CHARACTERISTICS OF ROD- AND CONE- MEDIATED VISION IN MICE: A PSYCHOPHYSICAL STUDY

A dissertation presented

by

Tricia Minerva Esdaille

to

The Department of Psychology

In partial fulfillment of the requirements for the degree of Doctor of Philosophy in the field of Psychology

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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychology in the Graduate School of Arts and Sciences of Northeastern University, July, 2008
ABSTRACT

The mouse has become one of the most important mammalian targets for modern basic and bio-medical research. This thesis project is one of many efforts that are underway in the vision research community to understand mouse visual function. The goal of the work was to apply behavioral techniques to the visual performance of wild type (WT) and certain mutant mice addressing the question whether mouse vision is governed by the same basic psychophysical laws as those established for humans. We developed a novel non-invasive behavioral instrument/method that yields first-ever visual sensitivity measurements while the mouse performs a natural behavior, running. Experiments were designed to collect increment threshold data for the rods and cones of individual WT and mutant mice, using the method of constant stimuli. The experiments were carried out with small well-calibrated targets that stimulated (for the first time) the retina locally.

Our results show that the absolute threshold intensity response of mouse rods was more sensitive than that of UV-cones by a factor of about 3000 and more sensitive than that of M-cones by a factor of about 12,500. The spatial integrating area of the mouse rod retina extended to about 450 rods. It was possible to register a threshold response mediated by no more than 25 rods imaged by the test flash. A brief, 500 nm light flash mediated by rods at threshold produced about 10-15 photoisomerizations in the dark-adapted mouse retina. Threshold versus intensity (t.v.i.) curves obtained on WT mice with test and background stimuli of various wavelength combinations were displaced horizontally and vertically as determined by the spectral sensitivity of the rods. As previously reported by others, the spectral sensitivity of Gnat-1\(^{-/-}\) mice, that lack functional rods, could be fitted with two pigment templates, one that peaked at about 360 nm, the other at 508 nm. Isolation of UV-
and M-cone responses in WT mice was achieved in the presence of backgrounds that produced about 25 - 50 and 200 - 300 photoisomerizations rod\(^{-1}\) s\(^{-1}\), respectively.

Due to limitations of the light source, the characteristics of the UV-cones could not be investigated. The M-cone increment threshold curve of the WT mouse showed a peculiar deviation from the Weber line. To shed light on this peculiarity, the threshold responses of \(\text{Gnat}-1^{-/-}\) mice to 510 nm stimuli and those of \(\text{Gnat}-2^{-/-}\) mice to 365 nm and 500 nm stimuli were determined. We found that the M-cones of \(\text{Gnat}-1^{-/-}\) mice desensitized profoundly in the presence of moderately bright lights suggesting that M-cones were not the mediators of vision in bright light. The increment threshold curve rose with a slope of 1.5 in the “Weber-adaptation” region. In \(\text{Gnat}-2^{-/-}\) mice all visual responses were mediated by rods. At a field intensity that produced 33,000 R* s\(^{-1}\) and that is known to saturate human rods, mice threshold responses did not deviate from the Weber line. However, the minimum Weber-Fechner fraction was 0.03 (in the high intensity region). For WT mice the Weber fraction was 0.09. The adaptive behavior of the mice to high intensity background light may be explained by the operation of a mechanism of light adaptation that was recently discovered (Nikonov et al., 2006). We propose that the mouse is very much a nocturnal animal whose vision is dominated by rods, over a wide range of light levels. The mouse’s rod system fundamentally resembles that of humans in its basic operations. It has achieved comparable absolute sensitivity as that of humans. The noise levels in the rod system of the mouse retina are about the same as in humans. Lastly, the rods of the mouse show Weber adaptation. Mouse rods are different from human rods in that they can operate in light conditions under which human rods would saturate. We believe that the present work has developed a number of critical
benchmarks that could be helpful in the evaluation of physiological data from single cell
and electroretinographical studies as well as the evaluation of data from molecular
biological studies.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor Frank Naarendorp without whom this work would not have been possible. Thank you for your continued support, particularly during the past three years. Your commitment to this work has been a driving force and inspiration to me.

I dedicate this thesis to my mother and my daughter both of whom have taught me the meaning of perseverance. Thank you mom for you unending love and support, the resolve that you have shown is something which I hope to exemplify in my own life.
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<tr>
<td>cd/m²</td>
<td>candela per meter square</td>
</tr>
<tr>
<td>log</td>
<td>logarithm</td>
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<tr>
<td>c/deg</td>
<td>cycles per degree</td>
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<td>cm</td>
<td>centimeter</td>
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<tr>
<td>μm²</td>
<td>micron square</td>
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<td>μm² s⁻¹</td>
<td>micron square per second</td>
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<td>scot.</td>
<td>scotopic</td>
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<td>nm</td>
<td>nanometer</td>
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<td>ms</td>
<td>millisecond</td>
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<tr>
<td>td</td>
<td>troland</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>μs</td>
<td>microsecond</td>
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<tr>
<td>R*s⁻¹</td>
<td>photoisomerizations per rod per second</td>
</tr>
<tr>
<td>rod⁻¹ s⁻¹</td>
<td>rod per second</td>
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INTRODUCTION

In this dissertation we attempt to make the case that the visual performance of mice can reliably be studied with behavioral techniques and that specific questions about mouse vision can be answered.

Since the completion of the mouse genome project in the late 1990’s, the mouse has become one of the most important mammalian targets for modern basic and biomedical research. The primary goal of the genome project was to decipher the entire mouse genetic code, the inherited instructions in its DNA. Given that the genetic code is now publicly available, large sections of the biological and biologically-oriented community have begun to take up the task of phenotyping the mouse’s genome. (Phenotyping refers to descriptions and/or analyses of expressions of the inherited genetic code in, for example, the development, morphology or behavior of the mouse). In the visual sciences, a wide range of techniques, including electro-physiological, histochemical, biophysical and biochemical methods, are being applied to phenotype the mouse’s visual system. Even though there are numerous examples of admirable efforts to document mouse vision with behavioral techniques (Daly et al. 2004; Hayes & Balkema, 1993; Jacobs et al. 1999; Nathan et al. 2006), progress in this area has been very slow. A few years ago, it was decided in our laboratory to enter the field of mouse psychophysics in order to contribute to the description and analysis of mouse vision.

Objective

The ideas and experiments of this dissertation deal with the behavioral measurement of visual thresholds in mice. The threshold concept originates from human
visual psychophysics, a discipline that examines relations between light stimuli and the perceptual attributes they evoke in observers. A special branch of psychophysics is devoted to the determination of threshold responses to simple stimuli. Over the course of at least 100 years, the threshold response has been documented under a wide range of experimental conditions, which has yielded a wealth of quantitative data describing human visual sensitivity robustly in terms of the physical properties of light stimuli (Boynton, 1979). Historically, psychophysical studies examining human rod and cone function have been a major source for physiological hypotheses about photoreceptor and retinal function, particularly those that bear on the physiological control of visual sensitivity. Over several decades, animal visual function has been explored and analyzed, in considerable depth, with various physiological and psychophysical techniques. We emphasize that psychophysics does not contribute to the unraveling of the physical mechanisms that mediate vision. Rather, new findings about the biological machinery implicated in threshold sensitivity, i.e. the photoreceptors and the retinal network, may help to explain the nature of psychophysical correlations (Barlow, 1972).

In essence, the study of human and animal basic visual psychophysics rest on three premises: (1) the concept of a visual threshold is an appropriate tool for the quantitative analysis of mammalian rod and cone vision, (2) visual thresholds reflect elementary output of the intact biological machinery that underlies visual function, and (3) specific genetic mutations expressed in the rod- and/or cone- systems can affect psychophysical performance qualitatively as well as quantitatively. In this thesis project we seek to measure the mouse’s visual thresholds elicited by ‘simple’ stimuli as opposed to visual
responses elicited by complex ‘perceptual’ stimuli. Understanding the latter responses requires deep knowledge about visual function in the brain that is yet not available.

The laboratory instruments and protocols currently in use for the study of mouse vision are based on learning principles that are rooted primarily in theories of operant conditioning (Blough & Yager 1972). From the literature (e.g. Breland & Breland, 1961) and based on results from preliminary experiments, we have come to appreciate that acquired/learned responses emitted by animals (in our case, mice) to light stimuli are difficult to maintain as these responses may conflict with natural behaviors. To overcome this difficulty, as well as other difficulties that shall be identified later, we developed a novel non-invasive behavioral instrument/method that yields first-ever visual sensitivity measurements while the mouse performs a natural behavior, running. A conscious mouse with all critical biological systems intact can now be examined under experimental conditions that: (1) do not require food/water deprivation or induction of stress/pain; (2) take into consideration the animal’s nocturnal lifestyle and related activity; (3) make the biological motivation to respond to light stimuli integral to the natural daily routine; and (4) allow for the measurement of visual performance using efficient mouse-computer interactions that also reduce the influence of human subjectivity.

Given the unique features of our method, we have chosen to make the objective of my thesis research: The application of the ‘two-color increment-threshold’ method to the visual performance of wild type C57Bl/6 mice, Gnat-1<sup>-/-</sup> and Gnat-2<sup>-/-</sup> mouse mutants in order to
explore the question whether mouse vision is governed by the same basic psychophysical laws as those established for humans.

The increment threshold technique (Stiles, 1959) allows for the presentation of rod and cone properties in three different data sets: (1) the spectral sensitivity to the test flash; (2) the spectral sensitivity to the adapting field, and (3) the (test) threshold versus the (field) intensity curve (Stiles, 1959). We have studied the properties of the rods in WT mice and those of UV and M cones in Gnat-1/- mice, mutants that lack functional rods. Weber fractions were determined and we evaluated whether W.S. Stiles’ test and field displacement laws held for mouse vision. We examined whether psychophysical rod saturation occurred in the Gnat-2/- mouse, a mutant with no functional cones, and we characterized the cone threshold response of the Gnat-1/- mouse at bleaching light levels.

If the answer to the question we posed is positive, we could begin to compare or link mouse vision, in particular rod vision, to that of human beings. This linkage could enable us to better understand dysfunctional human rod vision because mouse genes can now be manipulated to mimic specific human diseases in the mouse retina. Also, quantitative evaluation of pre-clinical retinal drug treatment may benefit from our novel method.

**Definition of terms and concepts**

The two-color increment threshold technique refers to a method developed by W.S. Stiles. It presents the human observer with a background of a given intensity and wavelength to alter the state of adaptation of the retina. The test and background stimuli may be of the same or different wavelength in order to selectively stimulate different classes of photoreceptor, hence the term two-color increment threshold. The test flash is presented
against the background and it is used to probe the altered state (i.e. sensitivity) of the retina. Sensitivity is inversely related to the threshold intensity of the flash. The two-color increment threshold technique produces threshold versus intensity (t.v.i.) data/curves; it describes the relationship between the test flash intensity and background intensity. This relationship is influenced by test and background wavelength. This procedure and its merits will be presented later in this introduction. Rod saturation refers to a large increment response to a relatively small increment in the adapting field intensity.

Psychophysical laws of interest are: Weber’s law, the law of spectral identity, the displacement laws of W.S. Stiles, and Ricco’s law. Weber’s law addresses the specific nature of the relationship between test flash intensity and background intensities. It states that the just noticeable difference (j.n.b.) or increment threshold is a constant fraction of the test flash and the background intensity. The law of spectral identity refers to the wavelength sensitivity of the mechanisms mediating the test and/or adapting field. It is assumed the mechanisms that mediate the sensitivity of the test stimulus and background stimulus are determined by the absorption properties of the visual pigments in the photoreceptors of the retina. Stiles’ displacement laws, as explained below, refer to the horizontal and vertical displacement of t.v.i. curves as determined by the spectral sensitivity of the rods and cones. The vertical displacement reflects the spectral sensitivity of the mechanism mediating the test flash. The horizontal displacement reflects the spectral sensitivity of the mechanisms mediating the background. Ricco’s law states that the intensity of stimuli and the area of stimuli at threshold are inversely related to produce constant energy.
To provide background information about the ideas advanced in this dissertation, we will review key literature regarding the prominence of mice in basic research, the fundamental role of photoreceptors in the visual process, animal visual psychophysics and the current status of mouse visual psychophysics.

**Background**

*The use of mice in modern biological and behavioral sciences.*

The prominence of the mouse in modern basic science has its roots in developments of the early 1900’s. Even though Darwin did establish in the late 1850’s that (see Crow, 2002; Guénet, 2005; Paigen, 2003) biological evolution was governed by the principle of natural selection (the tendency of some members of a species to produce more offspring than other members do), he did not explain how adaptations, ‘the sum of traits and tendencies inherited from parents by offspring’ were transferred. By cross-breeding pea plants, in the mid 1860’s Gregor Mendel (see Guénet, 2005; Paigen, 2003) discovered the principles of heredity, that is, how, for example, characteristics of plants, such as color, height, and seed shape were transferred from generation to generation. Mendel formulated two basic laws of inheritance: namely, the law of segregation and the law of assortment. Briefly, the law of segregation states that alleles are alternative versions of genes and account for variations in inherited traits. The law of independent assortment states that the inheritance pattern of one trait is independent of that of another trait. Interestingly, Mendel’s laws were in disrepute during his lifetime but were ‘rediscovered’ independently by three scientists in 1900.
Are animals subject to the same inheritance patterns as plants? This question was answered in the affirmative by Cuenot in 1902 using mice, and is taken to be the beginning of mouse genetics (see Guénet, 2005). Two other events led to the use of mouse genetics in understanding mammalian physiology and development, especially human disease processes: (1) E.E. Tyzzer demonstrated the inheritance in mice of resistance to the growth of transplanted tumors. (2) In 1909 C.C. Little, founder of the Jackson Laboratories in Bar Harbor Maine, began to mate mice in order to construct inbred strains, reproducible genetic crosses, that would serve as experimental material (background) in tumor transplantation experiments (Crow, 2002; Paigen, 2003). Onwards, mice became the choice of species in genetic research: they sexually reproduce with high frequency, reach maturity fast, are inexpensive, and are easy to maintain.

Retinal degenerations: the mouse as a genetic system.

The first report on an inherited retinal degeneration in mice was published by Clyde Keeler in 1924 (see LaVail, 1981). Thereafter, only gradually it was appreciated that mutations resulting in photoreceptor degeneration were widespread among animal populations. Using histological techniques and genetic crossing, investigators established that there was no single type of retinal degeneration (LaVail, 1981). For example, in the mouse, applying genetic linkage techniques, the retinal degeneration \((rd)\) mutation was found to segregate in probably more than 30 lines of mice. Thus, effective tools for mouse phenotyping were needed to adequately describe the various forms of retinal degeneration. It is in this context that an interest in mouse visual behavior developed in the 1930’s and 1940’s: to describe, among other things, the loss of visual sensitivity that
may occur in the course of a month, six months or perhaps a year depending upon the
type of retinal degeneration under study. The behavioral responses of mice to light have
since been studied using different techniques that will be reviewed later in this
dissertation.

The development of the mouse as a genetic system, however, was driven by cancer
research, up until the late 1950’s (Crow, 2002; Paigen, 2003). In the following decades,
applications of mouse genetics were adopted in other areas of mammalian biology, which
led to the development of more genetically uniform stocks of inbred strains. By the
1980’s the genotypic and phenotypic variety of mouse strains exceeded that of the human
population. The mouse was set to become the premier genetic system. With the advent
of molecular biology, not in the least due to the discovery of the structure of DNA, and
the availability of new (bio)technologies to rapidly mark genes and determine DNA
sequences, it became possible to track in great detail the inheritance of chromosomal
regions in crosses. Thus, the genetic structure of living organisms could be mapped and
sequenced.

