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# Bachelor Thesis

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## Implications of photoreceptor transplantation on visual function in mouse models of retinal degeneration

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Fakultät Angewandte Computer und Biowissenschaften

# BACHELORARBEIT

# Implications of photoreceptor transplantation on visual function in mouse models of retinal degeneration

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## Abstract

The loss of photoreceptors is a major course for visual impairment and blindness with no cure currently established. Photoreceptor replacement into mouse models of retinal degeneration is currently investigated as a potential future therapy. To evaluate visual function in mice before and after treatment two vision-based behavioral tests (optomotor tracking and the light/dark box) were investigated including their feasibility to distinguish between rod and cone photoreceptor function. Both methods turned out to be an objective and reliable readout for vision ability in wildtype mice and mice with vision impairment due to retinal degeneration. The capability of the methods to assess slight vision improvements have to be further evaluated.

Therefore options for improvement of the established tests and an idea for a new test paradigm have been introduced.

## Acknowledgment

First of all I would like to thank Professor Wünschiers. Without his encouragement, friendliness, creative time management and a little push once in a while the quality of this bachelor thesis would not have been the same. Special thanks belong to the people travelling all the way with me. Without the friendship of Jürgen, Richard and Jeton I would have been lost away from home and my beloved family. Besides them I would like to thank my family. Without any doubt I had always all the support and love a son and brother could dream of. Special thanks belong to the awesome guys of the CRTD. Professor Ader for giving me a chance to work on this project and especially the guys I spent the last 6 month with. Thank you for all your help, your open mind and all your patience. You really made my internship an unforgettable time. In particular Oliver Bosch who did not only spent countless hours helping me but also became a true friend.

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# Structure

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# Abbreviations

| | |
|---|---|
| AMD | Age Related Macula Degeneration |
| AOS | Accessory Optic System |
| CPFL1 | Cone Photoreceptor Function Loss 1 |
| DC | Dark Compartment |
| DKO | Double Knockout Organism |
| dLGN | dorsal Lateral Geniculate Nucleus |
| ERG | Electroretinography |
| IHC | Immunohistochemistry |
| LC | Light Compartment |
| LD Box | Light-dark Box |
| NRL | Neural Retina Leucine zipper |
| OCT | Optical Coherence Tomography |
| OKT | Optokinetic Tracking |
| OKR | Optokinetic Reflex |
| P347S | Proline 347 to Serine |
| RGC | Retinal Ganglion Cells |
| RP | Retinitis Pigmentosa |
| Rho-/- | Rhodopsin Knockout |
| SC | Superior Colliculus |
| SD | Standard Deviation |
| SEM | Standard error of the mean |
| Tg | Transgene |
| VOS | Vestibular Ocular Reflex |
| VS | Vestibular System |
| WT | Wildtype |

# 1 Introduction

> "*Everything must be taken into account. If the fact will not fit the theory, let the theory go*." (Agatha Christie)

Even if Agatha Christie was talking about criminalistics her quote is not less true regarding scientific approaches. Or as Douglas Adams put it: "But the reason I call myself by my childhood name is to remind myself that a scientist must also be absolutely like a child. If he sees a thing, he must say that he sees it, whether it was what he thought he was going to see or not. See first, think later, then test. But always see first. Otherwise you will only see what you were expecting." What both authors have in common is their understanding of problems in scientific methods. As a human being it is hard to be objective. What we see is always influenced by our hopes, wishes and fear and therefore biased by human interpretation. For example when retinal pigment epithelium (RPE) was transplanted in visually impaired patient retinas (done by Moorfields eye hospital, London, in 2015) no measurable improvement of visual functionality could be found but most voluntary patients stated that they "saw" better afterwards. This points to the necessity of having methods that give feedback about a certain therapeutic outcome objectively and reliably.

## 1.1 The eye

The human body is capable to provide five different senses. Besides hearing, smelling, tasting and feeling there is seeing, which is probably the most important one to most human beings. Vision alone accounts for almost 30% of the total sensory input perceived by the human mind (Swaroop et al., 2009). Throughout the animal kingdom a great diversity was discovered in composition and functionality of the eye, from the easiest motion-triggered senses till the complex spatial and colored vision of humans. We´re not only able to perceive light and dark, colors, shades and contours but also measure distance, moods or other important information. In fact the human vision also plays a key role in our

communication and interaction. Therefore a blind or visual impaired person is limited in many ways.

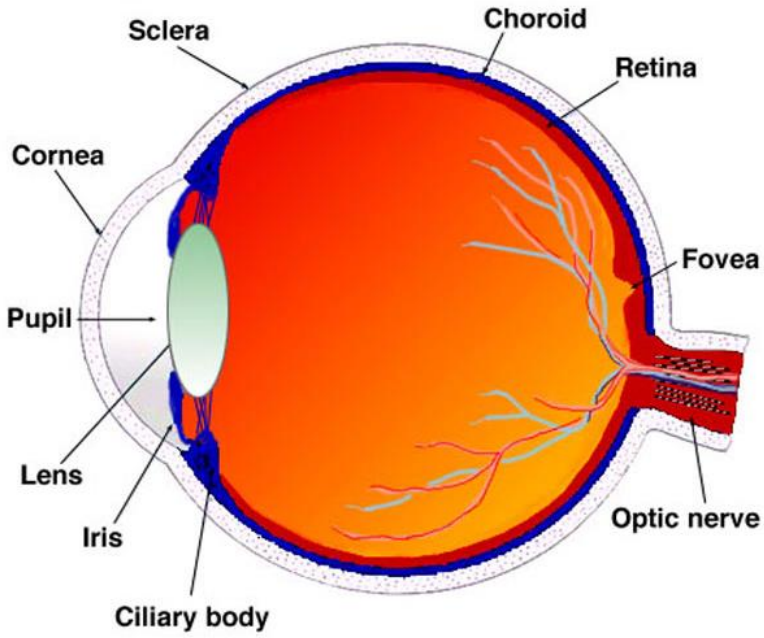



**Figure 1: Anatomy of the human eye**
(http://webvision.med.utah.edu/)

The human eye is a complex organ composed of different structures. The external layer of the anterior part is the cornea whereas the sclera constitutes the outermost layer of the posterior part of the eye. The intermediate layer is divided into two parts: anterior (iris and ciliary body) and posterior (choroid).The iris with the ciliary body controls the contraction of the pupil and thereby controls the amount of light entering the eye. The retina is a layered tissue coating the inner surface of the eye and forms the sensory part (http://webvision.med.utah.edu/)

## 1.2 The retina

The retina is the light sensing tissue located in the posterior region of the eye. It is composed of six different neuronal cell types, which are rod and cone photoreceptors, horizontal cells, bipolar cells, amacrine cells and retinal ganglion cells and three glial cell types being Müller glia, microglia and astrocytes (Fig. 2).

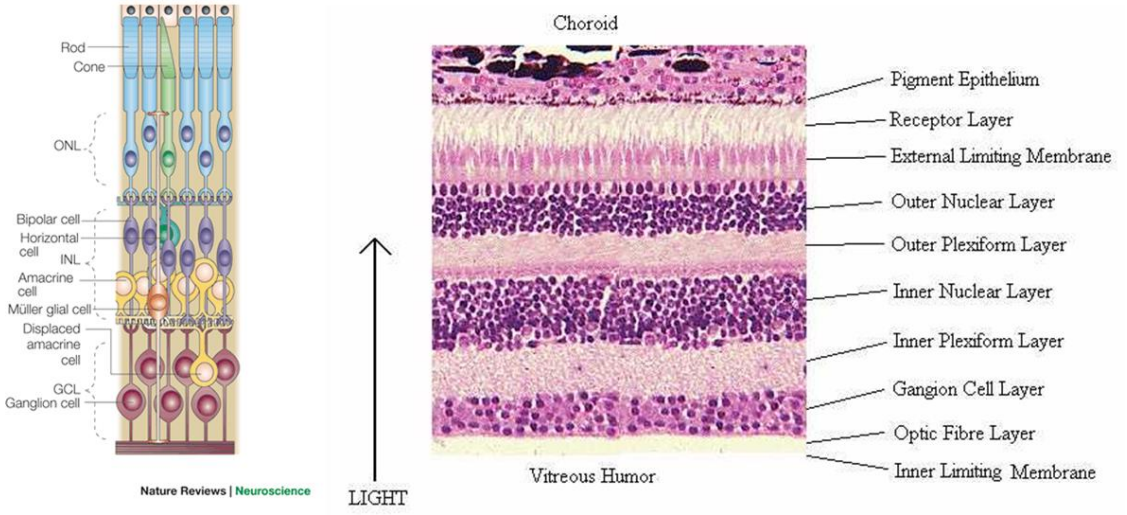



**Figure 2: Layered organization of the mammalian retina**

Left side: schematic structure of the retina; Right side: light microscopy picture of an adult mouse retina (HE staining) (Dyer and Cepko, 2001)

Rod and cones photoreceptors transform the light signal in an electrical/chemical signal that is further processed within the retina and transmitted to the corresponding brain areas where it is finally processed into our vision.

Regarding the general setup of upcoming approaches the distinction between rod and cone function is a crucial factor, since for humans cone-function is of particular importance for daylight-vision (Fig. 3).

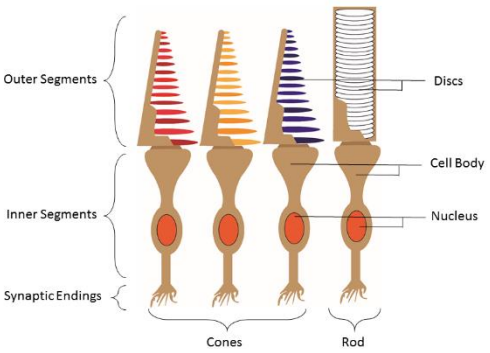



**Figure 3: Rod and cone photoreceptors**

Differences in structure leading to differences in light perception. There is only one type of rod photoreceptor needed for orientation in dim light conditions and perception of movement. Cone photoreceptor can distinguish colours and are needed for sharp vision.

General differences in morphology, photo-pigments and topography across the retina reflect the specialization of photoreceptors. While the rod system is very sensitive to light but has a low spatial resolution the cone system has a high resolution but is relatively insensitive to light. Therefore rods are activated in dim light conditions (scotopic < 1,5 Lx). If illumination increases the cone system becomes stepwise more dominant determining what is seen till the point of photopic conditions (>29 Lx) where the contribution of rods to vision diminishes.

The range of 1,5 – 29 Lx between those two conditions is called mesopic where both photoreceptor types are active (e.g. twilight) (Fig. 4) (Alam et al., 2015).
Human and mouse retina mainly differs at the morphological level. The mouse retina does not have a macula or area centralis in mice resulting in a decentralized distribution of cone photoreceptors. Also the rate of cones is smaller (3%) when compared to human retinas with circa 4.7 % (Jacobs et al., 2004; Purves et al., 2001). Mice are dichromates, lacking sensitivity for long wave light while short and middle wavelength can be detected including UV light (Jacobs et al., 2004).

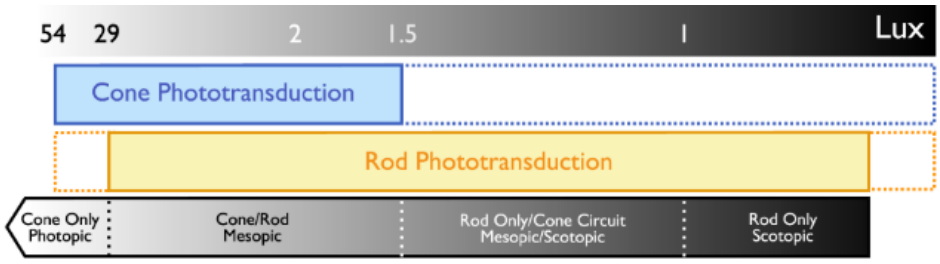



**Figure 4: Luminance range of rod cone functionality**

Light conditions from about 29 lux and higher (photopic conditions) are the luminance range where only cones are responsive. The range between 29 and 1.5 lux (Cone/Rod mesopic) is a luminance range within which cone and rod circuits are both active in WT mice. Rod-only responsiveness can be found at scotopic conditions below 1,5 lux (adapted from Alam et al., 2015).

## 1.3 Visual Pathways

Higher mammals have several pathways projecting the visual information form the retina to the brain. Of particular importance is the pathway from retinal ganglion cells to the dorsal lateral geniculate nucleus (dLGN) to the primary and secondary visual cortex. As seen in figure 5 the optical nerves cross direction in the chiasm leading to contralateral visual processing. The dLGN works as a relay station, sending information to the cortex and other regions. Besides optical fibers going through the chiasm, ipsilateral connections have been found leading to the assumption of possible ipsilateral visual processing (Huberman and Niell, 2011).

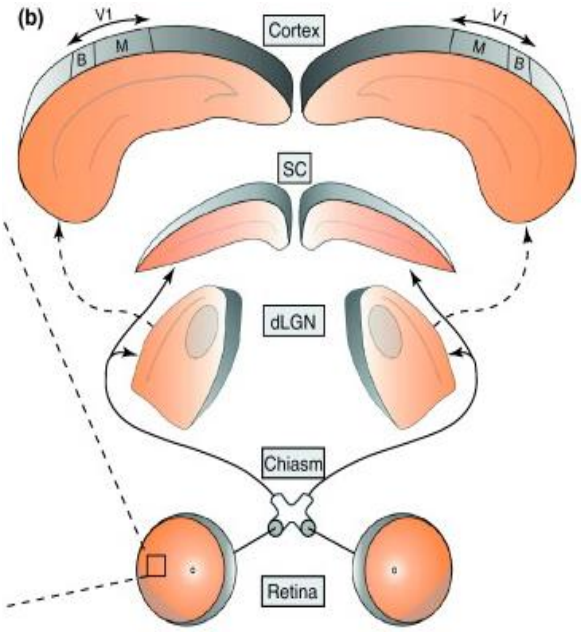



**Figure 5: Visual pathways in the mouse brain**

Solid lines representing direct retinal projections. Dashed lines displaying the geniculo-cortical pathway. By shaded parts of the retina, retinal ganglion cells (RGC) are indicated which project ipsilateral vision by not crossing the chiasm. The M and B section in the visual cortex show the Monocular (M) and Binocular (B) parts of visual processing. The oval parts in the dLGN are connected to the ipsilateral RGC axons (Huberman and Niell 2011). dLGN = dorsal lateral geniculate nucleus; SC = superior colliculus.

