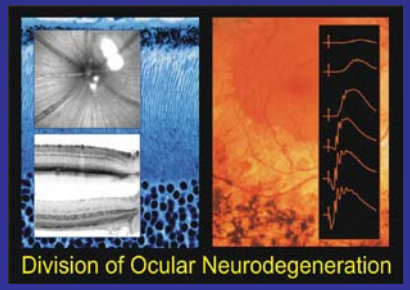
16.00 - 18.00

Restoration of cone vision in the Cnga3-/- mouse model of congenital complete lack of cone photoreceptor function using AAV-mediated gene replacement

Regine Mühlfriedel, PhD

Division of Ocular Neurodegeneration,
Centre for Ophthalmology, Institute for Ophthalmic Research
Schleichstr. 4/3, 72076 Tuebingen, Germany



Agenda

- Introduction: vector systems, entery pathway
- Investigation of the acute phase and long-term effects of subretinal gene transfer
- Restoration of cone vision in the CNGA3 knock out model: proof of principle

Gene therapy („gene replacement therapy“) is a technique for correcting corrupted genes that is responsible for disease development.

The technique aims to replace the corrupted gene with a correct version in the cells were it is needed.

Challenges of a gene replacement therapy

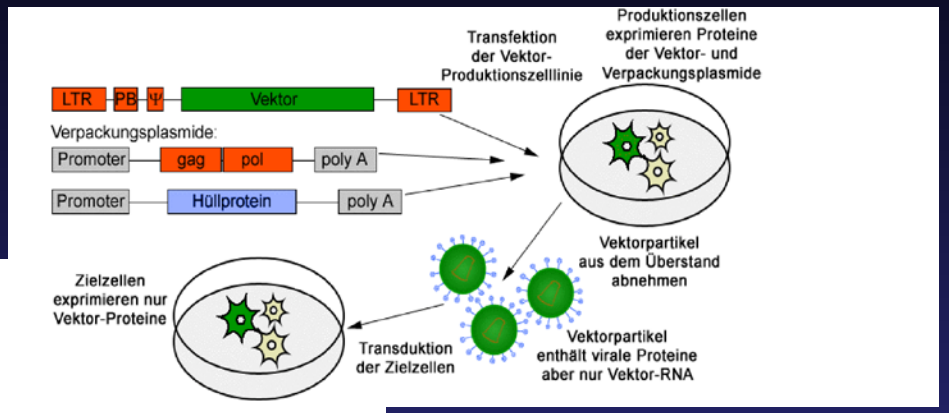
- Successful transfer of genetic information (e.g. transduction of a potent gene)
- Targeted transfer in specific tissues / cells and at the same time the guarantee of safety
- High compatibility/ tolerance and safety (e.g. documentary report about clinical trials, phase I)
- Clinical potency (e.g. control/ validation of clinical trials, phase II/III)

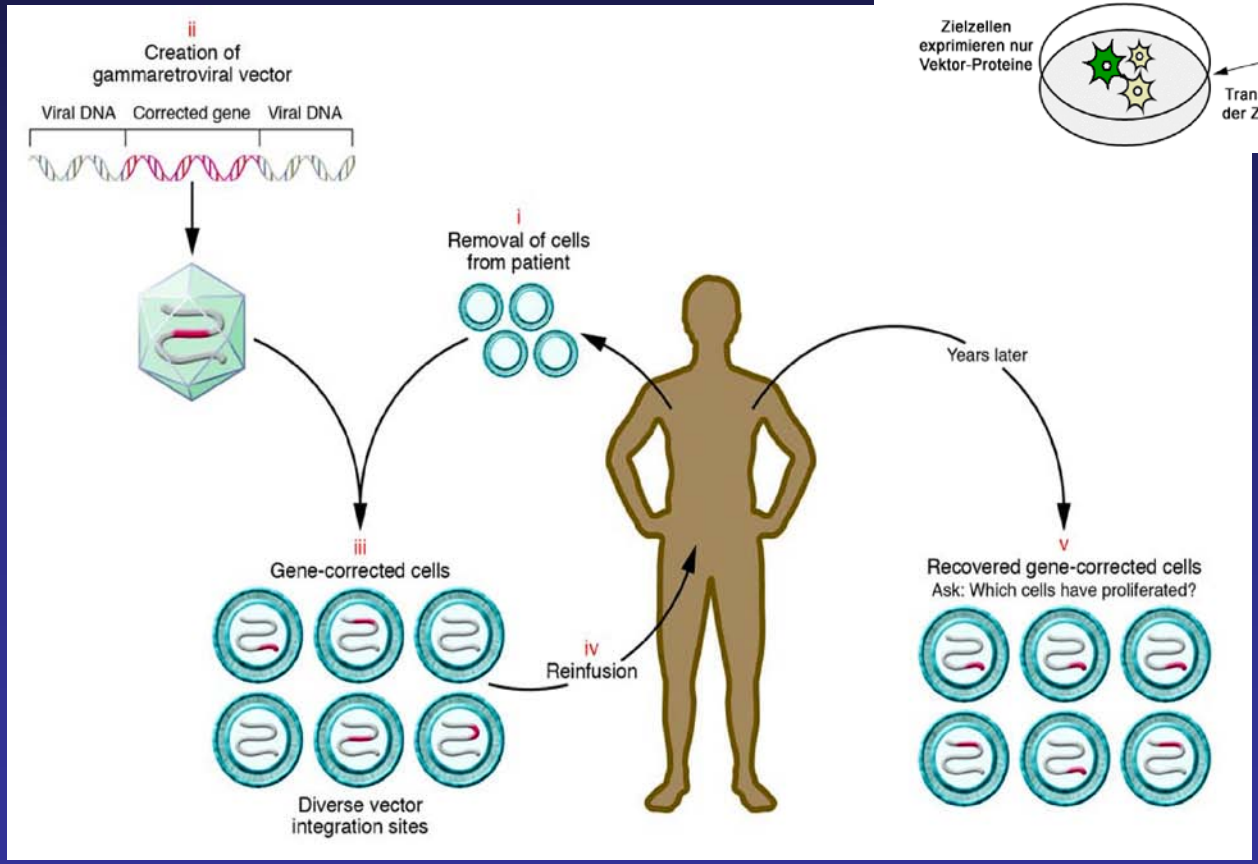
The technology of gene therapy

Ex vivo gene therapy:

- The cells come either from the same patient (autologous treatment) or from a donor (allogeneic treatment).
- Gene transfer is carried out in culture by many different techniques, involving viral or non-viral vectors.
- Due to limitations in growing, manipulating, and readministering cells from many tissues and organs, *ex vivo* gene transfer is today limited to blood, skin and liver cells, to cells of the immune system, or to tumor-derived cells used as cancer vaccines.

Ex vivo gene replacement:





Bushman F.D., ***The Journal of Clinical Investigation*** 2007

Gene therapy in patients:

- X-linked SCID (SCIDX1)
- ADA-SCID
- Chronic Granulomatous Disease (CGD)
- Neurological disorders (e.g. AD)

In vivo gene therapy:

- A therapeutic gene is administered to a specific tissue or organ for a defined appplication (e.g. direct transfection of somatic cells)

- The administration can take the form of particles derived from disabled viruses (viral vectors), artifical particles (synthetic vectors), or „naked“ DNA.

- The route of administration can be intravenous, intramuscular, by inhalation, or by direct injection into the target organ.

Indications addressed by gene therapy clinical trials 2010 (worldwide)

Indications	Gene Therapy Clinical Trials	
	Number	%
Cancer diseases	1060	64.5
Cardiovascular diseases	143	8.7
Gene marking	50	3
Healthy volunteers	38	2.3
Infectious diseases	131	8
Monogenic diseases	134	8.2
Neurological diseases	30	1.8
Ocular diseases	18	1.1
Others	40	2.4
Total	1644	

The eye is an ideal organ for correcting corrupted genes that are responsible for disease development:

- Anatomy: small, confined, paired, accessible
- Function: privileged immunology, established objective tests (e.g. ERG)
- Fairly good understanding of disease process
- Animal models of hereditary eye disorders

Gene Therapy Clinical Trials

- Stargardt's disease (*ABCA4*)
  - most common inherited juvenile macular degeneration
  - progressive visual loss starting in early life

  - Stargen® Oxford Biomedica

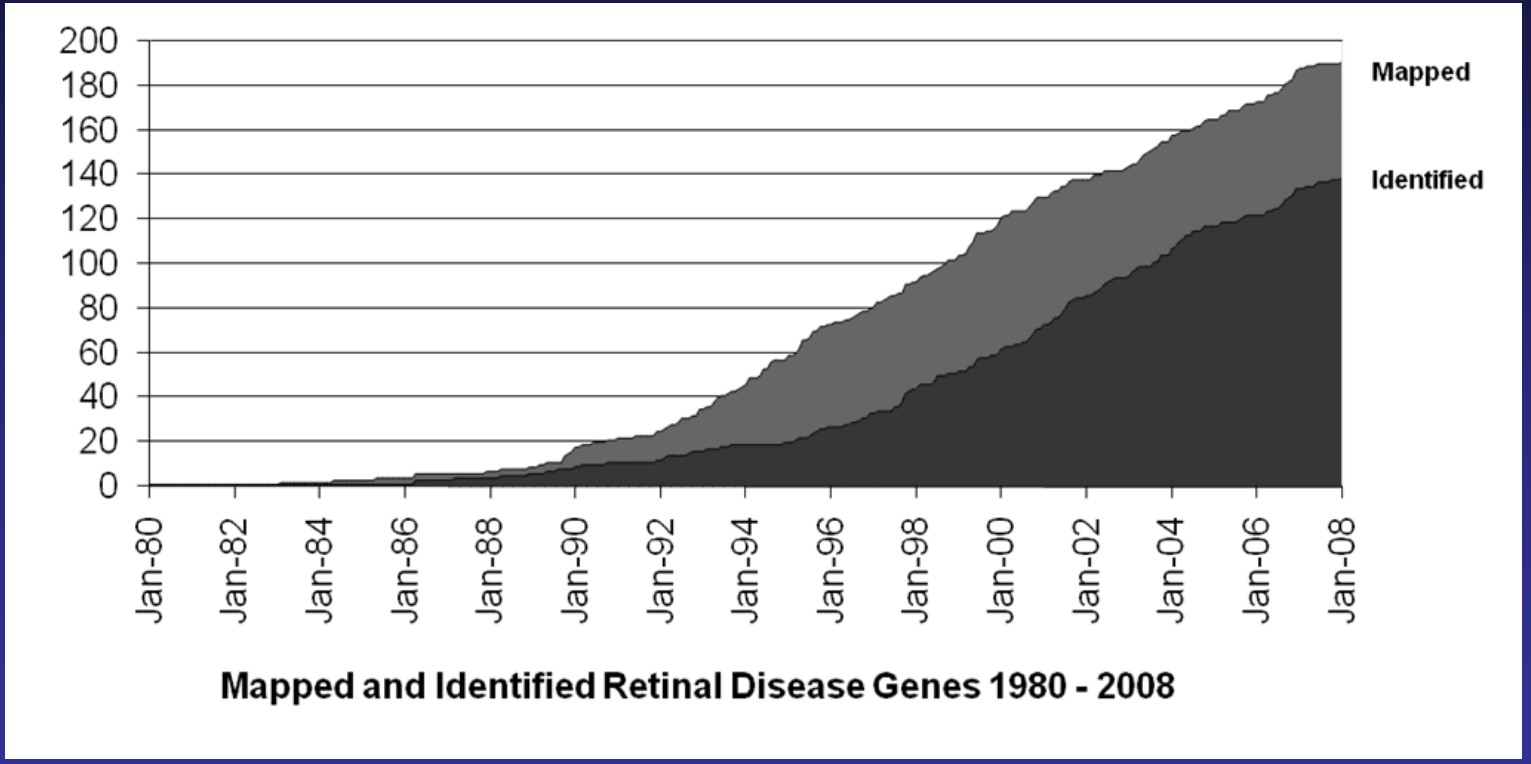
Gene Therapy Clinical Trials

- Leber's congenital amaurosis (LCA2: *RPE65* deficiency)
  - early loss of visual acuity
  - structure largely intact, function can be monitored
  - RPE as target tissue is phagocytotic
  - successful treatment in dogs with continuous benefit

LCA2 patients enrolled in clinical trials

- University College London (UCL)
- University of Pennsylvania (UPenn)

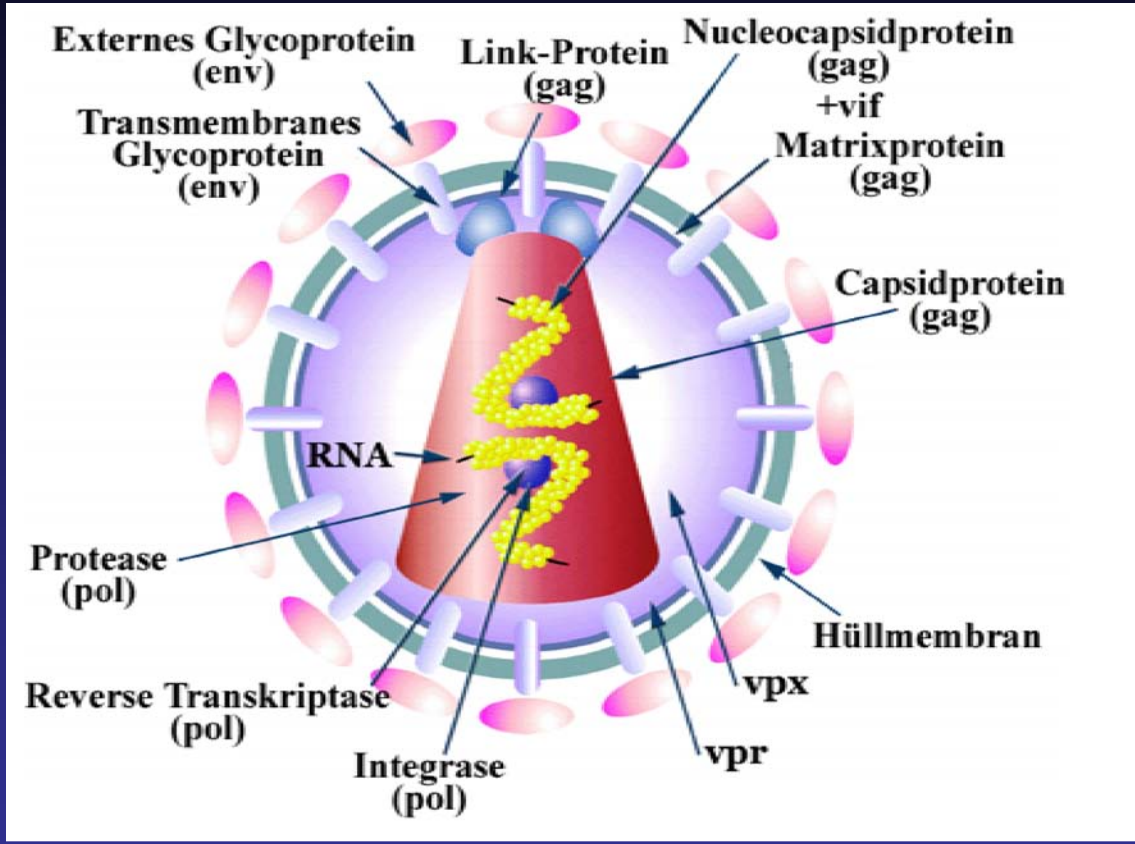
Inherited retinal diseases



Source: http://www.sph.uth.tmc.edu/retnet/

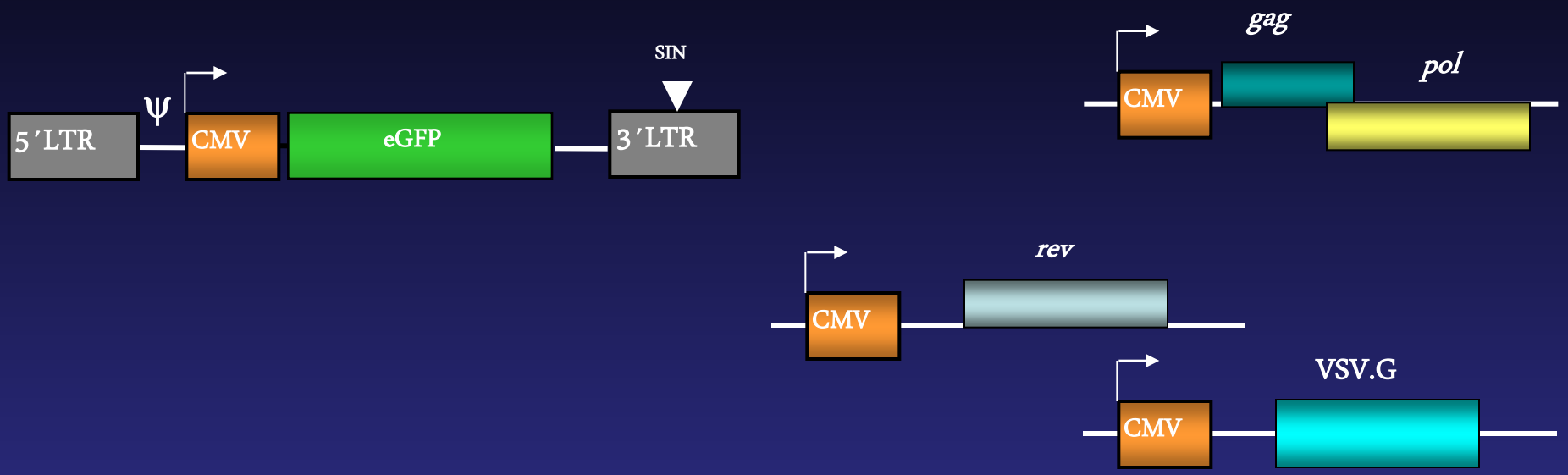
Vector	Gene Therapy Clinical Trials	
	Number	%
Adeno-associated virus	75	4.6
Adenovirus	392	23.8
Adenovirus + Retrovirus	3	0.2
Adenovirus + Vaccinia virus	3	0.2
E. coli	2	0.1
Flavivirus	8	0.5
Gene gun	5	0.3
Herpes simplex virus	56	3.4
Lactococcus lactis	4	0.2
Lentivirus	29	1.8
Lipofection	109	6.6
Listeria monocytogenes	3	0.2
Measles virus	4	0.2
Naked/Plasmid DNA	301	18.3
Naked/Plasmid DNA + Adenovirus	2	0.1
Naked/Plasmid DNA + Vaccinia virus	1	0.1
Newcastle disease virus	1	0.1
Poliovirus	1	0.1
Poxvirus	66	4
Poxvirus + Vaccinia virus	27	1.6
Retrovirus	341	20.7
RNA transfer	26	1.6
RNA virus	5	0.3
Saccharomyces cerevisiae	6	0.4
Salmonella typhimurium	3	0.2
Semliki forest virus	1	0.1
Sendai virus	2	0.1
Shigella dysenteriae	1	0.1
Simian virus 40	1	0.1
Sleeping Beauty transposon	3	0.2
Streptococcus mutans	1	0.1
Vaccinia virus	102	6.2
Venezuelan equine encephalitis virus replicon	2	0.1
Vesicular stomatitis virus	2	0.1
Vibrio cholerae	1	0.1
Unknown	55	3.3
Total	1644	

The lentiviral (LV) based vector system



- Enveloped virus containing a single stranded RNA molecule.
- Following infection, the viral genome is reverse transcribed into double stranded DNA.