By the late 1990’s and early 2000, the mouse had become the species of choice for
a significant number of visual scientists. The publication of the mouse genome sequence
(Waterston et al., 2002) revealed that the gene sequences in mouse and human that
encode the proteins to carry out important organ functions, including retinal functions,
are shared to a high degree: the sequence identity is as high as 90%. [DNA sequences in
the vast regions between genes are much less similar (50% sequence identity or less)]
Thus, the DNA sequence of the mouse genome may provide an essential tool to identify and study the function of human genes as well as the genetic origin of diseases. For example, about hundred mutations in the rhodopsin gene have been shown to cause the death of rod photoreceptors, leading to blindness or severely impaired vision. How any of these mutations, which produce ‘faulty’ protein(s), ultimately leads to photoreceptor death is not understood. It is hoped that by genetically engineering rhodopsin mutations in mice and by extensive phenotyping, which will include psychophysical tests of mouse vision, the course of action of the ‘faulty’ protein(s) can be established.

*The fundamental role of photoreceptors in vision.*

The great importance of the photoreceptors in the retina for the visual process was recognized by the mid-1800’s. By then, biologists and observers of natural life had noticed that most vertebrate retinas contained both rods and cones. Based on comparative anatomical studies of different vertebrate retinas, in the mid 1860’s Shultz proposed the Duplicity Theory of Vision, which stated that vertebrate vision is mediated by rods and cones only (see Rodieck, 1974). In general, the rod system mediates vision under scotopic (low) levels of illumination and the cone system mediates vision under photopic (high) levels of illumination.

Much of what we know about the retinal cells of vertebrates came from the work of Santiago Ramon y Cajal who employed the Golgi silver method to the study of retinal cells (see Rodieck, 1974). Vertebrate retinas are formally divided into five subcategories: teleost, amphibian, reptilian, avian, and mammalian retinas. All vertebrate
photoreceptors have in common the presence of an outer- and inner-segment, a cell body, an axon and terminal. Although they share the same basic anatomical design, the photoreceptors of the various vertebrate classes can differ greatly in terms of morphology, type of visual pigment contained in the outer-segment and connectivity with other retinal cells. For example, the amphibian retina contains two types of rods whereas other vertebrate retinas typically contain one type of rod. The retinas of fishes, amphibians, reptiles and birds often contain double and single cones with or without oil droplets and may express three or four cone opsins. There are no double cones or photoreceptors with oil droplets in the retina of placental mammals (Bowmaker & Hunt, 2006).

Within the mammalian branch, the photoreceptor pigments differ as well. For example, mouse rhodopsin absorbs light maximally at 498 nm (Lyubarsky et al., 1999) whereas human rhodopsin absorbs maximally at 507 nm (Wyszecki & Stiles, 1982) reflecting presumably ecological differences. Mouse rods are slightly thicker and shorter than those of human (Carter-Dawson & LaVail, 1979). In humans, rods outnumber cones by a factor of approximately 20 (Jeon, Strettoi, & Masland, 1998). There are, generally, two cone pigments in the mammalian retina, except in that of humans and a few other primates. The human retina contains three different types of cone that are called L-, M-, and S-cones, respectively. Their outer-segments house unique pigments that are maximally sensitive to long-, middle- and short- wavelength light, respectively (Rushton, 1953). Mice retina contains mid-wavelength sensitive (M-) cones that peak around 510 nm and ultra-violet sensitive (UV) cones that peak around 360 nm (Deegan & Jacobs, 1993; Jacobs et al., 1991; Lyubarsky et al., 1999). The mouse cone system is
expected to function in the same manner as that of other small mammals, such as the rabbit (Li et al., 2004; Streotti et al., 1994). Mouse cones make up 3% of the photoreceptor population (Carter-Dawson, & LaVail, 1973). In the mouse the distribution of the rods is fairly even, however the UV-cones that make up 80% of the cone population are located predominantly in the ventral retina, whereas M-cones are found mostly in the dorsal retina (Szel et al., 1992). The mouse retina lacks a fovea but it does have a central area that is more densely populated by cones.

*Psychophysics and the duplicity theory.*

The duplex theory is not only supported by anatomical studies but receives much of its validity from psychophysical studies on humans. The best known psychophysical data pertaining to the duplicity theory are classical dark adaptation data provided by Hecht et al., (1937); and Hecht et al., (1938). As dark adaptation is usually studied, the observer is presented with a bright bleaching light, and the absolute threshold of a test flash is subsequently determined during the time course of recovery. Bleaching refers to a larger than normal amount of light delivered to the eye which causes greater than 1% of visual pigment to be photoisomerized. The results typically show a double-limbed or duplex curve when the experiments are conducted in the peripheral retina. The upper limb of the duplex curve is attributed to cone function, the lower limb to rod function. An alternate way to show this difference in rod and cone function is to determine “a light adaptation curve” or increment threshold function (See Figure 2). Accordingly, the threshold of the test stimulus is determined as a function of the luminance of a continuously presented background. As expected, the light adaptation function consists of a lower rod and an
upper cone limb (e.g. Crawford, 1947). As used today, the term “duplicity theory” is really a summary of empirical data related to differences in rod- and cone- vision of vertebrates with major emphasis on the human psychophysical observer (Hecht, 1937; Ripps & Weale, 1976; Wyszecki & Stiles, 1982). In general, for humans, it can be said that scotopic vision is mediated by rhodopsin in rods, has low spatial and temporal resolution, has a relatively high Weber ratio, does not support color discrimination, is best a few degrees from the fovea, and has a spectral sensitivity curve that peaks at about 507 nm. Rods are insensitive to the angle or direction under which light strikes its longitudinal axis. Photopic vision is mediated by three types of cones that contain different pigments. Photopic vision has high spatial and temporal resolution, is best in the fovea, mediates color discrimination, has generally a lower “Weber ratio”, is directionally sensitive and its spectral sensitivity peaks at approximately 555 nm.

*Several important physiological hypotheses stem from psychophysical work on humans.*

Prior to, during and up to about two decades after the Second World War, psychophysiologists dominated the study of rod and cone function. Despite the indirect relation between the human psychophysical response and the biological machinery, from which the responses are thought to originate, psychophysiologists successfully revealed several fundamental properties of the photoreceptors that were many years later confirmed with single cell and suction electrode techniques. For example, the hypothesis that rods are responding to single photons was advanced based on sound psychophysical work (Hecht et al., 1942); the hypothesis was confirmed 37 years later in a physiological study of single rod responses (Baylor & Lamb, 1979). Psychophysical hypotheses about
‘dark light’ generated by rods (Barlow, 1957), rod light-adaptation and saturation (Aguilar & Stiles, 1954) are still very much pursued in physiology (Schnapf et al., 2005). Similarly, the spectral identity of the three cone types in the human retina were first established in psychophysical work combined with retinal densitometry (Rushton, 1953) and later confirmed by microspectrophotometry. (Marks et al., 1964). The temporal and spatial properties of the different human cone types as defined by psychophysics were confirmed by single cell recordings (Schnapf et al., 1990). The psychophysical studies, most successful in generating viable physiological hypotheses, have been those employing threshold responses.

**Visual experiments involving animals**

Animal visual psychophysics has been practiced for many decades yielding an important database. The list of animals that have been studied most thoroughly with visual psychophysical methods include fish (color vision), pigeon (dark adaptation, flicker sensitivity, color vision), monkey (flicker sensitivity, color), cat (absolute sensitivity, color vision), rat (rod spectral sensitivity) and dog (color vision). As pointed out by Blough and Yager (1972) in a review of animal visual psychophysics, it is assumed that human and animal visual psychophysics are essentially the same, provided the appropriate experimental conditions are adopted. A compelling reason to undertake the work with animals is that it makes possible “the comparison of anatomical, physiological and behavioral information in the same species, and comparison across species; the selection of subjects [is] uniquely suited to a given problem; the undertaking of experiments [is] too damaging, too dangerous or arduous for human subjects” (Blough
& Yager, 1972). Despite many methodological difficulties, the field enjoyed productive years, in the 1950’s and 1960’s, along side human visual psychophysics.

**Reflexive methods, the optokinetic response**

The main methods for data collection on animals, still in use today, involve either “no” training or require some form of training commonly based on classical and/or instrumental conditioning. There are few known reflexive behaviors in animals that can be used to characterize the visual system. The optokinetic response is perhaps the most well known reflexive response. It is elicited by spinning a drum, with stripes painted on the inner wall, around the animal subject (for example, a cat or mouse) that induces the animal to turn its head and/or body in the direction of the moving stripes (the visual stimulus). A substantial number of studies have used this response to describe; in particular, the spatial frequency properties of various animals including the mouse (Blough & Yager, 1972). The problem with the optokinetic response is that failure to elicit the response could be due either to limitations of the visual system or lack of interest/attention on the part of the animal. Also, in practically all available instruments, the experimenter must wait for a moment when the animal is motionless to initiate a trial. During the daytime mice tend to be behaviorally inactive and there is no guarantee that a mouse will be prepared to respond reliably to a light stimulus. At night, when mice are active, motionless moments are more difficult to find. Nevertheless, several studies have demonstrated that it is possible to behaviorally measure the mouse’s spatial vision using the optokinetic response (Douglas *et al.* 2005; Prusky *et al.* 2004; Umino *et al.* 2008).
However, as Pinto and Enroth-Cugell (2000) have pointed out, it requires considerable experimenter expertise.

Classical conditioning and the conditioned suppression response

Visual psychophysical experiments that use the classical conditioning method usually let a light stimulus acquire the power to elicit some measurable response from the animal subject. Thus, the light stimulus initially is a neutral stimulus; paired or associated with a positive/negative unconditioned stimulus (UCS), the neutral stimulus becomes a conditioned stimulus (CS). The number of vision experiments that exclusively employed classical conditioning techniques is rather small, perhaps due to the requirement that the CS must reliably elicit a measurable physiological or motor response. Electric shock seems to be the preferred UCS since it evokes an easily measured withdrawal or avoidance response. But there are limits to electric shock, as it tends to traumatize animal subjects, particularly mice. In visual behavioral studies, classical conditioning is most often used in combination with an operant behavior. An example of this mix is the method of “conditioned suppression” in which an operant behavior (such as the rate of licking, respiration, the heart beat, bar pressing, key pecking) is suppressed by a CS [light stimulus associated with a shock (UCS)]. Studies on conditioned suppression by L. Kamin, for example, in the 1960’s, have shown that conditioned suppression obeys the laws of classical conditioning (see Blough & Yager, 1972).

Hendricks (1966) elegantly applied this method in a study of the ‘critical flicker frequency’ threshold response in pigeons. The pecking rate was maintained by a
variable interval (VI) schedule of reinforcement. It was convincingly demonstrated that, to avoid a shock, the rate of pecking was powerfully suppressed by various flicker frequencies of a 20 sec light stimulus, the CS. The pecking rate was maintained throughout by a variable interval (VI) schedule of reinforcement. A limitation of this method is that the pigeon’s pecking rate may vary considerably. The birds, apparently, could not be tested beyond one hour per day. In fact, Hendricks undertook the study to explore whether withholding food (reinforcement) during the flicker presentation would more effectively maintain the pecking rate and more firmly establish suppression of the pecking rate. With respect to maintaining the pecking rate the results were inconclusive. As noted by Breland and Breland (1961), operant responses are often only temporarily available for experimentation and may, over time, become more difficult to condition. Ray (1970), using the conditioned suppression method and the lick response of mice in a study of auditory threshold responses, could induce the mice to respond to the stimuli only after they had lost 25% of their body weight due to water deprivation. Thus, despite the availability of an easily quantifiable operant response, its utility may depend on other factors, in this case, internal physiological homeostatic conditions.

Operant conditioning

The two-choice/multiple-choice method. The most common instrument used with the two-choice method is an apparatus in which animals run along a Y-shaped alley. At the V-junction, the animal must chose to enter one of the alleys, one of which displays a light stimulus. A water- or food-deprived subject learns to associate the presence of the light stimulus with the availability of water or food. Once the association is established,
a characteristic of the visual stimulus, for example, the intensity or spatial frequency, is systematically varied and its effect on the animal’s visual performance is determined. Prusky et al., (2004) and Umino et al., (2008) have used this technique to study the spatial visual capabilities of mice. Daly et al., (2004) have studied the visual sensitivity of mice using an apparatus that provided mice with six alleys to choose from. Jacobs et al., (1999) have also used a multiple-choice method to study mouse color vision. In their studies, mice were trained to approach variously illuminated panels that they poke with their nose to indicate their choice of a color light stimulus. There is little doubt that certain aspects of mouse vision can be quantified with these operant conditioning methods. However, serious concerns about the validity, reliability and efficiency of these methods have been raised.

*Single response method.*

Animals may display spontaneous operant behaviors that serve no obvious physiological function and that are easy to evoke and maintain. A good example of this is lever pressing by rats. These animals can be trained to express lever-pressing behavior at a rate that is unique to the individual animal. The lever-pressing rate is easily maintained by delivering food pellets on a VI schedule. The behavior can be brought under control of a light stimulus: that is, the food pellet is delivered only if the lever-press occurs timely in response to the presentation of a light stimulus. The unique spontaneous rate of lever pressing is documented to examine whether lever-press and light presentation are truly associated (Naarendorp et al., 2001). As only one response lever is usually available to the animal, the method is identified as a ‘single response method
using rate’. Herreros de Tejada et al., (1997) used the response rate lever-pressing method to measure the absolute sensitivity of mice. They report that it was exceedingly difficult to train mice to lever press reliably. To our knowledge, there are no other reports in the literature that have attempted to study mouse vision with a response-rate-based method. Although operant conditioning procedures have been successfully applied to the study of animal vision, in rats, progress in the study of mouse vision has been unremarkable. Blough and Yager (1972), now several decades ago, correctly observed that even if an operant behavior is available and can be conditioned, using it may be ill-advised.

Current studies of mouse visual responses.

Studies that involve the measurement of absolute visual sensitivity. We describe here briefly maze-based tests since they are used for sensitivity measurements. In maze-based instruments mice move around freely and are trained, using operant conditioning procedures, to respond to a visual stimulus located on a wall or computer screen.

Daly et al., (2004) examined the relationship between ambient light conditions and the dark-adapted threshold of black, himalayan, and albino mice. Behavioral thresholds were determined using a water maze, (hexagonal in shape with six T-shaped compartments). Mice were dropped in a water bath at the center of the maze and trained to exit the bath by climbing on a white removable escape ramp attached to a black GP acrylic plastic background. During an experiment the ramp was randomly inserted in one of the six maze compartments that were all evenly illuminated by an incandescent light source mounted above the center of the maze. Light levels were lowered over the
course of several experimental sessions until the mouse could not see the ramp. The mouse’s response was considered correct when the animal’s first choice was the compartment containing the escape ramp. In order to examine threshold differences in albino and pigmented animals, rhodopsin levels were equalized by maintaining albino mice at light levels of $2 \times 10^{-4}$ cd/m$^2$ (dim light) and black mice at $2 \times 10^2$ cd/m$^2$ (bright light). The authors found similar thresholds of -6.38 log cd/m$^2$ and -6.47 log cd/m$^2$ for albino and black mice respectively with equivalent rhodopsin levels. This suggests that differences in visual sensitivity were not the result of mouse strain but rather the amount of rhodopsin present in the eye.