Vision-based reflexes as the optokinetic reflex on the other hand are triggered by the transmission of the retinal information to the assessory optic system (AOS) (Schmidt et al., 2001). The AOS consists of dorsal, medial and lateral temporal nuclei (Büttner-Ennever et al., 2014). Together they detect image drifts across the retina by obtaining information from the photoreceptors. Lateral and medial nuclei detect vertical movements while the dorsal nuclei process horizontal movement (Benkner et al., 2013). Two different kinds of ganglion cells located in the retina are responsible for sending movement information: ON – and ON-OFF direction selective retinal ganglion cells (DSGC). While the ON DSGC are focused on compensation of self-movement, the ON-OFF DSGC perceives motion of single objects and send this information to the superior colliculus (SC) (Pushchin, 2013). Douglas et al. 2005 claimed that large cortical lesions have an effect on the OKR of mice. Therefore only subcortical pathways are relevant to trigger the OKR.

## 1.4 Retinal degenerative diseases

Retinal degenerative diseases affect 285 million people (WHO Fact sheet 2014). Unfortunately, lost photoreceptors within the human retina cannot be intrinsically regenerate so that the loss is permanent in affected patients. In the western society people's lifespan was immensely increased due to modern medicine,

health and hygiene standards and the invention of new drugs like antibiotics. Vice versa the frequency of age-related diseases is increasing in consequence of the longer lifespan. One of these diseases is age related macula degeneration (AMD). Which is the leading cause for blindness in industrialized countries (> 2 million patients in Germany). One symptom of AMD is the deposition of so called drusen between choroid and retina leading to an primary degeneration of the retinal pigmented epithelium followed by the degeneration of the photoreceptors in the macula (cone-rich point of high acuity vision) and finally to blindness.

Retinitis Pigmentosa (RP) on the other hand is a genetically inherited retinal degenerative disease leading to a loss of rod photoreceptors in the outer region of the retina. Often developing as a form of tunnel-like vision with subsequent complete loss of vision due to the secondary death of cone photoreceptors. In Germany about 35,000 suffer from this disease.

### 1.5 Cell replacement therapies

Classical treatment approaches focus on delaying/stopping the progression of retinal degeneration. Although important, these attempts lack the ability to repair the vision loss. Therefore cell replacement approaches represent a promising new treatment strategy. Indeed, recent studies demonstrated the successful integration, maturation and functionality of primary photoreceptors transplanted in the adult mouse retina (MacLaren et al., 2006; Bartsch et al., 2008; Pearson et al., 2012; Santos-Ferreira et al., 2015). Moreover, protocols have been developed that allow the generation of photoreceptors from pluripotent stem cells in vitro (Lamba et al., 2006; Eiraku et al., 2011; Nakano et al., 2012; Zhong et al., 2014; Sarah Decembrini, 2015). Such stem cell-derived photoreceptors are currently investigated for their capacity to restore vision function following transplantation into mouse models of retinal degeneration. In relation to that the evaluation of functional vision improvement by these new therapies is of major importance. Morphological and electrophysiological tests are important means to validate the integration and maturation of donor photoreceptors beside stimulation by light. However, such measurements do not demonstrate whether

improvement on that level is also reflected in a functional improvement of vision in treated mice. Therefore it is of prime importance to have objective and reliable testing methods for assessing possible vision improvement after therapeutic treatments in mice.

## 1.6 Vision-based animal tests

A frequently used test to assess functionality of retinal cells in mouse models of retinal degeneration is the electroretinogram (ERG). This method records light stimulated electrical changes in the retina and is therefore a readout for retinal functionality (Peachey et al., 1989). Dependent of light intensity rod and/or cone driven reactions can be distinguished (http://webvision.med.utah.edu/). Yet the ERG can only give a readout about functionality of retinal cells but not about complex vision processing. In that regard optokinetic tracking (OKT) and the light-dark box paradigm are promising approaches to overcome this limitation.

### 1.6.1 Optokinetic tracking (OKT)

Movement *per se* can be separated into two groups; either the animal is moving or the surrounding environment. The visual perception of the environment while the animal is moving is stabilized on the retina by the vestibular ocular reflex (VOR) based in the vestibular system (VS). While environmental movement (by concurrent stationary behavior) is compensated by the optokinetic reflex (OKR) relying on the AOS (Pushchin, 2013). Together the two systems enable gaze stabilization, triggering stabilizing reflexes for head and body (Masseck and Hoffmann, 2009).

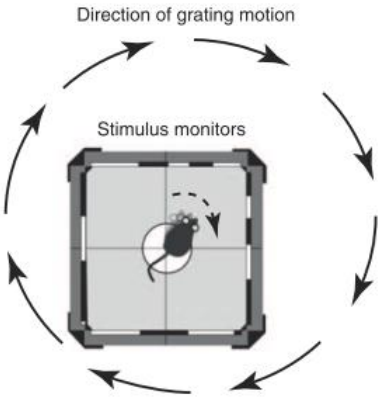



**Figure 6: Fundamental principles of the optokinetic tracking**

Involuntary head movement following global drifts across the retina are triggered by a moving stripe stimulus pattern (Huberman and Niell, 2011).

The optokinetic reflex can be observed in most animals and is described as an involuntary head and eye movement following global movements in the environment. To keep the image on the retina center, the animal´s eye and head is moving in direction of the movement (Benkner et al., 2013). This reflex can be triggered without prior training by presenting a drifting regular stripe pattern (Mitchiner et al., 1976). Based on the mononuclear characteristics of the OKR it is possible to test each eye individually. In contrast to behavioral analysis the OKT is based on a simple reflex instead of complex behavior and should be therefore unbiased by human interpretation and mishandling.

### 1.6.2 Light-dark box (LD Box)

Behavioral tests can be divided into two major groups: on the one side conditioned and on the other side un-conditioned models. Unconditioned tests (anxiety tests) include ethologically based paradigms that consider the animals' spontaneous or natural reactions and do not explicitly involve pain or discomfort. In conditioned tests it is necessary to train the animal to perform a certain behavior before the actual test is undertaken.

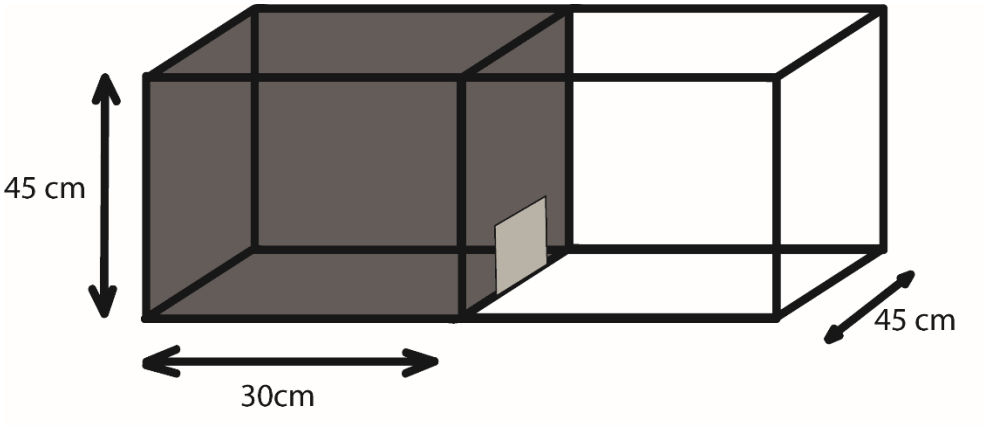



**Figure 7: Setup of light/dark box**

The LD Box consists of two equally sized compartments connected by a door. One is compartment is dark, whereas the other one is brightly illuminated. Mice are placed in the illuminated side of the apparatus and after a short habituation time the door between the compartments is opened. By infrared motion detectors surrounding the box several parameters like time in each compartment rearing-events or jumping is automatically recorded..

The LD Box represents such a test paradigm without the need of conditioning because it relies on a natural behavior of mice. Due to their nocturnal nature mice show light-aversion behavior when placed in the LD Box paradigm reflected in a preference for dark locations despite a their also present explorative drive. The apparatus consists of two equally sized compartments with one brightly illuminated (light compartment (LC)) and one dark compartment (DC). The two compartments are connected by a moveable door which allows the animal to freely transit from one compartment to the other. As a nocturnal animal wild-type (WT) mice prefer the DC over the light one. But due to their explorative drive they will also stay for some time in the LC. The higher the individual anxiety level the more time the animal will spend in the “safe” DC. Conversely, if the animals are less anxious they will show a stronger explorative behavior. Light induced avoidance behavior is based on retinal function and a shifted exploratory pattern is expected in mouse models of retinal degeneration compared to the WT. Since the aversion behavior is triggered by light conditions > 400 Lx (Costall et al., 1989) mice models of rod or cone degeneration might be distinguishable in the LD Box.

## 2 Aim of this study

The evaluation of visual repair after a therapeutic treatment is of major importance. Here, we evaluated whether the light/dark box paradigm and optokinetic tracking represent a reproducible and feasible test method for visual function in experimental mice. Moreover the methods are used to characterize vision ability in untreated and cell-transplanted mouse models of retinal degeneration.

# 3 Material and Methods

## 3.1 Mouse models

In this study six mouse models of retinal degeneration have been analyzed and compared to a wildtype strain to depict different states and symptoms of retinal degeneration. All mice were fed ad libitum with standardized food pellets and water before and after the test. They were group caged (up to five animals per cage) and ranged between 8 and 15 weeks of age.

### 3.1.1 C57BL6/J Wildtype (BL6)

C57BL6/J (BL6) is the most widely used WT strain in animal testing. Across all the strains involved, BL6 mice are included in nearly 30% of all anxiety studies (Bouwknecht and Paylor, 2008). It is also the background strain for most degeneration models used at the CRTD and therefore a suitable control group for comparison with mouse models of retinal degeneration in terms of vision ability regarding the behavioral analysis.

### 3.1.2 Rhodopsin knockout mouse ($Rho^{-/-}$)

Homozygous $Rho^{-/-}$ mice carry a replacement mutation in exon 2 of the rhodopsin gene, leading to a complete absence of rhodopsin (Toda et al., 1999) Rhodopsin deviancy causes absence of rod outer segments and rod degeneration over time. Eventually, this condition leads to a secondary cone photoreceptor loss, a common feature also observed in RP patients (Jaissle et al., 2001). The homozygote rhodopsin knockout ($Rho^{-/-}$) mice show complete rod dysfunctionality by the age of 4 weeks and cone function is completely absent by the age of 13 weeks (Jaissle et al., 2001).

### 3.1.3 Cone photoreceptor function loss 1 mouse (CPFL1)

CPFL1 mice carry a spontaneous mutation in the phosphodiesterase 6C gene leading to cone functional impairment and degeneration; thus the name 'cone photoreceptor function loss 1 (CPFL1)'. By 4 weeks of age, CPFL1 mice show no significant changes in the ERG under scotopic conditions but absent cone responses under photopic conditions (Fischer et al., 2010).

### 3.1.4. CPFL1/Rho$^{-/-}$ double transgenic mouse (Tg(CPFL1,Rho$^{-/-}$))

Tg(CPFL1;Rho$^{-/-}$) carry the rhodopsin knockout gene in combination with the spontaneous mutation in the phosphodiesterase 6C gene leading to cone and rod degeneration within 12 weeks.

### 3.1.5 Proline-347-to-Serine mouse (P347S)

Proline 347 to Serine (P347S) transgenic mouse suffers from severe rod photoreceptor degeneration due a dominant mutation in the rhodopsin gene. At 4 weeks only a thin layer of the photoreceptor layer (ONL) is left in homozygous P347S mice. Yet the remaining cone photoreceptors can still trigger ERG response at that age. (Li et al., 1996). The mutation leads to an aberrant transport of rhodopsin from the inner segments to the nascent disc membranes of the outer segments (OS). Therefore the rate of renewing the OS could be influenced and a progressive shortening of the OS occurs. It is also possible that a loss of cellular content to the extracellular space could lead to the loss of function or it is a combination of both (Li et al., 1996).

### 3.1.6 Rd1 mouse

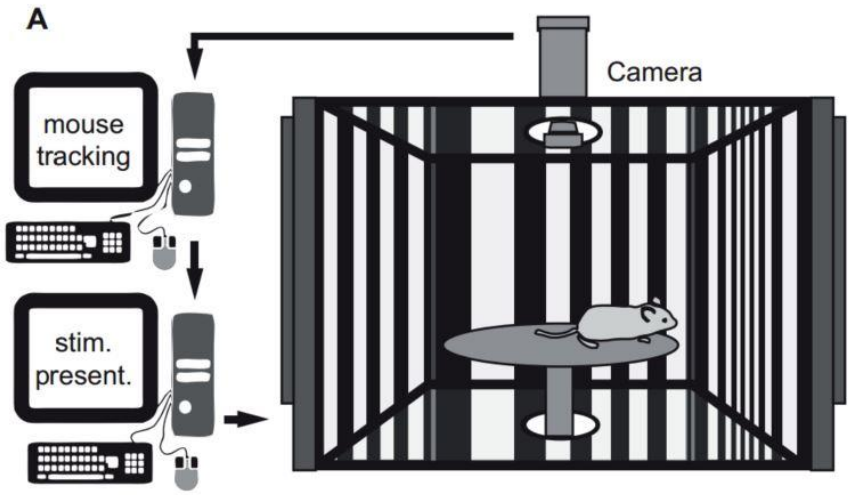
The retinal degenertation1 (rd1) model suffer from a severe and early onset loss of visual function caused by a mutation in the phosphodiesterase 6B (PDE6B) gene leading to the degeneration of photoreceptors (Chang et al., 2002). Photoreceptor nuclei begin to become pyknotic in the rd1 retina at postnatal day 10. These changes in the nuclei are leading to rod cell death the next few days. This results in rapid thinning of the outer nuclear layer with 18 days of age (Farber et al., 1994). Additionally this line is known for an extra mutation in the ON-bipolar cells Gpr179 leading to dysfunctional visual pathways (Nishiguchi et al., 2015). The rd1 mutation carrying mouse line in this study was the C3H wildtype strain. The mouse line was tested if it was suitable as a negative control group for the OKT.

### 3.1.7 Neural retina leucine zipper knockout mouse ($Nrl^{-/-}$)

The neural retina leucine zipper (Nrl) is a rod-determining transcription factor and thus exclusively expressed in rods (Mears et al., 2001). The mutation of exon 2 and 3 and entire coding sequence of the Nrl gene leading to a replacement by a Neo-PGK cassette (Mears et al 2001) and has been associated to retinitis pigmentosa as it shows degeneration over time. These cone-like cells produce some cone specific proteins and respond to short wavelength light in photopic ERG measurements (Mears et al., 2001).