-> Integration into the host genome and expression as proteins



3rd generation HIV-1 based lentiviral system:

+ replication-deficient lentiviral particles => increased **biosafety (level 2)**
+ large packaging capacity => up to 10kb
+ high viral titers possible ($\geq 10^9$ transducing units / ml)
+ infects neurons => infection of photoreceptors possible
+ integrates into genome => long lasting expression
+ fast onset of gene expression

— large particle size (80-100nm) => impairs tissue penetration

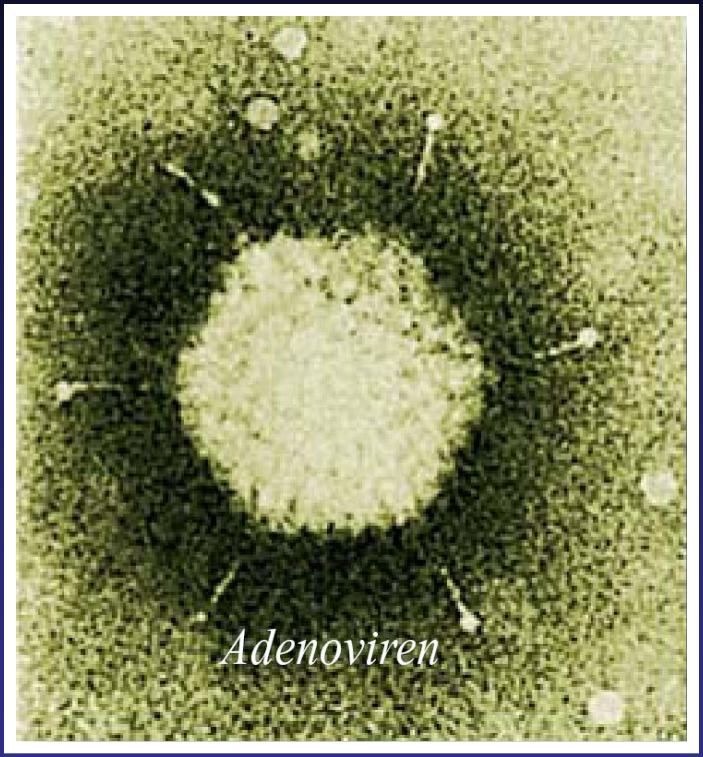
Stylianos Michalakis

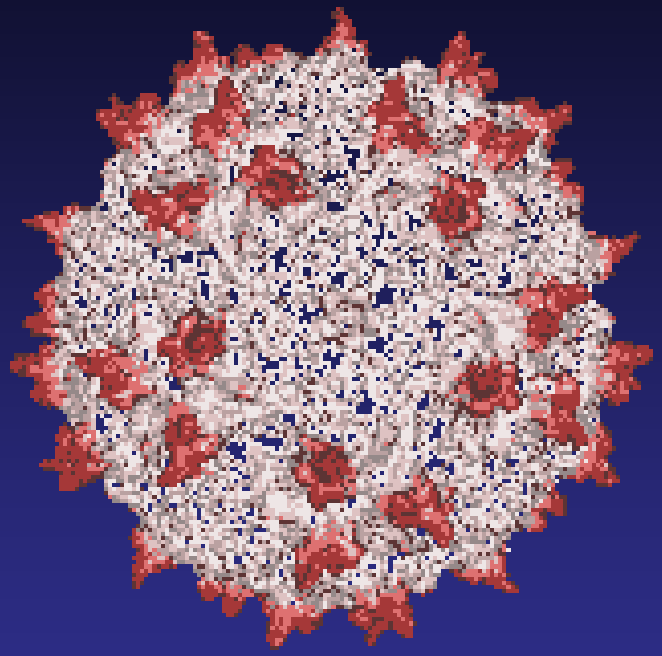
Gene therapy for neurodegenerative and ocular disease using lentiviral vectors

Disease	Approach	Gene target	Target tissue/cell type
AD	Gene silencing	β- and γ-Secretase, Tau, GSK-3 and Cdk-5	Cortex and hippocampus
	Overexpression	NGF and Nep	
PD	Gene silencing	α-Synuclein and LRRK2	Substantia nigra
	Overexpression	GDNF and dopamine biosynthesis enzymes	
HD	Gene silencing	Huntingtin	Striatum and cortex
	Overexpression	CNTF	
ALS	Gene silencing	SOD1	Spinal cord and brain stem motor neurons
	Overexpression	VEGF and IGF-1	
AMD/diabetic retinopathy	Gene silencing	VEGF	Retina
	Overexpression	Endostatin, angiostatin and sFlt1	
RP	Overexpression	PEDF and PDEβ	Photoreceptors
FED	Gene silencing	COL8α2	Corneal endothelium

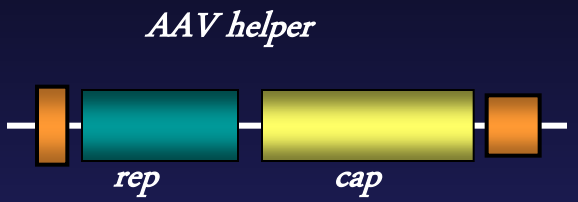
Ralph G. S. et al., ***Clinical Science*** 2006

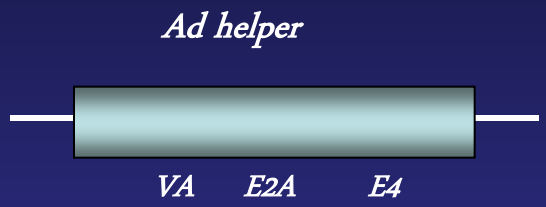
The adeno-associated viral (AAV) based vector system





- AAVs are non-enveloped viruses containing a linear single stranded DNA genome.
- AAV vectors are very efficient at transducing target cells in vitro & vivo.
- The life cycle does not normally involve integration into the host genome.
- They rather replicate as episomal elements (no risk of insertional integration).





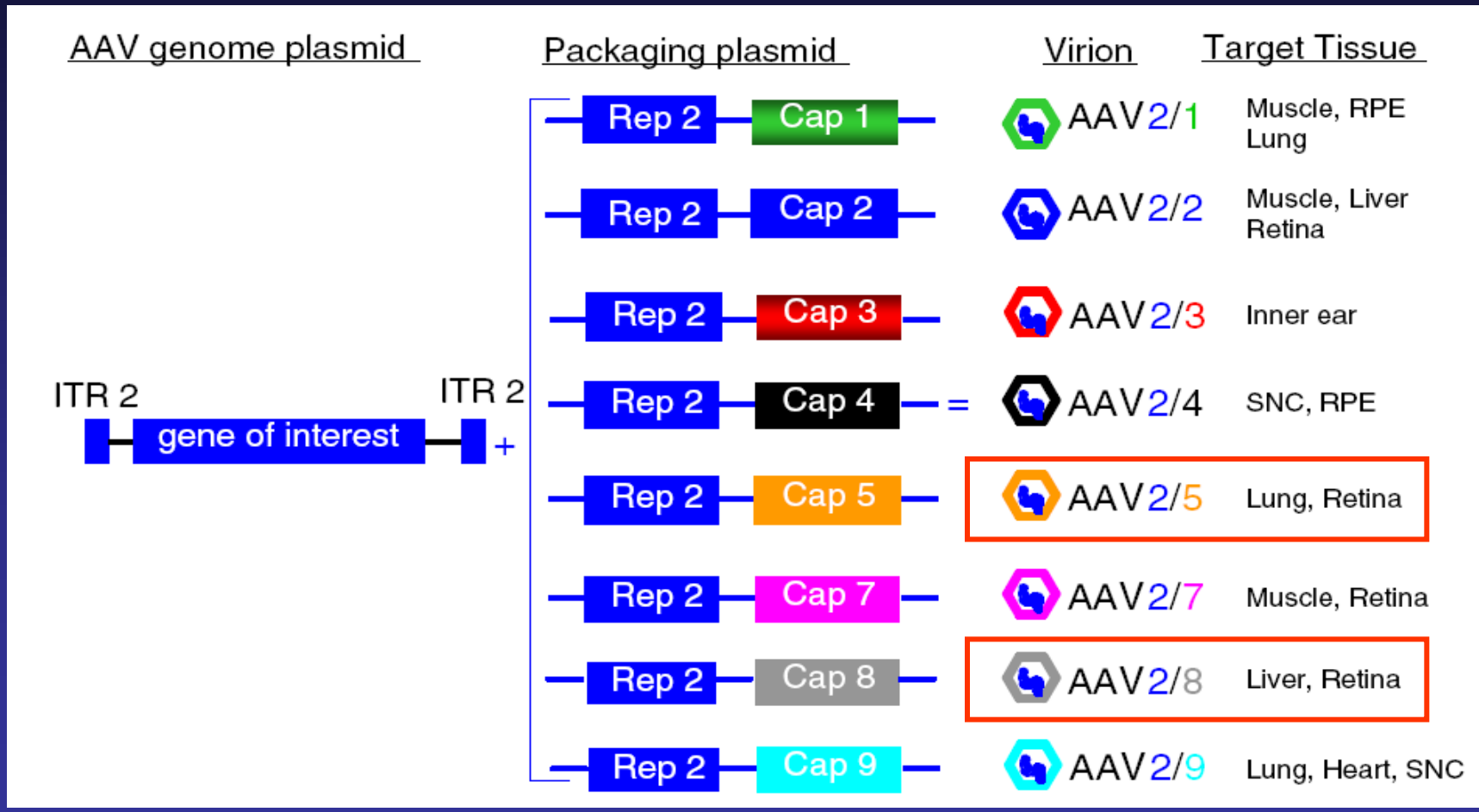
Stylianos Michalakis

biosafety level 1

+ infects neurons => infection of photoreceptors possible
+ does not integrate => but long lasting expression observed (>1 year)
+ small particle size (20-25nm) => good tissue penetration
+ many different serotypes possible => increased variability of tropism
+ high viral titers possible ($\geq 10^{13}$ genomic particles / ml)

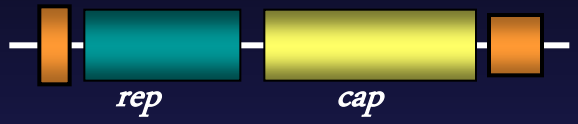
— difficult production method
— low packaging capacity (<5kb; except AAV5)
— single stranded AAVs: slow onset of gene expression in vivo

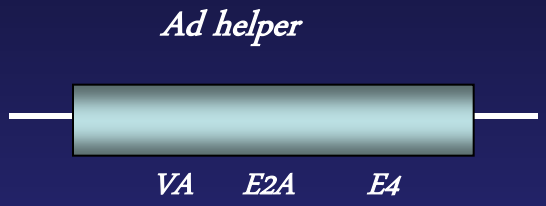
The recombinant adeno-associated viral vector system

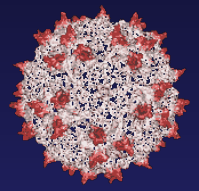


Auricchio A. , ***Vision Research*** 2008

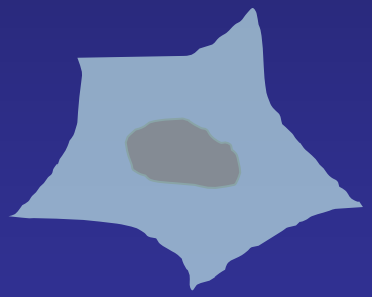

ITR
CMV
eGFP
ITR

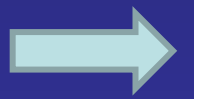

AAV helper
rep
cap

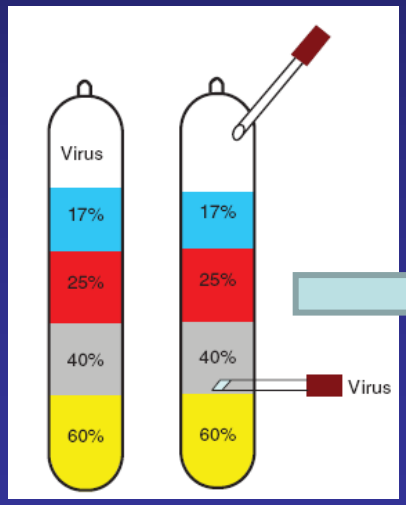

Ad helper
VA
E2A
E4


AAV


Transfection


HEK293T

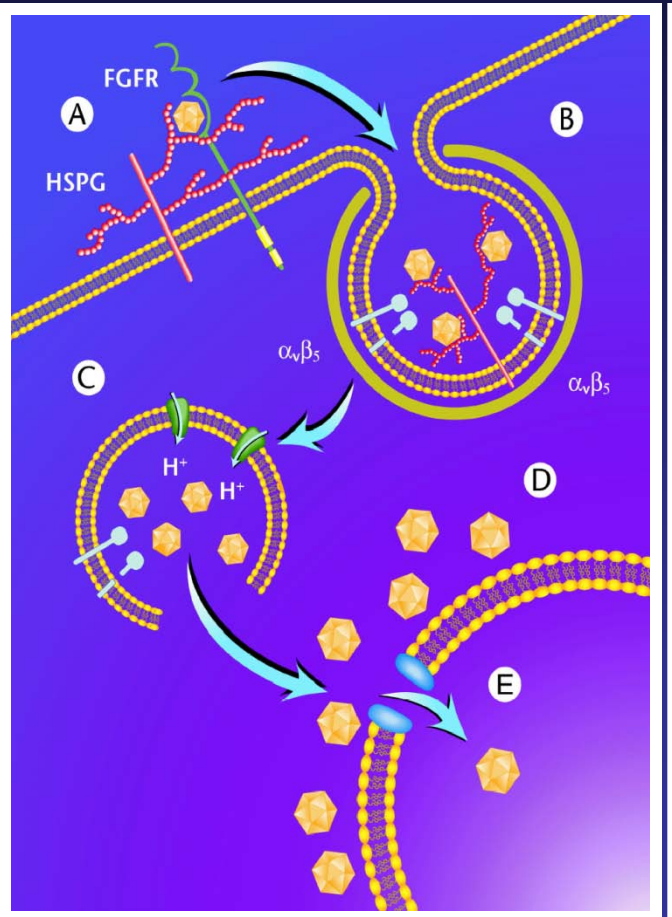

48h, lysis

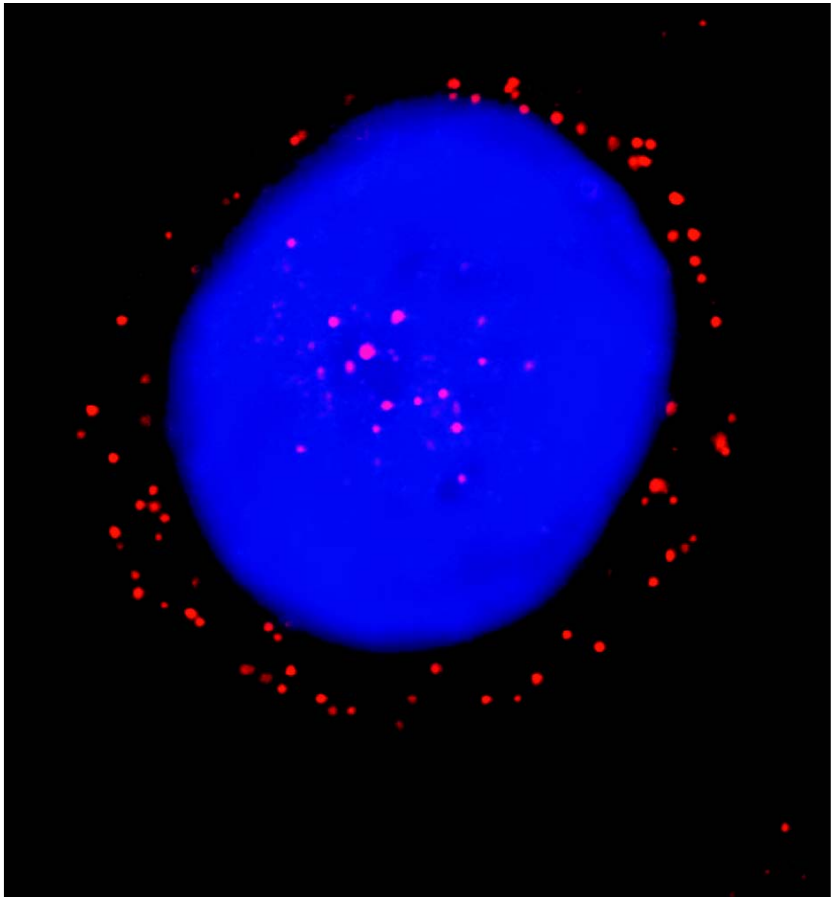

Virus
17%
25%
40%
60%
17%
25%
40%
Virus
60%
iodixanol-gradient
ultra-centrifugation