*Studies that involve measurement of visual acuity.* Gianfranceschi, *et al.*, (1999) examined the behavioral visual acuity of wild type and *bcl2* transgenic mice (*bcl2* stands for B-cell lymphoma 2). The mouse mutant used in this study was engineered to express the human gene *bcl2* which plays a role in protecting neurons of the central nervous system (CNS) from cell death. The *bcl2* overexpressing mouse has a retina which possesses twice as many ganglion cells as that of the WT mouse. As visual acuity is mediated by ganglion cell density, the authors tested the hypothesis that the visual acuity of *bcl2* mice would be greater than that of the WT. The apparatus was a T-maze, which consisted of a start box with a sliding door and a choice area comprised of two parallel alleys. Stimulus cards 15x15 cm high, with gratings of a contrast (75%) with a mean luminance of 25 cd/m$^2$ were placed at the end of both alleys. A tungsten lamp illuminated the stimulus cards. Mice were trained to discriminate between horizontal and vertical gratings of the same spatial frequency. The “correct” choice, (vertical gratings) were rewarded with
food. Gianfranceschi et al. (1999) reported a mean acuity of 0.51 cycles per degree (c/deg) for the wild type (WT) mouse and 0.61 c/deg for the transgenic \textit{bcl2} mouse. The visual acuity of WT and \textit{bcl2} mice was of the same order of magnitude; as such the authors concluded that increased ganglion cell density does not increase visual acuity.

Nathan et al., (2006) examined the spatial and temporal acuity of the WT mouse and \textit{Gnat-1}-/- and \textit{Gnat-2}-/- mice in a water maze. Mice were trained to find a black wall in a white water maze and the search time recorded. Stimulus intensity was lowered by 0.5-1.0 log units per day until the search time increased significantly. For the WT mouse, a 500 nm stimulus produced at absolute threshold 0.03 photon per $\mu m^2$ s$^{-1}$.

Prusky et al., (2000) studied mouse visual acuity with sine-wave gratings. Mice were placed in a trapezoidal pool, on one end of which two computer monitors were placed. Midline dividers were placed in the pool and an escape ramp was positioned below one of the monitors. To gain access to the escape ramp mice had to choose the side of the pool displaying a grating. Acuity of .49 c/deg was found for the WT mouse. Using the optokinetic response, Prusky et al., (2004) studied the visual acuity of the mouse. A virtual cylinder comprised of a vertical sine wave was generated on a computer monitor arranged in square around a platform. Mice were placed in the center on a platform. The experimenter followed the mouse with a crosshair superimposed on the video image. To determine whether an animal tracked the grating, the image of the cylinder, mouse, and crosshair were examined simultaneously. When the mouse tracked the movement of the cylinder it was seen as movement against the arms of the crosshair. A threshold of 0.4 c/deg was found for the WT mouse using the optomotor system.
Grating thresholds obtained using reinforcement-based tasks are higher than those obtained using the reflexive optokinetic response. Prusky in previous work using a water visual task reported grating thresholds between 0.5 and 0.6 c/deg compared to the threshold acuity of 0.4 c/deg found using the optomotor system. The authors noted that differences in the behavioral task might account for this disparity.

Umino et al., (2008) examined the contrast sensitivity and visual acuity of wild type, Gnat-1\(^{-/-}\) and Gnat-2\(^{-/-}\) mice utilizing the optokinetic response. Mice were placed on a pedestal in the center of computer monitors arranged to form a square. The mouse was monitored with an infrared camera positioned on top of the testing chamber. The camera allowed an observer to view only the mouse, the rotating grating could not be detected. The experimenter initiated trials (5s duration), however the direction of the grating was determined via a computer-controlled protocol. Grating contrast was also changed via computer using a staircase paradigm. The threshold was defined as 70% correct observer responses. Using a bright background (1.8 log cd/m\(^2\)), the authors found that the spatial contrast sensitivity function (CSF) of Gnat \(-1^{-/-}\) mice matched those of wild type mice. Under dim conditions (-4.5 log cd/m\(^2\)) Gnat\(-2^{-/-}\) mice and WT mice exhibited similar temporal and contrast sensitivities. The authors report that rods mediate mouse vision at light levels less than -4.0 log cd/m\(^2\) and cones at levels greater than -2.0 log cd/m\(^2\). Additionally, the visual acuity of the mouse is 0.48 +/- 0.03 c/deg under photopic conditions, which is similar to the value of 0.4 c/deg reported by Prusky et al., 2000.

*A study of cone-less mice.* Williams et al., (2005) examined rod and cone function in transgenic mice engineered to be coneless, using the forced-choice
discrimination task described in Jacobs et al., (1999). Mice were placed in a chamber containing three circular test panels, 2.5 cm in diameter, positioned in a line against a single wall. They were trained to detect the presence of a test light on one of three panels. Correct choices (nose poke at illuminated panel) were reinforced with soy milk. The panels were illuminated with a tungsten-halide lamp, creating a diffuse and homogeneous background. A test light light stemming from a 75 W Xenon-arc lamp was passed through a grating monochromator (half energy passband of 16 nm) and randomly presented on one of three panels during experiments.

Williams et al., (2005) report that, at low intensities, the sensitivity of the coneless mouse is the same as that of the wild type mouse for test lights of 500 nm and 600 nm on dim backgrounds. Increment thresholds were measured using a 500 nm test on a total of 15 background intensities. The authors found that at background intensities ranging from -1.37 log scot. cd/m$^2$ up to about 0.40 log scot. cd/m$^2$ the threshold values for coneless and wild type mice were essentially the same. However, at background intensities greater than about 1 log scot. cd/m$^2$ the threshold of the coneless mouse rose more rapidly than that of the WT mouse. Linear regression fit for both groups yielded a slope of 1.16 for the coneless mouse and 1.03 for wild type mouse. At the highest background intensity (2.37 log scot. cd/ m$^2$) the authors found no evidence of rod saturation. Spectral sensitivity functions were determined for both WT and coneless mice for wavelengths over 480 nm. The peak of the spectral sensitivity functions for the coneless mouse was 500.3 nm, in contrast the spectral sensitivity function for the WT mouse was 508.3 nm. Together, these findings suggest that sensitivity over middle to long wavelengths were mediated by different mechanisms: rods in the coneless mouse
and M-cones for the wild type mouse. In addition, the authors set the long wave component to have the aforementioned two peak values and examined whether the curve fit could be improved by adding contributions from a second spectral mechanism, the UV cones. The fit to the data array for the WT mouse showed peaks at 508.3 and 363 nm with relative proportions of 85.7% and 14.3%. In the coneless mouse, the fit to the data array showed photopigment absorption curves with peaks at 500.2 and 363 nm respectively, the relative proportions were 98% and 2% respectively. These findings demonstrated that some UV cones did survive.

The need for improvements in measuring techniques and instrumentation

Reviewing maze-based tests, we became aware of two major problems: (1) the behavioral tasks assigned to the mice in the visual experiments hardly took advantage of natural or spontaneous behaviors. In preliminary experiments we tested the feasibility of linking spontaneous wheel-running behavior to light stimuli. (2) The mouse’s head (eye) position relative to the light stimulus was not known in any of the reported studies. The stimuli were customarily ‘ganzfeld-like’ and kept steady (time-invariant), rendering, in our view, the visual sensitivity measurements ‘meaningless’ in psychophysical and physiological terms. Despite severe water- or food- deprivation, cold-water swims and the like, the mice in the studies we reviewed were rarely motivated to attend and respond to the visual stimuli.

In our opinion, meaningful threshold measurements require the stimulus to be brief, well within the integration time of the photoreceptors, and local on the retina. Moreover,
the mouse need not only be at a specific location where it can be stimulated with small flashed stimuli, it also must have the motivation to attend and respond to the stimulation.

We have developed a non-invasive behavioral technique that yields first-ever visual threshold measurements while the mouse wheel-runs. We will show that mice generate replicable responses, including absolute threshold responses to less than 15 photons delivered at the cornea. The method affords study of WT and mutant mice during their entire life time without using anesthesia, chemical or physical restraint, food or water deprivation.

To determine whether mouse vision is governed by the same basic psychophysical relationships as those established for humans, we propose to apply the psychophysical “two-colour increment threshold method” developed by W.S Stiles to C57Bl/6 (WT) to wheel-running WT and Gnat-1/- Gnat-2/- mutants. We expect the basic ‘laws’ to hold for rod vision as the retinal mechanisms underlying mouse rod vision closely resemble those of humans.

*A brief review of the increment threshold method.*

Aguilar and Stiles (1954) have defined the conditions for isolating the rod response, by psychophysical means, in the human retina which allowed them to describe important properties of the rods. For their human observers, they used a sizeable (9°), 200 ms test probe flash, delivered 9° away from the fovea, superposed on a large (20°) adapting field of variable intensity. The rod-isolating conditions were the following: (1) The flash entered the eye near the edge of the dilated pupil to strike the photoreceptors off-axis. Cones respond poorly to light that strikes them off-axis; rods are insensitive to
the direction of the incoming light. Thus, the rods had an advantage over the cones to
detect the test flash. (2) The adapting field entered the eye through the center of the pupil
stimulating rods and cones on-axis, therefore, equally. However, the adapting field
wavelength was so chosen that it would have a greater desensitizing effect on the L- and
M- cones than on the rods. The authors used a filter that caused the field not to contain
light of wavelengths shorter than 600 nm and its peak wavelength occurred at about 630
nm. (3) The wavelength of the test flash was so chosen that neither the S- cones nor the
M- cones would detect the flash at intermediate or high (photopic) adapting intensities.
The test flash wavelength was 530 nm. (Thus, although the rods are color blind their
proper isolation requires the test and background to be of different ‘color’, hence the term
“two-colour increment threshold”). Under these isolation conditions, Aguilar and Stiles
(1954) succeeded in measuring the threshold responses mediated by the rods in bright
light. They found that the curve relating the test flash threshold intensity to the adapting
field intensity could be divided in four sections (see Figure 1).

In the first section, up to an adapting intensity of 0.001 scotopic td, the increment
threshold was unaffected despite the presence of a dim veil of light. [Barlow (1971) has
hypothesized that this veil of light may be indistinguishable from the noise in the retina
that results from spontaneous thermal isomerizations of rhodopsin.] In the second
section, covering a 4-log unit adapting intensity range, the rod threshold increased in
direct proportion to the intensity of the adapting field. The rise of the increment
threshold is well-described by Weber’s law:

\[ \Delta I = c \times I \] 

Eq. 1
Figure 1. Rod-mediated increment threshold responses (Aguilar & Stiles, 1954). The rod threshold was measured over an 8-log unit range of background light intensity on four human subjects. All the curves are correctly placed with respect to the scale of abscissa, but the scale of ordinate is correct only for the curve in the lowest position. The other three curves have been displaced upward by 0.5, 1.0 and 1.5 log units.
where $\Delta I$ is the increment threshold and $I$ is the background intensity. Note that $\Delta I/I = c$. The constant $c$ is known as the Weber fraction, provided $\Delta I$ and $I$ have the same wavelength and are expressed in the same intensity units. In humans, the constant $c$ equals about 0.2. The third section covers a narrow adapting range in which the rods show psychophysical saturation, that is, the increment threshold rises rapidly in response to small changes in the adapting field intensity. In the fourth section, the test flash threshold is mediated by cones, most likely M-cones. The solid curve relating the test flash intensity to the adapting field intensity in [section a] and [section b] was designated by Stiles as $F$ and is described by the hyperbolic relation:

$$\Delta I = (f^s + I_{0.5}^s)$$

where $\Delta I$ is the increment threshold, $I$ is the background intensity and $I_{0.5}$ the background intensity that raises $\Delta I_{\text{dark}}$, the absolute threshold intensity, by a factor of 2. The exponent $s$ defines the slope of the curve.

Stiles (1949, 1959) showed that by varying the wavelength combination of test flash and background and by varying some other characteristics (for example, size and retinal location), the individual classes of photoreceptors could be isolated psychophysically and a curve, $F$, relating the test flash threshold intensity to the adapting field intensity could be obtained for each type of photoreceptors class or subclass. He discovered that the shape of the curve relating test flash threshold intensity to adapting field intensity was the same for all photoreceptors types, including the rods. Based on this finding he proposed the following general psychophysical relationship (Stiles, 1949):
\[
\log (s_\lambda U_\lambda) = F[\log (S_\mu W_\mu)], \quad \text{Eq. 3}
\]

where \(s_\lambda U_\lambda\) and \(S_\mu W_\mu\) represent the increment threshold and adapting field intensity, respectively. Furthermore, \(U_\lambda\) refers to the intensity of the test flash at threshold measured in the presence of a background having the wavelength \(\mu\) and the intensity \(W_\mu\).

The symbols \(s_\lambda\) and \(S_\mu\) refer to the normalized test- and field spectral sensitivity, respectively. (Note that sensitivity = 1/threshold.) Depending upon the wavelength of the test flash, the curve \(F\) is displaced vertically along the ordinate; depending upon the wavelength of the adapting field (background), the curve \(F\) is displaced horizontally along the abscissa.

The threshold properties of the rods and the specific classes of human cones are thus described by three curves: (a) the spectral sensitivity curve of the test stimulus; (b) the spectral sensitivity curve of the field stimulus and (c) the curve relating increment threshold and field intensity.

**Isolating rod and cone responses in the mouse retina.**

Psychophysical isolation of rod and cone responses in the mouse cannot be achieved in the same manner as in human beings. In human psychophysics, to deliver a light stimulus along the edge of the pupil or through its center requires that the light stimuli are focused in the plane of the pupil (that is, the stimuli must be in Maxwellian view); and the head must be stable which cannot be achieved with a wheel-running mouse. However, isolation of the rods in the mouse retina may be achieved in a different manner. For isolation of the mouse rods, we need to be concerned with UV-cones and M-cones detecting the test flash. If we choose to use a 500 nm stimulus, based on the
Figure 2. W. S. Stiles’ schematic illustrating how the positions of rod - (A) and cone mediated - (B) threshold versus intensity (t.v.i) curves are determined by the respective spectral sensitivity curves of rods and cones shown in auxiliary diagram. $N_\lambda$ is the test flash radiance. $M_\mu$ is the background radiance.
absorption spectrum of the UV-pigment, UV-cones are unlikely to detect a 500 nm test flash. The M-cones, however, will at higher intensities respond to a 500 nm stimulus. Because the spectral sensitivities of rods and M-cones are very similar, it is not possible to affect the sensitivity of these two classes differentially by chromatic adaptation. But given that there are relatively few M-cones in the WT mouse retina, these cones can be expected to have a ‘high threshold’ allowing for measurement of the rod threshold on relatively high background intensities. It is doubtful that the rod response can be studied on intense backgrounds in WT animals.

Recently, it was discovered that a spontaneous mutation had occurred in a mouse line bred at the Jackson Laboratories at Bar Harbor, Maine (Chang et al., 2006). Apparently, the cones of this mouse mutant, not the rods, have lost the ability to transduce light as the alpha-subunit of the cone-specific G-protein has become inactive. The cones themselves having intact outer segments are physically still present in the retina but do not function physiologically (Chang et al., 2006). This mutant mouse which has been designated the “Gnat-2/-” mouse, as it is distinct from the Gnat-1/- mouse (Calvert et al., 2002) that lacks functional rods due to an engineered inactivation of the alpha-subunit of the rod-specific G-protein. (Gnat stands for Guanine Nucleotide-binding protein, Alpha Transducing activity polypeptide). We intend to study the rod response characteristics of WT mice and a group of Gnat-2/- mice and their controls, generously given to us by Dr. Bo Chang at the Jackson Laboratories. We will also study the cone responses of Gnat-1/- mice and their controls, a generous gift from Dr. Janis Lem at Tufts University.
We will determine: (a) the spectral sensitivity of the rods to the test flash and to the adapting field; (b) \( F \), the curve relating the (test) threshold to the (field) intensity for rods and M-cones. The Weber fractions will be determined for rods and we will evaluate whether W.S. Stiles’ test and field displacement laws hold for mouse vision. We will examine whether psychophysical rod saturation occurs in the \( Gnat-2^{-/-} \) mouse. We will characterize the cone threshold response of the \( Gnat-1^{-/-} \) mouse at high adapting light levels.

