation of this pathway might restore the blood-retinal barrier[165]. Manipulation of EPAC1 or EPAC2 activity with inhibitory compounds has been shown to promote cell survival in retinal ganglion cells[164] and neuronal cells in the cerebral cortex[166]. However, activating EPAC2, instead of inhibiting its activity, can likewise prevent cell death within the CNS[167]. This may indicate that distinct neurons react differently depending on the changes within EPAC2 activity. However, common for the studies is that EPAC2 activity may be associated with the disease mechanism. To elucidate whether EPAC2 also might be associated with retinal degeneration in RP, we investigated its connection to cGMP. In Paper IV, EPAC2 was localized within, *e.g.,* rods' inner segments and EPAC2 was also found to be in proximity with cGMP

in the photoreceptors (Paper I and IV). These associations increase EPAC2's relevance to the secondary cell death mechanism where rods accumulate cGMP and initiate cell death prior to cones in RP degenerating retina[168]. In addition, significant temporal augmentation of EPAC2 expression was also seen in the photoreceptors within the *rd1* and *rd10* models and when compared to their respective healthy counterparts (Paper IV). It can, therefore, be speculated that EPAC2 is connected to retinal degeneration and that cGMP in some way, perhaps through binding, may influence EPAC2 activity. The latter was supported by the indication of the *rd10* retina having lower EPAC2 activity compared to a healthy retina.

The link between EPAC2 and cGMP was further supported in Paper IV with PLA, which revealed a significant increase in the number of interactions between cGMP and EPAC2 in the ONL within the *rd1*. Interestingly, no such significance in interaction was seen between EPAC2 and its main mediator, cAMP[102], within any of the *rd* models or the healthy counterparts. In contrast to *rd1* and *rd10*, the *rd2* model did not show a significant outcome in any experiments performed in Paper IV. However, this may be explained by their different mutations, where *rd2* has a mutation in the PRPH2 protein, whereas *rd1* and *rd10* have mutations in the PDE6β, as described previously. Furthermore, it can be speculated that if cGMP can mediate EPAC2 activity in the retina, then because of the lower cGMP level within *rd2*[44,147], cGMP could not influence EPAC2 as much as otherwise seen in the *rd1* and *rd10*.

Since EPAC2 can interact with both cAMP and cGMP[102,169], EPAC2's activity might be affected by changes in the nucleotide levels. In *rd10* and *rd1,* the mutated PDE6β leads to accumulated cGMP[44], and in Paper IV, we showed that such accumulation is also actual for cAMP. Accumulated cGMP, which is known to be an early marker of RP cell death[134], overlapped with accumulating cAMP in some photoreceptors. Meanwhile, accumulated cAMP was the only nucleotide coinciding with TUNEL[+] photoreceptors. In addition, cells having elevated EPAC2 expression also seemed to co-localize with accumulating cGMP (Figure 10). However, co-staining with EPAC2 and cAMP was not conducted because both antibodies were raised in rabbits. Nonetheless, it could be hypothesized that the accumulating cAMP could be brought about from the activatory or inhibitory cGMP actions on the cAMP, mediated by the hydrolyzation via cGMP-regulated PDEs (PDE2 and/or PDE3)[170] or adenylyl cyclases involvement in rod death[171]. This would be consistent with our findings that the increase in cGMP in a respective photoreceptor seems to appear before cAMP, and finally TUNEL positivity. Furthermore, cAMP accumulation may also be the photoreceptors' action to initiate cell survival[172] due to the increasing cGMP levels[44]. Although, ultimately, the elevated cAMP levels may likewise become toxic and result in photoreceptor cell death[172]. Even so, it can be hypothesized that the discrepancy in EPAC2 bindings, described above, with cGMP or cAMP, may depend on the level of the nucleotides within the respective photoreceptor and its current state in the cell death progression. This would be in

agreement with at least the significant increase of interactions seen between EPAC2 and cGMP due to their overlap within the *rd1*.