### 3.2 Optokinetic tracking

The OKT (Striata Technologies, Tübingen, Germany) consist of four computer monitors circular placed in a custom made Box (48 x 48 x 48) sealed of incident light besides a hole for the camera centered on top of the box. Floor and roof are made of mirrors to ensure global display of the stripe pattern shown on the monitors. A 20cm high platform (diameter 9cm) was placed in the center of the apparatus. A thin unstable wire enclosed the platform to prevent jumping or unintentional falling of mice from the platform. Tests took place during the light cycle of the animals between 8 am and 8 pm. They were fed *ad libitum* with standardized food pellets and water before and after the test. They were group caged (up to five animals per cage) and between 8 and 12 weeks of age. The mice were gently placed on the platform with minimal tail restraint. When placed in the box the measurement was started (Fig 8).

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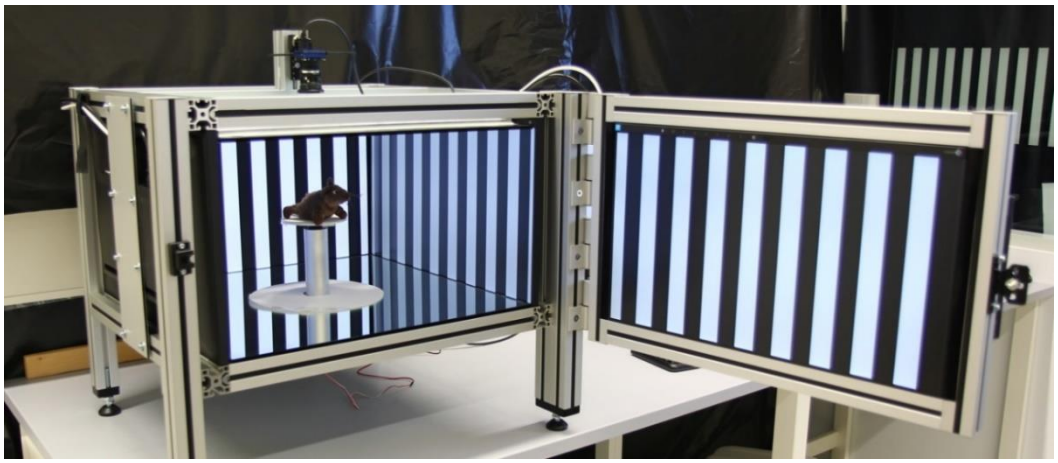

**Figure 8: Setup of the optokinetic tracking apparatus**

The custom made box consists of four monitors surrounding a platform for the test animal. At the bottom and roof mirrors are mounted to ensure gapless representation of the stimulus pattern. A hole in the center of the upper mirror allows the camera to record the animal movements. An algorithm assigns points to the main body parts (e. g. tail or corpus). It automatically detects ears and nose tip and calculates from the distance between these three points where the nose is located. If the position of the nose is relatively constant (mouse not moving) the stripe pattern is presented on the surrounding monitors. Exemplary presented stripe pattern (lower image) is equivalent to 0.061 cycles per degree (c/d) (Adapted from Benkner et al., 2013).

Parameters were set for pattern rotation speed at 12 °/s which is the optimum to elicit the optomotor response (Abdeljalil et al., 2005). The width of the stimulus pattern, consisting of one white and one black bar, was set to 22 (0,061 c/d). Minimum trial time was set for 35s. Maximum phase time was 5s and Michelson-contrast was set to 100% to begin with. The Michelson-contrast is defined as maximum illuminance subtracted by minimum illuminance divided by the sum of

maximum illuminance and minimum illuminance (Benkner et al., 2013). For simultaneous analysis of both eyes the rotation direction was set as randomized switching between left and right direction. For single eye analysis the rotation direction was set right for left eye and left for the right eye which is possible due to the monocular reflex characteristics of rodents (Benkner et al., 2013). Light intensity was set at 45 lx (measured on platform). The OKR was identified when the animal's head movement velocity is equal to the angular velocity of the rotating stripe pattern. The degree of correlation (rotation speed and consistence) between these velocities is then rated as a behavioral score (Fig. 8) (Benkner et al., 2013). The stimulus pattern was displayed automatically only when the mouse stood still and therefore an OKR could be detected by the system.

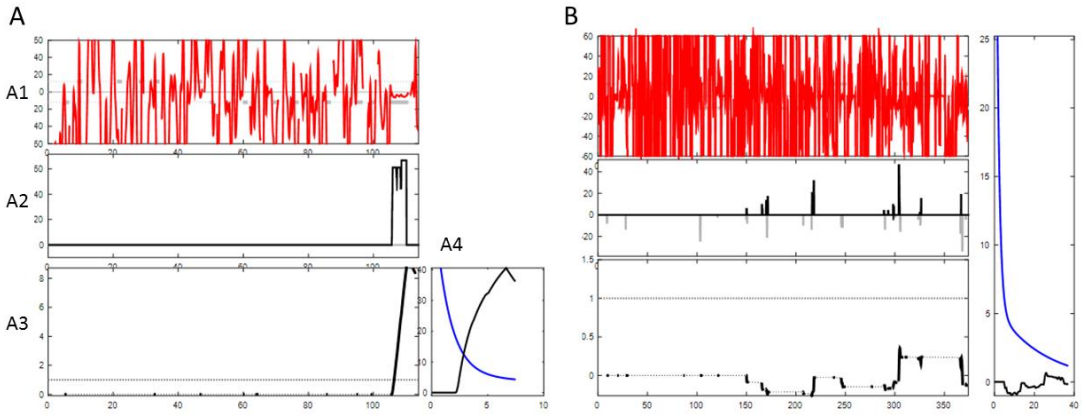


**Figure 9: Exemplary OKT analysis**

(A): Exemplary graph of successful OKT trial. (B): Exemplary graph of failed trial.
(A1): Red lines indicating head movement of mouse, grey lines show display of stripe pattern. Lines above the median show movements in left direction and lines below movements to the right side. (A2): indicates tracking moments by swings of the black line (grey lines indicating head movements antithetical to the stripe pattern). (A3): displaying triggered OKR according to real time. (A4): red lines shows the level of tracking events; blue lines indicate the to-reach-threshold. If the lines cross each other the OKR is seen as triggered.

The testing procedure for each animal was as follows: For every animal (every eye) the lowest visual threshold (Michelson-contrast) was evaluated where OKR could still be triggered. Every experiment starts with a contrast of 100%. Afterwards the contrast is reduced step-wise in this scheme: 50%, 30%, 20%, 10%, 5%. For every threshold the mice have three trials with fixed settings. If a mice repeatedly didn´t react to the drifting pattern in any given contrast the threshold was seen as reached and the test was aborted. Trials were repeated for the actual threshold if the animals showed cleaning behavior that can be

misinterpreted by the system as tracking events. Tests were aborted if a mouse showed high activity or the trials took longer than 45 minutes. The animals were caged separated from the others and retested with at least one hour pause between trials.

### 3.3 Light-dark box

The LD Box system used (TSE Systems, Bad Homburg, Germany) consists of two equally sized compartments (45cm x 45cm x 30cm). The two compartments are made of acrylic glass; one side is brightly illuminated while the other half is darkened. Two rows of infrared beams encircle the apparatus to measure every move, jump or rearing-event of experimental animals (Fig. 10). The light intensity at the center of the light compartment has a value of about 620 Lx in the LC while the DC is only enlightened by incident light through the connection between the compartments (Varying between 2.5 and 6 lux, which is close to optimum for mouse orientation and movement) (Thompson et al., 2010).

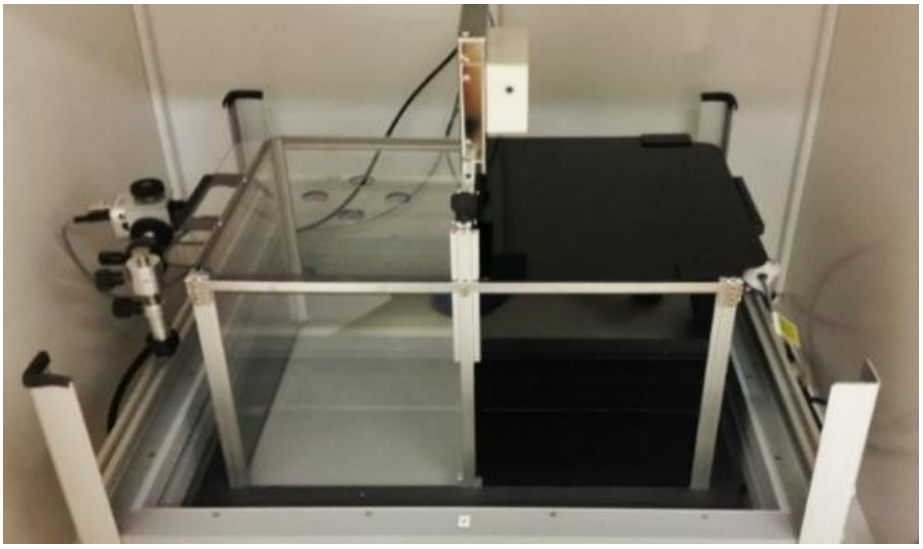

**Figure 10: LD Box apparatus**

The LD Box consists of two equally sized compartments. One is brightly illuminated by a roof lamp while the other one is darkened. Infrared sensors within the metal frame record the mouse behavior during the trials. Additionally two cameras, one centered above and one located on the left side allow the live observation of the animal. . The compartments are divided by a movable door allowing access to both areas.

Seven indicators were considered as readout parameters: Mean speed in each compartment, rearing events, overall distance, transitions between compartments, latency for first transition from light to dark side, time spent in the LC and distance covered in the LC. The first four parameters describe the general activity of mice in the apparatus. It is expected that general motor activity is suppressed due to the light conditions in the illuminated compartment and therefore increased in the DC in comparison. The three additional indicators are based on the assumption that a mouse will prefer to spend more time in the "safer" DC when it can freely choose.

The mouse was gently placed in the LC of the box. After 10 secs of habituation without tracking, a movable door opens and allows access to the DC. From then on the animal can move freely. The Box was cleaned with ethanol-free Mikrozid sensitive (Schülke & Mayr GmbH, Nordersted, Germany) before each experimental round to remove any distorting scent marks from prior test animals. Mice were dark-adapted overnight (over 12 hours) before the trial to provide maximum light sensitivity. Tests took place in the morning from 8 to11 am in a quiet, red illuminated environment.

### 3.4 Transplantation into P347S mice

Fluorescent photoreceptors were isolated from retinas of postnatal (PN) day 4 - 6 donor mice and enriched by CD73-based magnetic associated cell sorting (MACS) (Eberle et al., 2011). 2 x $10^4$ donor cells were injected into the subretinal space of adult P347s mice (done by a post-doctoral researcher in the lab, Dr. Ferreira). Sham-injected and untreated P347s mice served as controls. LD Box and OKT analysis where done 3 weeks post transplantation.

### 3.5 Statistical analysis

Experiments consisting of two groups were analyzed with two-tailed t-tests with a 95 % confidence interval by using GraphPad Prism software (GraphPad Software Inc, La Jolla, USA). Results with a p-value <0,05 were entitled

“significant”. For experiments consisting of three groups or more Tukey´s multi comparison test was used to compare the means of every single group. The confidence level was automatically adjusted for each group using GraphPad Prism software (GraphPad Software Inc, La Jolla, USA). Error bars in resulting graphs indicate the standard error of the mean (SEM) or standard deviation (SD) which shows the empirical measured variances in an analyzed group.

# 4 Results

At first it was assessed if the outcome of the tested methods is indeed driven by the visual function of the animals. Secondly, the methods were used to analyze different mouse models of retinal degeneration in relation to wildtype mice. And thirdly, it was investigated whether rod and cone mediated vision can be distinguished via the aforementioned tests.

## 4.1 Optokinetic tracking

First we tested if the tracking system provided by Striata Technologies triggered the optokinetic reflex in wildtype mice.
During the first trials (n=8) it was found that the OKR in wild-type mice could be triggered down to a 5% Michelson-contrast. Therefore, a visual threshold of 5% was set as full visual function baseline regarding the OKR. This is in accordance with Benkner et al. (2013) stating that in BL6 wildtype mice the OKR can be triggered by a stripe-pattern with a Michelson-contrast of 3.8% minimum. In a second step we analyzed for differences between WT mice and mouse models of retinal degeneration (Fig. 11).

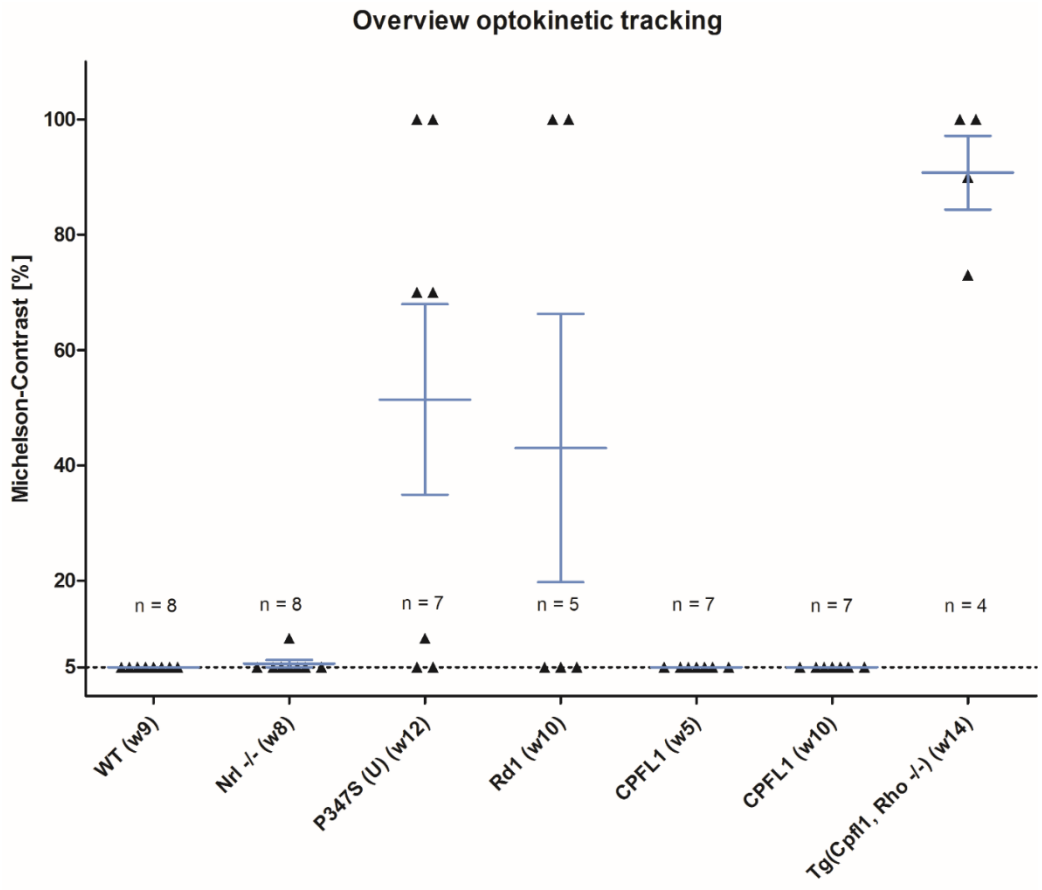


**Figure 11: Overview optokinetic tracking**

Every point indicates the smallest contrast animals have reached. The tickled line indicates the lowest threshold reachable in course of this experiment. 100 percent equals no measurable visual function. Thus no optokinetic response could be triggered in these mice. T = Transplanted; S = Sham-transplanted; U = Untreated. Mean with SEM (blue).