**METHODS**

*Animals*

All experimental procedures were performed in accord with protocols approved by the Institutional Animal Care and Use Committee at Northeastern University. Breeding pairs of WT C57Bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and Jackson Laboratories (Bar Harbor, Maine). Twelve male and female C57Bl/6 mice were employed in experiments that measured the absolute sensitivity, the increment threshold response, and spectral sensitivity. The mice were on average 35 days old before they were used in experiments. Three mice that were 18 days of age have participated in experiments. Three \( Gnat-1^{-/-} \) mice, donated by Dr. Janis Lem at Tufts University (Boston), and four \( Gnat-2^{-/-} \) mice, donated by Dr. Bo Chang at Jackson Laboratories (Bar Harbor), also participated in these experiments.

*The apparatus*

Individual mice were placed in a standard cage (9.3” x 13.9” x 7.7”) equipped with a running wheel (Lafayette Instrument, Lafayette, IN) that was suspended from a
custom-made clear plastic cage-cover. Wheel revolutions were monitored with a photocell mounted above the rungs, near the edge of the wheel. The wheel moved in either direction, and the photocell counted the wheel revolutions in either direction. The signal from the wheel was sent via an electronic interface to a PC. A custom-made electrically-wired water spout penetrated one of the cage walls, at a location approximately 10” away from the wheel, 1.5” above the floor. Water flowed by force of gravity from a 35-ml syringe via a filter and electronic micro pump (Lee Company; Westbrook, CT) through polyethylene tubing to the waterspout. Contact between the mouse’s tongue and the spout, on the one hand, and between its feet and a set of metal bars on the cage floor, on the other hand, completed an electrical circuit that caused the pump to open. With every lick at the spout a signal was transmitted via an interface to the computer. The timing of water delivery was controlled by computer. Lab chow was permanently available, distributed on the cage floor with the bedding (See Figure 3).

*The light stimulus.*

An LED assembly was centered above the wheel. The assembly consisted of two hollow equal-length opaque cylinders that differed in diameter. The larger-diameter cylinder could be slid over the narrower cylinder. The former was tightly capped at one end and contained the LED. The latter served as a filter holder and was press-fit to the cage cover, in an opening located above the wheel. To direct the light stimulus onto a wheel-running mouse, the LED cylinder was slid over the filter holder until it rested on an opaque circular platform that served to block stray light. An opal glass filter was used
Figure 3. Main sequence of mouse behaviors in experimental cage. A. (1) Mice spontaneously wheel running; (2) learn to associate the presence of a light flash with the availability of water; (3) and exit the wheel within one or two seconds to visit the water spout [w.s]. B. Three key elements in the cage are visible: the licking spout (mouse is nearby), the LED assembly and running wheel. Food is distributed on the floor of the cage. C. A light-diffusing box normally covers the cage; regulated spot lamps illuminate the diffusing cover for bleaching or light adaptation. The LED is imaged on the mouse’s retina as a disc of about 5.3 deg of visual angle, and subtends ~ 8300 rods.
Figure 5

[A] 1. LED
   2. W.S.
   3.

[B] 

[C]
to diffuse the light stimulus before it exited the assembly. The LED/diffuser target had a diameter of 0.7\textdegree.

**Stimulus calibration: retinal image size, photon flux.**

The distance between the stimulus target and the retina was 7.5\textdegree, yielding an image size of 5.3 deg \[\tan^{-1}(0.7\textdegree/7.5\textdegree) = 5.3\textdegree\]. As the mouse’s retina can be taken to lie on a sphere having a radius of \(r = 1.69\) mm (Lyubarsky et al., 2004), the diameter of the target image was calculated to be \((5.3/180) \times \pi \times 1.69 = 0.156\) mm. Thus, the retinal surface occupied by the target was \(\pi \times r^2 = \pi \times (0.156/2)^2 = 0.0191\) mm\(^2 = 19,100\) \(\mu\)m\(^2\).

As the average density of rods in the mouse retina is \(4.37 \times 10^5\) mm\(^2\) (Jeon et al., 1998), the target covered about \(8500\) rods.

Test flashes were provided by interchangeable LEDs purchased from Philips Lumileds (San Jose, CA) and Nichia (Tokyo, Japan). LED intensity was controlled in three ways: (1) by pulse-width drive, \textit{i.e.} flash duration. The pulse applied to the LED is a square wave that can be varied in duration, from 4.8 \(\mu\)s to 1016 \(\mu\)s in steps of 0.8 \(\mu\)s or multiples thereof. Differences in duration between very brief flashes are imperceptible to the human observer; flash discrimination is instead based on differences in flash quantum content; (2) by selecting either a low or high amplitude level for the square wave pulse driving the LED. In the low amplitude setting, the flash is 7 times less intense than at the high amplitude level; (3) by manually inserting calibrated neutral density filters in the LED beam.

For radiometric calibrations the appropriate narrowband filter (Edmund Scientific, Barrington, NJ) was placed in front of the LED. The flashed stimuli were incident on a
Figure 4. A. Stimulus calibration arrangement. The photodiode (plus built-in amplifier) was placed on the running wheel, slightly off-center so that its light sensitive surface was homogenously covered by incident light from a flashing LED. The signal was stored on a disk for further processing. To obtain estimates of the flash intensity at the location of the filled circle the inverse square law was applied. B. Waveform and duration of light stimulus (upper panel). The intensity was controlled by varying the flash duration in either one of two possible amplitude settings (lower panel, see arrows).
$\lambda = 500 \text{ nm}$  
Flash duration: 21.6 $\mu$s

Figure 4
high-speed photo-detector (PDA 500; Thorlabs, Newton, NJ). The detector was placed in the location where the mouse’s head would be, when the animal traverses the lowest position on the running wheel (see Figure 4). The flash intensity \( Q_\lambda \) was measured in volts. Using equations specified by Wyszecki and Stiles (1982), \( Q_\lambda \) was expressed in units of “photons \( \mu \text{m}^{-2} \) at the cornea”. The maximum intensity values obtained with 1 ms flashes with no neutral density filter in place are shown in column 2 of Table I. The area of the pupil of the dark-adapted mouse was taken to be 2.5 mm\(^2\). The maximum photon flux captured by the pupil from an un-attenuated light flash is equal to the product of the listed intensity (Table I, column 2) and the pupil area (expressed in \( \mu \text{m}^2 \)). For example, for the un-attenuated 365 nm flash, the maximum flux through the pupil would be 1.75 \( \times \) \( 10^7 \) photons (= 7 photons \( \mu \text{m}^{-2} \) \( \times \) 2.5 \( \times \) \( 10^6 \) \( \mu \text{m}^2 \)). Assuming that all photons delivered at the pupil plane were transmitted to the retina, the flux density on the retina was about 916 photons \( \mu \text{m}^{-2} \) [\( = (1.75 \times 10^7)/(19100) \)].

A potentially significant factor influencing the stimulus intensity at the retina is that the effective cross section of the mouse pupil is much less than 2.5 mm\(^2\), due to the position of the LED relative to the optic axis (the mouse pupil assumes its maximal cross-sectional area only for sources that lie on the optic axis, which for our purposes can be defined to be perpendicular to the pupil). If the angle between the optic axis and the surface of the LED-assembly is 45°, there will be an attenuation of the flash intensity due to the smaller effective pupil area of \( \cos (45^\circ) = 0.71 \). We assume an angle of 60°, thus, an attenuation of 0.5 (= \( \cos (60^\circ) \)). Since there are at present no good data available for loss in the pre-retinal media we did not take these effects on the stimulus intensity at the
Table I. LEDs and test flash intensities

<table>
<thead>
<tr>
<th>Interference filter, nominal peak wavelength (nm)</th>
<th>1ms flash, intensity: $Q_\lambda$ (photons $\mu m^{-2}$ at cornea)</th>
<th>1ms flash, intensity: $Q_\lambda$ (photons $\mu m^{-2}$ at retina)</th>
<th>Manufacturer label; $\lambda_{\text{max}}$ (nm)</th>
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</thead>
<tbody>
<tr>
<td>365</td>
<td>6</td>
<td>450</td>
<td>UV; 365 nm [1]</td>
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<tr>
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<td>4</td>
<td>271</td>
<td>UV; 395 nm [2]</td>
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<td>436</td>
<td>1.2</td>
<td>78</td>
<td>White; 440 and 560 nm [3]</td>
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<tr>
<td>455</td>
<td>28</td>
<td>1849</td>
<td>Royal Blue; 455 nm [3]</td>
</tr>
<tr>
<td>470</td>
<td>0.9</td>
<td>59</td>
<td>Blue; 470 nm [3]</td>
</tr>
<tr>
<td>500</td>
<td>11</td>
<td>718</td>
<td>Cyan; 505 nm [3]</td>
</tr>
<tr>
<td>510</td>
<td>11</td>
<td>720</td>
<td>Cyan; 505 nm [3]</td>
</tr>
<tr>
<td>532</td>
<td>3.6</td>
<td>238</td>
<td>Green; 530 nm [3]</td>
</tr>
<tr>
<td>550</td>
<td>4.5</td>
<td>298</td>
<td>White; 440 and 560 nm [3]</td>
</tr>
<tr>
<td>590</td>
<td>7.7</td>
<td>506</td>
<td>Amber; 590 nm [3]</td>
</tr>
</tbody>
</table>

For all interference filters (Edmund Optics, Barrington, NJ), nominal bandwidth at half-max transmittance was 10 nm. LED manufacturer: [1] Nichia, Tokyo, Japan. [2] Roithner Laser Technik, Vienna, Austria. [3] Philips Lumiled, San Jose, CA.
retina into account. Thus, the flux density on the retina from the 365 nm flash was \((916 \times 0.5) = 458\) photons \(\mu m^{-2}\).

During both light- and dark-adapted experiments, the cage was always covered by an ‘upside-down’ white translucent polyethylene box (19” x 12” x 14”; McMaster-Carr; Princeton, NJ). Two different light sources were used to homogeneously illuminate the top surface of the cover box. One source was a 150-Watt Xenon arc lamp located in an adjacent room. Its light was first collected at a small aperture by a collimating lens. The collimated beam was then focused by a second lens on the entrance aperture of an optical fiber. A mechanical shutter positioned just in front of the optical fiber aperture electronically interrupted the beam. Interference and calibrated neutral density filters placed manually in a filter rack, in the collimated portion of the beam. The optical fiber was sent to the neighboring room where the mouse unit was housed. The exit aperture of the optical fiber was placed 1.5 meters away from and perpendicularly oriented to the box’s top surface (see Fig 5). Before illuminating the top surface the light passed through diffusing glass. Radiometric measurements were made at the location of the wheel. Readings were expressed in Watts/cm\(^2\). The number of photons \(\mu m^{-2} \, s^{-1}\) at the cornea for each wavelength background was calculated using standard equations provided by Wyszecki and Stiles (1982).

The background wavelengths used were, in nm: 400, 440, 470, 500, 530, 550, 570 and 590. Only one background intensity level and one wavelength were used per session. The light remained on until all 40 or 60 trials were completed. The dark-adapted sensitivity was intermittently probed, usually after completing measurements on two
Figure 5. Schematic of optical arrangement: light from a 150W Xenon-arc lamp was incident on the translucent surface of the cover box placed over the mouse cage. Initially, the light passed through a 2-mm aperture that was positioned one focal length away from a collimating lens. The collimated beam passed through narrow-band and neutral density filters and was then focused on a shutter vane positioned in front of the entrance aperture of an optical fiber. The exit aperture of the fiber was covered with diffusing glass and it was oriented perpendicularly to the surface of the cover box. The light was distributed homogeneously on this surface.
Xenon-arc lamp

small aperture

collimating lens

interference, neutral density filters

focusing lens

shutter

wall between rooms

optical fiber

diffusing glass

cover box placed over experimental cage

Figure 5
three or different background intensities. The mice dark-adapted every day for at least 12 hours. The other background light source was provided by four white 30-Watt or 100-Watt 45° flood lamps, arranged around the cover box in a square, and tilted so that they pointed toward the ceiling of the room. The reflected light illuminated the cover box homogeneously. Timing of the light stimuli and their intensities were controlled by computer. The luminance of the adapting field was measured with a radiometer/photometer (UDT Model S370; San Diego, CA) in four different locations inside the cage. The maximum local luminance difference was again less than 0.15 log units. The light intensity was controlled by varying the current applied to lamps, which inevitably affected the wavelength composition of the background light. Since we planned to use the background light to primarily light-adapt the rods, we were not too concerned about the wavelength changes as rods respond to luminance changes rather than changes in wavelength. The luminance provided by the four 30-Watt lamps ranged from 0.0002 – 6 phot cd/m². The four 100-Watt lamps were calibrated over the range of 0.03 - 550 phot cd/m². Both sets of lamps were calibrated at 15 digital settings. Below 1 cd/m², one phot cd/m² was taken to correspond to 0.7 scot cd/m²; at intensities > 1 cd/m², one phot cd/m² was taken to correspond to 1.5 scot cd/m². A stimulus luminance of 1 scot cd/m² passing through a pupil area of 2.5 mm² produces about 450 photoisomerizations rod⁻¹ s⁻¹ (see Lyubarsky et al., 2004).

**Training**

The training of the mice was carried out in two stages. In the first stage the goal was to establish in the behavior repertoire of the mouse an association between light flash
occurrence and the availability of water. In the second stage the mouse was trained to work for water.

Stage 1: Untrained mice were individually placed in the experimental cage. Access to food and water was unrestricted. The mice consumed on the first day between 5 and 7 ml water and traversed distances ranging from 6 to 12 km. After about 30 hrs in the cage, mice triggered, by wheel running, a bright flash programmed to occur at every 20\textsuperscript{th} or so revolution. While access to food remained free, water was available only during a short period after the flash. At first, it was available during a 600s interval that was gradually shortened to 12 s (intermediate intervals were 300 s, 120 s, 60 s, 30 s, and 15 s). At the end of the interval in which water was available, the trial concluded; counters and clock were reset, and a new trial began, irrespective whether the mouse was on or off the wheel. Experiments usually started between 5 pm – 7 pm; animals dark-adapted each day for more than 15 hrs. The mice could wheel-run during the day, however, the flash trigger was disabled denying them access to water. A mouse was deemed to detect the flash if it interrupted running within one wheel revolution after the flash. Mice learned to associate the presentation of a light flash with the availability of water, within 10 hrs after the first trial (i.e. initial presentation of a bright flash). Figure 6a shows histograms of flashes (filled bars) generated by wheel-running mice and their early responses to these flashes (white bars). The abscissa indicates the time of day in two-hour intervals. Six to ten hrs after the first flash presentation, the mice sporadically exited the wheel within one revolution after the flash. Over the course of 16 hrs, WT mice responded “correctly” on 12 - 20\% of the trials and reduced or stopped the running after they consumed ~ 5ml of water.
Figure 6. A. Histogram of flashes generated by wheel running mice, and their early responses to these flashes. The abscissa indicates the time of day in two-hour intervals. Six to 10 hours after the very first flash presentation, the mice occasionally began to exit the wheel within 1 revolution after the flash. Water availability after the flash was first set for 600s, 300s, 120s, 60s, and then 30s. Overnight it was set for 15s. Number of wheel revolutions per flash: 30. B. Visual performance of a mouse measured over the course of six days, following initial placement in the experimental cage. Bold numbers above the bars are random numbers of wheel revolutions required before the flash is triggered.
animal 1

number of trials on which mouse exits wheel at 0 or 1 revolutions after flash

0 20 40

animal 2

number of flashes generated

0 20 40

participation in experiment (number of days)

1 2 3 4 5 6

percent of trials on which mouse exits wheel at 0 or 1 revolution

0 20 40 60 80 100

A

B
Stage 2: In the 6-day period following establishment of the link between flash and water availability, the ‘correct response’ rate of the mice gradually improved, as shown in Figure 6b. On the third day, the mouse had a ‘correct response’ rate of about 40% compared to 17% on the first day. Since the flash was intense and was triggered frequently (once every 20th wheel revolution), the mice may have simply ignored its occurrence, on most occasions. To increase the cost for skipping a flash, we forced the animal to run longer distances before gaining access to water. In the second stage of training, increasing the number of wheel revolutions per flash was the most essential step. The average number of wheel revolutions per trial required to trigger a flash was set to be random. By the sixth day the performance was near perfect. We have found that, starting from Day 2 or Day 3, the flash intensity may also be gradually reduced (not shown), so that by Day 6 the flash intensity needed is only about 1 - 1.6 log units above the animal’s absolute threshold. As the flash intensity was further reduced, the number of errors made by the animal inevitably increased. To insure that it would get enough water, bright light flashes were inserted toward the end of the session.