HiTrap-Q column
ion-exchange
chromatography


Amicon ultra
filter centrifugation

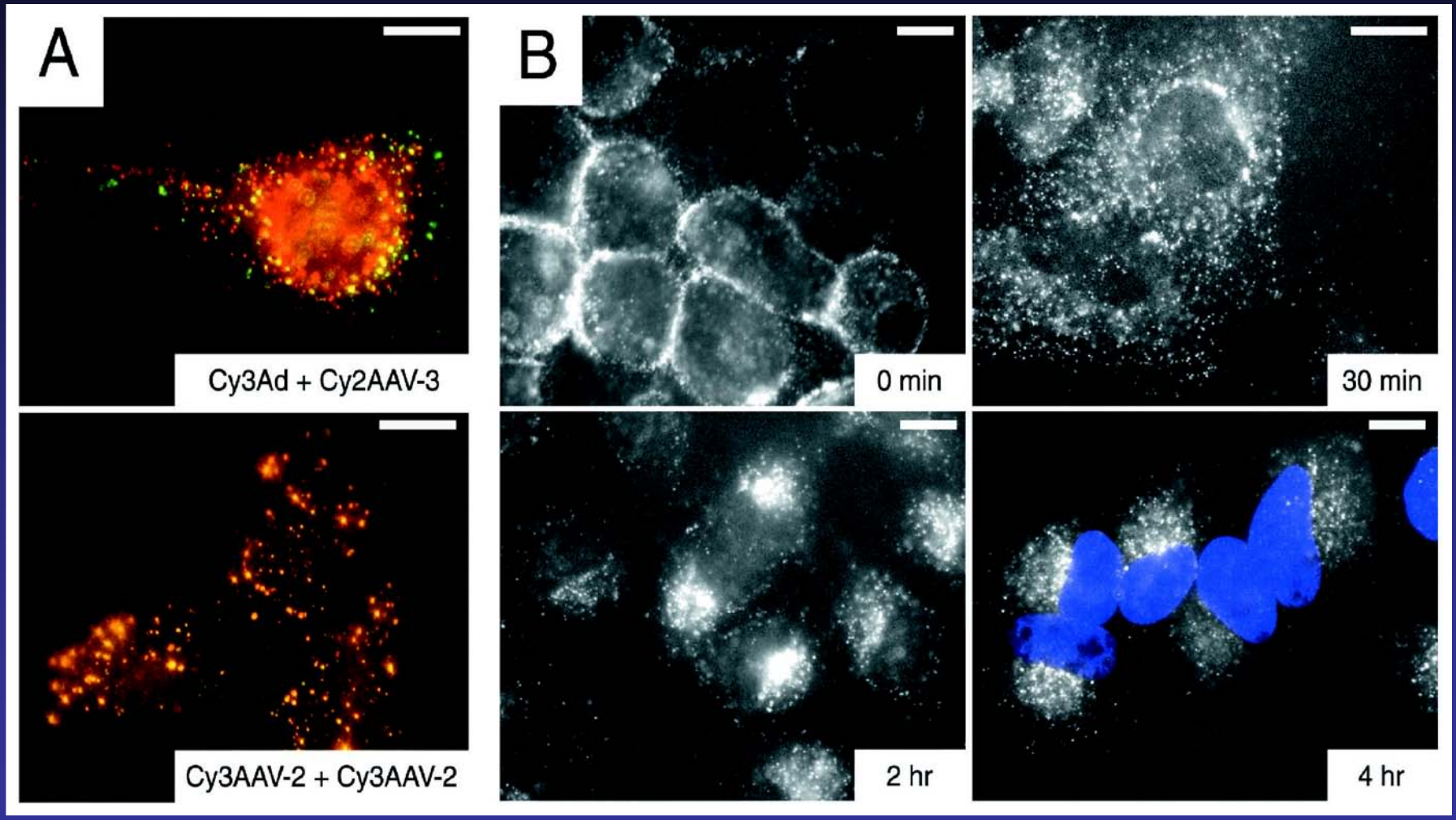
Infectious entry pathway of Adeno-Associated Virus and Adeno-Associated Virus vectors





Bartlett J. et al., ***Journal of Virology*** 2000

Pulse-labeling evaluation of fluorescent AAV distribution in HeLa cells



Bartlett J. et al., ***Journal of Virology*** 2000

Agenda

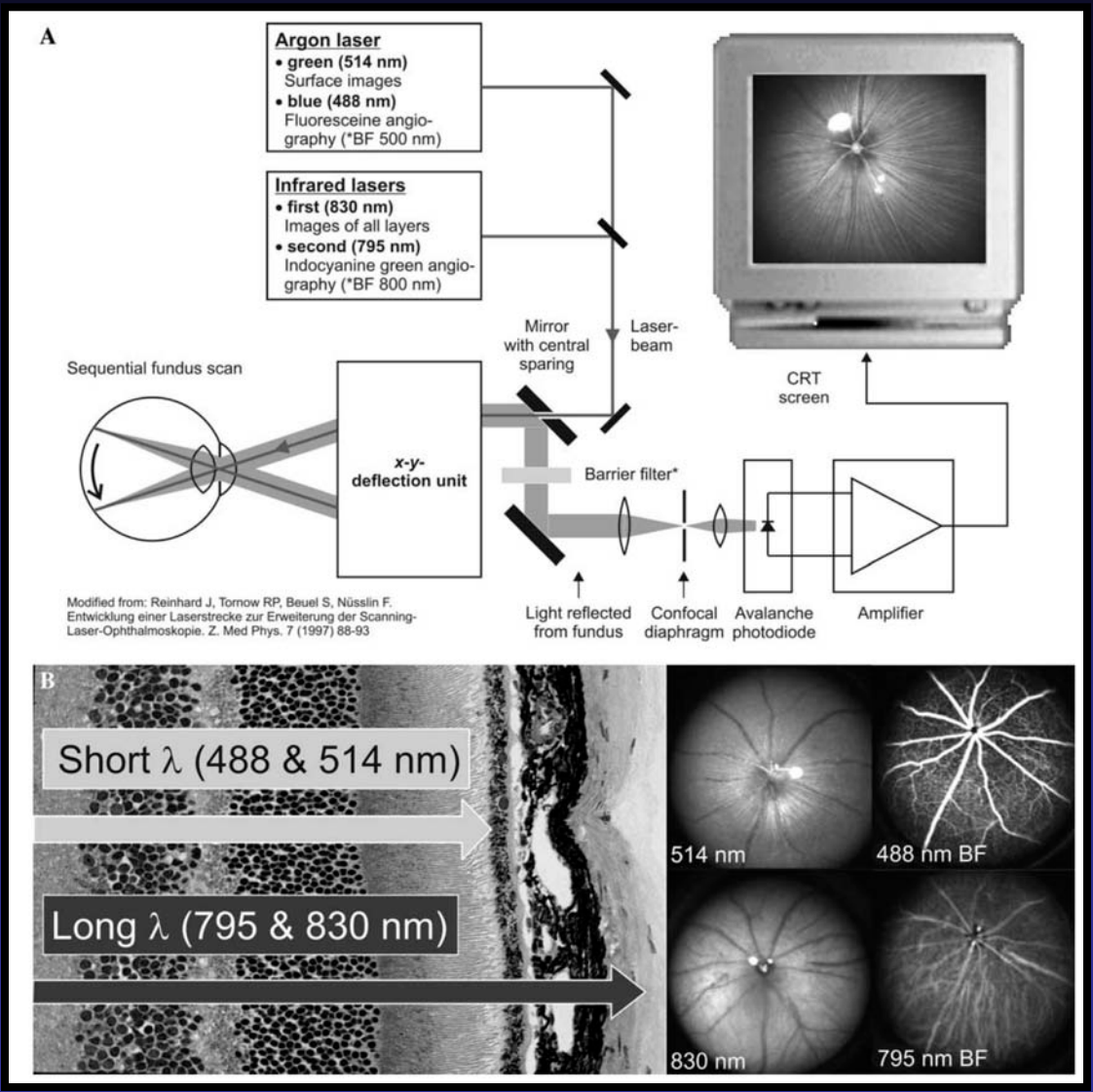
- Investigation of the acute phase and long-term effects of subretinal gene transfer

Aim of the study

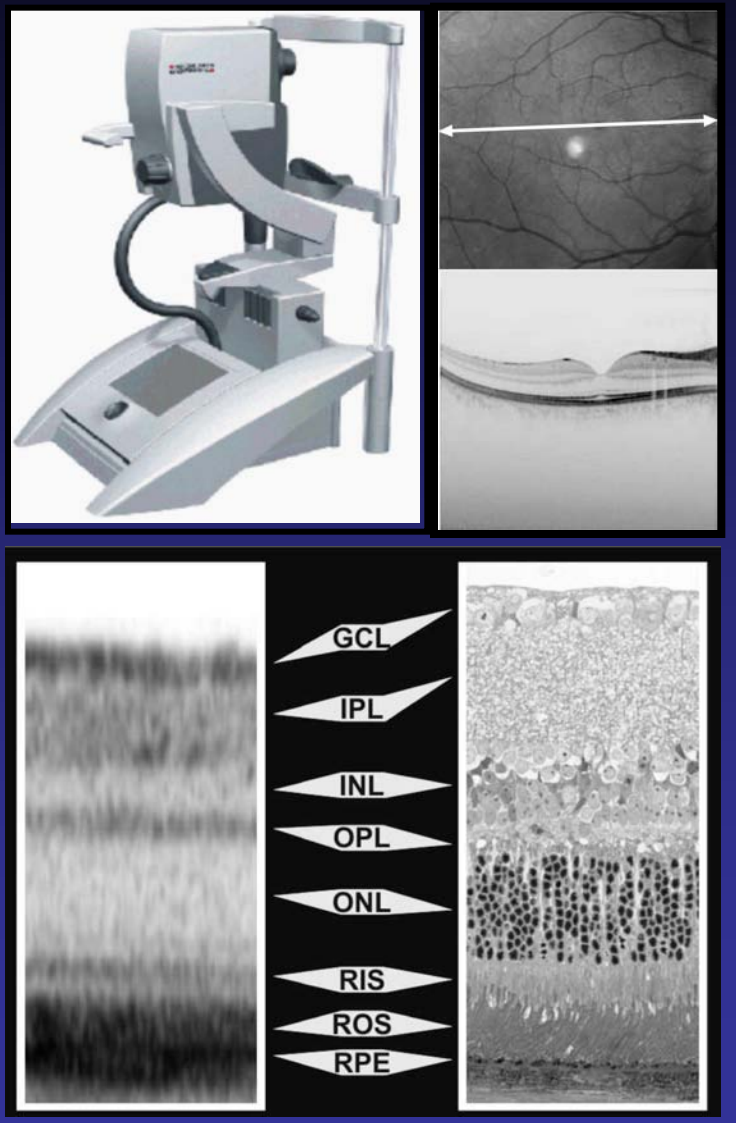
- Monitoring of subretinal injections and side effects (e.g. retinal detachment) using non-invasiv imaging techniques: cSLO imaging (fundus overview, autofluorescence) and SD-OCT (retinal thickness map, A-scans)

- In vivo studies addressing expression efficiency and promoter specifitcity followed up by eGFP expression patterns and intensities

- Evaluation of a long-term eGFP expression under the control of cone- and rod-specific promoters



Seeliger *et al.*, 2005, Vision Research



Fischer *et al.*, 2009, PLOS one

Experimental procedure

> Subretinal injection of different viral vectors in mice (WT, CNGA3-/-):