The nucleotide levels within degenerating photoreceptors might mediate EPAC2 based on their contradicting abilities, where cAMP can activate[102] and cGMP may inhibit EPAC2 activity[101] in the retina. To see how dysregulation of EPAC2 activity would affect the *rd* retina, we performed organotypic retinal cultures of *rd10* retinas in a long treatment paradigm to ensure pharmacological manipulation before photoreceptor death[44] (Paper IV). It showed that an EPAC2 activatory compound significantly promoted cell survival when compared to EPAC2 inhibiting conditions. This coincides with some of the above-mentioned abilities of EPAC2[167], including that activating EPAC2 can improve the situation, and thus it may be implied that EPAC2 activity may have neuroprotective abilities and thus may be a therapeutic target against retinal degeneration.

Despite the current information, EPAC2's role in RP remains obscure. Although it can very well be that altered EPAC2 activity may influence various EPAC2-related pathways in the retina like the EPAC2/Rap1-MAPK[173]-, EPAC2/Rap1-Akt-system[167] (Rap 1 signaling; KEGG version 2021), or the MAPK-signaling, the latter through the Ras-like GTPase family members Rap1 and Rap2[157] (Figure 11). These pathways may somehow govern the degeneration[68] and, therefore, proteins relevant to the cGMP-system like EPAC2 and others (described below) may be promising targets to promote cell survival.
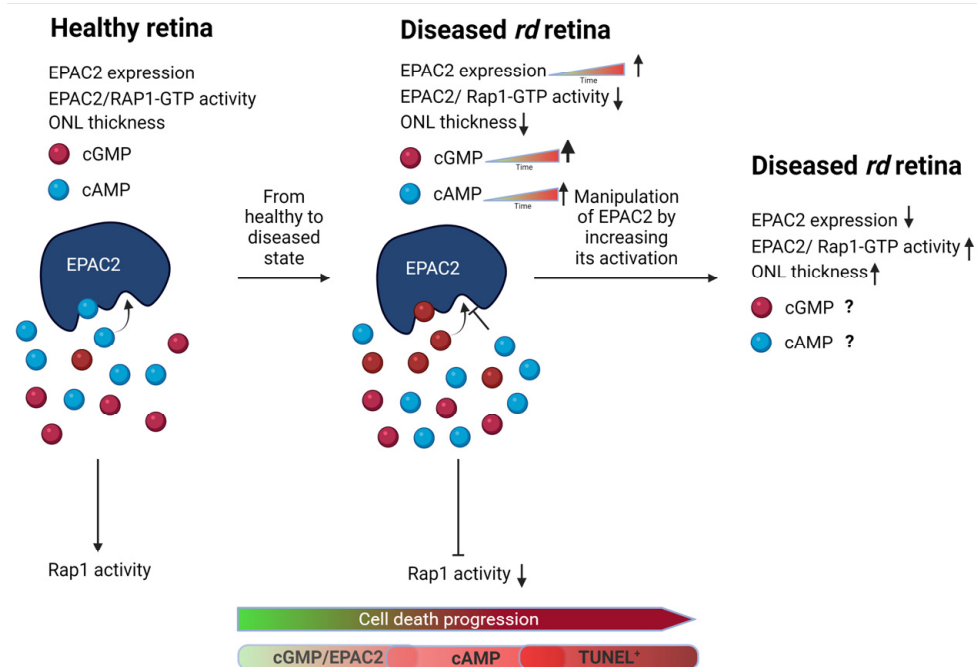
Figure 10 I An illustration of EPAC2's potential role in diseased *rd* retina. In some diseased *rd1* and *rd10* retinas, it is indicated that the EPAC2 expression temporally increases within the ONL. This increase may be due to the elevated nucleotide levels (*i.e.,* cGMP and cAMP) in the early stage of cell death. Furthermore, it is seen that cGMP interacts more with EPAC2 than cAMP, creating a scenario where cGMP thus could impede EPAC2's activity and potentially promote cell degeneration. Moreover, manipulating EPAC2 activity by increasing its activity in the *rd10* models suggests that EPAC2 may have a neuroprotective effect by impeding ONL reduction. Created with BioRender.com.