Efficient and reliable data collection on individual mice

Our method overcomes two crucial problems: readiness and motivation. Firstly, the mouse’s extensive daily wheel-running is monitored by a computer and is used to generate experimental trials in which light flashes are presented randomly and the availability of water is time-controlled. In terms of readiness, the mouse is always up and running, in a known location, when a flash occurs. Secondly, the mouse readily associates the presentation of the light flash with the availability of water at a spout such
that the light flash signals the availability of water. It was never necessary to deprive the mouse of water. For whatever reason rodents wheel-run, once the association is established, wheel running is now partly undertaken to obtain water. The goal of obtaining water constitutes a powerful motivating factor. In our setup, the mouse can only obtain water if it detects the light flash, which is presented only if the animal wheel runs. Note that the wheel running itself is not under control of the light stimulus. We do not attempt to shape the wheel running in any way: the method does not involve ‘conditioned suppression’.

The strength of our method then rests on the use of, and control over, three critical variables: (1) Control of the animal’s motivation. By letting the mouse work for water, we succeeded in making its motivation to respond to the light stimulus integral to its natural daily routine. Provided the animal is allowed to obtain at least 5 ml of water over the course of 24 hrs, we argue that it is not water deprived. (2) Timing of water availability after the stimulus. It is very important that water is made available immediately after the stimulus presentation to establish a strong association, i.e. apply the principle of temporal contiguity from operant conditioning theory. The availability of water must be rather brief (a 12 s-period after the flash) so that the proper level of motivation is maintained, preventing premature satiation. About 2-3 μl of water was made available per lick; mice produce 6-10 licks/s. Wheel-running mice consume anywhere between 5 and 7 ml water/day. If for some reason the mouse does not obtain enough water, we give it free access for a few hours. The association between the light stimulus and the availability of water is ‘permanent’, that is, it can be reactivated, usually
within an hour or two, after many months of not having lived in the experimental cage.

(3) Characterization of the spontaneous behaviors. Distances run by the mice range from 7 to 12 km per day and most of the running is done at night. The animals do not require special encouragement to wheel run. The flash frequency is determined by: (a) the average number of wheel revolutions/flash set by the experimenter and (b) the mouse’s activity level. The latter can be influenced by flash intensity. [For example, if no limit is placed on the number of trials, mice will produce a large number of trials when flashes are very dim, and a much smaller number of trials when flashes are bright (which allow for higher water intake)]. Thus, it is essential to complement the presentation of a series of dim stimuli with more easily visible stimuli to avoid exhaustion and dehydration of the mouse.

The relationship between wheel-running and test flash intensity

The relationship between wheel-running and test flash intensity near the visual threshold was further examined. Over the course of six days, dark-adapted mice (n = 4) were presented each night with a different but fixed flash intensity. The flash wavelength was 365 nm. There was no restriction on the number of trials the mice could generate; a session ended when the mouse had taken in 5-6 ml water. The average number of wheel revolutions required to trigger a flash on a trial was set at 400 for the three highest flash intensities and at 250 for the three lowest. Fig 7A – 7F show histograms of trials completed in six experiments with a fully dark-adapted mouse. The abscissa shows the number of wheel revolutions completed in the after-flash period (12 s). The label “0” indicates that the mouse completed less than one wheel revolution after the flash; label
“1” indicates that it completed more than one but less than two wheel revolutions and so on. The total number of trials generated in the experiment and the flash intensity used are listed in each panel. In the experiment of Fig. 7A, in which the highest flash intensity was used, the animal generated 34 trials and exited the wheel 32 times within one or two wheel revolutions after the flash, earning 5-6 ml of water. Based on these results, and similar data from three mice, the following criterion was adopted: the mouse was deemed to detect the flash if it interrupted running within two wheel revolutions after the flash. The running speed is, on average, 0.85 (± 0.14, n = 4) wheel revolutions s⁻¹. In Fig 7A, the fraction of ‘correct responses’ plotted at “0” and “1” on the abscissa were 0.59 (p = 20/34) plus 0.35 (p = 12/34), respectively. In subsequent experiments, the flash intensity was further lowered. Fig 7B shows that the animal performed as well as in Fig 7A. The visual performance in the experiment of Fig 7C was, compared to that in Fig 7B, poorer; presumably due to the difference in flash intensity. Even though the mouse consumed about the same amount of water in the two experiments, it traversed a much longer distance (number of trials x average number of wheel revolutions set per trial) in the experiment of Fig. 7C. With further decreases of the flash intensity in the experiments of Figs 7D, 7E and 7F, respectively, the mouse responded progressively less frequently to the flash. Under these circumstances, the animal did not earn sufficient amounts of water, it therefore kept returning to the wheel increasing the number of trials. To prevent dehydration and exhaustion, moderately intense flashes were inserted on every 8-9th trial and the average number of wheel revolutions required to trigger a flash on a trial was reduced from 400 to 200. We ended these experiments after the p-value had stabilized. The low number of required wheel revolutions has no obvious effect on the mouse’s
Figure 7. A-E. Histograms of trials in five experiments with a fully dark-adapted mouse. The abscissa indicates the number of wheel revolutions produced in the after-flash period (12 s). The flash wavelength was 365 nm. The flash duration is indicated in each panel. The flash intensities were (photons µm$^{-2}$ at the cornea): $4.2 \times 10^{-5}$, $6.4 \times 10^{-5}$, $1.1 \times 10^{-4}$, $1.9 \times 10^{-4}$, $6.4 \times 10^{-4}$. In A, the intensity was extremely low; the histogram is indistinguishable from that obtained in the absence of a stimulus (not shown).

F. Behavioral sensitivity of a well-trained mouse derived from 5 experiments carried out over 5 days.
a = photons $\mu m^{-2}$ at cornea (365 nm)
b = number of trials

Figure 7a-f
visual response to low intensity flashes. On the contrary, frequent presentation of high intensity flashes leads to early satiation that affects the animal’s motivation and that, in turn, leads to a tendency to ‘ignore’ flash presentations. In the experiment of Fig 7F, the flash intensity was extremely low. On only 2 trials out of 90, the mouse exited the wheel ‘correctly’ \( p = 2/90 = 0.02 \). This histogram is indistinguishable from that obtained in the absence of a light stimulus (not shown). In Fig 7G, the fraction of ‘correct’ responses is plotted as a function of the flash intensity based on the experiments of Figs 7A-7F.

The solid gray curve fitted to the data points represents the sigmoidal function:

\[
y = \frac{a}{1 + e^{-(x - x_0)/b}}
\]  

(Eq. 1)

where \( y \) represents the fraction of ‘correct responses’; \( a \) is the normalized maximum number of ‘correct responses’ and is equal to 1; \( x \) is the flash intensity; \( x_0 \) is the ‘50% threshold’ flash intensity and \( b \) is a weighing factor that affects the slope of the curve. Sigmaplot for Windows was used to fit Eq. 1 to the data and to determine the slope, \( s \), of the curve, taken to be the first derivative at \( (x_0, y = 0.5) \). The mouse’s 50% threshold was \( 3.4 \times 10^{-5} \) photons \( \mu m^{-2} \) at the cornea; \( s = 1.45 \).

‘Frequency of seeing’ data were collected on individual mice mostly during a single nighttime session, with the method of constant stimuli. Usually, four different flash intensity levels were used. The average number of wheel revolutions required to trigger a flash on a trial was the same at all flash intensities during a single night’s session. Each intensity level was presented 15 times, on 60 trials (some times, the number of trials was reduced to 40 or increased to 80). The trials were organized in 12 blocks; each block contained 5 trials of fixed flash intensity. The blocks were presented
Figure 7G. Behavioral sensitivity of a well-trained mouse derived from 6 experiments carried out over 6 days. The fraction of correct trials plotted as a function of flash intensity is shown. The data represented was obtained from experiments shown in Fig 7a-f. The solid gray curve fitted to the data points represents Eq 1. The mouse’s 50% threshold was $3.4 \times 10^{-5}$ photons $\mu m^2$ at the cornea.
The graph shows the relationship between the test flash threshold intensity (photons $\mu$m$^{-2}$ at cornea) and the fraction of trials marked as "correct". The intensity values range from $10^{-5}$ to $10^{-4}$, and the fraction ranges from 0.0 to 1.0. The data points are marked with black circles, and a curve fits the data, indicating a sigmoidal response pattern.
in random order. The intensity range was occasionally extended to six flash intensities spanning a range of approximately 1.6 log units. The grand average of the correct responses per intensity level was calculated along with the SD and SEM. The ‘50% threshold’ intensity was found by linear interpolation or by curve fit and is reported as mean (± 2*SEM; r = number of replications). The threshold was considered adequately measured if the interpolated mean and the threshold intensity of the sigmoidal curve differed by less than 0.15 log units, and if the curve’s slope fell between 1.25-1.75.

Increment threshold responses were measured with narrow-band test flashes on narrow- or broad-band adapting fields. At each field intensity level, at least two frequency of seeing data-sets were collected and the ‘50% threshold’ values were extracted as described above. The increment threshold responses were evaluated with the general hyperbolic relation designated \( F \) by W. S. Stiles (see Eq. 2, pg 34).

Spectral sensitivity data were expressed as \( \Delta I_\lambda/\Delta I_{\lambda,\text{max}} \) and \( I_\mu/I_{\mu,\text{max}} \). \( \Delta I_\lambda \) is the dark-adapted threshold intensity obtained with a test flash containing wavelength, \( \lambda \). \( \Delta I_{\lambda,\text{max}} \) is the dark-adapted threshold intensity obtained with a test flash containing \( \lambda_{\text{max}} \), the peak wavelength. \( I_\mu \) is the adapting field intensity at a given wavelength, \( \mu \), that raises the test flash threshold one log unit above \( \Delta I_{\lambda,\text{max}} \). \( I_{\mu,\text{max}} \) is the intensity of the adapting field that contains \( \mu_{\text{max}} \), the peak wavelength, and that raises the test flash threshold one log unit above \( \Delta I_{\lambda,\text{max}} \). The spectral sensitivity data were characterized with Lamb’s (1995; see also Lyubarsky et al., 1999) photopigment template curve, given by:
\[ y = \frac{1}{\exp[a(A-x)] + \exp[b(B-x)] + \exp[c(C-x)] + D} \quad \text{(Eq. 3)} \]

When the spectral sensitivity of the mechanism mediating the test flash is considered,
\[ y = \frac{\Delta I_{\lambda}}{\Delta I_{\lambda,\text{max}}} \text{ and } x = \frac{\lambda_{\text{max}}}{\lambda} \] where \( \lambda \) is the wavelength of the test flash. For the fit to the background data,
\[ y = \frac{I_{\mu}}{I_{\mu,\text{max}}} \text{ and } x = \frac{\mu_{\text{max}}}{\mu} \] where \( \mu \) is the adapting field wavelength.

The parameter values defining the shape of the template for mouse photopigments were adopted from Lyubarsky et al. (1999). The values were: \( A = 0.88, B = 0.924, C = 1.104, D = 0.655, a = 70, b = 28.5 \) and \( c = -14.1 \).

The experiments that are key to the dissertation.

We have determined: (a) the spectral sensitivity of the rods to the test flash and to the adapting field; (b) \( F \), the curve relating the (test) threshold to the (background) intensity for rods and M-cones. Weber fractions were determined for rods and we evaluated whether W.S. Stiles’ test and field displacement laws hold for mouse vision. We examined whether psychophysical rod saturation occurs in the \( Gnat-2^{-/-} \) mouse, and we characterized the cone threshold response of the \( Gnat-1^{-/-} \) mouse at high adapting light levels.

RESULTS

Dark-adapted threshold responses of WT mice measured with small stimuli.

Dark-adapted WT mice (\( n = 4 \)) were presented with 5.3 deg, 500 nm flashes that were attenuated to deliver: \( 6.7 \times 10^{-5}, 3.2 \times 10^{-5}, 1.5 \times 10^{-5} \) and \( 5.4 \times 10^{-6} \) photons \( \mu \text{m}^2 \) at the cornea. The corresponding flash durations were: 100, 48, 21.6 and 8 \( \mu \text{s} \). Each intensity level was presented 15 times, on 60 trials. The trials were organized in twelve blocks; each
Figure 8. Absolute threshold responses obtained from dark-adapted WT mice (n= 5).

Each symbol represents data from a different WT mouse. At least two frequency of seeing curves were collected from each animal. The stimulus size on the retina was 5.3 degrees of visual angle; $\lambda_{\text{max}} = 500\text{nm}$. The mean “50% threshold” intensity was $3.0 \times 10^{-5}$ photons $\mu m^{-2}$ at the cornea and produced about $1.7 \times 10^{-3}$ R* corresponding with 14 photoisomerizations per flash.
Figure 8

Fraction of trials correct vs. flash intensity (photons $\mu$m$^{-2}$ at cornea).
block contained five trials of fixed flash intensity. Occasionally, the flash intensity range included a 216 μs, 32.8 μs or 8 μs flashes. At least two frequency of seeing curves were collected from each animal. The results are shown in Fig. 8, where the fraction of ‘correct responses’ is plotted as a function of the flash intensity. Each symbol represents data from a different WT mouse. All data sets are well described by a sigmoidal function. The mean “50% threshold” intensity of the 500 nm flash was 3.0 x 10^{-5} photons μm^{-2} (± 2.7 x 10^{-6}, n = 4) at the cornea and produced about 1.7 x 10^{-3} R* corresponding with 14 photoisomerizations per flash (see Methods).

To verify whether the mouse could have responded to stimulus light reflected from reflective surfaces nearby, the entire wheel assembly was painted black except for a narrow strip on the outer surface of the rungs to allow the emitter/photodetector assembly to be activated by IR light reflected off the rungs. The open inverted triangles in Figure 8 represent data obtained on a mouse running on the painted wheel; these data are well within the absolute sensitivity range for other mice indicating that the animals were not responding to reflected light.

To explore the effect of stimulus size on the mouse’s absolute sensitivity, the diameter of the 500 nm test flash was varied. The following sizes, given in degrees of visual angle, were used: 5.3, 3.3, 1.8, 1.2, 0.6, 0.45 and 0.3. Target sizes on the retina (μm^2) and the number of rods imaged by each target are listed in Table II (columns 1 and 2). Stimulus intensities were calibrated in situ expressed in numbers of photons μm^{-2} at the retina (Table II, column 3) and in photoisomerizations rod^{-1} as well. The target intensities were
Table II: Stimulus spatial parameters and rate of photoisomerization.