Titer: 6-9 10^9 genomic particles/ ml

Application site: ventral/ dorsal

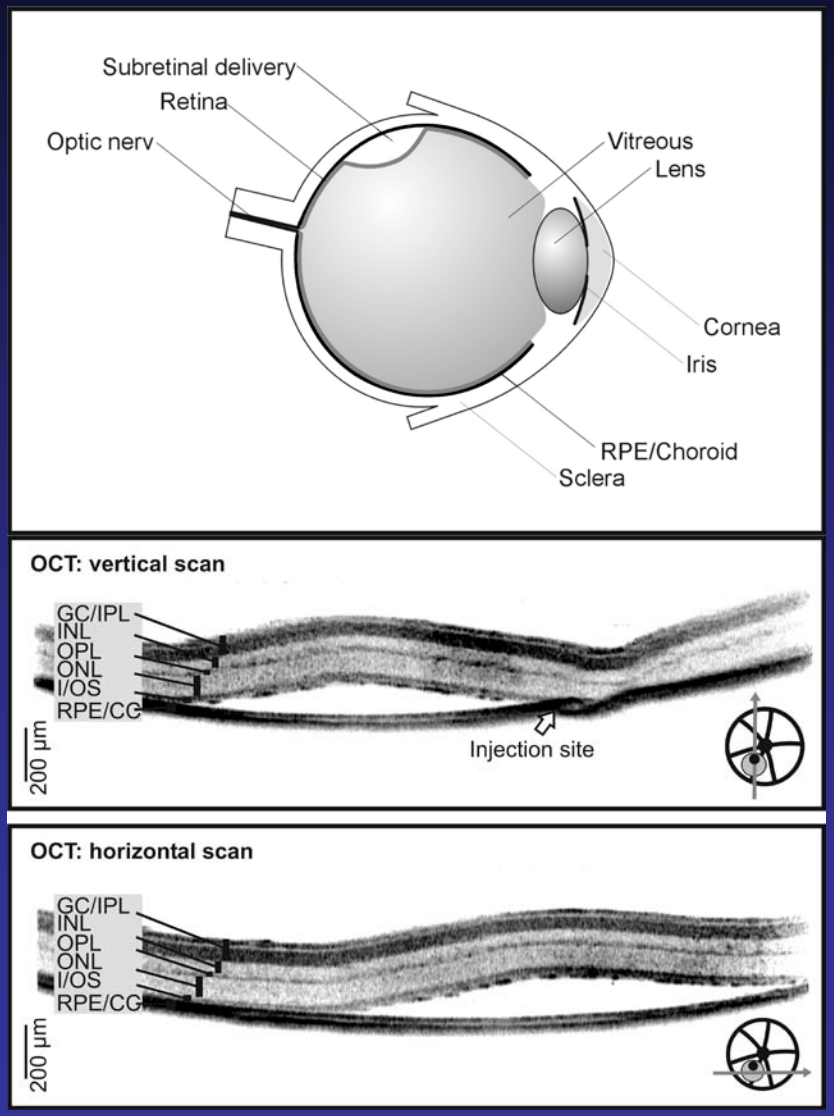
Injection volume: 1.0-2.0 µl

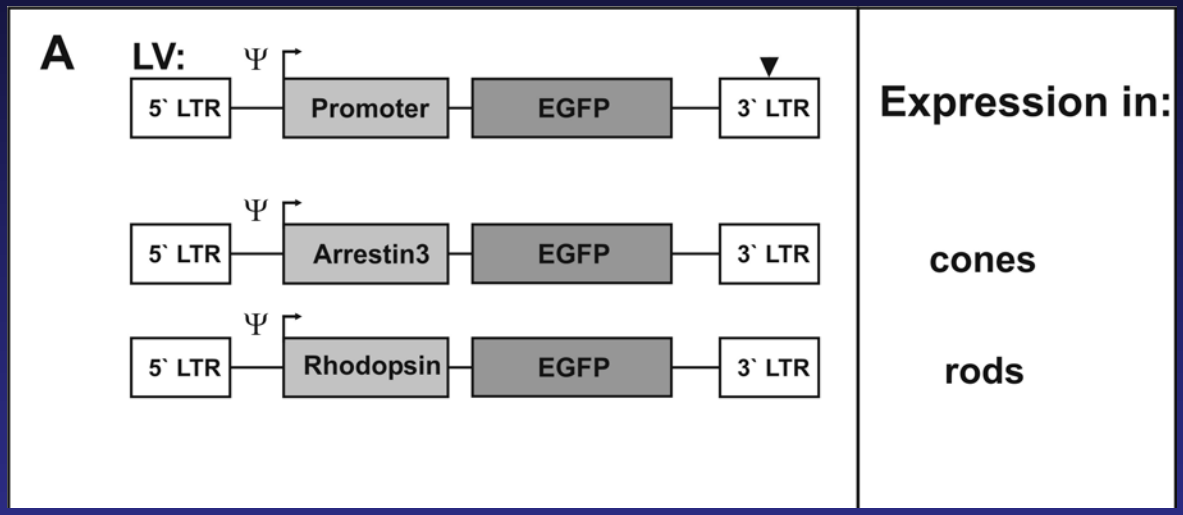
Age: PN 12d-PN 3m

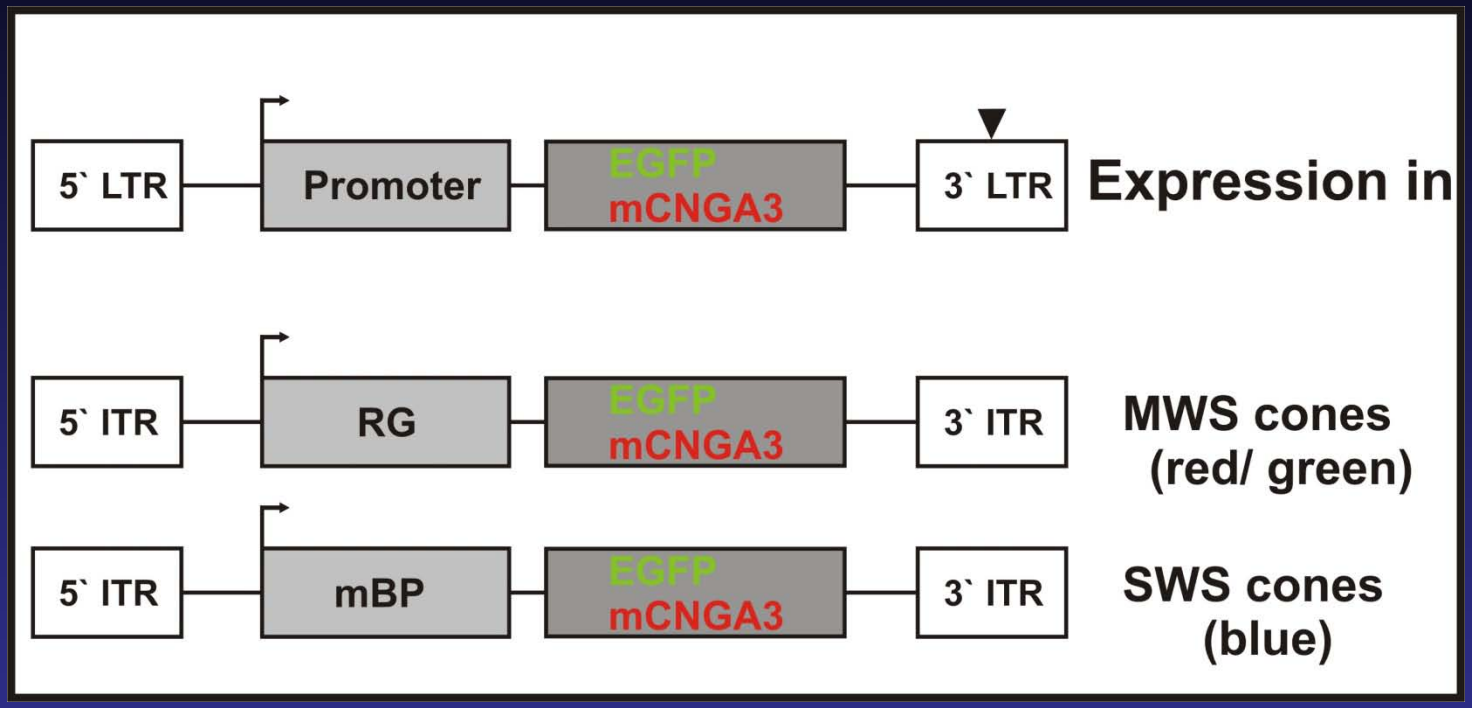
Time course: from PI 4d-16w

✓Quality control: SD-OCT

✓Assessment of EGFP expression: SLO




A
LV:
Ψ
5` LTR
Promoter
EGFP
3` LTR
Ψ
5` LTR
Arrestin3
EGFP
3` LTR
Ψ
5` LTR
Rhodopsin
EGFP
3` LTR
Expression in:
cones
rods



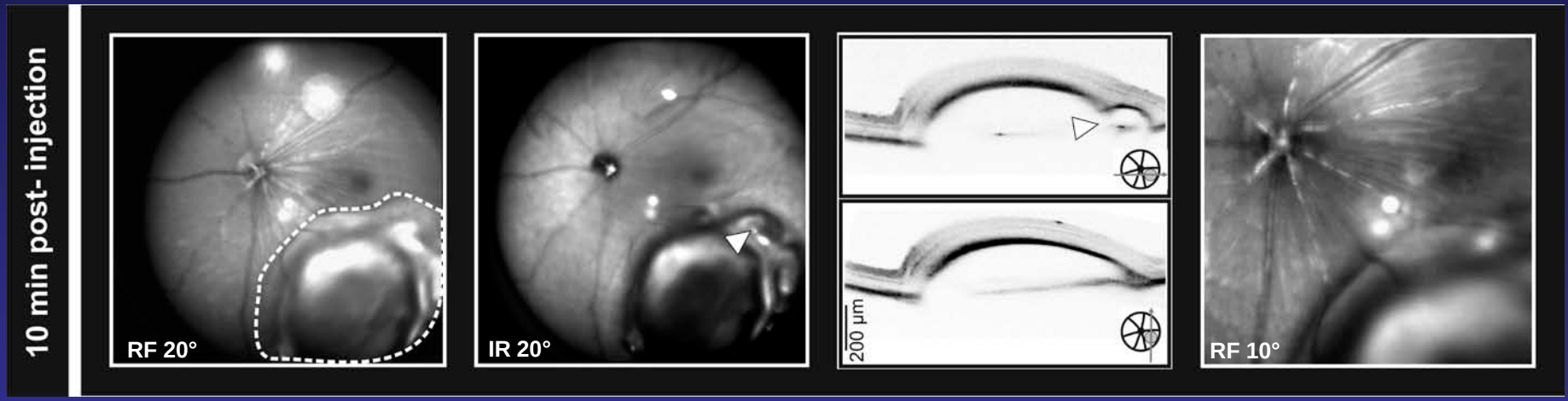
EGFP:

rAAV2/5-SWS-EGFP vector construct (n=3)
rAAV2/5-RG-EGFP vector construct (n=3)
rAAV2/5-sc-SWS-EGFP vector construct (n=3)

rAAV2/8-SWS-EGFP vector construct (n=7)
rAAV2/8-RG-EGFP vector construct (n=3)

Monitoring of injection and retinal detachment: 10 min post injection

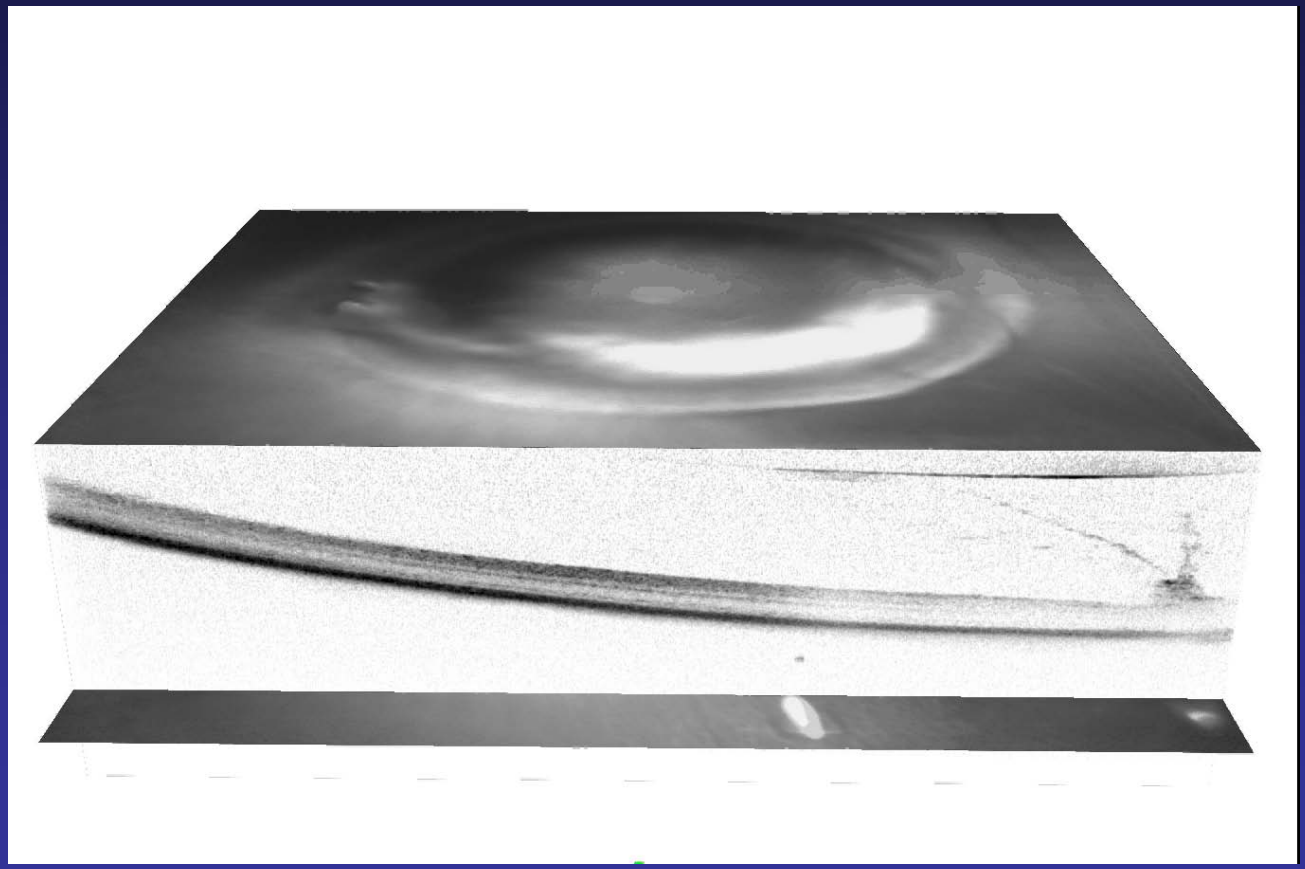
1.5 µl AAV-RG-eGFP dorsal-nasal inj., #2, DOB: 17.08.09, @ PN 14d inj.



Susanne C. Beck, Gesine Huber

Monitoring of injection and retinal detachment: 10 min post injection

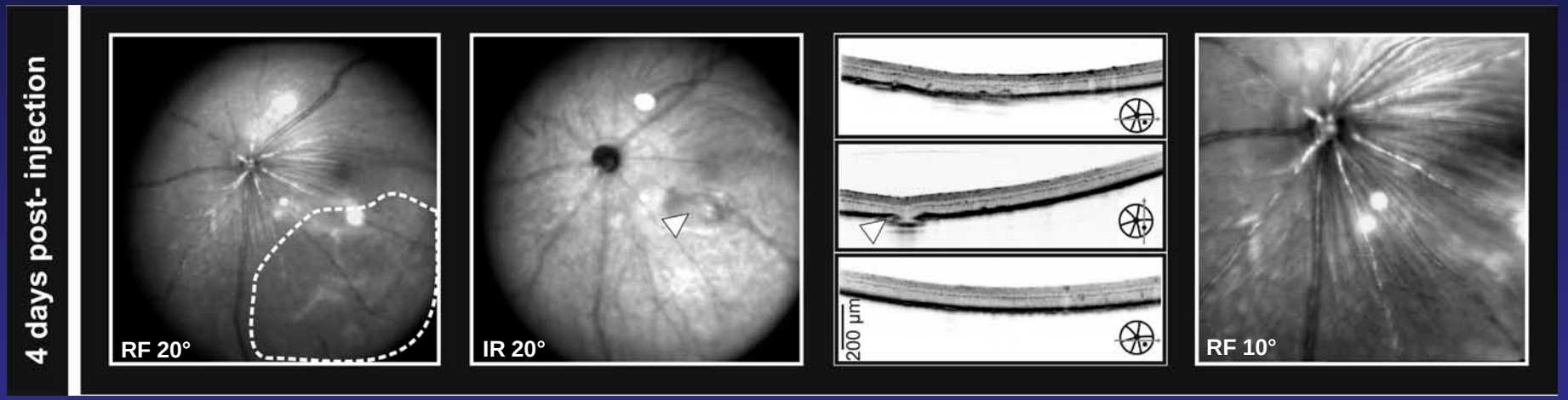
1.5 µl AAV-RG-eGFP dorsal-nasal inj., #2, DOB: 17.08.09, @ PN 14d inj.



Gesine Huber

Monitoring of injection and retinal detachment: 4 days post injection

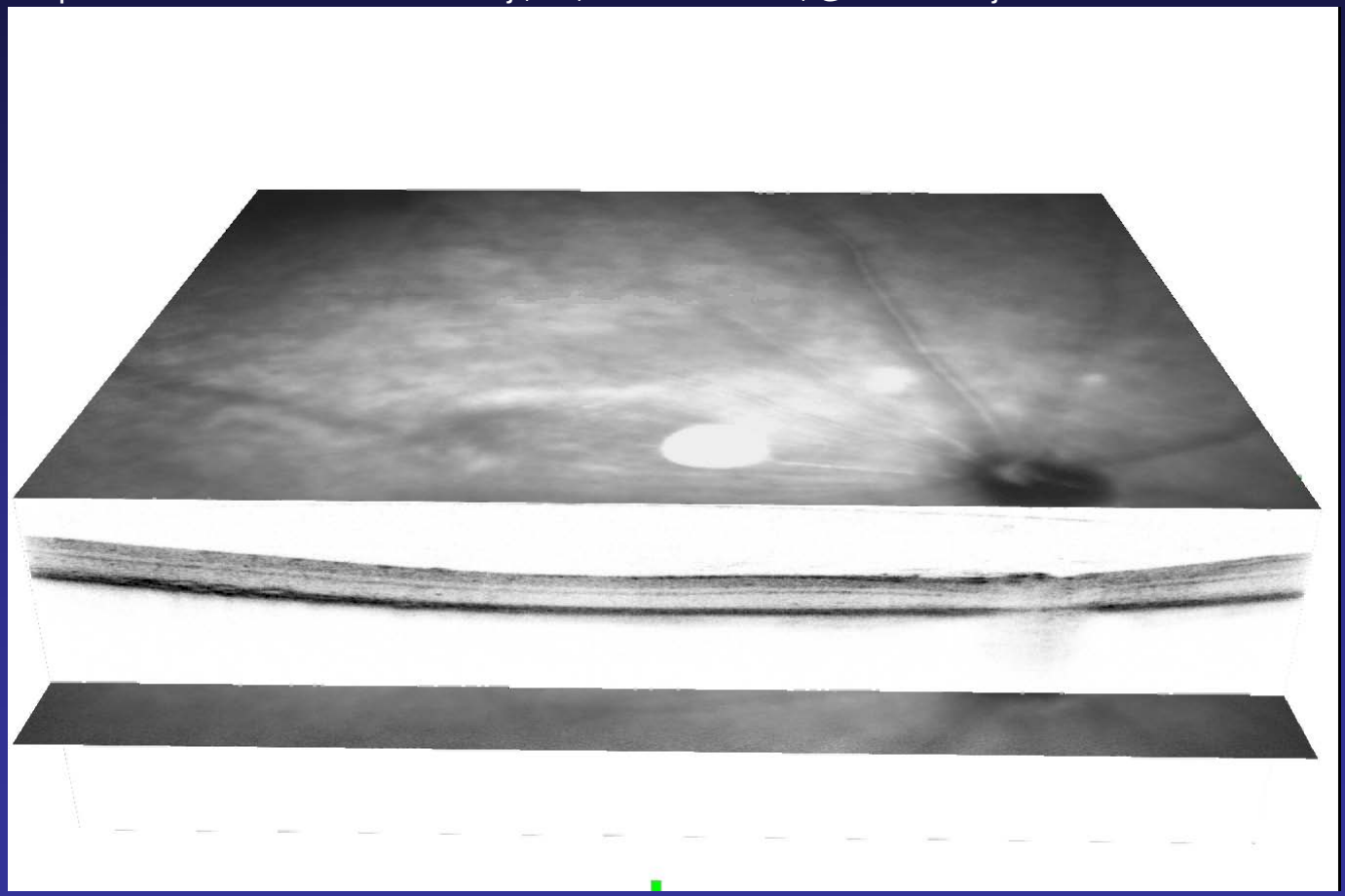
1.5 µl AAV-RG-eGFP dorsal-nasal inj., #2, DOB: 17.08.09, @ PN 14d inj.