MAPK - The MAPK-pathway proteins MAPK1 and MAPK3 were, like EPAC2 (Paper I), found relevant to photoreceptors since the loss of MAPK1/3 function causes thinning of the two layers, namely ONL and INL[86]. Therefore, it can be assumed that these MAPKs play an important role in photoreceptor survival[86]. MAPK1/3 relevance to the cGMP-system was further supported when an association was found with regular cGMP and modified cGMPS' (Paper I and II), as well as CN03-analogs (Paper III). However, the likelihood of MAPK1/3 binding these nucleotides directly may be minor since in Paper I, the non-phosphorylated MAPK1/3 did not show numerous interactions with cGMP within the photoreceptors[114] like, *e.g.,* EPAC2 or PKGI. This may be because non-phosphorylated or phosphorylated MAPK1/3 exists in a complex with cGMP, perhaps including PKG[84,85]. It has previously been inferred that cGMP could interact directly with MAPK kinases[83], although a direct binding between PKG and MAPK1/3 has, to the best of our knowledge, not been verified yet.

Nevertheless, MAPK1/3 may still be relevant to the cGMP-system and retinal degeneration. A study by Roy et al., 2022, showed that the MAPK1/3 activity is not significantly altered within the whole *rd1* retina when compared to a healthy

retina[68]. However, the study revealed that when using CN03 to manipulate PKG activity, PKG may govern MAPK3 activity within the *rd1* model[68]. Though, this study quantified the MAPK activity from the entire retina, and thus it did not reflect the current situation within the photoreceptors. In Paper III, we showed more particularly that such MAPK1/3 activity does not seem to be substantially altered within the ONL in the cross-sectional stainings of *rd1*, *rd10,* and their respective healthy counterparts, which in some way is comparable to the above-mentioned outcome[68]. However, neither did such activity show to be considerably governed by CN03 manipulation within the ONL of cultured *rd1* and *rd10* retinas (Paper III).

On the other hand, in Paper III, the segments within *rd1* and *rd10* generally seemed to express more MAPK1/3 and activated MAPK1/3 than the ONL, though the *rd2* segments showed significantly higher expression and activity per μm^2 compared to its ONL and healthy counterpart. This discrepancy between MAPK1/3 expression and activity between the models may be linked to their different disease mutations and characteristic of the genetic backgrounds[174,175]. However, we could not analyze the effect of the CN03 treatment on the segments from the cultured retina due to their poor morphological condition. Therefore, we could not elaborate on whether perhaps the MAPK1/3 in the segments could have been significantly impacted by such manipulation and thus, in some way, would have reflected the outcome in Roy et al., 2022.

Even though MAPK1/3 activity may not be significantly upregulated within photoreceptors, the cGMP-PKG-systems might, together with other pathways like the cAMP-PKA-[176,177] and PI3K-Akt-pathway, still modulate MAPK-signaling[68,98]. MAPK1/3 could eventually then influence other downstream systems where MAPK-signaling likewise is involved, *e.g.,* mTOR-pathways and thus HIF-signaling (KEGG version 2021). Notably, the three systems' (*i.e.,* MAPK-, mTOR-, and HIF-signaling) activities were all found increased in the *rd1* retina and downregulated after CN03 treatment[68,178]. However, how significant effect MAPK1/3 may have on these systems, as well as what effect the systems may have on photoreceptor degeneration, is elusive (Figure 11).