### 4.1.2 OKT measurements of retinal degeneration mouse models

Nrl$^{-/-}$ mice showed normal vision by the age of 8 weeks. The same applies for CPFL1 animals which showed no age dependent decrease in OKR. Results of 12 week-old P347S and 10 week-old Rd1 mice were heterogeneous. Some reached the 5% hurdle while other animals with the same age and degeneration did not react to the stripe pattern at all. Only Tg(CPFL1,Rho$^{-/-}$) mice showed a consistent loss in their ability to display an OKR at 14 weeks of age, none of them passed the 70% hurdle. (Fig. 11).

Only one experimental animal of the Nrl$^{-/-}$ showed slight visual impairment. A clear shift in the visual threshold was first recorded regarding the untreated P347S mice displaying visual impairment in the OKT. Yet two of them reached the 5% hurdle. Another one reached at least a contrast sensitive of 10 %. Two reached

70% while the last two did not react to the stripe pattern at all. Similar odd results have been displayed for Rd1 degeneration model. Three displayed a wild-type behavior while the remaining 2 showed no visual function.

### 4.1.3 Rod photoreceptor transplantation

In addition rod-transplanted and sham-injected adult P347s mice were analyzed for their ability to show an OKR compared to untreated mice (Fig.12 A).

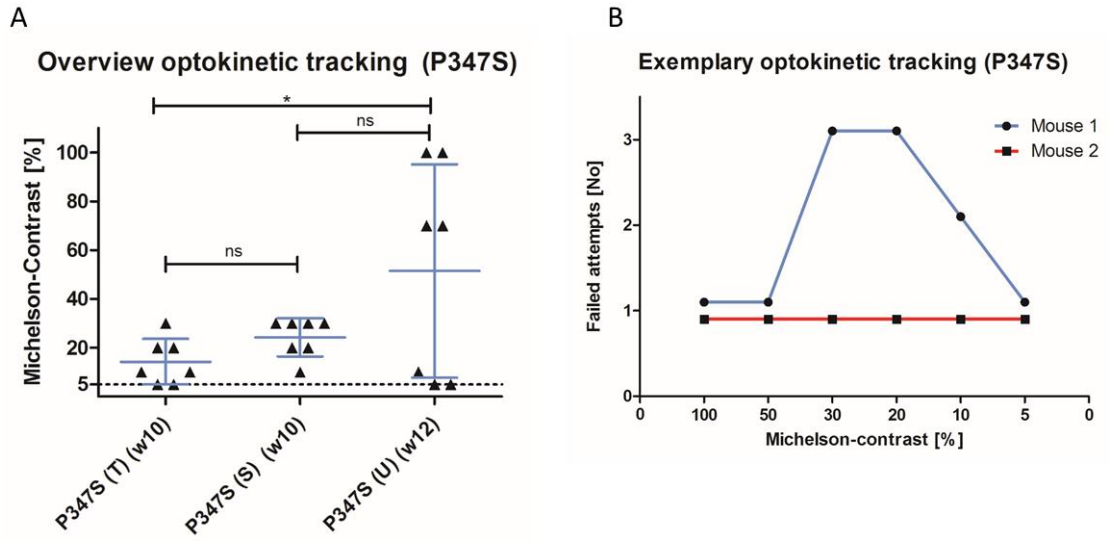


**Figure 12: Overview optokinetic tracking (P347S)**

(A) Every point indicates the smallest contrast animals have reached. The tickled line indicates the lowest threshold reachable in course of this experiment. 100 percent equals no measurable visual function. No optokinetic response could be triggered in these mice. (A) T = Transplanted; S = Sham-injected; U = Untreated. N = 7; Mean with SD (blue). (B) Exemplary optokinetic tracking of 2 rod-transplanted P347s mice. Although both reached the wildtype threshold of 5%, mouse 1 needed far more attempts to pass the particular threshold levels. For instance 3 attempts to pass the 30% and the 20% threshold.

Transplanted and sham-injected animals reached lower threshold in average than the untreated equivalents. In contrast to the prior tested untreated animals all experimental mice reached at least a visual threshold of 30%. Some of P347s mice which reached the wildtype threshold differed remarkably in the number of trials they needed to pass the “threshold ladder” (Fig. 12 B). That could be used as an additional performance marker, when the animal’s performance cannot be distinguished by the reached contrast threshold alone.

### 4.1.4 Single eye analysis

The results of the OKT were later divided into two major groups: single and both eyes analysis. While the single eye analysis should be more sensitive by doubling the number of tested objects as well as doubling the minimum phases per trial (single eye analysis at least 7 phases per trial, both eyes analysis average minimum 3.5 phases per trial) the both eye analysis is more practicable in terms of time needed to perform the test. As a baseline control group the vision ability of WT mice was tested (n = 8). Likewise the both eye analysis if they have reached a visual threshold of 5% (Michelson-contrast) their vision ability was set as full functional. WT animals consistently reached this threshold in single and both eye analysis. For further assessment of the results two groups that have been evaluated for conventional OKT have been retested for single eye analysis. Since there are no habituation factors in the monocular reflex properties for mice the results of the single eye analysis should increase the power of the both eye analysis (Benkner et al., 2013). In a first trial it was found that no significant differences regarding the OKR were present. Nonetheless a slight tendency was displayed pointing to slightly better vision in transplanted animals. To validate this trend, the host mice were re-analyzed with single eye analysis.

Such tendency was not displayed again in terms of single eye analysis (Fig. 13 B). Also the resulting variance was increased. This leads to the conclusion that single eyes in the same animal can perform differently.

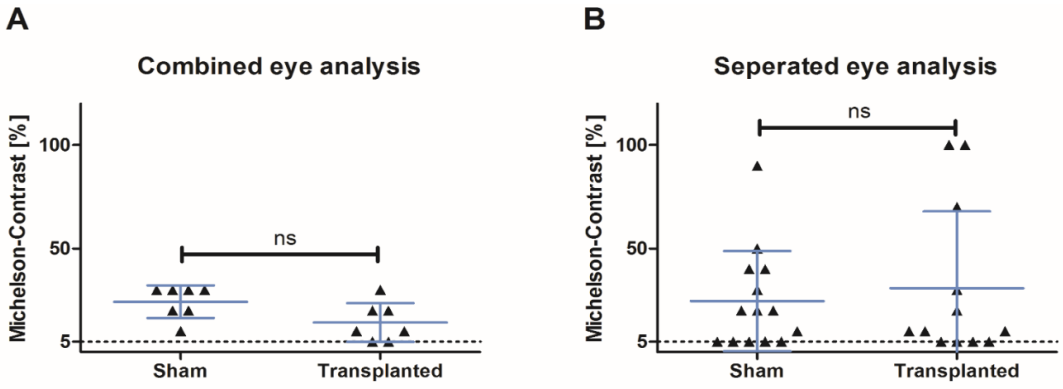


**Figure 13: Combined and single eye analysis of rod transplanted P347S mice**

(A) Two transplanted animals reaching the 5% threshold for wildtype vision but overall no significant difference were found comparing sham- and cell transplanted animals. The two groups, underlined by. (B) Analysis of individual eyes of the animals plotted in A. T = Transplanted; S = Sham-transplanted; U = Untreated. Mean with SD (blue).

## 4.2 Light-dark box

The LD Box was introduced during this study to test visual function and is a commonly used test-paradigm without the requirement of animal conditioning prior to tests. At first it was tested which parameters are strong predictors for the presence or absence of vision. Afterwards wildtype animals were compared to different mouse models of retinal degeneration

### 4.2.1 Assessment of readout parameters

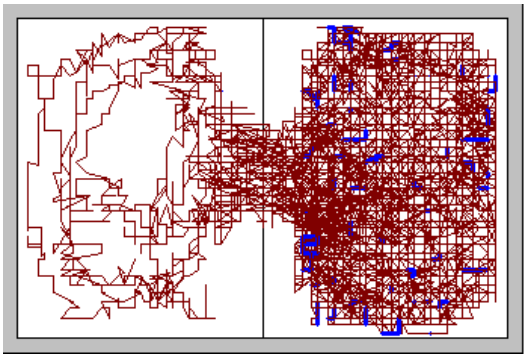

**Figure 14: Wildtype pattern of movement**

Representative movement (red lines) and rearings (blue) in a trial (10min) with a wildtype mouse in the light-dark box. WT mice mainly localize to the DC (right) and rarly explorate the LC (left).

The first question we addressed was whether a measurable difference in the behavior of WT mice could be observed within the two compartments. As a first indicator a pattern of movements over time was analyzed (Fig. 14). The distribution of time spend, distance covered and rearing events in the compartments, was strongly different. In following experiments several parameters were analyzed to identify feasible readout parameters that might be useful to distinguish between normal and visually impaired mice. Regarding this it was tried to point out countable and significant parameters as evidence for light aversion. Of the tested parameters the time spent in a particular compartment, the distance covered in the compartments, the mean speed in the compartments, transitions and explorative rearing-events turned out to be feasible markers to distinguish wildtype behavior from degeneration models (Fig. 15).

Throughout the literature it was shown that time spent in the particular compartments is a crucial parameter reflecting light-aversion. As expected the nocturnal nature of mice forces them to spend on average over 70% of trial-time in the DC (Fig. 15).

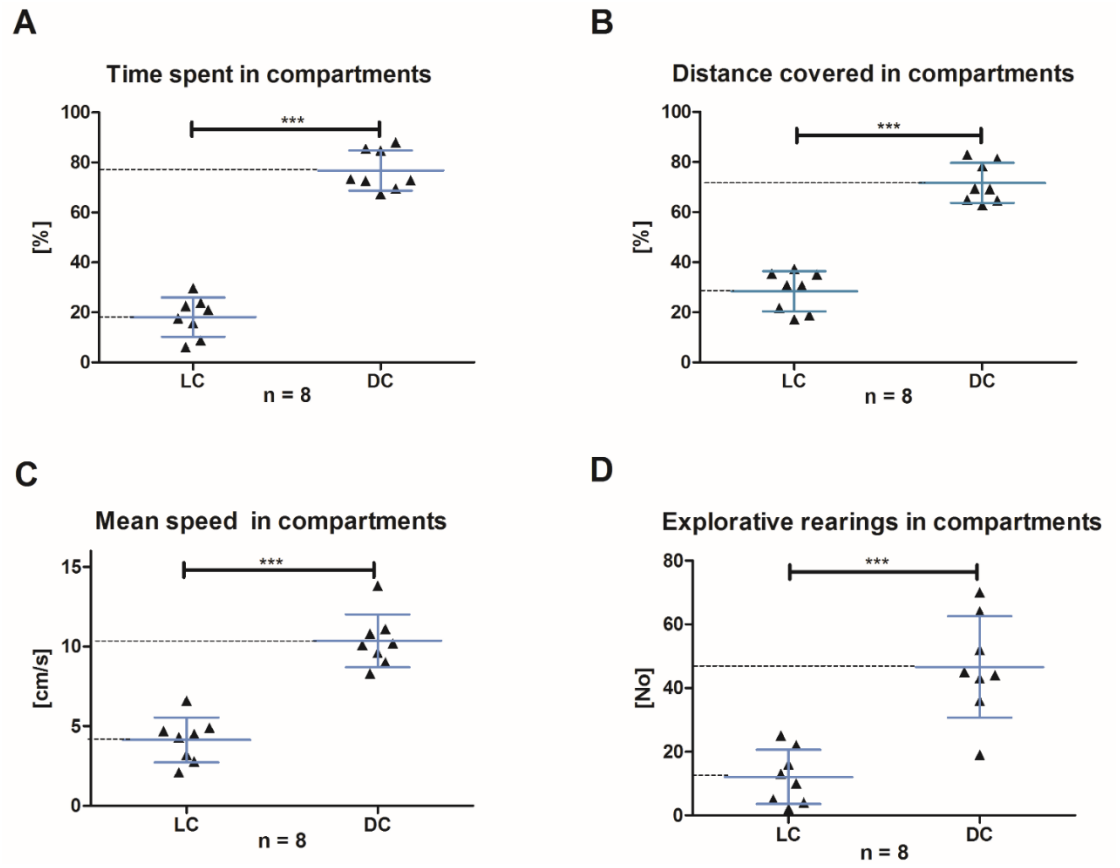


**Figure 15: Wildtype light aversion**

(A) WT mice spend significantly more time in the DC than the LC. WT animals spent on average 20 percent of their time in the enlightened area. (B) Experimental mice covered significantly longer distances in the DC when compared to the LC. (C)The mean speed was significantly increased in dim light conditions compared to the brightly illuminated area. (D) Rearing events were significantly increased in dim light conditions compared to the brightly illuminated area. Mean with SD (blue).

Interestingly, these differences were found throughout all measured parameters. This lead to mean speed as an alternative parameter (Fig. 15 C) since the pattern of mouse movement strongly depends on their surrounding conditions. The experimental animals tend to find a “home base” (e.g. the DC). When the “home base” is found it is the starting point for cautious exploration and quick returns. Also the number of explorative rearing events was strongly increased in the DC (Fig. 15 D), which is an indicator for higher activity.