Susanne C. Beck, Gesine Huber

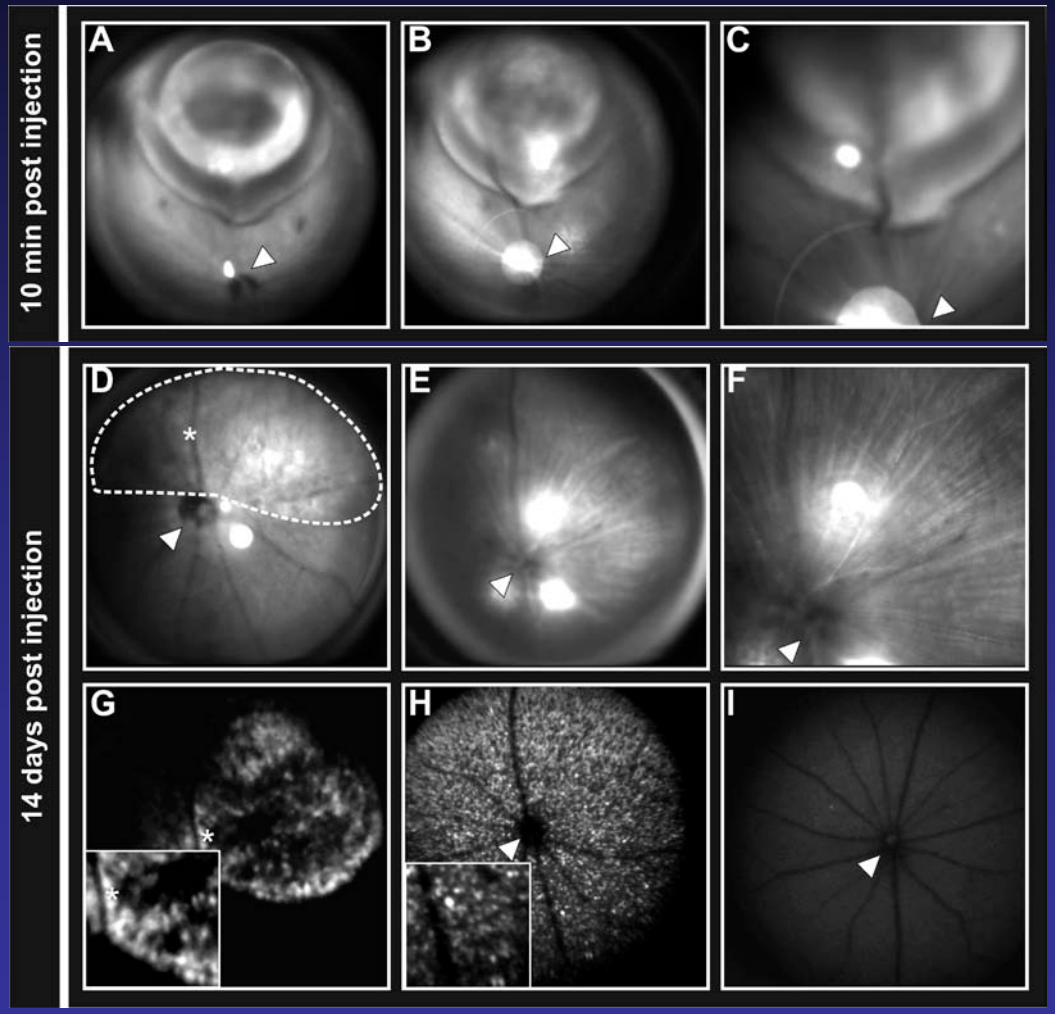
Monitoring of injection and retinal detachment: 4 days post injection

1.5 µl AAV-RG-eGFP dorsal-nasal inj., #2, DOB: 17.08.09, @ PN 14d inj.

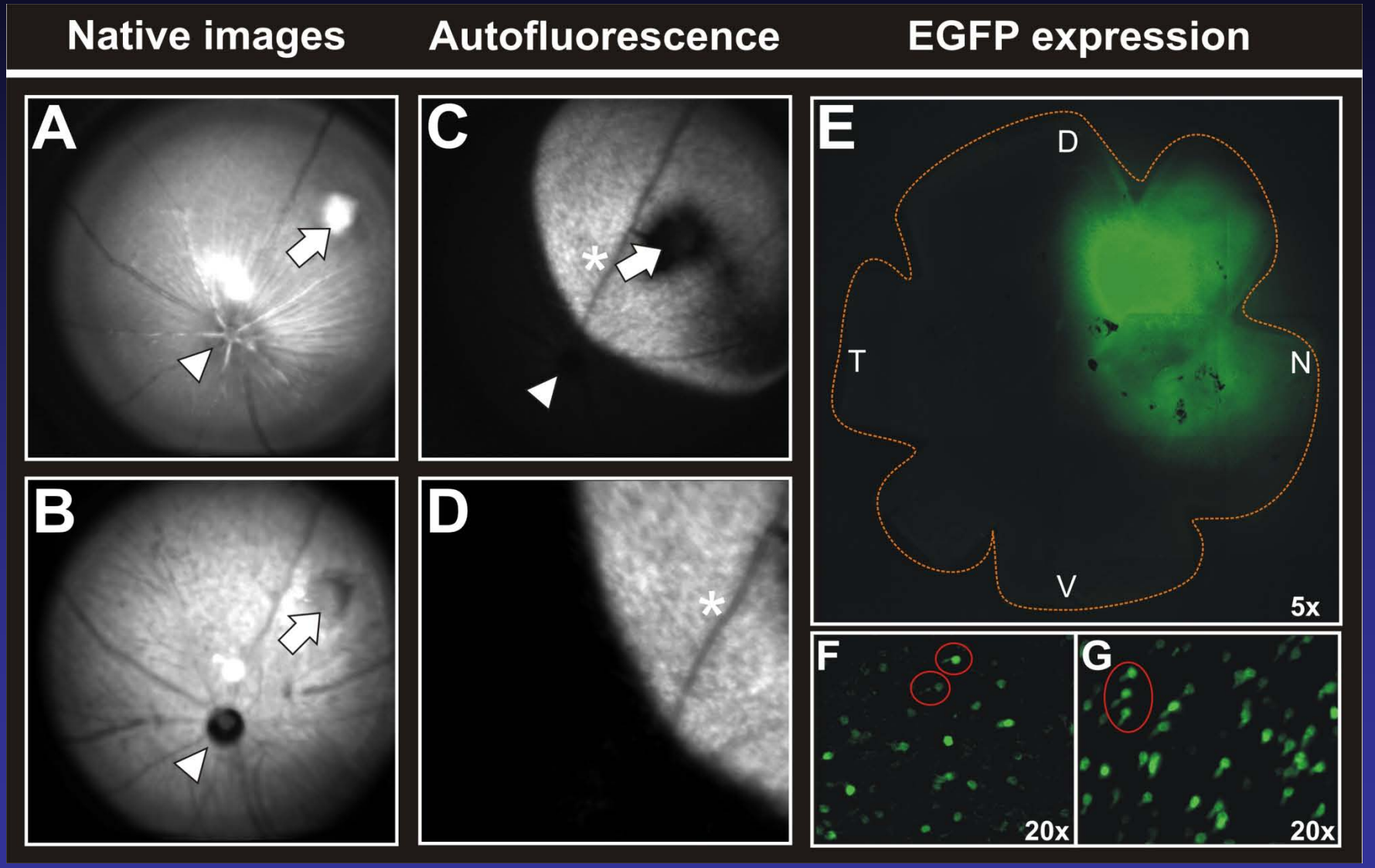


Gesine Huber

The approach of lentiviral vector system leads to an early onset of expression

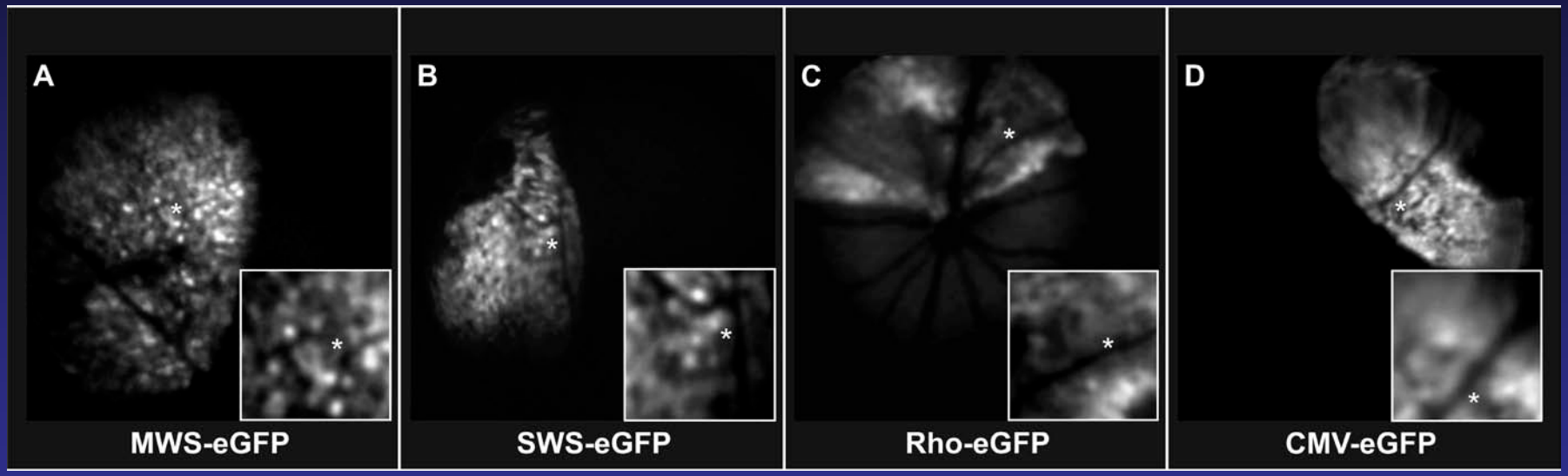


1,5 µl LV- Arrestin3-eGFP dorsal-nasal inj., #10, DOB: 10.10.07, P15



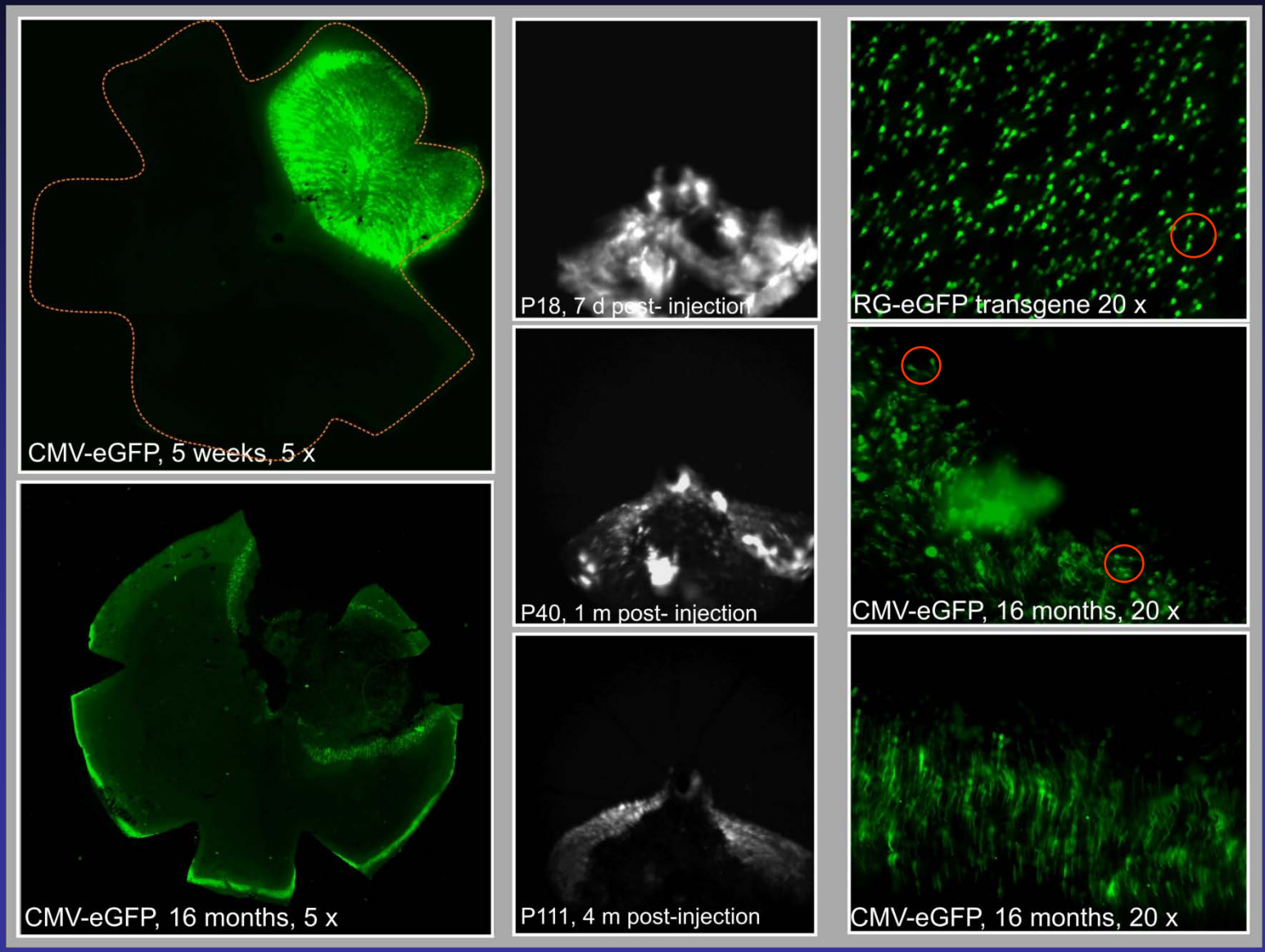
1.5 µl AAV2/8-RG-eGFP dorsal-nasal inj., #10, DOB: 13.07.09, @ PN 14d inj.

Lentiviral plasmids drive specific eGFP expression under control of cone- and rod-specific promoters

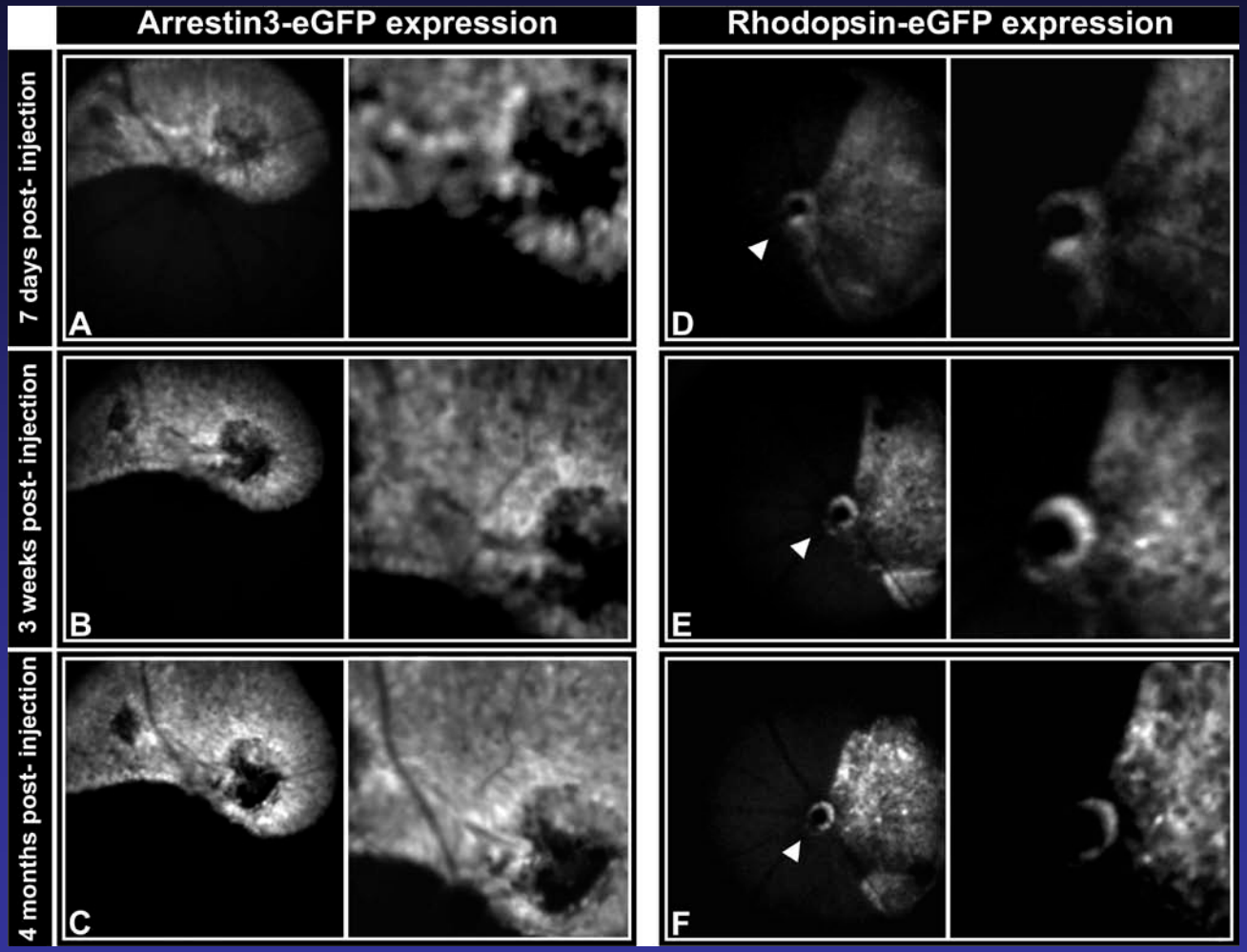


All promoters induce eGFP expression!