<table>
<thead>
<tr>
<th>Stimulus diameter in mm. [target diameter on retina, in deg.]</th>
<th>Image size at retina (μm²)</th>
<th>Numbers of rods imaged by target</th>
<th>Max. intensity (photons μm⁻²) at retina.</th>
<th>50% threshold intensity (R* ± SEM) n = 3</th>
<th>Photoisomerizations (± SEM) at threshold* n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.8 [5.3]</td>
<td>19100</td>
<td>8300</td>
<td>260</td>
<td>1.8 x 10⁻³ ± 4.5 x 10⁻⁴</td>
<td>16 (± 3.5)</td>
</tr>
<tr>
<td>11 [3.3]</td>
<td>7300</td>
<td>3250</td>
<td>290</td>
<td>1.1 x 10⁻³ ± 2.8 x 10⁻⁴</td>
<td>7 (± 2.2)</td>
</tr>
<tr>
<td>6 [1.8]</td>
<td>2175</td>
<td>975</td>
<td>300</td>
<td>5.5 x 10⁻³ ± 1.2 x 10⁻³</td>
<td>5 (± 2.5)</td>
</tr>
<tr>
<td>4 [1.2]</td>
<td>975</td>
<td>430</td>
<td>290</td>
<td>2.6 x 10⁻² ± 3.6 x 10⁻³</td>
<td>9 (± 3.6)</td>
</tr>
<tr>
<td>2 [0.6]</td>
<td>230</td>
<td>110</td>
<td>280</td>
<td>1.1 x 10⁻¹ ± 1.4 x 10⁻²</td>
<td>12 (± 2.9)</td>
</tr>
<tr>
<td>1.5 [0.45]</td>
<td>140</td>
<td>60</td>
<td>250</td>
<td>2.2 x 10⁻¹ ± 2.5 x 10⁻²</td>
<td>14 (± 2.2)</td>
</tr>
<tr>
<td>1.0 [0.3]</td>
<td>60</td>
<td>25</td>
<td>240</td>
<td>5.1 x 10⁻¹ ± 6.1 x 10⁻²</td>
<td>13 (± 3.1)</td>
</tr>
</tbody>
</table>

*Number of photoisomerizations at threshold equals number of rods imaged at retina x 50% threshold intensity.
attenuated until a frequency of seeing curve was measured with the flash durations given above. At least two curves were obtained per target. Fig 9a shows, in log-log coordinates, the 50% threshold intensities (R* or photoisomerizations rod\(^{-1}\)) for the targets plotted against the target area (μm\(^2\)) occupied on the retina. The fit of a straight line, having a slope of -1, to the data indicates that for target sizes \(\leq 0.6\) deg (area = 230 μm\(^2\)) the threshold intensity and area are inversely related. The data from Fig 9a are re-plotted in Fig. 9b as the number of photoisomerizations produced per flash (product of threshold intensity and number of rods imaged by the target) versus the target area, in μm\(^2\). The 3.3 deg and 1.8 deg test flashes elicited the most sensitive responses from the mice. The results obtained with the three smallest targets were, however, most meaningful in that the retinal area over which the stimulus energy is integrated contained 110 rods or less. Similar results were obtained on two other C57Bl/6 mice; on a fourth mouse, it was found that area and threshold intensity were inversely related with a 1.2 deg stimulus that imaged about 450 rods.

**Dark-adapted threshold responses of Gnat-1\(^{-/-}\) mice.**

Dark-adapted cone threshold responses of Gnat-1\(^{-/-}\) mice (n = 3) to 365 nm and 510 nm flashes were also measured. The stimuli were presented in twelve blocks of five trials. The 365 nm flash was attenuated to deliver 6 x 10\(^{-1}\), 2.6 x 10\(^{-1}\), 1.4 x 10\(^{-1}\) and 8.7 x 10\(^{-2}\) photons μm\(^{-2}\) at the cornea, respectively. The 510 nm flash delivered 3, 1.4, 0.6 and 0.2 photons μm\(^{-2}\) at the cornea. For both the 365 nm and the 510 nm flashes, the intensity range was expanded occasionally by doubling the intensity of the brightest flash or reducing that of the dimmest flash by half. Gnat-1\(^{-/-}\) mice completed the experiments in the same time frame as did WT mice. The 365 nm and 510 nm data from the knock-out mice are shown in
Figure 9. The effect of stimulus size on the absolute threshold response. Each data point represents a frequency of seeing curve. Frequencies of seeing curves were obtained with stimuli 5.3°, 3.3°, 1.8°, 1.2°, 0.6°, 0.45° and 0.3° of visual angle. A. The 50% threshold intensity, expressed in R* is plotted as a function of the size of the target area. B. Test flash threshold intensity, given in R* is multiplied by the number of rods imaged in the target. The result, the number of photoisomerizations at threshold, is plotted as a function of the number of rods imaged by the target.
area on retina occupied by target (μm²)

A. 

test flash threshold (R*)

10² 10³ 10⁴ 10⁵

number of rods imaged by target

B. 

number of photo-isomerizations per flash

10 20 30

10 10² 10³ 10⁴

4 5 8 20 30

A. B.
Figure 10 and are represented by the open and gray symbols, respectively. For the 365 nm test flash, the mean “50% threshold” intensity was 0.12 photons $\mu$m$^{-2}$ ($\pm 1.1 \times 10^{-2}$; n = 3); for the 510 nm flash, the mean value was 0.5 photons $\mu$m$^{-2}$ ($\pm 1.58 \times 10^{-2}$; n = 3). Thus, the absolute threshold response of the WT mouse to a 500 nm stimulus was about 3000 times more sensitive than that of the Gnat-1$^{-/-}$ mouse to a 365 nm flash; and it was about 12,000 times more sensitive than the mutant’s response to a 510 nm flash.

The effect of target size on the threshold responses of dark-adapted Gnat-1$^{-/-}$ mice (n = 2) was examined with 3.3, 1.8, 1.2 and 0.6 deg targets, using procedures described above. We found that the 1.2 deg stimulus was the smallest 365 nm target size for which a threshold response could be obtained. When the wavelength of this target was changed from 365 nm to 532 nm the mouse failed to make intensity discriminations, no matter what the intensity settings were. However, both Gnat-1$^{-/-}$ mice responded successfully when the size of the 532 nm target was increased to 1.8 deg. Assuming that the 1.2 deg, 365 nm target stimulated primarily UV cones in the ventral retina of the Gnat-1$^{-/-}$ mouse, about 12 cones must have been activated per flash. The 1.8 deg, 532 nm target imaged about 30 cones; 20% of which, about 6, could have been M-cones. Given that the majority of M-cones are concentrated in the dorsal retina, it is more likely that less than 6 M-cones contributed to the threshold response. The 1.6 deg, 532 nm target, and by extension the 5.3 deg, 510 nm target, possibly activated UV-cones in the ventral retina that contain significant amounts of mid-wavelength sensitive pigment.
Figure 10. Dark-adapted cone threshold responses of Gnat-1<sup>−/−</sup> mice (n=3). Mice were presented with test flashes of either 365nm or 510nm represented by the gray and open symbols respectively. Each symbol represents data from a different Gnat-1<sup>−/−</sup> mouse. The stimulus size on the retina was 5.3 degrees of visual angle. The stimuli were presented in twelve blocks of five trials. The mean “50% threshold” intensity was 0.12 photons µm<sup>−2</sup> for test flashes of 365nm and 0.5 photons µm<sup>−2</sup> for test flashes of 510 nm.
Figure 10

Flash intensity (photons $\mu$m$^{-2}$ at cornea)

Fraction of trials correct

Gnat-1$^{-/-}$
365 nm

Gnat-1$^{-/-}$
510 nm
The t.v.i. curve and the spectral sensitivity of the rod mechanism in WT mice

In human threshold psychophysics, well-defined test flash stimuli that are local on the retina are commonly used to probe the state of retinal regions as they are affected by the presence of a larger adapting (conditioning) field. Benefiting from biting boards and fixation points, humans can direct small stimuli to almost any retinal area of their regionally diverse retina. Wheel-running mice cannot be stimulated with any precision in a specific retinal region but fortunately, mouse visual psychophysics stands to benefit from three unique circumstances: (1) The rods are naturally homogenously distributed throughout the mouse retina diminishing the need for precise stimulus delivery; (2) The availability of Gnat-2\(^{-/-}\) mice that lack functioning cones could allow for the study of rod threshold responses at high adapting intensities unencumbered by cone activity; (3) Gnat-1\(^{-/-}\) mice allow for the study of cone vision in absence of rod activity. The study of cone vision in mice is expected to be more challenging than that of the rods given that the patterns of M-cone and UV-cone distribution are highly dissimilar and that UV-cones contain more than one type of active visual pigment. In the interest of a consistent analysis of mouse vision using the wheel running mouse, we thought it to be appropriate to first establish the general psychophysical principles by which the rods operate as these principles may serve as a frame of reference for future analyses of cone vision.

The mouse rod spectral sensitivity is a known quantity that has been studied with different techniques, including behavioral techniques. In this study, the spectral sensitivity was determined not only to the test flash but to the background stimulus as well; we thereby described the characteristics of the curve that relates the test flash to the background, and
combined with measurements on Gnat-1-/- mice, we estimated at which background intensities cone responses can be isolated in WT mice.

Three slightly different procedures were adopted to determine the spectral sensitivity of mouse rods. We first assessed the general applicability of W.S. Stiles’ (1959) vertical and horizontal displacement laws by measuring t.v.i. curves with three different chromatic combinations of test and adapting stimuli. Secondly, the spectral sensitivity to the test flash was additionally derived from the dark-adapted absolute threshold to another seven test flash wavelengths. Thirdly, for an additional selection of five field wavelengths we measured the field intensity that raised the threshold of a 500 nm flash by one log unit above its dark-adapted threshold value. Two WT mice were employed in the experiments involving t.v.i. curves. Data were collected with a 500 nm flash on a 500 nm adapting field, a 470 nm flash on a 590 nm field, and a 365 nm flash on a 440 nm field. During a session, forty trials organized in eight blocks were presented; each block contained five trials of a fixed intensity. The adapting light remained on between trials until the animal completed the experiment. At least two frequency of seeing data sets were collected per adapting field intensity level, usually, over the course of two or three nights. The mice ran about the same distance daily in the light as they did in the dark. The dark-adapted sensitivity was probed once every 3 - 4 days.

The results are shown in Figure 11. The intensity of the test flash, ΔIλ, is given in photons μm⁻² at the cornea and that of the adapting field, Iμ, in photons μm⁻² s⁻¹ at the cornea. The 500 nm field intensities ranged from 8.0 x 10⁻⁴ – 17 photons μm⁻² s⁻¹; those of the 590 nm field ranged from 7.0 x 10⁻³ – 29 photons μm⁻² s⁻¹. The filled symbols represent data obtained with the 500 nm test/500 nm adapting field combination; the open symbols
Figure 11. Threshold versus intensity (t.v.i) curves obtained with a 500nm test flash on a 500nm background and a 470nm test flash on a 590nm background. The solid line, function $F$, was fitted to the 500 nm test/500 nm background data; it had a slope of 1. The curve fitted to the 470 nm test/590 nm background had a similar shape but was vertically and horizontally displaced (see text). Two animals participated in these experiments; their data are represented by the open and closed symbols respectively.
I, background intensity (photons \( \mu m^{-2} \) at the cornea)

\[ \Delta I_\lambda, \text{ test flash threshold (photons } \mu m^{-2} \text{ at cornea) } \]

\[ \lambda = 500 \text{ nm} \]
\[ \mu = 500 \text{ nm} \]

\[ \lambda = 470 \text{ nm} \]
\[ \mu = 590 \text{ nm} \]

Figure 11

\[ I_\mu, \text{ background intensity (photons } \mu m^{-2} s^{-1} \text{ at the cornea) } \]
represent the 470 nm test/590 nm adapting field combination. (Data points collected on the highest field intensities are not shown). The solid curves fitted to the data sets are the graphic representation of Eq. 2; both had slopes, \( s \), of about 0.88. Extrapolation of the horizontal asymptote of the two curves to the ordinate yielded threshold values for the 500 nm and 470 nm flash of \( 4.7 \times 10^{-5} \) and \( 6.9 \times 10^{-5} \) photons \( \mu m^{-2} \) at the cornea, respectively. The threshold ratio of the extrapolated test flash intensities, \( \Delta I_{470}/\Delta I_{500} \), was: 1.47.

According to Stiles’ displacement law (1959), the extent of vertical displacement between the two \( t.v.i. \) curves, expressed as a ratio, corresponds with the difference in spectral sensitivity of the mediating mechanism to the 500 nm and 470 nm test flashes. The two curves are also horizontally displaced. Following Stiles, we arbitrarily adopted as points of comparison, the adapting field intensities that raised the dark-adapted test flash intensity by one log unit above its dark adapted threshold value; the adapting field intensities are indicated at the intersection of the dotted vertical lines and the abscissa. The threshold ratio of the 590 nm to 500 nm adapting field, \( I_{590}/I_{500} \), was, \( 6.25/0.44 = 14.29 \). The horizontal displacement between the two fitted curves, expressed as a ratio, reflects the difference in sensitivity of the mediating mechanism to the two wavelength backgrounds. The \( t.v.i. \) curve measured with a 365 nm test flash on a 440 nm background (not shown) was also compared to the 500 nm test/500 nm adapting field data.

The 500 nm data sets of Fig. 11 are re-plotted in the central coordinates of Fig. 12. The horizontal asymptote was extrapolated to the lower-left coordinates where the test flash threshold ratios are plotted as a function of the test flash wavelength. The threshold value of the 500 nm test flash was set to 1. The data points at 365 nm, 500 nm and 470 nm
represented by filled symbols had been obtained by extrapolation of the horizontal asymptote of \( t.v.i \) curves to the ordinate. The other data points represented by open symbols are dark-adapted absolute threshold intensities for different wavelength-stimuli (394 nm, 436 nm, 532 nm, 550 nm, 579 nm and 590 nm) expressed relative to the dark-adapted 500 nm test threshold intensity. The solid curve fitted to all data points is the graphical form of Eq. 3 with the peak sensitivity set to unity at 500 nm (see also Lyubarsky et al., 1999).

Toward the short wavelength end, the photopigment template does not extend beyond 460 nm. Below 460 nm, a fifth order polynomial was fitted to the data points by least-square regression. The ratio of the 470 nm (from Fig 11) to 500 nm and 365 nm to 500 nm threshold intensities are plotted as a function of test flash wavelength with both the test flash threshold intensity and adapting field intensity that raised the threshold by one log unit set to unity, to reflect the maximum sensitivity of the mediating mechanism.