### 4.2.2 Aversion behavior is light-dependent

Wildtype mice were tested, if the lack of light in both compartments prevents light-aversion and thus leads to a similar movement pattern in both compartments (Fig. 16). Significant behavioral differences were observed when comparing the LC with and without light. Without light the same animals spend increased lime periods in the LC (Fig.16 A) and showed higher activity in terms of (i) covered distance (Fig. 16 B), (ii) transitions (Fig. 16 D) and (iii) overall speed (Fig. 16 C).

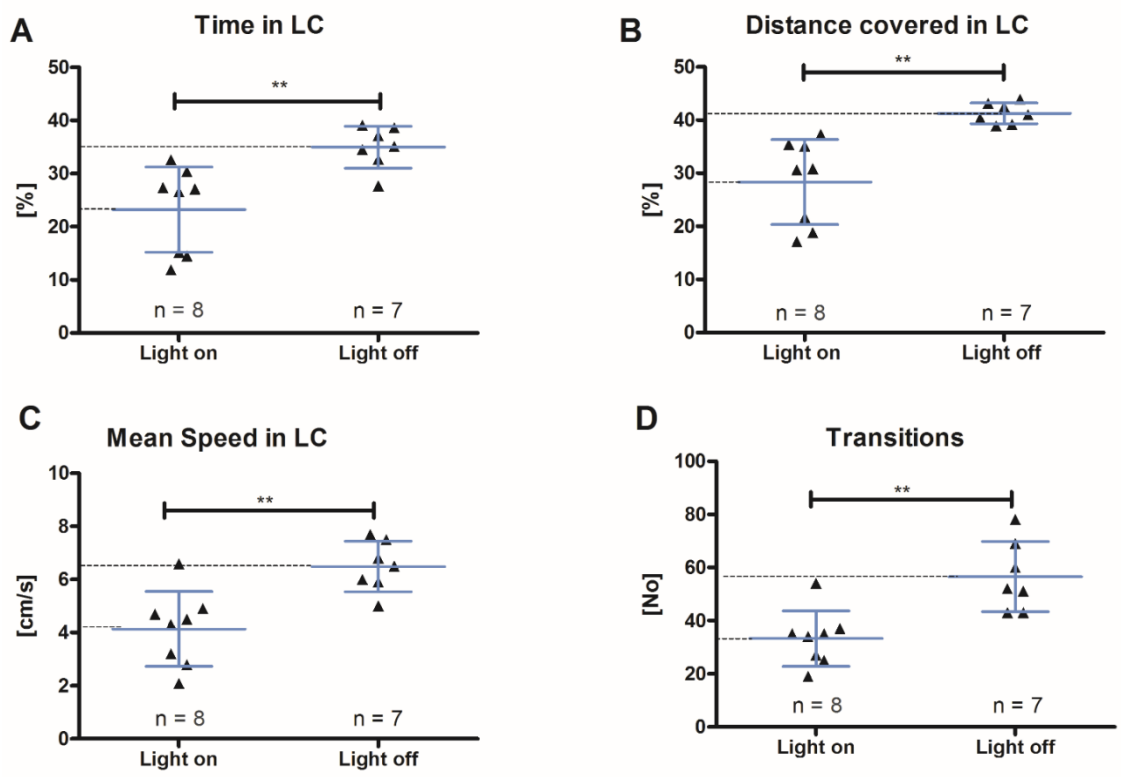


**Figure 16: Light dependence of aversion behavior**

(A) The time spent in the LC was significantly increased by erasing the aversive factor (i.e. light was switched off). (B) The distance covered in the LC was significantly increased proportional to the time spent in the LC. (C) The suppression of the mean speed was not displayed without the aversive factor. (D) The amount of transitions between the compartments was significantly increased by erasing the aversive factor. Mean with SD (blue).

### 4.2.3 Mouse models of retinal degeneration

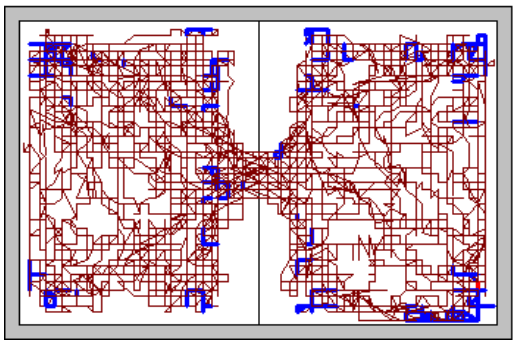

**Figure 17: Rho$^{-/-}$ pattern of movement**

Typical movement (red lines) and rearing-events (blue) of a Rho$^{-/-}$ mouse in the light-dark box with similar exploration pattern in the LC (left) and DC (right).

In course of this study it was found that WT mice showed a distinct pattern of movements independent of gender, increased stress level or adaption to the environment (see supplementary information: S1, S2, S4). Next different mouse models of retinal degeneration, i.e. Rho$^{-/-}$, P347S, and Nrl$^{-/-}$, were analyzed with the LD Box.

**Rho$^{-/-}$ mice:** Within the LC and DC Rho$^{-/-}$ mice showed significant differences regarding the distribution of movement and rearing events compared to WT controls (Fig. 17). Rho$^{-/-}$ mice spend equal amounts of time in each compartment and showed equally behavior on both sides (Fig. 18). This behavior is indicative for a lack of light aversion and thus impaired vision.

**CPF1 mice**: Five week-old CPFL1 showed a behavioral pattern similar to WT controls in regard to time, distance and speed within the compartments (Fig. 18 A-C), 10w old CPFL1 mice showed a significant increase in the analyzed parameters in the LC (Fig. 18).

**P347S mice:** The P347S mice showed significant differences regarding the distribution of time spent, distance covered and speed deviation compared to WT controls (Fig. 18). P347S mice spend equal amounts of time in each compartment and showed equally behavior on both sides (Fig. 18).

**Nrl$^{-/-}$ mice:** The Nrl$^{-/-}$ mice showed significant differences regarding the distribution of time spent, distance covered and speed deviation compared to WT controls (Fig. 18). Nrl$^{-/-}$ mice spend equal amounts of time in each compartment and showed equal behavior on both sides (Fig. 18). Yet the variability of each parameter analyzed was increased compared to the other degeneration models.

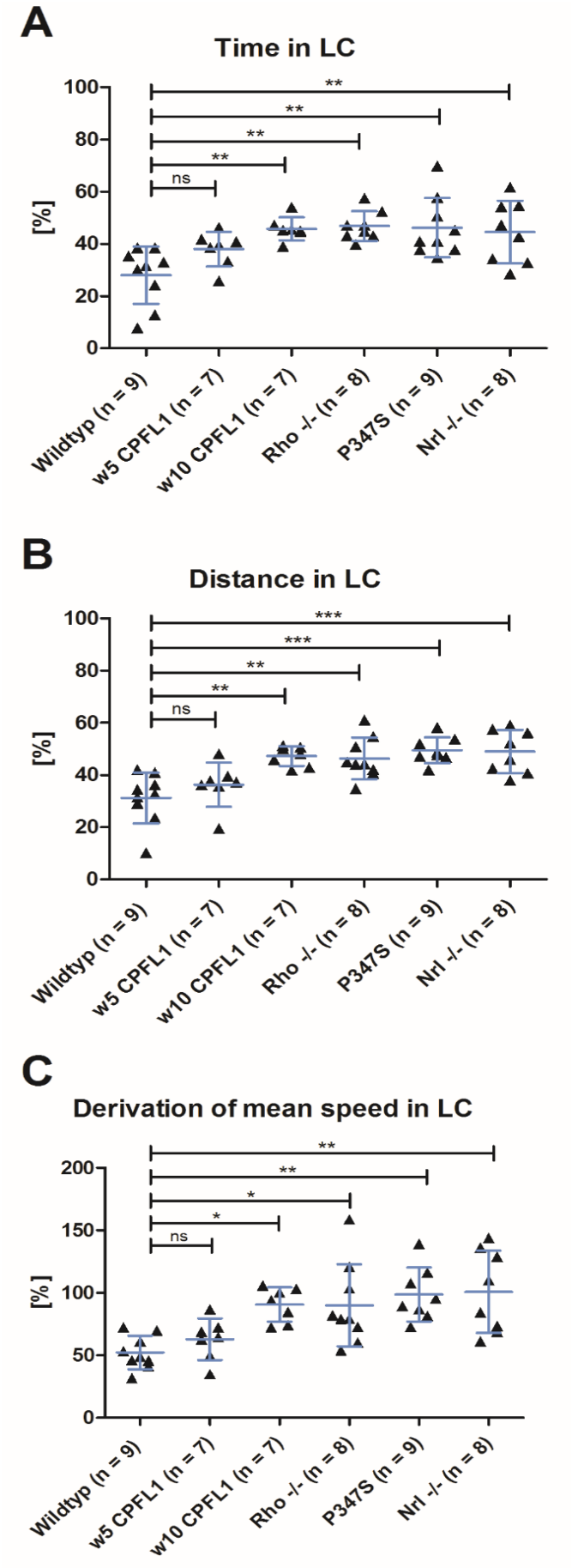


**Figure 18: Overview LD Box results**

In the parameters compared only 5 week old CPFL1 showed a behavioural pattern similar to that of the wildtype group. Neither adult CPFL1 nor the other degeneration models any of the other animals displayed clear light-aversion behaviour. Age: WT = 10 w; $Nrl^{-/-}$ = 8 w; CPFL1 = 10 weeks; $Rho^{-/-}$ = 9 w; P347S = 10 w. Mean with SD (blue).

While time and distance in LC are simple readout parameters the mean speed comparison is more complicated. Since the average speed of every group of mice is not similar and depends on numerous factors not their total speed was compared but the deviation of average speed.

100 percent threshold indicates that the average mouse is exactly as fast in the LC as in the DC. Values below 100 % display a speed reduction in the LC. Vice versa values above 100 % display mouse were quicker in the LC than in the DC.

As it is displayed in figure 18 the differences between wildtype animals and five week old CPFL1 mice were not significant. However the comparison between wildtypes and adult degeneration models showed significant differences by comparing their means. Nonetheless the disparity between wildtypes and adult CPFL1 respectively $Rho^{-/-}$ was less significant than the comparison to P347S and $Nrl^{-/-}$ on consideration of distance covered and deviation of mean speed in the LC (Fig. 198B, C).

The $Nrl^{-/-}$ showed no light aversion behavior when compared to wildtypes. (Fig. 18). CPFL1 were tested at the age of 10 weeks to differentiate between rod and cone driven behavior since results showed ERG signal is not completely absent at the age of 5 weeks (Fischer et al., 2010) which is in line with the LD Box results (Fig. 18).

No significant differences were present between adult CPFL1, $Rho^{-/-}$, P347S and $Nrl^{-/-}$. Comparison between rod and cone degeneration models showed no significant differences in the tested parameters. The only group not significantly differing from the WT was the young CPFL1-group (Fig 18).

### 4.2.4 Rod photoreceptor transplantation

Rod-transplanted P347S mice were tested in the LD Box to assess whether transplanted rod photoreceptors could lead to functional, i.e. visual, improvement. OKT results were presented prior in chapter 3.1. Since no light aversion behavior has been recorded in models of rod degeneration as it was shown in chapter 3.2 the demonstration of light aversion due to photoreceptor transplantation would be a hint for functional improvement. No significant differences have been found between the untreated, sham-injected and rod-transplanted P347S groups. All of them showed no light aversion behavior reflected in more time spent and distance covered in the LC. Their behavior was significantly different from the wildtype group (Fig. 19).

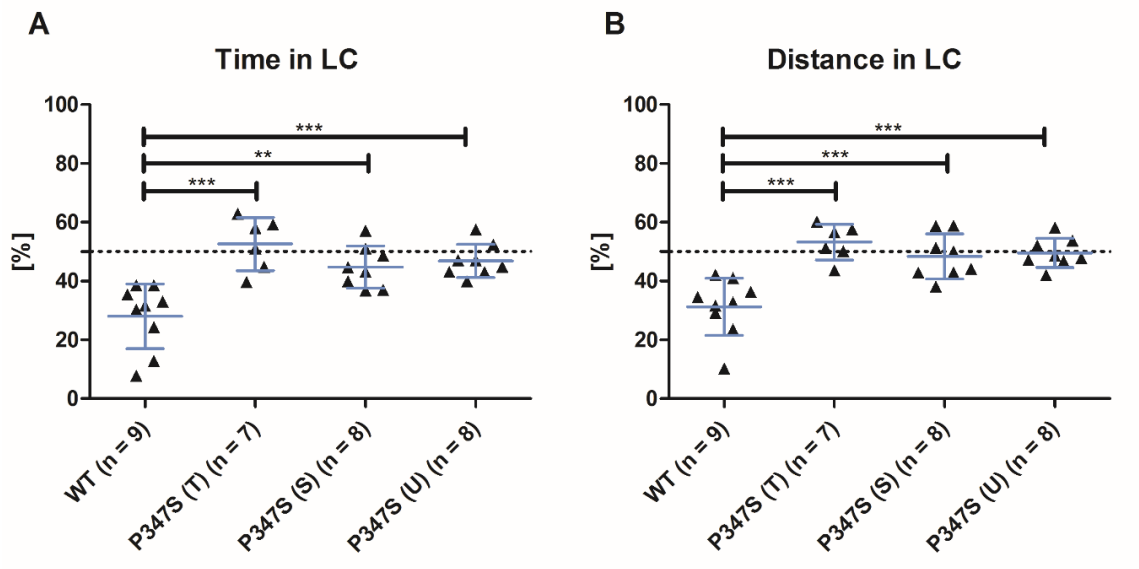


**Figure 19: Rod transplanted P347S**

(A) WT mice spend significantly more time in the DC than the LC compared to all groups of P347S. At least significant was the result regarding the sham transplanted group. WT animals spent in average 20 percent of their time in the enlightened area. (B) WT mice covered significantly longer distances in the DC when than in the LC. Simultaneously P347S mice showed no light influenced behavioral deviation. T = Transplanted; S = Sham-transplanted; U = Untreated. Mean with SD (blue).