CMV-promoter drives a strong expression that results in a loss of retinal cells



Serial assessment of eGFP fluorescence using cSLO: Lentiviruses induce a long term and stable expression



1,5 µl LV-Arrestin3-eGFP dorsal inj., #4,
DOB: 15.02.08, @ PN 11d inj.

1,5 µl LV-Rho-eGFP temporal inj., #2,
DOB: 15.02.08, @ PN 11d inj.

Conclusion I

- A defined local retinal detachment was present immediately after subretinal injection. The detachment resolved quickly within the first week post injection. Alterations around the site of injection were observed.
- The presented data indicate that all the utilized vectors are able to drive eGFP expression.
- The time course experiments suggest stable efficient and long-term expression of the transgene in the injected area using LV and AAV vector systems. Differences in the latency period of eGFP expression were observed among both vector systems (LV vs AAV) and among different promoters due to their specificity.
- About ¼ of the whole retina cells are transduced by a single injection.
- We show for the first time a conclusive series of sequential examinations focused on size of detachment, resorption time, morphological integrity and transduction properties.

- ✓ Refinement of subretinal injections and
- ✓ Improvement the reproducibility of obtained results

Agenda

- Restoration of cone vision in the CNGA3 knock out model: proof of principle

Monitoring Disease Dynamics

Structure

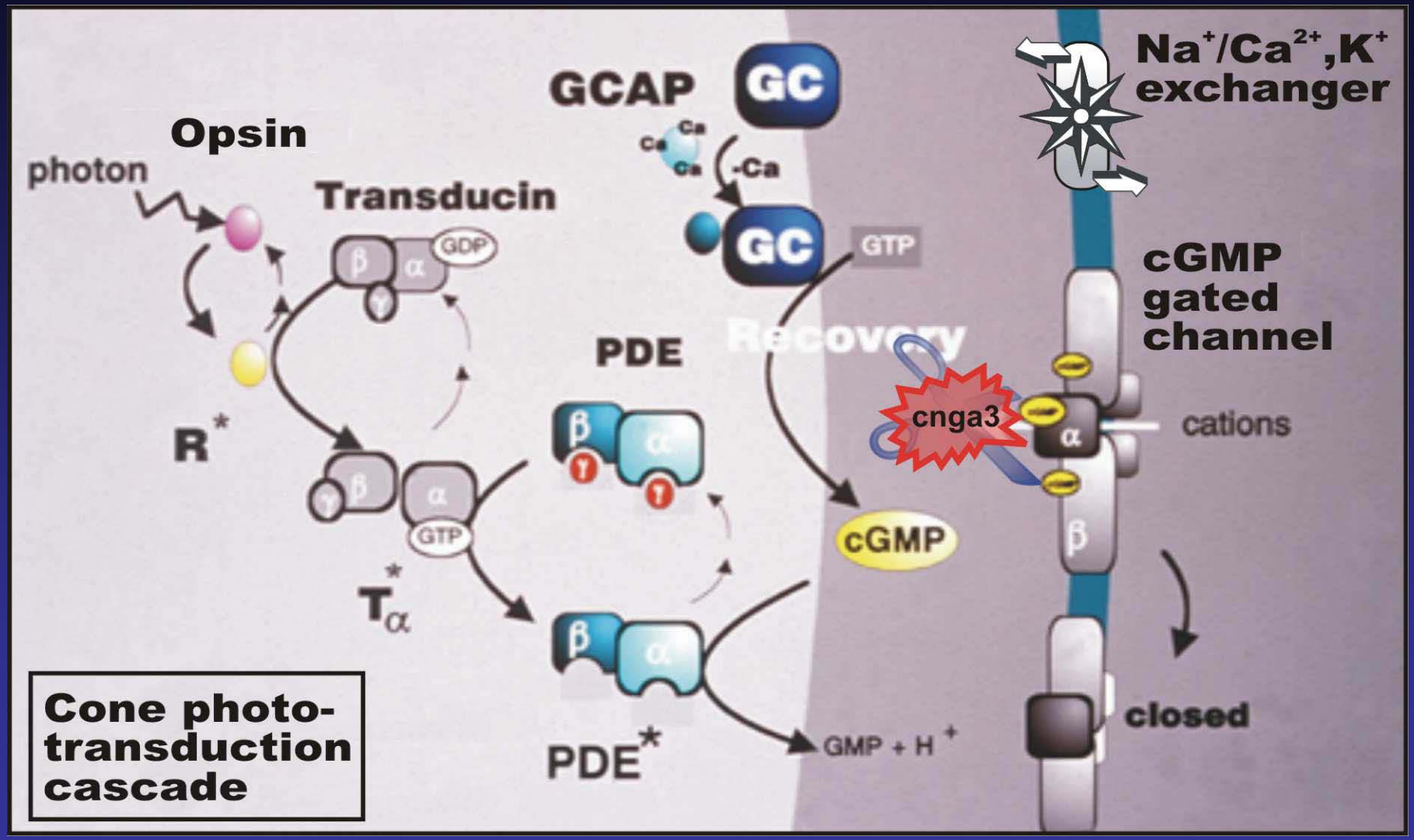
- En face imaging (Fundoscopy, cSLO)
- Cross sectional imaging (OCT, Ultrasound)

Function

- Electrophysiology (Ganzfeld ERG, mfERG)
- Pupillary response, microperimetry, psychophysical tests

Aim of the study

- Application of the viral gene replacement therapy in a retinal degenerating mouse model: CNGA3$^{-/-}$
- In vivo studies of treated animals using imaging methods (e.g. SD-OCT, cSLO)
- Functional analysis of treated animals using the ERG
- Investigation of signal transmission to the brain (e.g. responsiveness of ganglion cells to photopic stimuli)
- Investigation of cone-mediated vision in a behavioral test (e.g. water maze test)


Opsin
photon
Transducin
GDP
GTP
R*
T*α
GCAP
GC
Ca
-Ca
GC
GTP
PDE
PDE*
Recovery
cGMP
GMP + H+
Na+/Ca2+,K+
exchanger
cGMP
gated
channel
cnga3
cations
closed
Cone photo-
transduction
cascade

Achromatopsia:

- cone dystrophy
- visual-acuity loss
- photophobia
- nystagmus
- Incidence 1:30 000

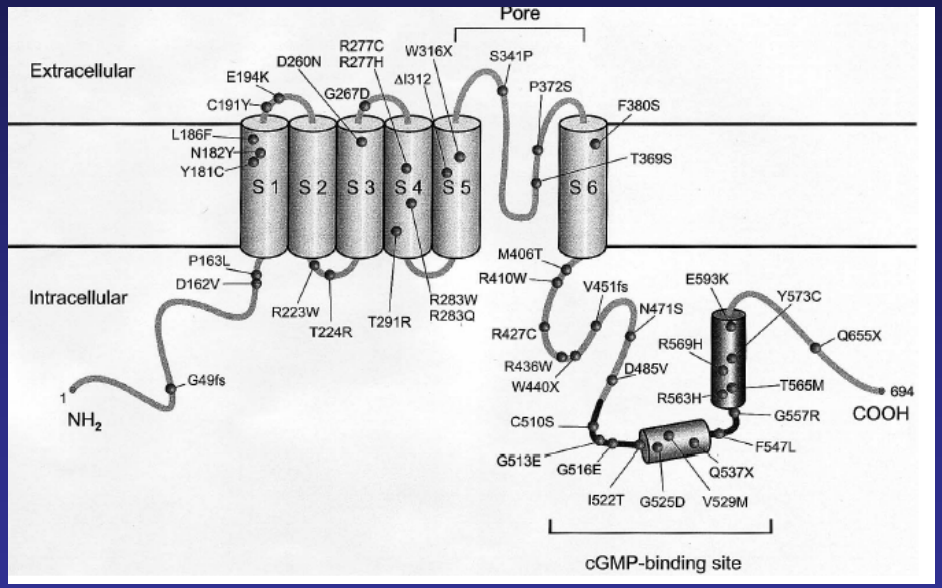
Genes causing Achromatopsia:

- *Cngb3* ~ 47 %
- *Cnga3* ~ 23 %
- *Gnat2* ~ 2 %
- *Pde6c* ~ 2 %

Am. J. Hum. Genet. 69:722–737, 2001

CNGA3 Mutations in Hereditary Cone Photoreceptor Disorders

Bernd Wissinger,[1,3] Daphne Gamer,[1,3] Herbert Jägle,[2,3] Roberto Giorda,[4] Tim Marx,[1,3] Simone Mayer,[1,3] Sabine Tippmann,[1,3] Martina Broghammer,[1,3] Bernhard Jurklies,[5] Thomas Rosenberg,[6] Samuel G. Jacobson,[7] E. Cumhur Sener,[8] Sinan Tatlipinar,[8] Carel B. Hoyng,[9] Claudio Castellan,[11] Pierre Bitoun,[12] Sten Andreasson,[13] Günter Rudolph,[14] Ulrich Kellner,[15] Birgit Lorenz,[16] Gerhard Wolff,[17] Christine Verellen-Dumoulin,[18] Marianne Schwartz,[6] Frans P. M. Cremers,[10] Eckart Apfelstedt-Sylla,[3] Eberhart Zrenner,[3] Roberto Salati,[4] Lindsay T. Sharpe,[2,3,19] and Susanne Kohl[1,3]



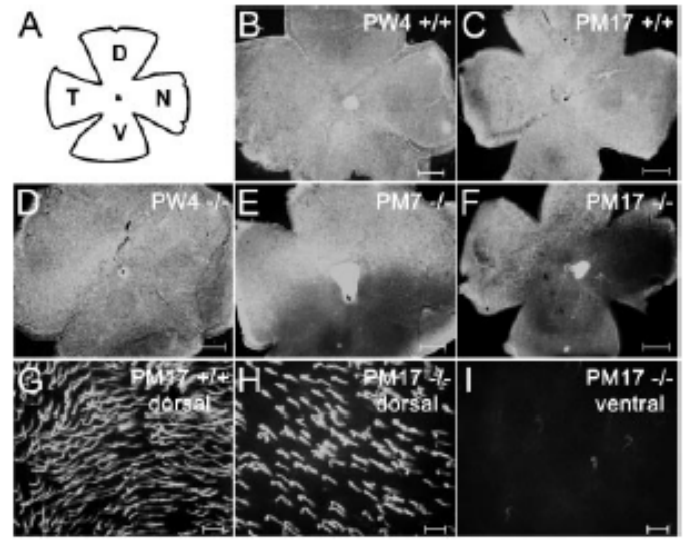
- **More than 40 mutations were found in the CNGA3 subunit in patients with achromatopsia.**

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 7553–7557, June 1999
Neurobiology

Selective loss of cone function in mice lacking the cyclic nucleotide-gated channel CNG3

MARTIN BIEL*†, MATHIAS SEELIGER‡, ALEXANDER PFEIFER*, KONRAD KOHLER‡, ANDREA GERSTNER*, ANDREAS LUDWIG*, GESINE JAISSLE‡, SASCHA FAUSER‡, EBERHART ZRENNER‡, AND FRANZ HOFMANN*

*Institut für Pharmakologie und Toxikologie, Technische Universität München, Biedersteiner Strasse 29, 80802 Munich, Germany; and ‡Abteilung für Pathophysiologie des Sehens und Neuroophthalmologie, Universitäts-Augenklinik Tübingen, Schleichstrasse 12-16, 72076 Tübingen, Germany



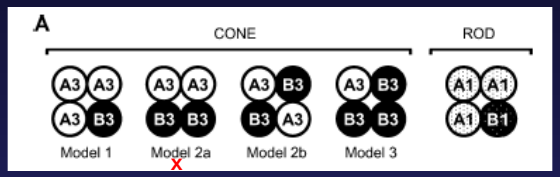
Morphological/ histological analysis:

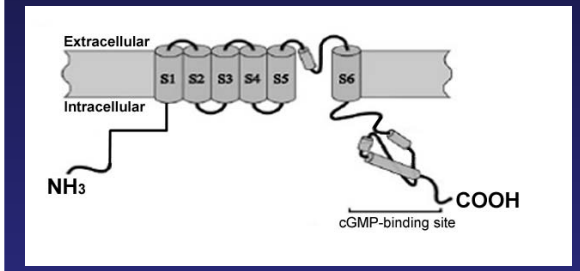
- Progressive degeneration of cones
- high intracellular concentration of cGMP

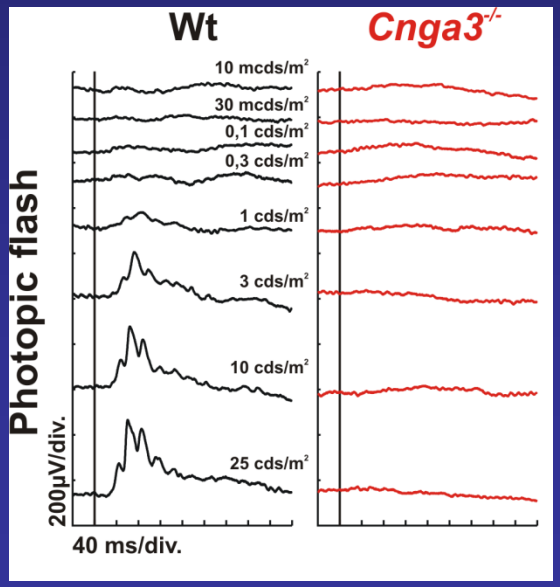
Functional analysis:

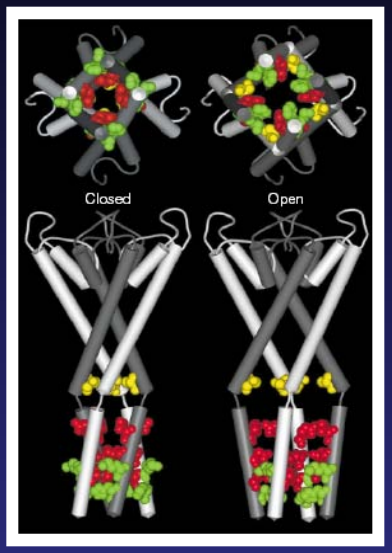
- No photopic ERG-signals
- Rod-mediated vision is unaffected

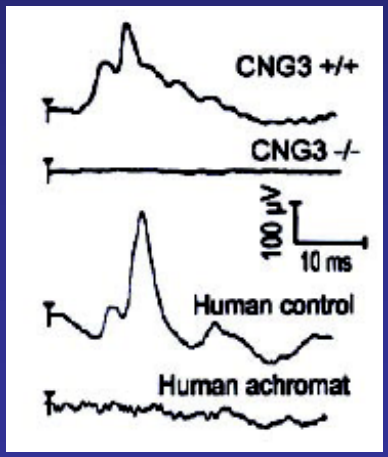
Channel protein is composed in the native form of A and B subunits