CaMKII - The multifunctional serine/threonine-protein kinase CaMKII (*i.e.,* α, β, γ, and δ) regulates various neuronal functions and is activated by Ca^{2+}/calmodulin[179]. The calmodulin-binding introduces a conformational change that allows autophosphorylation, which turns the CaMKII isoform, like CaMKIIα, into a constitutively active form (*i.e., p*-CaMKIIα)[180]. Both CaMKII and *p*-CaMKII are localized in photoreceptors, where CaMKII has been suggested to be involved in photoreceptor degeneration in the *rd1* model[130]. However, CaMKII isoforms are localized to different retinal cell types[181], and in Hauck et al., 2006 no discrimination was made between the various CaMKII isoforms[130]. Thus it remains a question whether one specific CaMKII isoform may be more critical for photoreceptor survival than others. However, Hauck et al., 2006 found that *p*-CaMKII was only localized in cone segments upon the healthy retina, whereas it was present in both

rod and cone segments in the *rd1* retina[130], reinforcing CaMKII's and, more specific, the isoforms; CaMKIIα, CaMKIIβ, and CaMKIIδ, role in the retina and their likelihood of having neuroprotective abilities[181–184]. In Paper I, II, and III, we showed that CaMKII also is connected to the cGMP-system, since cGMP(S)-nucleotides and CN03 pulled down CaMKIIα/β/γ/δ, although the analogs only isolated some of the CaMKII isoforms[110] (Table 3). In Paper I, CaMKIIα's relevance to the cGMP-system and thus the degeneration was further reinforced since *p*-CaMKIIα showed proximity with cGMP in the ONL and INL[114]. However, whether cGMP interacts directly with general CaMKII is unknown, though it may be that cGMP is in a complex including PKA[185] or that CaMKII interacts with MAPK1/3[186], where the latter also is associated with the cGMP-system (Paper I, II, III). Furthermore, general CaMKII is involved in the Long-Term Potentiation and Circadian entrainment pathways (KEGG pathways version 2021), which were upregulated in the *rd1* model and downregulated after CN03 manipulation[68] (Figure 11). Through these pathways, CaMKII could potentially, and maybe CaMKIIα as well, influence MAPK- and PKG-signaling (KEGG pathways version 2021). Nonetheless, further studies are required to verify the association between the CN03 analog and CaMKII as well as CaMKIIα's role in the degenerating photoreceptor and whether it could be a therapeutic target.

GSK3β - The last protein to be emphasized is the GSK3β isoform of the constitutively active glycogen synthase kinase-3[110,114]. GSK3β was likewise associated with general cGMP, modified cGMPS' and the CN03 analog in Paper I, II, and III, respectively. GSK3β have been localized to inner segments of photoreceptors[187], and the levels of GSK3β^{ser9} are elevated in the *rd10* model compared to its healthy counterpart[188]. Interventions with compounds that can target and inhibit general GSK3 have shown cell survival abilities[189] and abilities to reduce photoreceptor cell death[188,190]. Together, this makes GSK3 a promising therapeutic target to impede retinal degeneration in *rd* mouse models. However, like MAPK1/3, GSK3β^{ser9} also indicated only little proximity with cGMP within the photoreceptors (Paper I). Hence, it is most likely that GSK3β^{ser9} does not interact with cGMP directly. Instead, it may be integrated into a complex including cGMP[114] and possibly PKGI since PKGI can phosphorylate GSK3β at the serine 9 position and thus inhibit GSK3β activity[191,192]. Albeit, whether the cGMP-PKG system can significantly govern GSK3β activity in the photoreceptors and thus influence, *e.g.,* the mTOR-signaling (KEGG pathways version 2001, Figure 11) through GSK3β mediation remains to be investigated.

To summarize, Papers I, II, III, and IV have aided in comprehending the cGMP-system by suggesting proteins that might be associated with the system and may propel the mediation of either photoreceptor degeneration or survival within RP.