In the lower-right coordinates of Fig 12, we plot the horizontal displacement of the 590 nm (from Fig 11) and 440 nm field relative to the 500 nm field intensity that raised the dark-adapted test threshold by one log unit as a function of adapting field wavelength. The filled symbols represent data obtained from full \( t.v.i \) curves. The open symbol data were collected by adjusting the intensity of different wavelength backgrounds (410 nm, 470 nm, 500 nm, 530 nm, and 580 nm) until the mouse generated a frequency of seeing curve with the 50% threshold intensity raised about one log unit above the dark adapted test flash threshold. The solid line is the mouse rod action spectrum with the peak at 500 nm.
Figure 12. Spectral sensitivity of mouse vision. The filled symbols plotted in the main coordinates were taken from Fig 9. (500 nm test / 500 nm background). The horizontal template of the function $F$ was extrapolated to the coordinates on the left and set to be one on the ordinate. **Left** coordinates (*lower part; action spectrum of test*): dark-adapted threshold intensities of various wavelength flashes (normalized relative to the threshold intensity at 500nm) are plotted as a function of wavelength. **Lower right** coordinates (*action spectrum of background*): intensities of various wavelength backgrounds that raised the flash threshold by one log unit (normalized relative to that of a 500nm background stimulus) are plotted as a function of background wavelength (see Fig 9.). The grey line fitted to the test and background spectral data is a template of mouse rhodopsin *in situ* ($\lambda_{\text{max}} = 498$ nm). The monochromatic background stimuli were not bright enough to isolate cones in the WT mice. Cone thresholds were measured on *Gnat-1*−/− mice in darkness (**left** coordinates, **upper** part). The grey lines represent the action spectra for UV-cones ($\lambda_{\text{max}} = 360$nm) and M-cones ($\lambda_{\text{max}} = 508$ nm), respectively.
Figure 12

$I$, background intensity
(photons $\mu m^{-2}$ s$^{-1}$ at cornea)

$\lambda$, test flash wavelength (nm)

$\mu$, background wavelength (nm)

normalized test flash threshold intensity (rel. to 500 nm threshold)

normalized backgr. int. (rel. to 500 nm backgr. raising threshold by 1 log unit)

$\Delta I$, test flash threshold intensity (photons $\mu m^{-2}$ at cornea)
Spectral sensitivities of the Gnat-1⁻/⁻ mouse

Because the intensities of the monochromatic adapting fields were not high enough to isolate cones in WT mice, we measured the dark-adapted cone absolute threshold of Gnat-1⁻/⁻ mice (n = 3) to various chromatic test flashes (upper-left coordinates). At least two frequency of seeing data sets were collected per test flash wavelength. The threshold intensity was lowest with a 365 nm flash. Previous research has shown that the peak sensitivity, \( \lambda_{\text{max}} \) of the mouse UV-cone pigment, is at 360 nm; the absorbance of the pigment at 365 nm is 0.985. Therefore, we first normalized the threshold intensities of the chromatic test flashes relative to that of the 365 nm test flash which was set to unity; then all relative threshold intensities, including that at 365 nm, were multiplied by \( 1/0.985 = 1.015 \). With \( \lambda_{\text{max}} \) set for 360 nm in Eq. 3, the template failed to adequately describe the spectral data above 394 nm. To fit the pigment template at 510 nm, \( \lambda_{\text{max}} \) of the midwavelength sensitive pigment, the test flash intensities obtained with wavelengths > 400 nm were normalized relative to that of the 510 nm stimulus which was set to 1. The spectral data are shown in the upper-left coordinates of Fig. 12. The threshold (1/sensitivity) of the UV-cones at 365 nm was set 3000 times higher than that of the rods; the cone threshold at 510 nm exceeded that of the rods by a factor of about 12,000. The solid lines fitted to the threshold data represent the photopigment templates for UV cones (\( \lambda_{\text{max}} = 360 \text{ nm} \)) and a mid-wavelength sensitive pigments (\( \lambda_{\text{max}} = 510 \text{ nm} \)), respectively. The points of intersection between the dashed horizontal lines and the hyperbolic curve indicate the lowest adapting field intensity at which the UV- and M-cone threshold responses of the WT mouse may be isolated.
Isolation of cone threshold responses in WT mice

To characterize the rod threshold response at high adapting intensities and determine the cone absolute sensitivity in the WT mouse, increment threshold responses were measured with a “white” broad-band adapting field and test flashes that were 500 nm, 532 nm or 365 nm in wavelength. The intensity of the “white” background ranged from $1 \times 10^{-5} - 7.0 \times 10$ cd m$^2$, corresponding to $4.5 \times 10^{-3} - 3.4 \times 10^4$ R* s$^{-1}$ (Lyubarsky, et al., 2004). ‘Frequency of seeing’ data sets were generated at the background intensity levels listed in the figure legend; only one intensity level was presented per session. Four WT mice were used in these experiments; the results for two animals represented by circles and triangles, respectively, are shown in Fig 13. The 500 nm data are represented by the filled circles and filled triangles “with crosshairs”, the 532 nm data by the open circles and the 365 nm data by the gray triangles. The test flash and adapting field intensities were expressed in terms of their effectiveness on the rods, specifically, in R* and R* s$^{-1}$. The purpose of this transformation was to reveal that threshold intensities for test flashes of different wavelength measured at any given background intensity are about equal provided the flashes are detected by rods. Thus, as the background intensity is increased all rod-detected test flash thresholds will rise along the solid line (slope = 0.88). Deviations from the line indicate either that the test flash is not mediated by rods or that the rods are saturating (Aguilar & Stiles, 1954). The filled circles are the 500 nm test flash/500 nm adapting field data from Fig. 11. This data set was used here to make calibration corrections (< 0.2 log units) to the 532 nm data (open circles) collected in the presence of a white adapting field that stemmed from the tungsten-halogen light sources. The latter sources proved difficult to calibrate at very low light intensities. The triangles
Figure 13. Increment threshold data obtained on WT mice with broad-band background stimuli and narrow-band test flashes, both expressed in terms of their effects on rods. The test flash wavelength was 500 (filled circles) nm or 532 nm (open squares) or 365 nm (grey triangles). The background was either 500 nm in wavelength or “white”. Test flash intensities were normalized relative to their respective dark thresholds and plotted as a function of background intensity. The solid line fitted to the rod-mediated data represents the hyperbolic function: $\frac{\Delta I}{\Delta I_{\text{dark}}} = \frac{I + I_{0.5}}{I_{0.5}}$ where $\Delta I$ is the increment threshold, $\Delta I_{\text{dark}}$ is the absolute threshold; $I$ is the background intensity and $I_{0.5}$ the background intensity that raises $\Delta I_{\text{dark}}$ by a factor of 2. Deviation of the grey symbol and open symbol data from the scotopically (i.e. rod-) matched data at higher background intensities indicates that vision is mediated by cones. $I_{0.5}$ was 0.005 R* s$^{-1}$. The short-dash lines represent the hyperbolic equation above with $I_{0.5}$ values of 35 R* s$^{-1}$ and 250 R* s$^{-1}$ for UV-cones and M-cones, respectively.
Figure 13
“with cross hairs” were obtained with a 500 nm test flash on a “white” field stemming from the 150W-Xenon arc source.

The data collected with the 500 nm, 532 nm and 365 nm test flashes show the following: (i) The dark-adapted test flash thresholds were equal irrespective of wavelength. (ii) The dark-adapted sensitivity of the rods was reduced by 50%, in the presence of an adapting field that produced about 0.007 R* s\(^{-1}\). (iii) At a background intensity of about 20-50 R* s\(^{-1}\), the threshold response to the 365 nm flash (gray triangles) began to deviate from the 500 nm and 532 nm data points suggesting that the 365 nm flash was presumably detected by UV-cones. The influence of the “white” adapting field on the threshold of the UV-cones was not further examined. (iv) At 200-300 R* s\(^{-1}\), cones began to detect the 500 nm (filled triangles “with crosshairs”) and 532 nm flashes (open circles). The threshold remained unchanged over a range of about 0.6 log units, then rose again at the adapting intensity of 1000 R* s\(^{-1}\). The curve positioned at the putative M-cone threshold response to the 532 nm data represents Eq. 2; \(I_{0.5}\) was 1000 R* s\(^{-1}\) and \(n = 0.88\). The UV-cone and M-cone threshold responses of the WT mouse were elicited with about the same corneal flash intensities as those used for \textit{Gnat-1\(^{-/-}\)} mice.

The deviation of the M-cone data points from the solid curve was surprising. To better understand the contributions of M-cones and UV-cones to the threshold response of the WT mouse at higher adapting levels, the increment threshold responses of \textit{Gnat-1\(^{-/-}\)} (\(n = 2\)) and \textit{Gnat-2\(^{-/-}\)} mice (\(n = 3\)) were further examined. \textit{Gnat-1\(^{-/-}\)} mice were presented with a
510 nm test flash on a white field. Even though \textit{Gnat-1}\(^{-}\) mice lack rods, for the purpose of comparison with WT and \textit{Gnat-2}\(^{-}\) mice, the flash intensity was expressed in R* s\(^{-1}\). Figure 14 shows the increment threshold response curve for two \textit{Gnat-1}\(^{-}\) mice. The solid line represents Eq 2; \(I_{0.5}\) was 280 R* s\(^{-1}\) and the slope 1.7. Thus, the M-cones or UV-cones that co-express mid-wavelength sensitive pigment desensitize strongly in the presence of background light. Due to test flash intensity limitations, we could not determine the fate of the M-cone response at very high adapting intensities.

\textit{Gnat-2}\(^{-}\) mice were studied with 365 nm and 500 nm test flashes, respectively, on “white” fields. The test flash intensity was expressed in R* and the field intensity in R* s\(^{-1}\). Figure 15 shows the rod increment threshold response of two \textit{Gnat-2}\(^{-}\) mice measured over a 7.6 log unit adapting range. The scotopically matched 365 nm and 500 nm data points rose with the same slope (n = 0.88) as the adapting field intensity was increased. Adherence of both data sets to the “Weber line” at adapting levels where UV-cones (20-50 R* s\(^{-1}\)) and M-cones (200 – 300 R* s\(^{-1}\)), respectively, may be expected to be active is consistent with the claim that the cones of \textit{Gnat-2}\(^{-}\) mice are inactive. The open triangle data were obtained on a second \textit{Gnat-2}\(^{-}\) mouse. The highest adapting field intensity used produced 33,000 R* s\(^{-1}\) and no sign of psychophysical saturation was observed. Due to difficulty with the calibration of the 100-Watt lamps, the open circle data obtained on a third \textit{Gnat-2}\(^{-}\) mouse deviate at lower background intensities from the solid line. The rods are practically at all adapting intensities more sensitive than the M-cones, except within a narrow range of field intensities between 200 – 800 R* s\(^{-1}\). Thus, under ambient light containing wavelengths > 410 nm, mouse vision will be mediated, more likely, by the rods than by M-cones.
Figure 14. The increment threshold response of \textit{Gnat-1\textsuperscript{-/-}} mice (n= 2). The test flash $\lambda = 510$nm was presented on a white background. The x-axis is expressed in R*s\textsuperscript{-1} for the purpose of comparison with WT and \textit{Gnat-2\textsuperscript{-/-}} mice. The solid line represents Eq 2; $I_{0.5}$ was 280 R* s\textsuperscript{-1} and the slope 1.5. The dashed line represents the Weber curve with a slope of $s = 0.88$. 
Figure 14

$\Delta I$, test flash threshold (photons $\mu m^{-2}$ at cornea) vs. $I$, background intensity ($R^* s^{-1}$).
Figure 15. Increment threshold curve of a single Gnat-2−/− mouse. The filled circle data were obtained with a 500 nm test flash on a 500 nm background. The grey square and white circle data were obtained with a 365 nm and 500 nm test flash, respectively, on a white background. The open triangle data were collected with a 500 nm test on very bright backgrounds. The slope of the curve was 0.88. The dashed line positioned at about 1000 R* s⁻¹ indicates the onset of human rod saturation. On the right ordinate, the Weber-Fechner fraction, ΔI/I, is indicated and plotted as a function of the background intensity. The symbols used in the ΔI/I vs I plot correspond with the symbols in the increment threshold plot. For clarity, the open circle data have been omitted from the ΔI/I vs I plot.
Figure 15

Gnat-2\(^{-/-}\)

$\Delta I$, test flash threshold ($R^*$)

$\Delta I/I$, Weber-Fechner fraction

$I$, background intensity ($R^* \text{s}^{-1}$)

Scale
Table III: Parameters of mouse rod- and cone-mediated vision

<table>
<thead>
<tr>
<th>Mice</th>
<th>a Abs. threshold at $\lambda = 500$ nm [1 R* (# rods)$^{-1}$]</th>
<th>b $I_{0.5}$ (at 500nm) (R* rod$^{-1}$ s$^{-1}$)</th>
<th>Cone threshold at $\lambda = 365$ nm (photons $\mu m^2$)</th>
<th>Cone threshold at $\lambda = 510$ nm (photons $\mu m^2$)</th>
<th>Minimum Weber-Fechner fraction for rods</th>
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</thead>
<tbody>
<tr>
<td>C57Bl/6</td>
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<td></td>
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<tr>
<td>M #1</td>
<td>630</td>
<td>0.007</td>
<td>$^{c}$ 0.25</td>
<td>$^{d}$ 0.90</td>
<td>0.09</td>
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<tr>
<td>M #2</td>
<td>1100</td>
<td>0.005</td>
<td>$^{c}$ 0.15</td>
<td>$^{d}$ 0.70</td>
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<tr>
<td>M #3</td>
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<td>0.004</td>
<td>$^{c}$ 0.20</td>
<td>$^{d}$ 0.50</td>
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<tr>
<td>M #4</td>
<td>840</td>
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<td>$^{c}$ 0.12</td>
<td>$^{d}$ 0.62</td>
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<tr>
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<td>660</td>
<td>0.007</td>
<td>--</td>
<td>--</td>
<td>0.04</td>
</tr>
</tbody>
</table>

$^{a}$ Target size: 5.3 deg; duration: 8 $\mu$s - 100 $\mu$s.  

$^{b}$ $I_{0.5}$, background intensity that raised threshold by a factor of 2. 

$^{c}$ Background intensity to isolate UV-cones: 20 - 50 R* rod$^{-1}$ s$^{-1}$.  

$^{d}$ Background intensity to isolate M-cones: $\sim$ 200 R* rod$^{-1}$ s$^{-1}$.  

The right ordinate of Fig 15 is labeled "ΔI/I, Weber-Fechner fraction". The connected data points represent the plot of ΔI/I versus I. For adapting intensities between 0.25 – 1400 R* s\(^{-1}\), the average value of ΔI/I, the Weber fraction, was 0.08 ± 0.02. All measurements were made with the pupil in its natural state.

**DISCUSSION**

A novel method for measurement of the visual sensitivity of mice was introduced. The method takes advantage of the vigorous spontaneous activity of a wheel-running mouse by collecting electronic signals from the wheel to activate computer software. Mice are trained to associate the presentation of a light flash that is triggered by the wheel running, with the availability of water. As the mouse runs, the tail is arched upward; the head is lifted up, and nearly horizontally aligned with the stretched back. The elevated position of the head affords 'Lambertian diffused' light stimuli, centered above the wheel, to be within the visual field of the running mouse. From the standpoint of visual psychophysics, the most significant feature of this novel method is the capacity to deliver stimuli that are local on the retina.