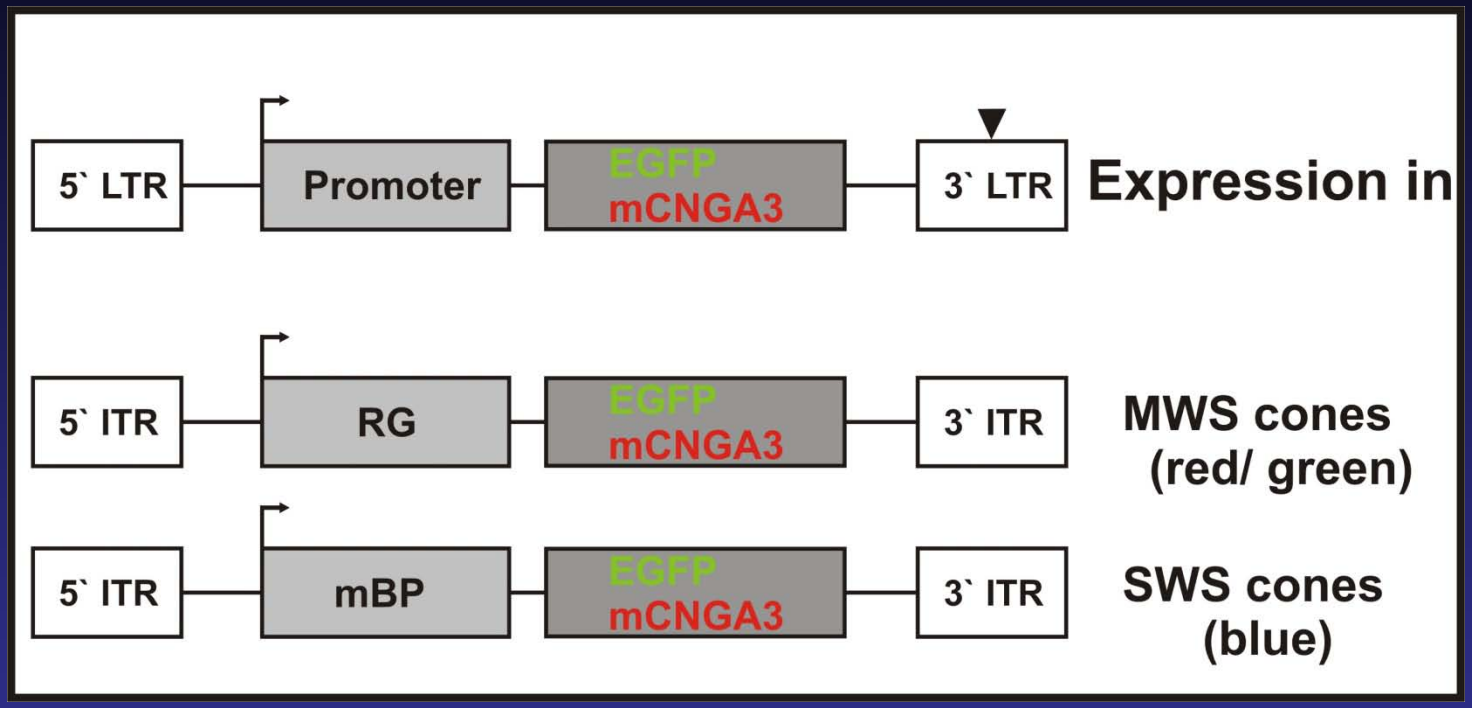








About ¼ of patients with Achromatopsia shows mutations in the *Cnga3* gene

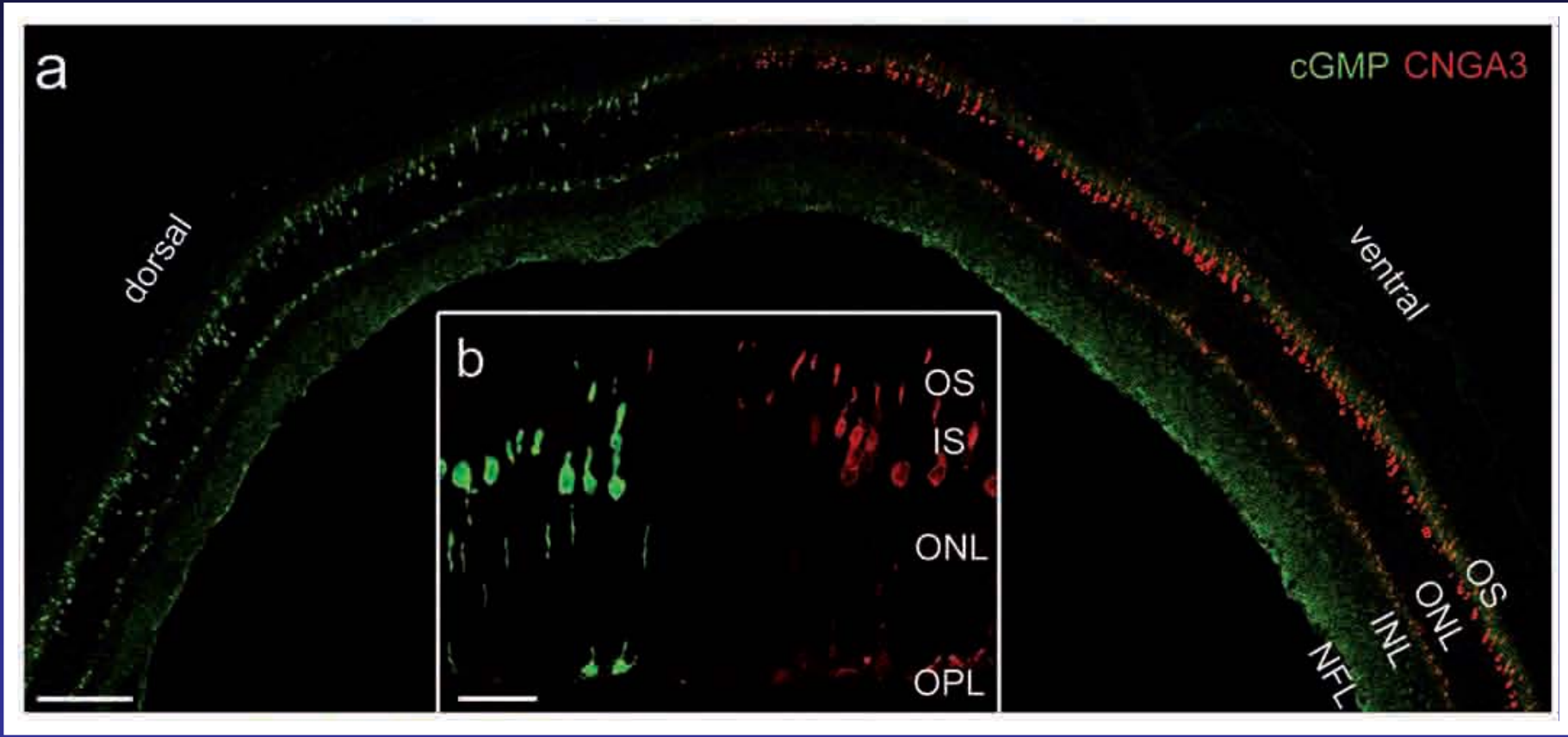


AAV:

rAAV2/5-SWS-mCNGA3 vector construct (n=12)
rAAV2/5-RG-mCNGA3 vector construct (n=4)
rAAV2/5-sc-SWS-mCNGA3 vector construct (n=7)

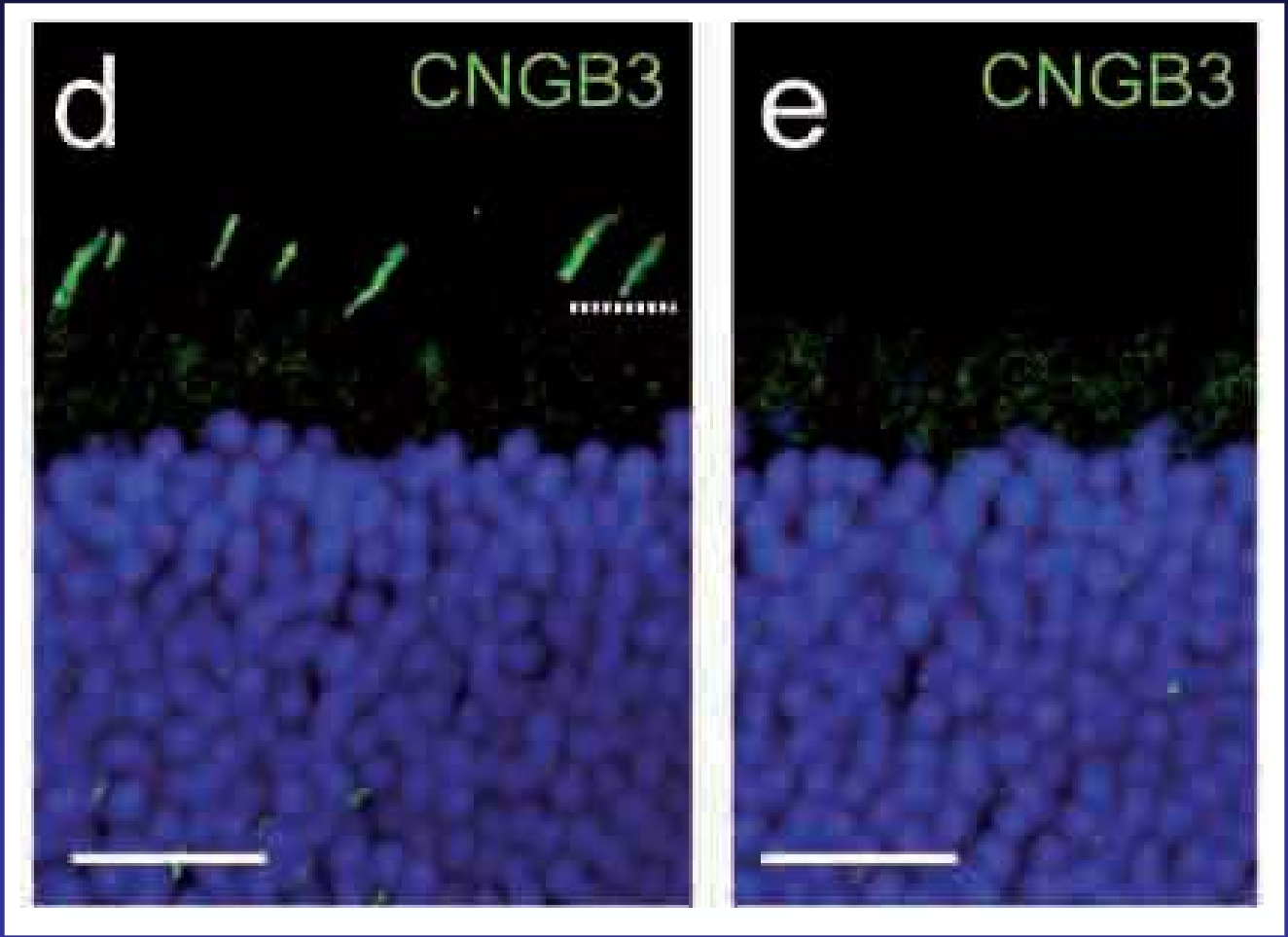
rAAV2/8-SWS-mCNGA3 vector construct (n=12)

Expression of CNGA3 protein normalizes cGMP level



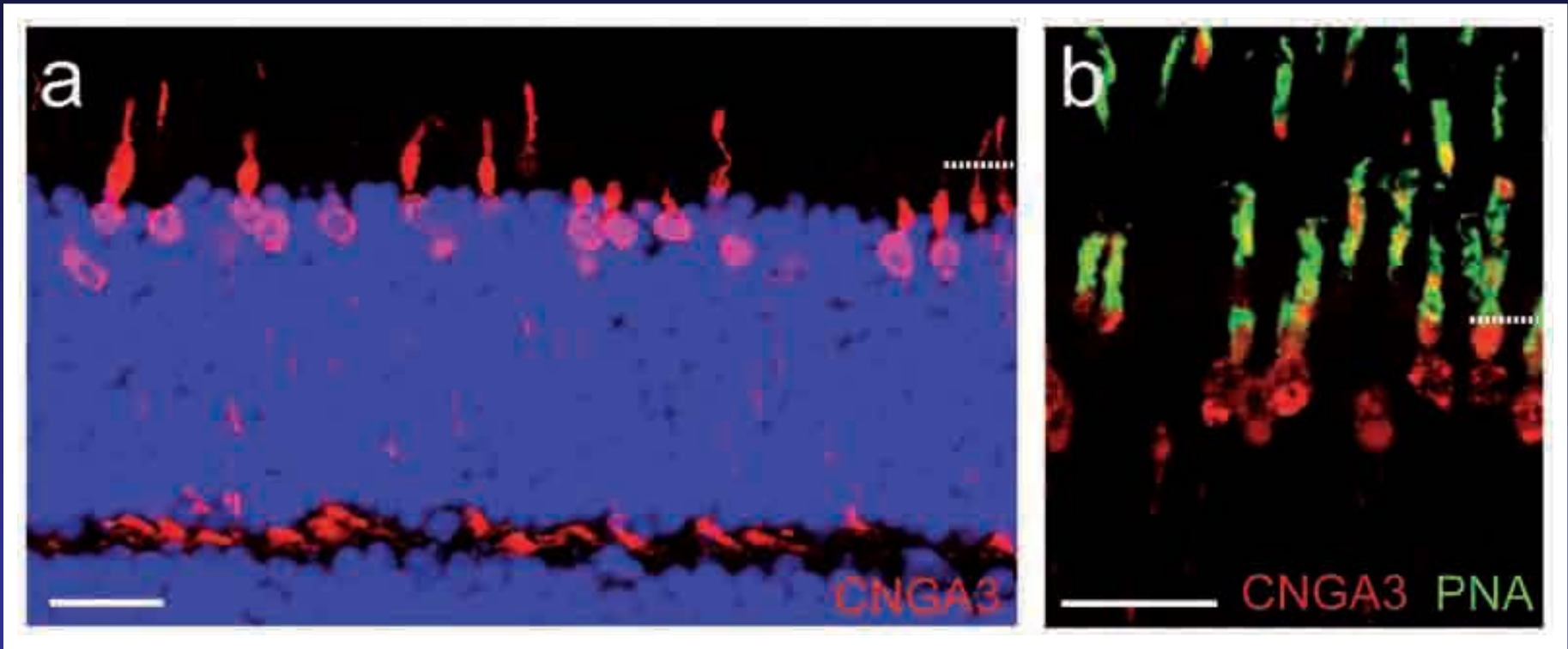
S. Michalakis

CNGA3-replacement-therapy restores normal outer segment localization of the CNGB3 subunit