A simplified map, mainly accentuating the proteins assumed to be connected to the cGMP system as well as previously designated to be associated with the CN03 analog (*e.g.,* PKA, PKG, CaMKII, EPAC2, MAPK, and GSK3), is depicted in

Figure 11. It should be clearly stated that numerous other proteins are also involved within these pathways, and some of them have also been governed by the degeneration and CN03 manipulation[67,68]. Nonetheless, it could be hypothesized that the proteins emphasized within this thesis may have a key role within the retinal degeneration mechanism(s) since some of the proteins are involved in pathways that were found to be reciprocally regulated in CN03-treated retinal cultures[68], *i.e.,* Long-Term Potentiation, Insulin resistance, mTOR-signaling, Circadian entrainment, and the HIF-signaling. However, whether all these proteins and pathways also represent the actual situation within dying photoreceptors and thus are involved in the RP disease should be further studied.
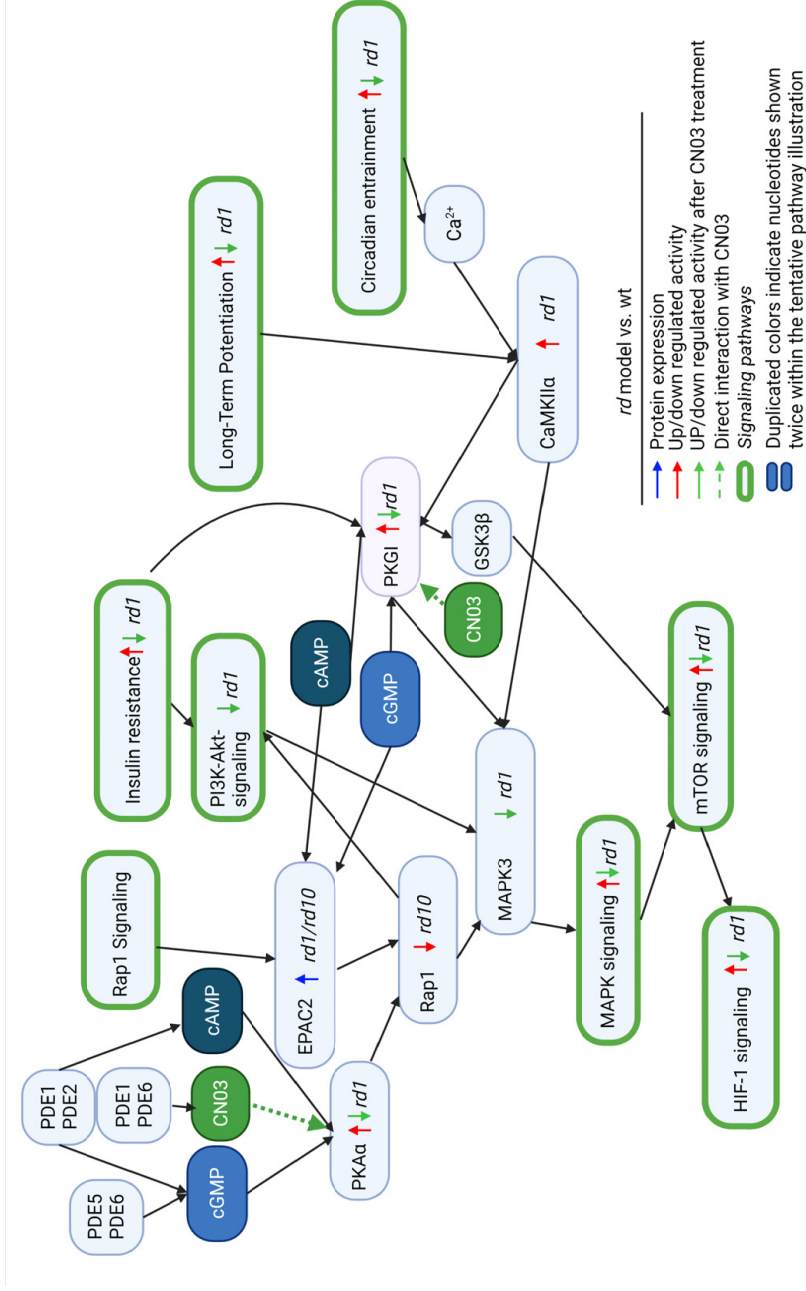
Figure 11 | A simplified tentative pathway illustration of cGMP and CN03 associated proteins that were found relevant to the retinal degeneration mechanism(s) in Paper I, II, and III. The proteins; PKAα, PKGI, CaMKIIα, EPAC2, MAPK3, and GSK3β, have previously been designated to be associated with the cGMP, the cGMP-system, and the CN03 analog, suggesting that they may be relevant to the retinal degeneration mechanism. Furthermore, these proteins are involved within various pathways like the Long-Term Potentiation, Insulin resistance, mTOR-signaling, Circadian entrainment, and the HIF-signaling, where all the aforementioned pathways activity have shown to be reciprocally regulated after CN03 manipulation in retinal *rd1* cultures (Roy et al. 2022). Created with BioRender.com.