*Absolute visual sensitivity: a single-photon event in mice.*

The dark-adapted rod system of mice is remarkably sensitive. Mice responded reliably to a small brief test flashes that produced at threshold about 13 photoisomerizations. This threshold value is very close to that reported for human observers by Hecht, Schlaer and Pirenne (1942). Those authors used in their classic study a 10 min, 1 ms 500 nm flash that imaged 400 – 500 rods and it was estimated that between 5 and 14 photoisomerizations
were required for threshold. Hecht et al. (1942) reasoned that the 5 or more
photoisomerizations could have occurred in any of the rods imaged by the target, however,
the chance that two or more photoisomerizations would occur in any one rod was thought to
be small. They calculated the probability that, say, two of ten photoisomerizations had
occurred in a single rod using the equation (Brindley, 1971):

$$1 - [e^{-s} (1 + s/r)^r],$$

where $s$ is the number of photoisomerizations and $r$ is the number of rods imaged by the
flash. The probability was 0.12, which was substantially less than 0.6, the criterion for
threshold. Hecht and his colleagues came to the important conclusion that at absolute
threshold each photoisomerization occurred in a separate rod; they concluded further that a
single photoisomerization by itself is sufficient to produce a rod signal. It is now a well-
documented fact, demonstrated by suction electrode technique that following the
isomerization of a rhodopsin molecule by a photon a light-related response is generated in
the rod. The photon-activated signal causes a 3% reduction in the magnitude of the rod’s
circulating current (Baylor et al.). Based on data obtained with the 1.2 deg stimulus, our
calculations for the mouse (with $s = 12, r = 450$) showed the probability that two of twelve
photoisomerizations had occurred in a single rod to be 0.14 which was substantially less
than the 0.5 criteria we had adopted for seeing. We conclude that single-photon events can
be indirectly documented in the alert mouse, as it was done for humans more than 65 yours
ago. Our method allows for the behavioral study of mice that are valuable preparations in
the important field of study that is concerned with mechanisms that underlie single-photon
events in rods. $Grk^{1-}$ and $Arr^{-}$ mice that lack rhodopsin kinase and arrestin, respectively,
would be interesting candidates for behavioral investigation.
It has long been understood that there must be more than one rod signal at absolute threshold given the substantial amount of noise present in the retina (Barlow, 1957). A mouse apparently needs at least 12 isomerizations to inform the brain that there is a light stimulus in the outside world. But even for these twelve signals, there must be mechanisms at work in the visual system dedicated to their survival, given the continuous noise in the system. It is generally agreed upon that the principal sources of retinal noise are: photon noise from the light stimulus; random transduction events in the photoreceptors and neural noise due to spontaneous activity in the visual pathway (see Van Rossum & Smith, 1998). It has been proposed that these natural sources of noise act independently but nevertheless combine to impose an intrinsic limit on the human visual sensitivity. The overall value of this noise or “intrinsic light” can be derived by estimating what is known as ‘dark light’, a quantity defined as the background light intensity that is required to raise the absolute threshold by a factor of 2. In the coordinates of the increment threshold curve, it is the background intensity that corresponds to the intersection of the extrapolated “Weber line” and extrapolated horizontal asymptote (Barlow 1957; Rushton, 1965). In the experiments of this study, this quantity is \( I_{0.5} \) in Equation 2. In humans, the intensity of “dark light” is estimated to be equivalent to one photoisomerization occurring once every 200 seconds per rod. In some human retinal diseases (Sieving et al., 1995) that result from mutations in the rhodopsin gene, the amount of dark light is excessive. Our measurements on WT mice showed that the “dark light” is equivalent to the occurrence of one isomerization once every 142 seconds per rod. Our study is the first psychophysical report about “dark light” in mice.
or any non-human mammalian species. Psychophysical tests provide the most sensitive measure of this parameter of rod function.

*Behavioral studies on mice may help us exclude the brain as a site where visual sensitivity is regulated.*

There is another reason why the measurement of dark light is important. It could tell us whether visual sensitivity is entirely regulated in the retina. Early studies of extra-cellular recordings from cat ganglion cells (Barlow, 1971) have indicated that the spontaneous rate of discharge coincide with the spontaneous rate of (thermal) isomerization in cat rods and with psychophysically measured “dark light” in human. Based on these findings, for many years now, ganglion cell firing has been considered to be a correlate of psychophysical sensitivity, such that there would be no difference in sensitivity between the two sites in the visual system, *i.e.* the retina and brain. Rieke *et al.* (2006) reported, based on recordings of mouse ganglion cell currents, an $I_{0.5}$ value of about 0.28 R* s$^{-1}$ differing by about 1.6 log units from the $I_{0.5}$ value reported in this study. Without solid benchmarks for the threshold sensitivities in the field of mouse retinal physiology and psychophysics, it will be impossible to successfully test the important hypothesis that visual sensitivity is entirely regulated in the retina. If visual sensitivity were entirely regulated in the retina, the question then becomes: where in the retina is sensitivity regulated? In the photoreceptors or in the retinal network? Studies by Dowling (1985) and others that recorded from single photoreceptors and other retinal neurons have suggested that the sensitivity to dim background light is regulated mostly in the retinal network and that the sensitivity to moderate and bright lights is regulated in the photoreceptors. Yau (1989) has also suggested that dim light visual
sensitivity is controlled in the network. However, Lamb (1981) and others proposed that the photoreceptors control the entire adaptive process. The idea has been advanced that the terminal of the rod photoreceptor and the synapse with the rod bipolar cell form the command center for control of the retinal sensitivity to dim light. Families of small molecules, at this time unidentified, of which there are many in the photoreceptor terminal, could be responsible for the sensitivity to dim ambient light. It is precisely under these circumstances that the alert WT mice, given their unusually high sensitivity, and mutant mice that lack one or more families of terminal molecules, could show their strength. Electrical recordings and biochemical studies may predict how visual sensitivity should change due to an engineered mutation. In our setup the change in visual sensitivity can actually be measured with the brain intact.

Weber adaptation in mice.

Over a narrow range of light adapting intensities, about 0.7 log units above the $I_{0.5}$ value, the threshold rises with a slope of 0.5. The relationship between the test flash threshold and this range of the background intensity is called the deVries-Rose or square root relationship. The rise of the threshold is thought to be due to photon noise. Thus, at or below $I_{0.5}$, the noise originates from biological sources as mentioned earlier. Just above $I_{0.5}$ the photons in the background light become more numerous in the eye as the background intensity increases and as such constitute a new source of noise called “quantum fluctuations”. The threshold is said now to be elevated by quantum fluctuations (see for review, Sharpe, 1990). As the background intensity increases and the test flash threshold is raised to almost one log unit above the dark-adapted threshold, the background light begins
to affect the state of adaptation of the rod photoreceptors. This is considered the beginning of true photoreceptor adaptation that is also known as “Weber adaptation”. At the physiological level, steady background light causes the closure of sodium channels in the outer segment of rod photoreceptors thereby reducing the number of open channels available to respond to a light flash. Further increases of the background intensity (and photon absorption) close more channels and if there were no particular adaptive mechanism operating, the rod would eventually be incapable of responding to a flash. Apparently, by speeding up its response, i.e. decreasing its integration time, the rod can avoid complete response compression and maintain its ability to respond at higher adapting intensities. That is the essence of light adaptation. Rods are equipped with a calcium feedback mechanism that allows for the adaptation to bright adapting intensities (Nakatani & Yau, 1988). When the background light becomes very bright, the rods finally succumb and saturate. For the mouse, presented with a 5.3 deg stimulus that imaged 8300 rods, the value of the slope of the Weber curve was 0.88; the Weber-Fechner fraction (in the Weber region) was 0.08. For Aguilar and Stiles’ (1954) subjects, using a 6 deg target that imaged 400,000 rods, the Weber slope was 0.9; the Weber-Fechner fraction was 0.2 in the Weber region. For the human rod achromat Kurt Norby, a well-trained psychophysical observer, the Weber slope was found to be 0.67 using a 100 ms, 1 deg target that imaged about 10,000 rods. Thus, in the Weber region the visual performance of mouse and human are remarkably similar.
The $M$-cone threshold responses of light-adapted $Gnat-1^{-/-}$ mice and the rod responses of $Gnat-2^{-/-}$ mice at higher adapting intensities are unexpectedly different.

Psychophysical studies show that in humans, long before the rods saturate, cones take over vision (Aguilar & Stiles, 1954). In the present study, our findings indicate that at an adapting intensity of about 50 $R^* \text{s}^{-1}$ the UV-cones instead of the rods, began to detect near-UV test flashes. We were not equipped to study the increment threshold response of the UV-cones psychophysically. But electrophysiological studies (Nikonov et al., 2006) using suction electrode techniques have shown that S-cones (UV-cones) which are most prevalent in the ventral retina, responded normally when stimulated with either near UV or mid-wavelength light. The argument was made that the UV- and mid-wavelength sensitive pigments within an individual cone cell must indeed function independent of each other. Based on the results obtained from $Gnat-1^{-/-}$ mice with very small stimuli delivered to the dark-adapted ventral retina, we have suggested earlier that responses to the mid-wavelength test flashes were likely mediated by UV-cones. This suggestion is consistent with the findings of Nikonov et al. (2006). “M-cones” of WT mice began to mediate vision on a background producing 300 $R^* \text{s}^{-1}$. As the background intensity was increased the cone threshold rose in a peculiar manner. The visual response seemed normal up to about 800 $R^* \text{s}^{-1}$ in the sense that the M-cones did not show the expected Weber adaptation at higher intensity levels. Instead, the threshold response rose with a slope much greater than 0.88. The likely contributors to this peculiar threshold response, rods and/or M-cones, were separately investigated in mutant mice, $Gnat-1^{-/-}$ and $Gnat-2^{-/-}$ mice. In both types of mutants unexpected results were obtained. In the $Gnat-1^{-/-}$ mouse, the increment threshold response rose with a slope of 1.7, again instead of the expected slope of 0.88, indicating a
profound desensitization of the M-cones. In the Gnat-2\(^{-/-}\) mouse, the rods showed Weber-like light adaptation to a background intensity that produced 33,000 R* s\(^{-1}\). Threshold responses measured in WT mice on a background of this intensity suggest that the threshold was mediated by rods. The data points obtained on the two brightest backgrounds in Figure 13 coincided with the extrapolated rod Weber line (not shown) and with corresponding data points from the Gnat-2\(^{-/-}\) mouse in Figure 15.

*Psychophysical and electrophysiological saturation of the rods compared.*

Aguilar and Stiles (1954) were the first to observe the profound loss of sensitivity of the rods in the presence of bright adapting lights. They defined the psychophysical saturation of the rods as the background intensity that caused the minimum Weber-Fechner ratio recorded in the Weber region, to increase by a factor of 100. In their observers, the rods saturated at an intensity of about 32,500 R* s\(^{-1}\). The onset of saturation is also an acceptable measure of saturation: it is the intensity of the background that doubles the minimum Weber-Fechner ratio. In normal human observers used by Aguilar & Stiles, saturation onset occurs at about 2300 R* s\(^{-1}\); in the rod monochromat KN (Sharpe, 1990) it takes about 3000 R* s\(^{-1}\) to induce the beginning of saturation. The manifestation of rod saturation in humans has led to fundamental conclusions about how rods function. Here, we shall briefly list some of the core conclusions. First, saturation is specific to rods. Cones and the cone pathways do not show a similar loss of sensitivity. The saturation mechanism must be intrinsic to the rod. Cones have little or nothing to do with the saturation process in the rods. Second, saturation is not due to pigment bleaching, that is, pigment depletion. The idea here is that the reduction in the rhodopsin concentration due to the bright adapting
lights would lead to the absorption of a smaller number of quanta. The reduced stimulation of the rod would then provide the receptor the opportunity escape saturation. It is well established that less than 1% of the rhodopsin is usually bleached when the rods saturate. Third, rod monochromats who do not possess cones show rod saturation. Rods seem to suppress themselves. Using current-source density analysis at the outer segments of rat rods, Penn and Hagins (1971) were the first to show physiological saturation in the rods and suggested it to be the correlate of psychophysical rod saturation seen in humans. Sharpe (1990) has compared the saturating responses recorded from single macaque rod photoreceptors with the psychophysical human rod saturating response. He points out that the maximal dark current at the rod outer segment is reduced to half by a background that produces 100 R* s⁻¹. [For the rat this value is 160 R* s⁻¹; see Nakatani, Tamura & Yau, (1990)]. According to Sharpe (1990) this measure is comparable to the effect of a background that causes the onset of saturation; more specifically, the intensity of a background that raises the minimum Weber-Fechner fraction by a factor of 2. In normal observers, such a background [507nm] produces about 2300 R* s⁻¹. To suppress the circulating current of the macaque rod completely requires the use of a background that produces about 700 R* s⁻¹. [To suppress the rat rod circulating requires a background that produces 4000 R* s⁻¹ according to Nakatani et al. (1990)]. The rods of psychophysical observers are completely saturated in the presence of a background producing 35,000 R* s⁻¹. It is true that the comparison between single cell recordings and human psychophysical performance may be flawed. Indeed, the calculations for the rate of photoisomerizations for a single rod and a human observer could be too imprecise: the stimuli are different; the units of measurement are different; one preparation is measured in an artificial medium, the other
in a biologically intact environment. Nevertheless, to the extent that some comparison is legitimate that the rods of the mouse appear to behave very much like those of humans; it should be added: except at higher adapting intensities.

Do rodent rods escape saturation at higher adapting intensities?

A recent study of rod light adaptation could provide a plausible explanation for the observation that Gnat-2/ mice do not show any sign of rod saturation. Indeed, Sokolov et al. (2002) have reported a new cellular mechanism of rod photoreceptor adaptation in vivo, which is triggered by day light levels of illumination. The mechanism involves a light-dependent translocation of the photoreceptor-specific G protein, transducin, between functional compartments of rods. Up to 90% of transducin translocates from rod outer segments to other cellular compartments on the time scale of tens of minutes. The reduction in the transducin content of the rod outer segments is accompanied by a corresponding reduction in the amplification of the rod photoresponse, allowing rods to operate in illumination up to 10-fold higher than would otherwise be possible. The amplification constant of the ERG a-wave was the measure used to provide evidence that in bright lights rod photoreceptors decrease the signal amplification in order to avoid saturation. Gnat-2/ mice that show no rod saturation can be expected to exhibit reduced signal amplification. Are Gnat-2/ rods different from WT rods? We think not. As remarked earlier, WT rods seem to mediate vision at the highest adapting intensities used in this study. Even though they do not show saturation, it cannot be concluded from the experiments in Fig 14 that these rods would not eventually saturate at even higher adapting adapting intensities. Cones are known not to saturate.
Some concluding remarks.

When we started this thesis project, the methodological difficulties associated with the psychophysical measurement of mouse visual sensitivity were considerable. Besides the delivery of small, brief stimuli to moving mice, the difficulties included the following. (i) The selection of suitable biological responses that facilitate the measurement of visual responses; (ii) Sustainment of the animal’s motivation to respond to the light stimuli; (iii) Control over non-visual behaviors that unpredictably (spontaneously) interfere with the visual response; (iv) subjective human influences on experimental outcomes as the mouse’s performance is being guided and/or judged. We succeeded to introduce a non-invasive behavioral method that yields first-ever visual sensitivity measurements while the mouse performs a natural behavior, running. We have shown that with this method mice can be thoroughly examined under experimental conditions that do not require food/water deprivation or induction of stress/pain. The method takes into consideration the animal’s nocturnal lifestyle and makes the biological motivation to respond to light stimuli integral to the natural daily routine. Lastly, the method allows for the measurement of visual performance using efficient mouse-computer interactions that strongly reduce the influence of human subjectivity. Throughout the thesis we demonstrated that the output behavior of a mouse in response to stimulus intensity variations, under a wide range of conditions, adheres closely to a steep-sloped sigmoidal function.

What are the weaknesses of the method? The most obvious weakness is that only one mouse can be tested at one time in a single box. For those researchers who need to
test the vision of large numbers of animals quickly this method may prove to be unpractical. Also, each box is a stand-alone unit and requires its own space and that could prove to be troublesome at some institutions. Ultimately, every user will have to make his or her own decisions how to best use the instrument for their purposes. We believe that the instrumentation will make it possible to examine visual function reliably and efficiently in important lines of mutant mice, in mice that have been produced as models of eye disease and mice that have been treated therapeutically. Importantly, the mice need not be sacrificed, so that the same mouse may be investigated repeatedly over days, months or its entire lifetime. A priceless advantage of the method is that the mouse does most of the work with no human assistance.
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