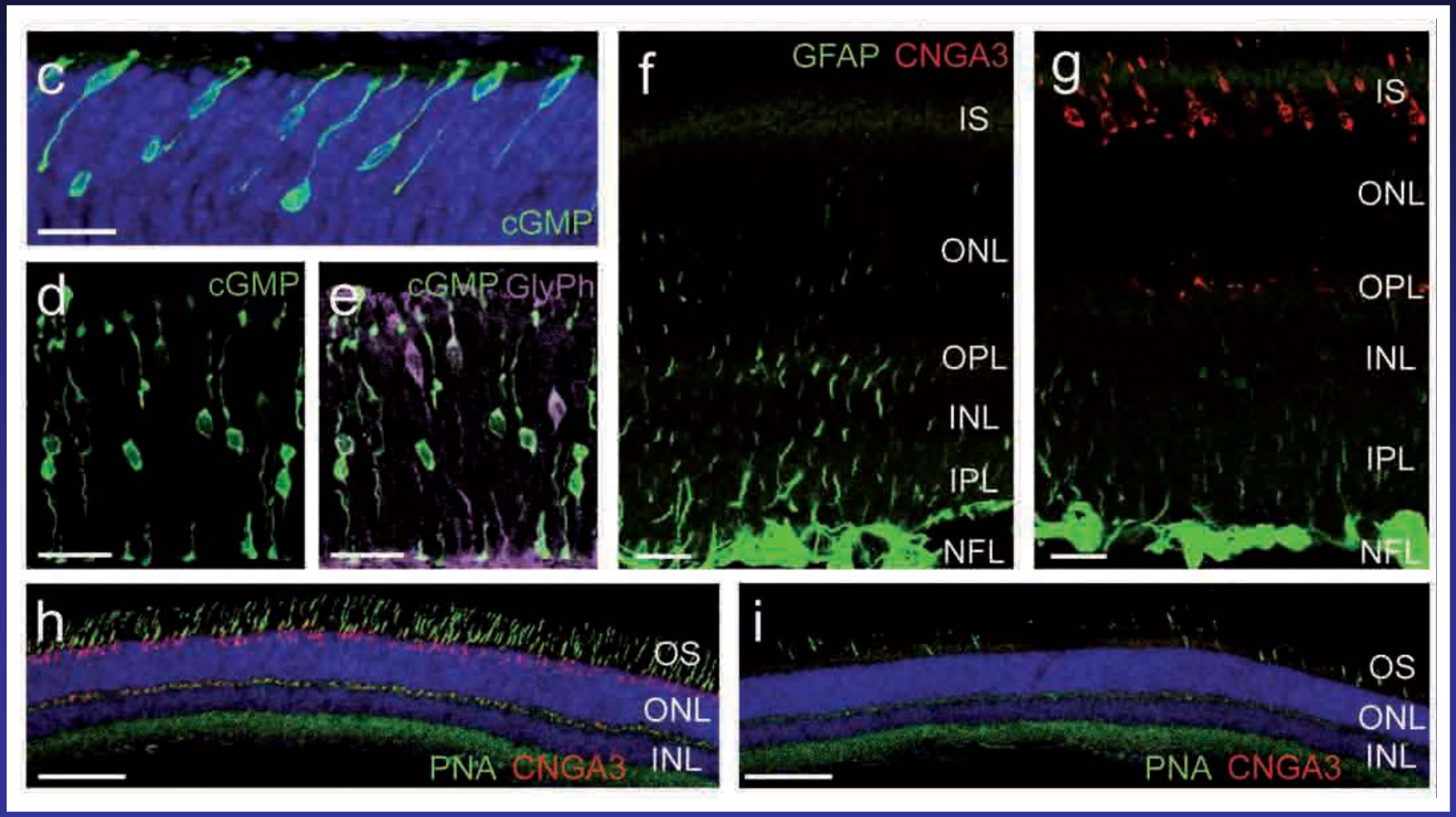
S. Michalakis

CNGA3 is reexpressed in cone photoreceptors



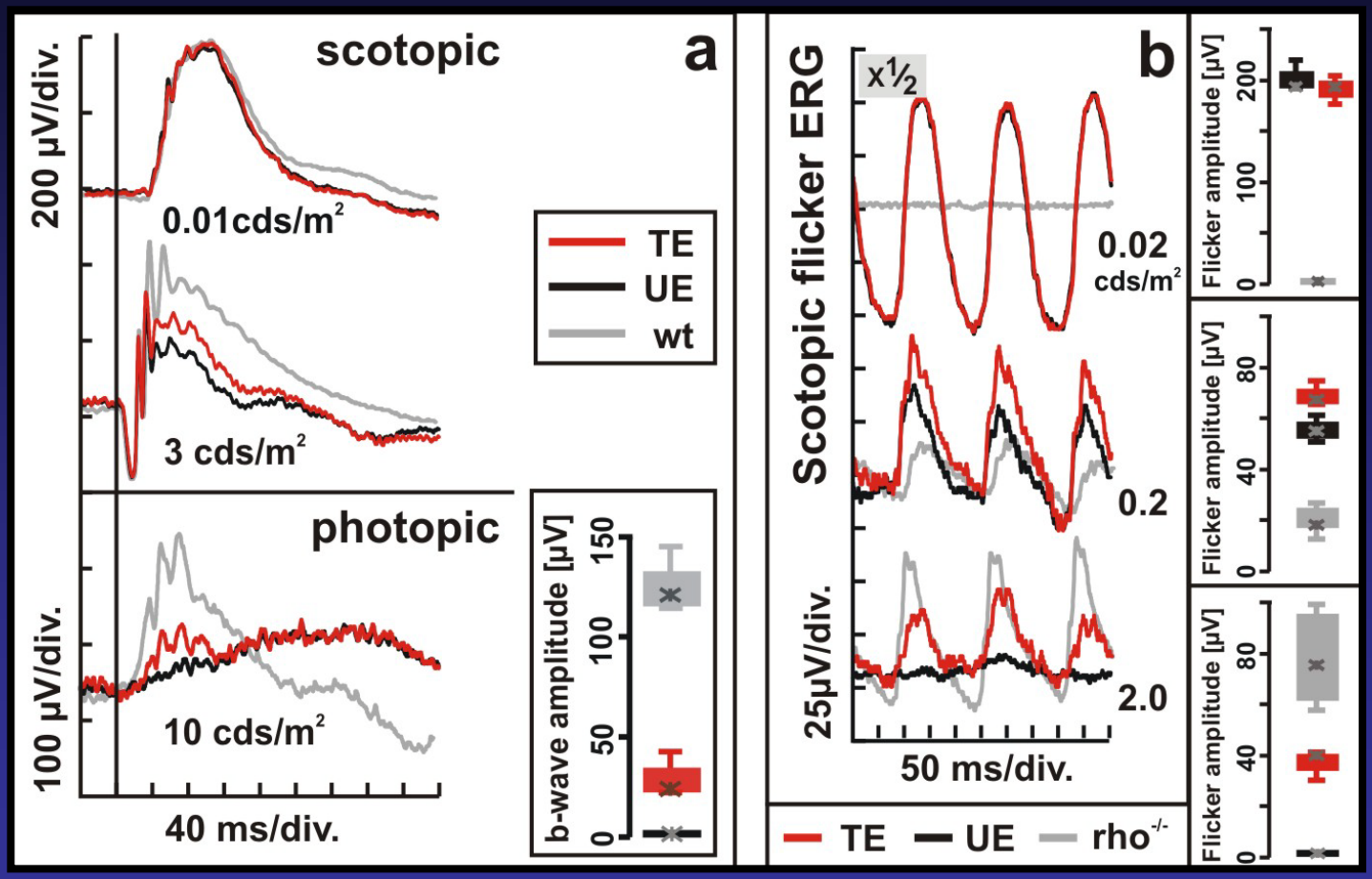
S. Michalakis

AAV-mediated gene replacement therapy reactives deregulated light cascade function



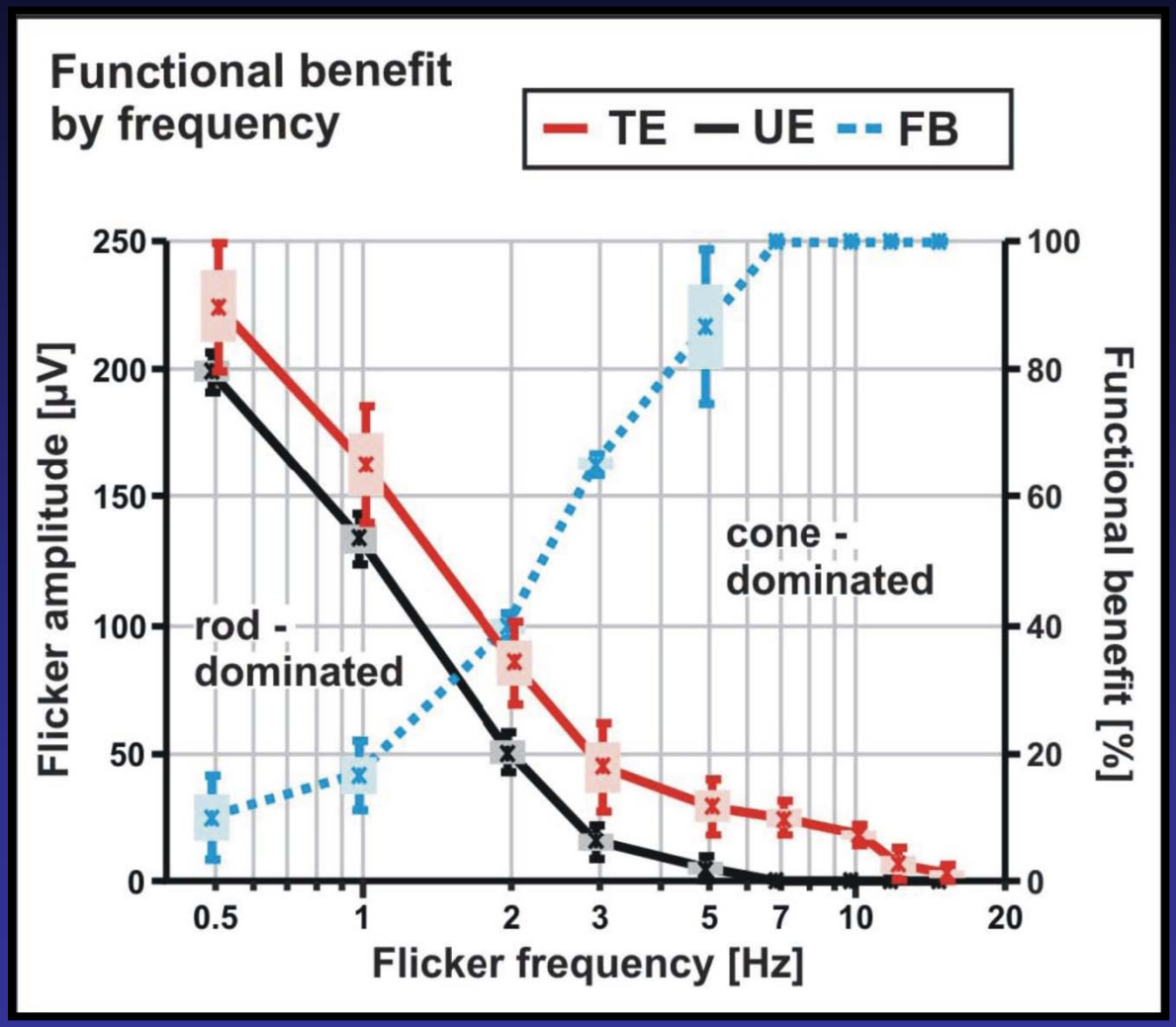
S. Michalakis

Restoration of cone-mediated ERG in treated CNGA3$^{-/-}$ mice



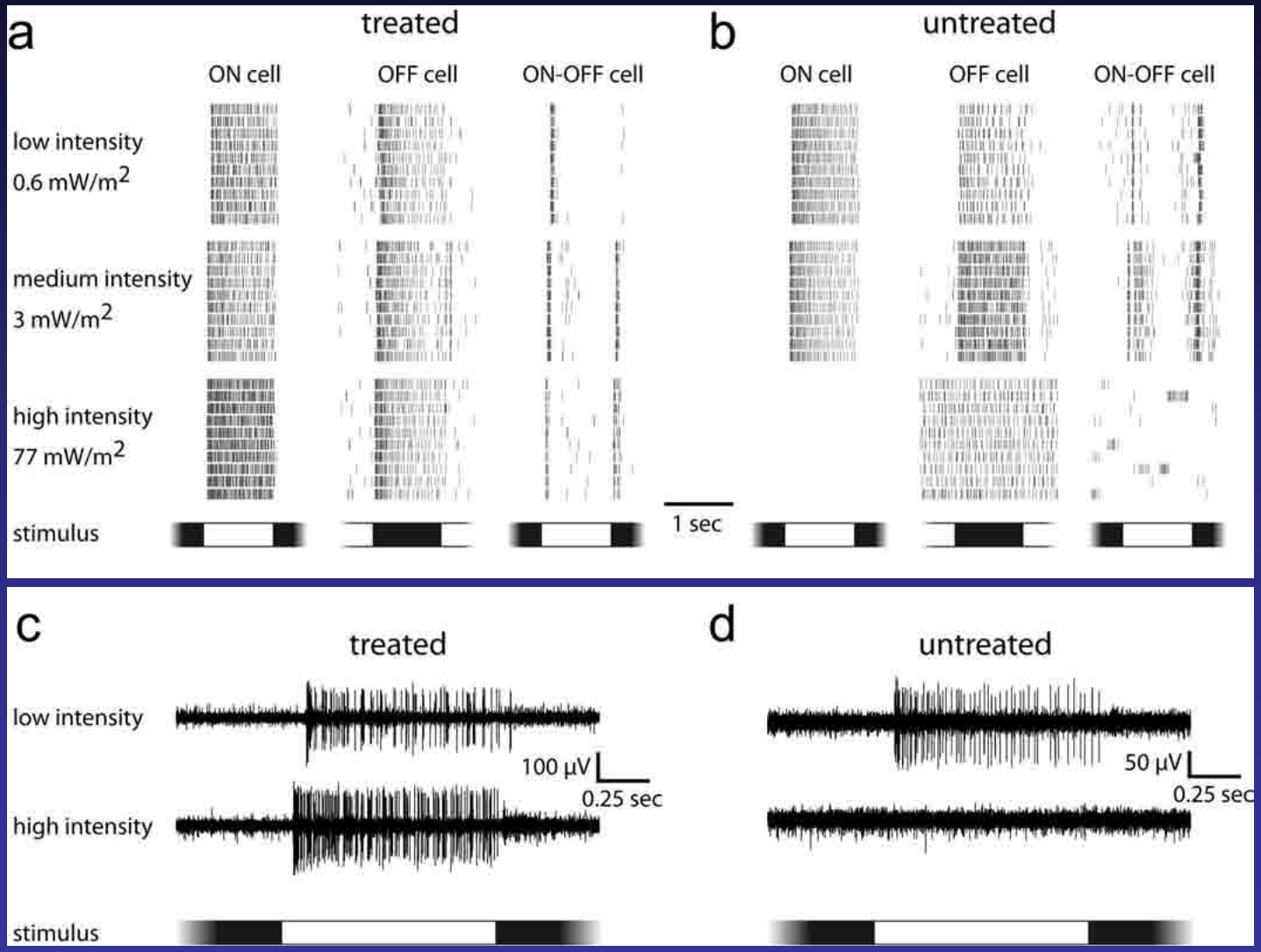
N. Tanimoto/ M. Seeliger

Functional benefit from treatment as a function of ERG flicker frequency



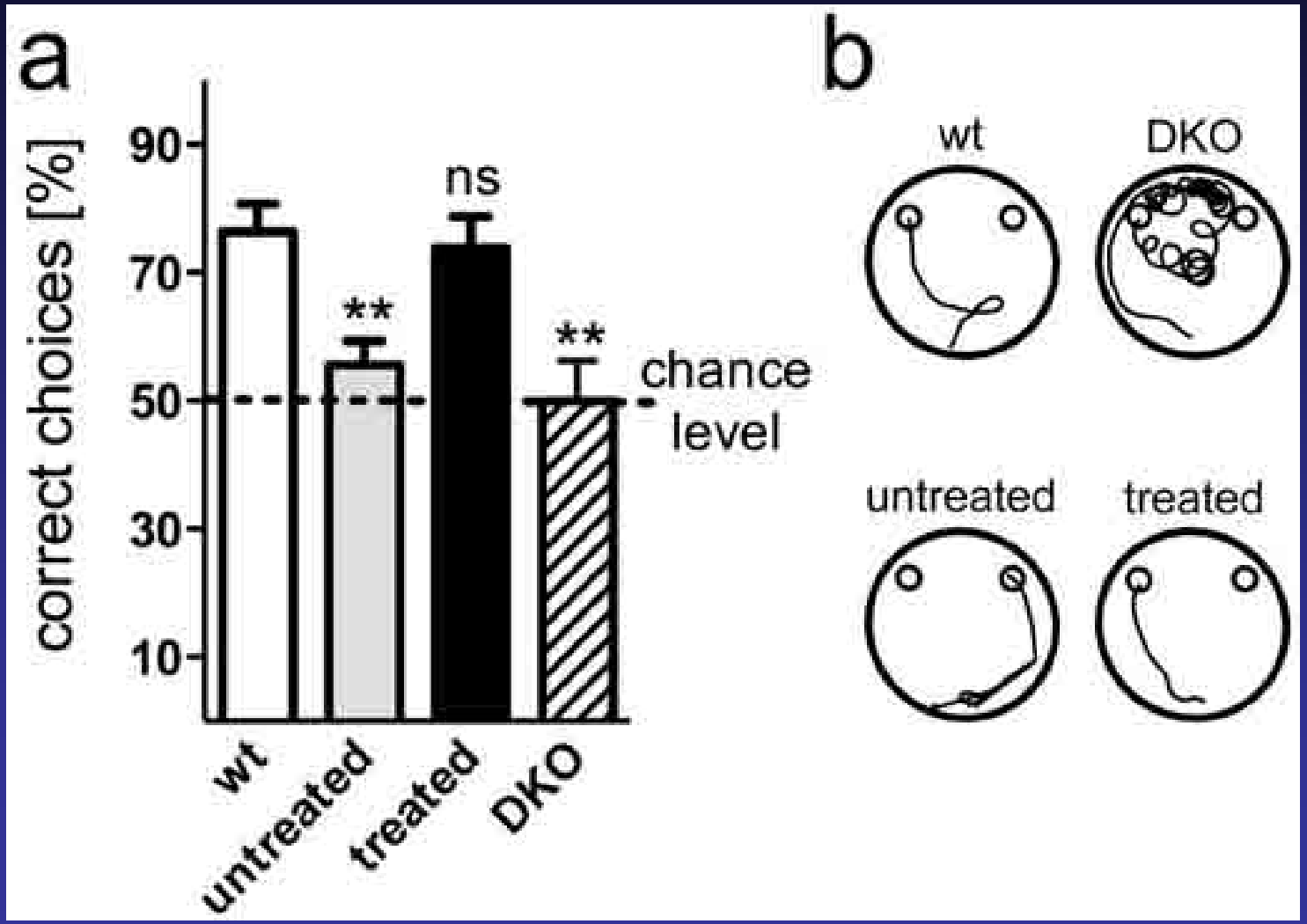
N. Tanimoto/ M. Seeliger

Analysis of cone driven light-evoked spiking activity



S. Koch

Restoration of cone-mediated vision-guided behavior



Stylianos Michalakis/ Susanne Koch

Conclusion II

The gene replacement therapy using rAAV2/5 vectors restores:

- ✓ The cone-mediated ERG (functional rescue) with a late onset (starting from PI 9w).
- ✓ The CNGA3 protein expression in all cone subtypes and results in a recovery of the CNGB3 expression in COS.

  The expression of the full length channel protein composed of both subunits normalizes the increased cGMP level in treated mice. CNGA3-replacement restores expression and correct localization of visual cascade proteins.
- ✓ The responsiveness of ganglion cells to photopic stimuli by analyzing the cone driven light-evoked spiking activity.
- ✓ The cone-mediated vision guided behavior (water maze test).

Restoration of cone vision in the CNGA3$^{-/-}$ mouse model of congenital complete lack of cone photoreceptor function

Stylianos Michalakis[1], Regine Mühlfriedel[2], Naoyuki Tanimoto[2], Vidhyasankar Krishnamoorthy[3], Susanne Koch[1], M. Dominik Fischer[2], Elvir Becirovic[1], Lin Bai[1], Gesine Huber[2], Susanne C. Beck[2], Edda Fahl[2], Hildegard Büning[4], François Paquet-Durand[5], Xiangang Zong[1], Tim Gollisch[3], Martin Biel[1], Mathias W. Seeliger[2]

Stylianos Michalakis and Regine Mühlfriedel contributed equally to this work. Martin Biel and Mathias W. Seeliger contributed equally to this work.

[1]Center for Integrated Protein Science Munich (CIPSM), Department of Pharmacy – Center for Drug Research, Ludwig-Maximilians-Universität München, Munich, Germany.
[2]Division of Ocular Neurodegeneration, Institute for Ophthalmic Research, Centre for Ophthalmology, Eberhard Karls-Universität, Tübingen, Germany.
[3]Visual Coding Group, Max Planck Institute of Neurobiology, Martinsried, Germany.
[4]Department I Medicine and Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany.
[5]Division of Experimental Ophthalmology, Institute for Ophthalmic Research, Centre for Ophthalmology, Eberhard Karls-Universität, Tübingen, Germany

Accepted: 15.06.2010 in *Molecular Therapy*

Acknowledgement

Mathias Seeliger (Tübingen)

Gesine Huber, Susanne Beck
Naoyuki Tanimoto

Gudrun Utz
M. Dominik Fischer
Edda Fahl

Francois Paquet-Durand

Martin Biel (Munich)
Stylianos Michalakis, Susanne Koch, Elvir Becirovic, Lin Bai

Tim Gollisch, Vidhyasankar Krishnamoorthy

Hildegard Büning (Cologne)