CONCLUDING REMARKS

This thesis successfully optimized and performed a proteomic approach (Paper I, II, III) which aided in comprehending known cGMP-interacting proteins as well as new cGMP-associations in healthy and *rd* retinas (*i.e., rd1, rd10,* and *rd2*). Paper II reveal that cGMPAAs can capture various known cGMP-binding proteins, *e.g.,* PKAs, PDEs, PKG, and EPAC2, albeit, the stereospecific requirements and the different cGMPAAs' compositions may influence the respective interactions. The cGMP nucleotides' selectivity and target specificity may be used for 1) finding new cGMP targets or 2) providing valuable knowledge in drug design in the field of cGMP-analog-based therapies. In Paper I, the approach helped find five photoreceptor-relevant proteins (*p*-CaMKIIα, GSK3β^{ser9}, MAPK1/3, and EPAC2), which revealed proximity with cGMP. This arguments in favor for that these proteins thus can be connected to the cGMP-system and potentially also to the degeneration. Notably, whether these interactions were potentially direct (*i.e., p*-CaMKIIα and EPAC2) or indirect (GSK3β^{Ser9} and MAPK1/3) could not be clarified. Nonetheless, Paper II and III further supported some of these proteins' relevance to the cGMP system. Paper III showed that the modified cGMP-analog CN03 binds seven known cGMP-interacting proteins (PDE1β, PDE1c, PDE6α, PKAα, PKA1α, PKAβ and PKGIβ) in contrast to regular cGMP, which was found to bind 12 known cGMP-interactors (Paper I). Furthermore, CN03 was also associated with GSK3β, MAPK1/3, and CaMKII isoforms, although not CaMKIIα. It can therefore be postulated that CN03 may impede degeneration through some of these proteins and the pathways they are involved in (*e.g.,* MAPK-signaling, Long-Term Potentiation, Insulin resistance, mTOR-signaling, Circadian entrainment, and HIF-signaling).

Overall, the results from Paper I, II, and III indicate that the above-mentioned proteins may be important to decipher the cGMP signaling pathway and hence the degeneration mechanism(s) as well as reinforces the idea that therapy for RP could target, for instance, cGMP-interactors. This was further tested in Paper IV in healthy and *rd* retinas (*i.e., rd1, rd10,* and *rd2*). Here it was revealed that EPAC2 is also located within the ONL and rods' inner segments. Furthermore, its expression increases significantly during the degeneration progression within the ONL in the *rd1* and *rd10* models. In the *rd1* photoreceptors, a significant augmentation in the interaction between EPAC2 and cGMP was seen. On the contrary, the binding between cAMP and EPAC2 was not significantly altered within the *rd* models or the healthy retina. Furthermore, organotypic *rd10* retinal cultures revealed that

manipulating the EPAC2 activity may affect the cell viability in the *rd10* retina. Overall, this may suggest that EPAC2 is connected with the cGMP-system and rod degeneration and that EPAC2 activity might thus have a neuroprotective effect.

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APPENDICES