# 5 Discussion

Vision impairment and blindness due to loss of photoreceptors represents the main cause for disability in industrialized societies. Thus, the development of novel treatment options has a high priority and pre-clinical studies provided evidence for successful replacement of photoreceptor by cell transplantation. In the majority of these studies mice were used as hosts as diverse transgenic mouse lines recapitulate retinal degeneration affecting humans. However, the mouse as a nocturnal animal does not depend on visual input as much as human beings and, thus, methods have to be developed that allow reliable and quantitative measurements of vision to assess therapeutic success. The aim of this study was therefore to establish two tests that allow the measurement of visual function in mice and find a feasible and streamlined protocol to assess visual repair in various mouse models of retinal degeneration. Therefore LD Box and OKT measurements were analyzed and quantified.

## 5.1 Optokinetic tracking

So far it is not completely clear if the OKR is solely cone-dependent under photopic conditions. Results of the OKT suggest, that the OKR can be triggered by rods and cones under photopic conditions since CPFL1 (cone knockout model) and $Nrl^{-/-}$ (functional transformation of rods into S cones) displayed no impaired functionality. Cahill and Nathans (2008) claimed that the OKR can be triggered by cones or by rods alone under photopic conditions (Fig. 20). Also Alam et al. (2015) showed that in CPFL1 (cone dystrophy) mice the OKR can be triggered despite the functional dropout of cone photoreceptors by the age of 5 weeks (M Dominik Fischer, 2010). A possible explanation for the rod function even under bright light conditions for rod stimulation could be that due to pupil contraction mesopic or even scotopic light levels reach the retina allowing rod functionality (Cahill and Nathans, 2008).

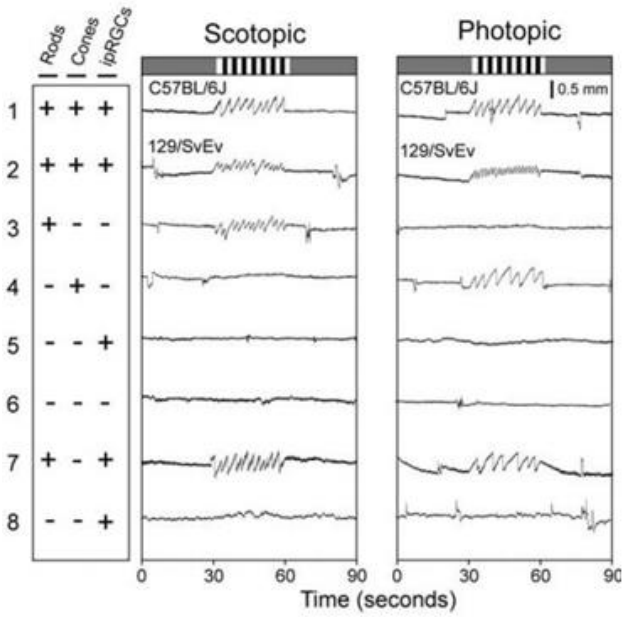


**Figure 20: Overview of OKT results of different photoreceptor and RGC knockout models**
(With different knockout combinations of rods, cones and retinal ganglion cells (ipRGCs**))**
(A) Abilitiy of different knockout mouse models to have a OKR triggerd (spiked line) under scotopic (0.3 average lux) or photopic (200 average lux) conditions after presentation of stimulus pattern (black and white pattern at the top). Under photopic conditions the OKR was triggered in a rod-deficient model (Line 4) as well as in a cone-deficient only model (Line 7) (Adapted from Cahill and Nathans, 2008).

Since the OKR can seemingly be triggered by rods and cones a double knockout mouse would represent a control where the OKR is absent. We analyzed Tg(CPFL1; $Rho^{-/-}$) mice which showed a clear distortion regarding the OKR with no or limited events at highest contrasts. However, more animals should be analyzed to confirm this finding as within this study only four mice were used. Due to breeding limitations no further double-knockout mice had been tested so far. Furthermore, it would be interesting to use additional rod and cone degeneration models like Gnat1 and Cnga3 double knockout mice which have been used in several studies ( e. g. Cahill et al. 2008) or generating new mouse lines by crossing a fast rod degeneration (e. g. Rd1 or P347S) with a cone dystrophy model (e. g. CPFL1).

Regarding the results of the P347S there is a wide variance of results in the untreated control group. They displayed visual impairment but were inconsistent during the trials Therefore the experimental animals should be re-genotyped to

avoid misinterpretation due to heterozygous mating partners as parents or animal selection. Since the OKT is dependent on mouse behavior another explanation of this outcome might be that the animals were transferred to a new experimental room prior to the test. The novel environment could lead to stress and hyper activity, thus bad or no measurable OKT results. In contradiction to that wildtype animals were transferred as well and displayed no impaired function. The variance of the results among the untreated P347S group have also implications on the rod photoreceptor transplanted P347S groups.

First, even with 10 weeks of age the cell-transplanted group shows still a good vision ability based on the OKT. So do the sham-injected animals, despite of having strongly degenerated retinas. Thus, although the primary rod degeneration in the P347S mice leads also to a strong secondary degeneration of cone photoreceptors there number seems to be still high enough to trigger the OKR at low spatial-contrasts at the tested age. These results indicate that further transplantations should be performed in a rod and cone double knockout model (e.g. tg(CPFL1, $Rho^{-/-}$)). There the assessment of possible vision improvement by OKT might be unambiguous, since ability to have the OKR triggered should be clearly worse than in wildtype mice. With such a baseline improvement by photoreceptor transplantation could be assessed properly.

Single eye analysis might be slightly more sensitive and would provide the possibility of having an animal-internal control eye (sham-injected or untreated). Nevertheless, this approach can't be recommended yet without further testing and optimization. The reliability of single eye analysis is questionable since it is based on the cross directional visual pathways. Yet ipsilateral pathways exist and could lead to misinterpretation that is in accordance with Alam et al. (2015) who claimed that the contra-lateral eye is not completely blind to the stripe pattern. Additionally the OKT device was optimized for both eye analysis. Another way to increase the sensitivity of the measurements would be to add visual acuity as a readout-parameter besides contrast-sensitivity. This could be achieved by finding the maximum stripes per degree a transplanted mouse could still distinguish at also different contrast values.

## 5.2 Light-dark box

Throughout all experiments performed significant differences in the readout parameters were not only found in speed differences, time and distance covered in the light side but also in rearing events, overall distance, transitions, latency and mean speed in the DC. The latter ones were not consistent for every experiment and could not be assigned to vision ability. Even though it was assumed that these factors are influenced by light these parameters showed no significant differences (see supplementary information: S1). Latency for the initial movement from the light compartment to the dark compartment seemed to be a promising indicator at first, but showed no clear differences between wildtype mice and mice with retinal degeneration. No habituation effects could be observed during the 6 day trial in time spent and distance covered. Nonetheless a slight decrease of latency was found as in the WT group (see supplementary information: S1) interestingly, the differences in mean speed occurred not only in the LC. Depending on the experiment these differences were found in both compartments (e.g. $Rho^{-/-}$ comparison to WT animals). A possible explanation for this parameter could be the influence of the individual differences from mouse to mouse. Therefore the mean speed comparison is assumed as most reliable if animals are compared within their own group. Besides the young CPFL1 mouse model the LD Box approach seems to be an on/off decision. Costall et al. (1988) showed in their experiment that a certain level of light intensity is needed to force a light aversion. Below a threshold of about 400 lux they found no behavioral changes (Costall et al., 1989). Indicating that pure rod-function is not sufficient to trigger light-aversion behavior.

With help of the LB Box paradigm it was tested if difference in vision capability between WT mice and several retinal degeneration models could be distinguished. In WT mice pattern of exploratory behavior are consistent and light dependent. Compared models of degeneration showed no clear light-aversion behavioral. These differences are present regarding the time spent in each compartment, the covered distance and mean speed in each area. A possible

explanation could be that beneath this threshold light intensities were perceived as twilight or dim light conditions. Thus no predator related anxiety behavior is displayed by the experimental animals. During the experiments it was also questioned why none of the degeneration models spent in average more than 47% of their time in the LC. Even if the 10 seconds of habituation are added to the total time, no equal distribution resulted. Yet even the light off control group showed reduced time spent and distance covered in the LC (see paragraph 3.2, P.16). This might be explained by two reasons: first, the initial placement (in the LC) might lead to habituation in this compartment and the memory effect which determines the favorite compartment. Because of that, additional trials with a changed start compartment and naive animals would be necessary to clarify this issue. Additionally, time needed for first transition to the opposing compartment has to be taken into account as well. In future projects it would be interesting to calculate time and distance after the experimental animal has discovered the entire LD Box. In this regard the mean speed comparison is the most reliable since it is not changed through choice of starting spot.

Prior discussed functional drop out of rod functionality in light intensities > 29 Lx leads to the assumption that the light aversion is based on cone functionality. This claim is seemingly confirmed by the CPFL1 animals, that show a decreasing awareness of light conditions, which it is in accordance with their cone degeneration (Comparison of 5 weeks and 10 weeks animals). Conversely to that P347S, $Rho^{-/-}$ and Rd1 mice are all rod knockout models of retinal degeneration. This observation might be explained by the disease progression, as mouse models of rod degeneration suffer from a secondary cone degeneration at late disease stages. The $Nrl^{-/-}$ mice are thought to be a model of pure cone function due to the transformation of rods into S cone-like photoreceptors. Yet $Nrl^{-/-}$ mice showed no light aversion behavior in adult stages, thus insensitivity to photopic light conditions. Interestingly, it has been shown that they produce a ERG response to photopic stimuli but no reaction to scotopic stimuli (Daniele et al., 2005). These results could indicate that the cone-like cells, although sensitive for photopic light conditions, could still facilitate rod-based reactions. A possible

explanation could be that the cone-like cells highjack the rod pathway, since they are still connected to rod bipolar cells (Daniele et al., 2005).

Due to the cone-driven character of the LD Box approach the transplantation of rod cells into the P347S mice was assumed to exert a beneficial effect on vision through indirectly. Loss of cone photoreceptors could be delayed due to rescue effects by donor rods which are known to support cones with several factors (Reichman et al., 2010). Additionally rod function as relay cells for cones in daylight conditions could help restore light aversion behavior (Szikra et al., 2014). Regarding the results it was found that no functional improvement has been achieved by rod transplantation in P347S mice. Possible explanations could be that 200,000 photoreceptors are just too little for functional repair. Therefore it would be important to comprehend different states of degeneration with the LD Box approach by continuously testing mouse models during their development to display whether light aversion is decreased parallel to the state of degeneration. Besides the CPFL1 mouse model the ability to trigger light aversion seems to disappear abruptly

At this point of research the LD Box seems to be a robust, reproducible and valuable approach to validate vision ability but the lack of sensitivity makes it questionable whether a slight improvement can be measured by this system. Still several questions have to be answered and some adjustments have to be done before this behavioral analysis can be seen as a fully established method. In case of small experimental groups the evaluation is difficult because the variances of WT animals can reach the visual impaired patterns. Therefore it is important to test large groups to reduce the random variance partially which occurs in most behavioral studies. Also improvements of the box and readout parameters should be considered, for example by changing the proportions of the Box. WT animals are expected to spend a comparable percent of their time in an enlarged LC while visual impaired mice should behave comparable to a random distribution. Therefore the ratio of DC and LC could be changed (Bourin and Hascoët, 2003). Another approach might be to add new parameters to the equation like the home

base behavior. As mentioned before the mice will find themselves a secure home base from where they start explorations in the surrounding area. An algorithm could be applied which automatically detects this home base spot which would be the region of longest stay and starting point of slow exploration with quick returns. Besides this functional improvement also other questions have to be answered. For example whether the light-aversion behavior can be introduced in animals blind from birth. If not: the general setup has to be re-evaluated for some degeneration models. OR how would a hypothetical restoration of vision change the animals' behavior per se and whether it is comparable to the WT behavior in the LD Box paradigm.

Yet in this study it is shown that the behavioral analysis of mice is a powerful tool to validate the functionality of vision in mice. The LD Box provides measurable and reliable differences regarding the discrimination between animals with normal phenotype and models with retinal degeneration. It was shown that the animal pattern of movement shifted due to the influence of light and therefore assumed that the behavioral analysis of mice could be a useful tool to evaluate major functional restoration of vision after photoreceptor transplantation.

### 5.3 Synthesis of results

Comparing both methods the first remarkable difference is that light-aversion in the LD Box could be triggered in wildtype animals and young CPFL1 mice but not in the retinal degeneration models. In contrast, the OKR could be still triggered until a quite low contrast-threshold in almost all animals. That indicates that the OKR can be triggered even if already large numbers of photoreceptors are degenerated, while the light-aversion behavior is already disrupted at the same time. The margin for detecting small differences in vision ability seems to be therefore wider with the OKT compared to the LD Box. Summarized the appropriate methods and there order of application for assessing possible vision improvement has to be chosen carefully. While the OKT seems to be more sensitive the LD Box is more practicable in terms of time needed per animal (10-

15min compared to 20-240min). The LD Box poses a promising method to assess possible vison improvement of cone function if for example cone photoreceptors are transplanted in a cone-deficient model. The decision which method should be used is also dependent of the degeneration model and the transplanted type of photoreceptors. So far the OKT can be used reliably to asses transplantation-based improvements of cone function if double knockout mice were used as hosts. To asses possible improvement of rod function the system would have to be adjusted to scotopic conditions (<1,5 Lx). Another improvement would be the fixation of an experimental animal on the platform. This would decrease the necessary testing-time per animal immensely, since the OKR can only be measured by the system when the animal is standing still so far On the other hand , the apparatus was created to display natural behavior. A fixation of mice would be contradict this paradigm. In this state of the establishment of the OKT it is necessary to improve the functional readout by adding the prior discussed parameters like visual acuity and number of failed trials.

# 6 Conclusion and future perspectives

A crucial goal of the cell replacement strategy to improve vision is to determine whether cell transplantation into the mammalian retina leads to functional improvement in vision ability. Therefore 7 transplanted and 8 sham-injected P347S were tested in the LD Box paradigm to assess whether lost light aversion due to photoreceptor degeneration can be triggered again by transplanting 200,000 rods in the subretinal space. It was found that no increased light sensitivity was triggered by cell transplantation. Neither time nor distance was reduced in the LD Box paradigm. Similar results have been found for the OKR. A possible explanation could be a still insufficient number of transplanted cells (200,000) to improve vision. Another explanation might be that these tests are cone driven despite other results (Cahill and Nathans, 2008) and therefore not suitable to display improvements by rod transplantations. Furthermore rods could have been transplanted into regions where they are not reached by appropriate amounts of luminance due be activated.

Concerning the assessment of higher cortical vision improvement the LD Box is particularly useful for its relatively easy setup and ability to give the experimenter feedback about the improvement of higher vision ability without the need to condition the animals beforehand. That is of particular importance since further transplantation studies are likely to use completely blind mice to assess the outcome of transplantations where visual conditioning would be impossible. Nevertheless, also the LD Box has its aforementioned disadvantages leaving for the conception new vison-based behavioral test paradigms. For that reason a new testing approach is presented here.

Suggestion of new conditioning-independent approach for testing vision ability in mice

Based on the visual cliff test mice have a natural anxiety of heights and there explorative drive the Jump-Decision method tries to combine these two factors (Bourin and Hascoët, 2003; Fox, 1965). A novel test system could be the forced decision to jump from a platform (Jump-Decision test) by shaking the platform or otherwise unpleasant conditions for the animal (e.g. heat or electric shock) to a lightened area. As for the Light-dark box no prior conditioning would be necessary. A single mouse would be placed on a platform in a complete dark environment. After a short time of habituation one quarter of the floor of the compartment is dimly enlightened. The mouse is expected to leave the platform eventually due to its size and uncomfortable setup. Since the likelihood to jump should correlate to the ability to visually assess the height and the location of the enlightened corner it is expected that a mouse with functional vision is willing to jump after short time and will eventually decide to jump in the direction of the light source (dim light conditions < 1,5 Lx) As another possibility the setup could be readjusted not basing on light but on adjustable floor height ( Fig. 21, 22) Therefore the light conditions would not be influencing the surrounding floor plates and photopic conditions could be used.

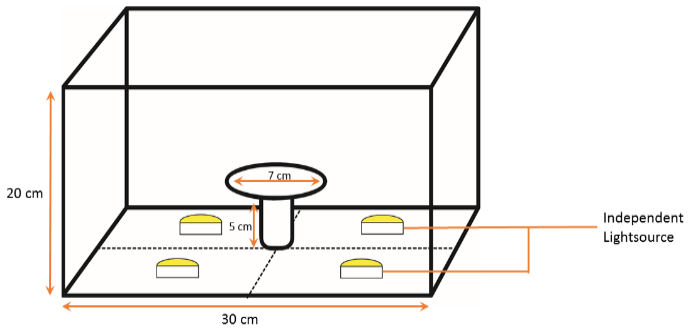


**Figure 21: Jump-Decision-Test (light based)**

Mice should differentiate the illuminated floor plate and therefore realize that she could leave the platform

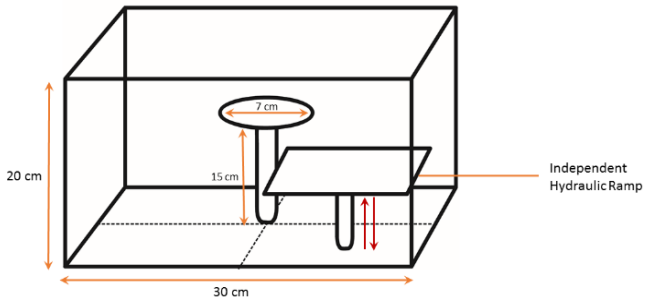


**Figure 22: Jump-Decision-Test (movement-based)**

This setup could be executed under different light conditions. Mouse should realize the decreased height difference of the platform compared to the floor and decide to leave the starting spot. Yet orientation by noise has to be minimized for optimal readout.

Possible readout indicators could be decision to jump on the illuminated / elevated platform and time needed to leave the platform. Since it implies active decision making instead of instinctive or reflexive behavior it would be also a readout for higher vision improvement.

# 7 Zusammenfassung

In den letzten Jahren wurden neue und komplexe Protokolle zu Wiederherstellung der Sehfähigkeit kreiert. Während die Transplantation und Orientierung der Zellen durch die Immunohistochemie dargestellt werden kann, können funktionelle Verbesserungen durch Zelltransplantationsansätze noch nicht bewertet werden. Das OKT oder die LD box stellen zwei vielversprechende Methoden dar um eine funktionelle Wiederherstellung der Sehfähigkeit zu belegen. Ziel dieser Arbeit war zu untersuchen ob diese zwei Geräte sinnvolle Testsysteme darstellen um Ergebnisse in weiteren Experimenten zu erzielen. Durch den Vergleich von WT Mäusen mit Modellen retinaler Degeneration wurde entdeckt, dass der Verlust von Sehfähigkeit mit den zwei Testsystem dargestellt werden kann. Die LD Box unterteilt die Mausmodelle grob in zwei Gruppen (normale Funktion und gestörte Funktion) durch die Parameter verbrachte Zeit, überbrückte Distanz und Abweichung der Durchschnittsgeschwindigkeit aufgrund von hellen Lichtbedingungen. Der OKT Ansatz hingegen scheint sensitiver zu sein wenn es um die Analyse einzelner Tiere (oder Augen) geht und ist deswegen passender um kleine Veränderungen der Sehfähigkeit zu detektieren. Daher scheint das OKT die logische Grundlagenmethode zu sein (neben Bildgebenden Verfahren) um erfolgreiche funktionelle Wiederherstellung zu beweisen oder zu negieren.

# 8 Summary

New and complex protocols regarding visual restoration were created in the last years. While transplantation and orientation of the donor cells can be displayed by immunohistochemistry the functional improvement is way more complicated to show. Methods like the optokinetic tracking (OKT) or the light dark Box (LD Box) can be used to assess visual improvements. Aim of this work was to investigate whether the two devices represent useful test systems as readout methods for further experiments. By comparing WT mice with models of retinal degeneration it was found that the loss of vision ability can be displayed with the two systems. The LD Box shows rough differences separating mouse models in just two groups (normal function and distorted function) by differences in time spent, distance covered and mean speed interference due to bright light conditions. The OKT approach seems to be more sensitive in terms of single mouse (+ single eye) analysis and is therefore more suitable to detect minor changes in vision ability. Therefore the OKT seems to be the logical baseline method besides imaging procedures to proof/disproof successful functional restoration.

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# Appendix

## 1. Supplementary information to the LD Box

Prior to the experimental work of this thesis a lot of experiments were made to test the reliability and reproducibility of the LD Box procedure. Therefore certain changes were made in the original setup to prove / disprove the influence of the following factors.

### 1.1 Habituation

To evaluate whether repeated testing can be performed (e. g. the light on/off paradigm, chapter 3.2) it was checked how habituaton factors influence the mouse behavior in the LD Box paradigm. In case the mouse behavior is changed through the adaption to the novel environment it would be necessary to have naive animals in each trial.

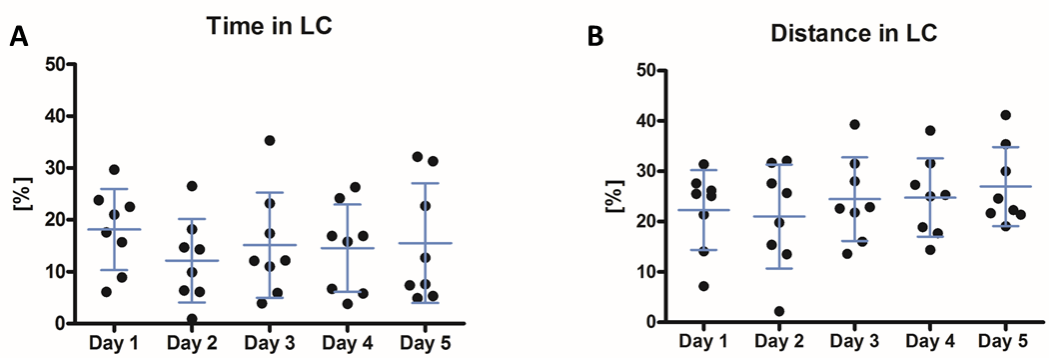


**Figure 22: Habituation effects of wildtype mice**

No habitational factors occurred during the 10 day trial in time spent (A) and distance covered (B) by experimental animals. Mean with SD (blue).

Among the tested parameters there were no significant differences. Time and distance in the LC can be seen as robust indicators which are not changed through habituation (Fig. 22). Also in a day to day comparison a strongly seen correlation of distance and time appeared likewise in the light off paradigm. Therefore it is assumed that repetitive testing is possible with these parameters.

## 1.2 Gender differences

It is stated by several authors that the gender of tested animals could have crucial influences on their behavior regarding the LD Box (Bourin and Hascoët, 2003). For this reason a group of female mice was tested under similar conditions as their male counterparts. No significant differences were found in all seven parameters recorded (Time, distance, mean speed, rearing-events and latency for first transition to the DC). Exemplary four parameters are shown in figure 23.

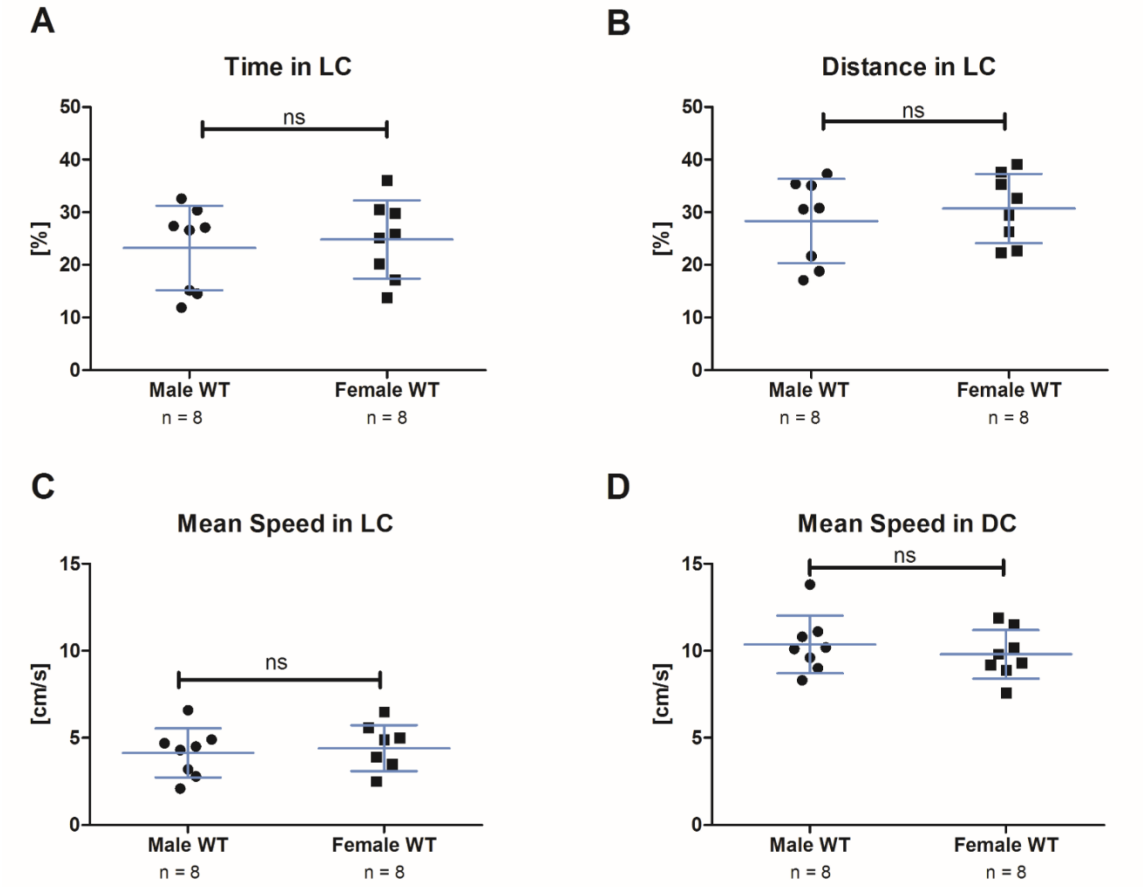


**Figure 23: Gender related differences**

No significant differences in the parameters could be observed between male and female mice. Mean with SD (blue).

### 1.3 Means to increase test sensitivity

After it was confirmed that there is a measurable difference between the two compartments in means of activity and duration of visits the next step was to try to increase the sensitivity of the LD Box.

Increased illuminance (1000 lux):

Therefore an external light source was provided capable of creating light intensities of 1000 Lx. No difference in light dependent behavior of WT mice was observed when comparing different light intensities, i.e. 600 and 1000 lux (Fig. 24).

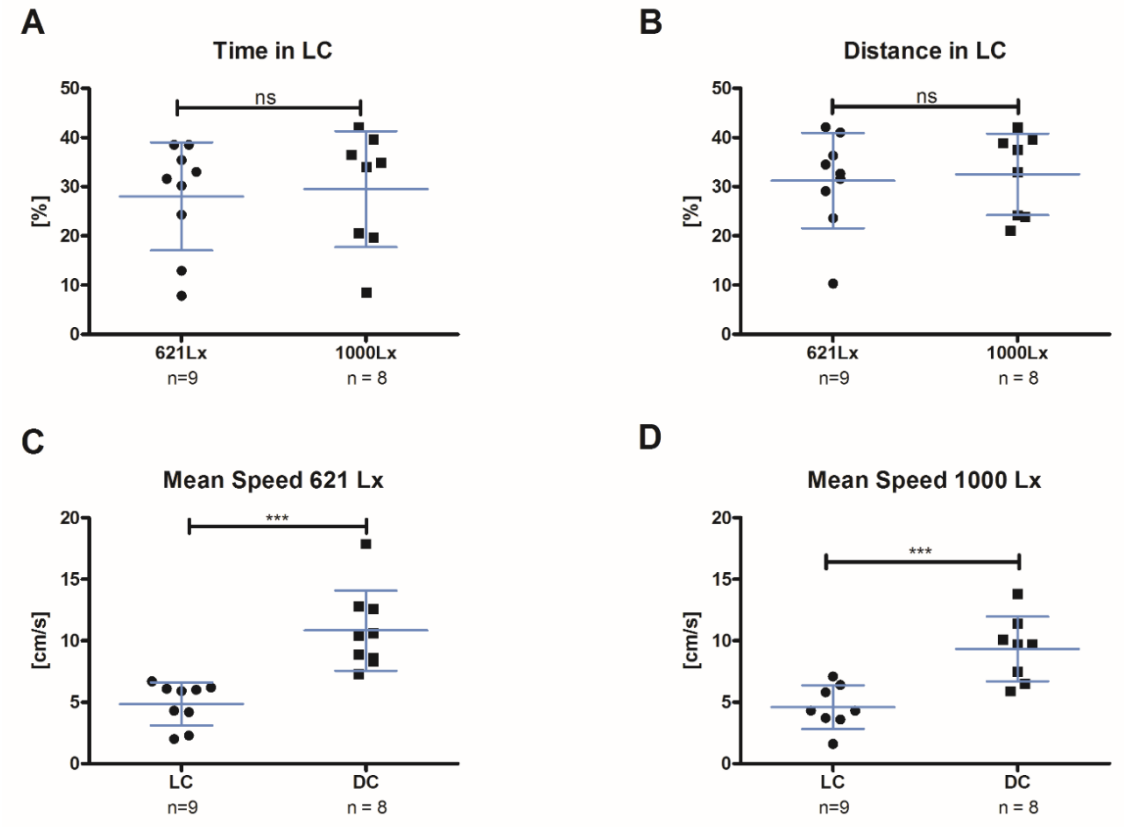


**Figure 24: Increased Illuminance**

No significant differences in the parameters could be observed between animals in 600 lux and 1000 lux conditions. Mean with SD (blue).

Pupil dilation via atropine:

With the pupil dilation via atropine the amount of light reaching the retina is increased. But due to the increased stress level the results should be taken with caution. For providing the dilation the animals have to be caught and fixed. Due to this treatment a higher anxiety behavior was expected.

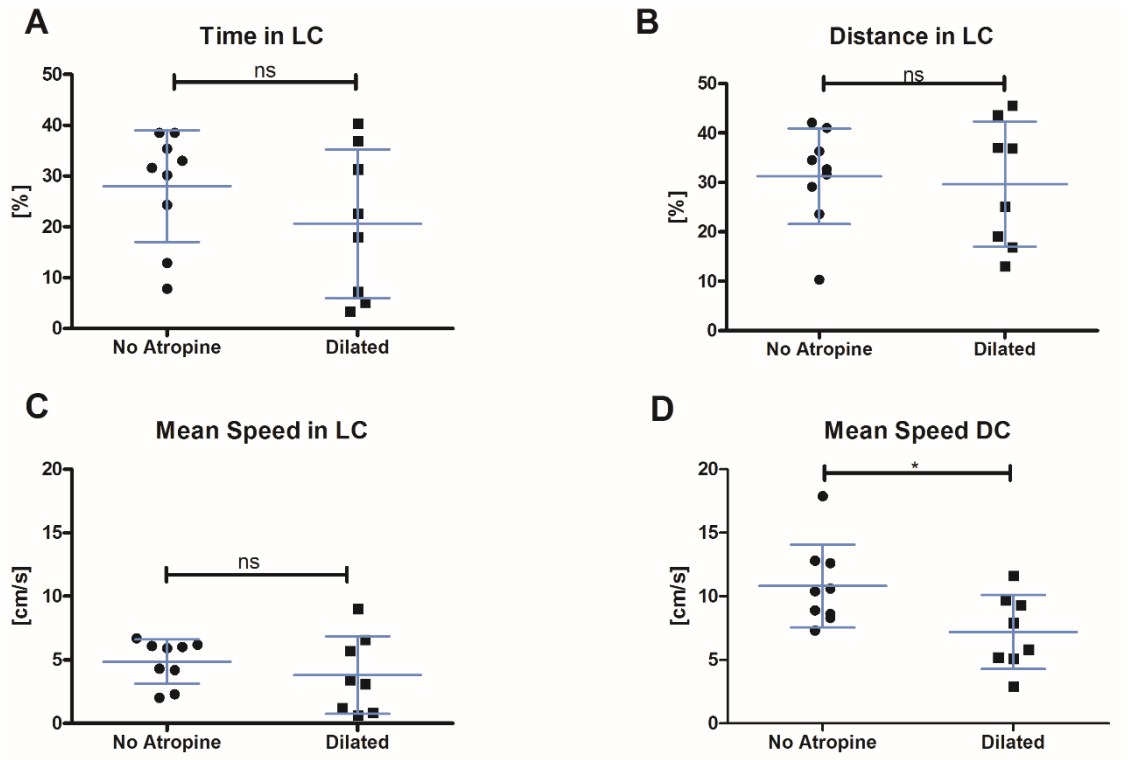


**Figure 25: Dilation of atropine**

No significant differences in the parameters could be observed between atropine dilated animals and the control group besides the mean speed in the DC. But a correlation between time in the LC and the overall activity was found. Mean with SD (blue).

With the dilution of pupils with atropine none of the three most consistent indicators were changed significantly (Fig. 25). A slight shift to more anxiolytic behavior (e.g. time spent in the LC (Fig. 25 A) can be shown which is influenced by the wide distribution of the results. The fluctuation is increased comparing to the control group which leads to the conclusion that it is not a general response to increased light aversion but to personal differences in stress reaction.

Prolonged time frame:

No significant differences were found due to longer experimental time per trial in the LD Box. Also the variances were not changed (Fig. 26). The elongation of the time frame seemed not to be suitable as a practicable improvement to the LD Box paradigm because neither the significance nor the variances were considerably changed.

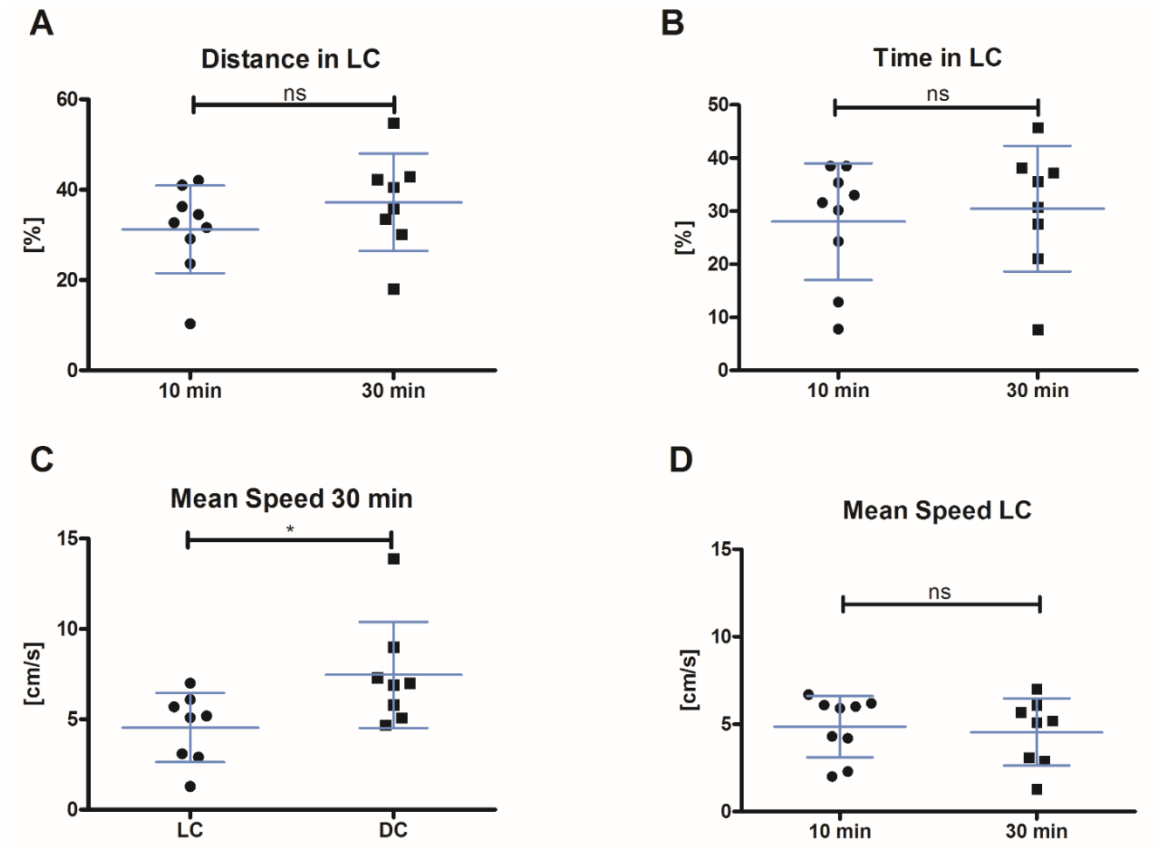


**Figure 26: Prolonged time frame**

No significant differences were found between the 10 min and the 30 min groups Mean with SD (blue).

It is also arguable that for the light aversion test the elongation of the time frame is a misconception due to the decreased level of activity occurring by prolonging the individual trials (Fig. 27) whereby the results got less informative. Since the LD Box is an unconditioned model the natural spontaneous behavior is measured. Therefore the decreased activity could be considered as a form of

adaptation. Regarding the elongation of the time frame the novelty of the surrounding should be seen as a reducible factor.

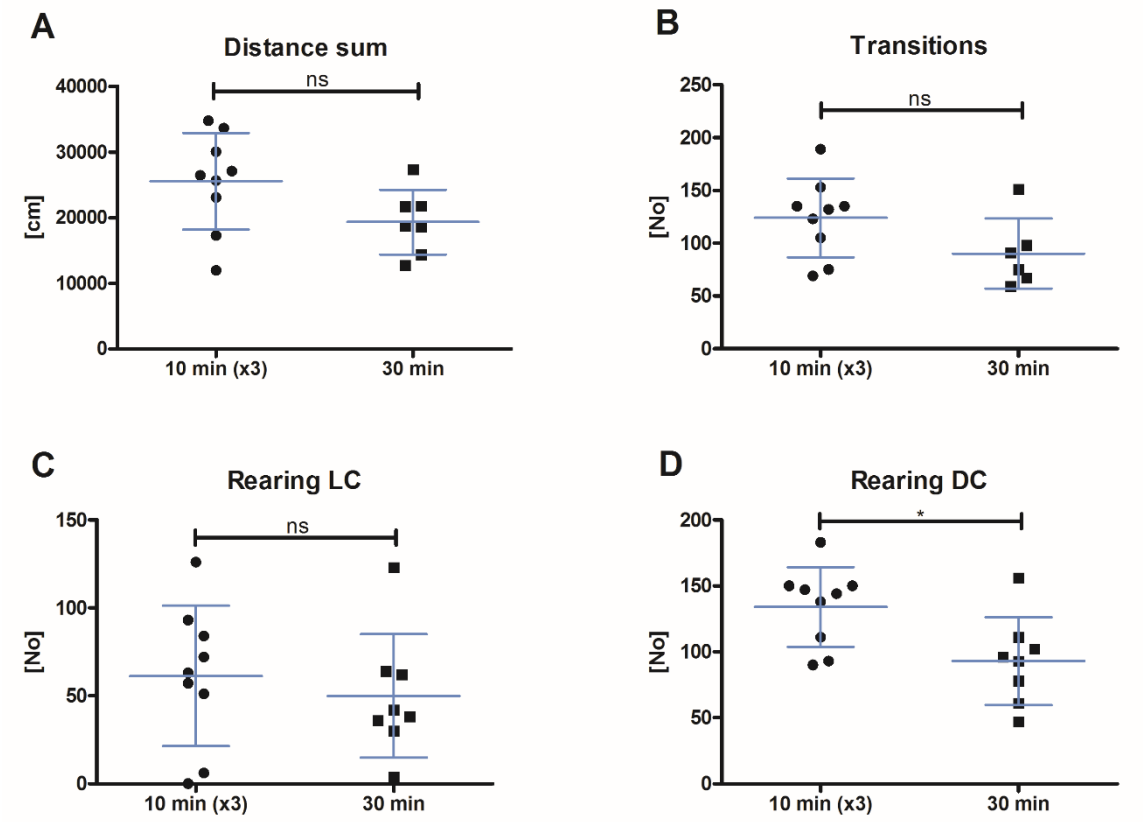


**Figure 27: Elongated time frame (decreased activity)**

The overall activity was decreased due to the longer experiment time. Shown in comparison to the extrapolated results in the 10 min trial. Mean with SD (blue).

## 1.4 Separating informative indicators

The multi-conditioning system of TSE systems is capable of recording several different indicators. During this study it was found that just time and distance in the LC and the deviation of mean speed are consistent values throughout all experiments. Yet some indicators seemed to be significant but with closer examination failed to indicate light induced behavior.

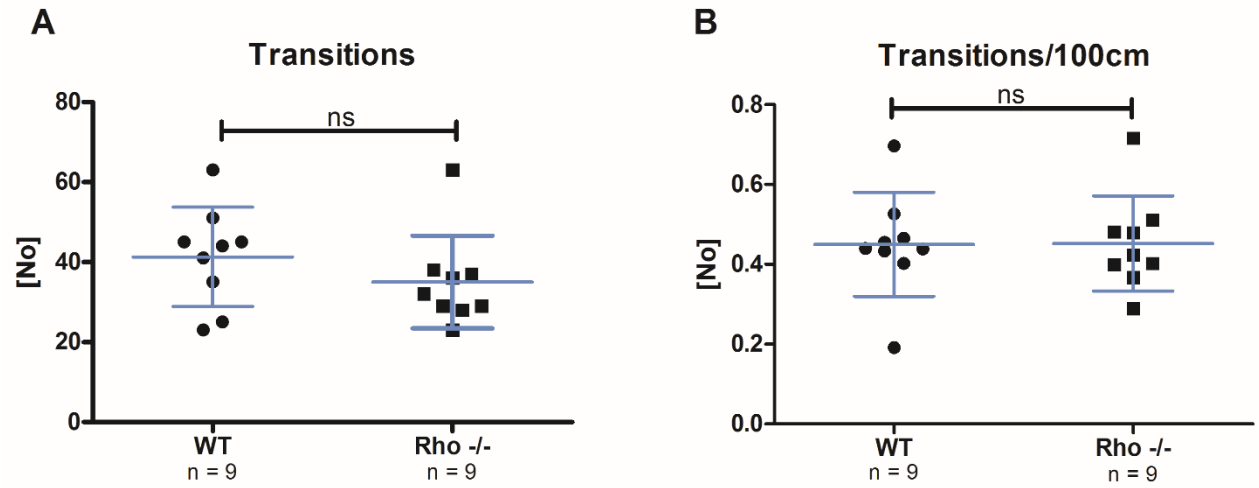


**Figure 28: Transitions between LC and DC**

In graph A, a tendency is displayed for fewer transitions by visual impaired mice. (B) If compared subjected to their overall activity (sum of distance covered) the tendency is negotiated. Mean with SD (blue).

## Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Stellen, die wörtlich oder sinngemäß aus Quellen entnommen wurden, sind als solche kenntlich gemacht.

Diese Arbeit wurde in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegt.

Dresden, den 28.01.2016

Hannes Jäger