vations (*15*) that several strains of HeLa or presumed HeLa have at least two X chromosomes as identified by banding techniques. These may of course be replicas of one chromosome.

SUSAN H. HSU, BERNICE Z. SCHACTER
NANCY L. DELANEY, THOMAS B. MILLER
VICTOR A. MCKUSICK
Division of Medical Genetics, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205
R. H. KENNETT, J. G. BODMER
D. YOUNG, W. F. BODMER
Genetics Laboratory, Department of Biochemistry, University of Oxford, Oxford, England

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Visual Adaptation: Effects of Externally Applied Retinal on the Light-Adapted, Isolated Skate Retina

Abstract. *Incubation with externally applied 11-*cis *retinal induces a marked increase of visual sensitivity within partially bleached skate photoreceptors. This activity of 11-*cis *retinal is duplicated by 9-*cis *retinal, but not by all-*trans *retinal. The sensitization of photoreceptors promoted by 11-*cis *and 9-*cis *retinal is accompanied by the formation of rhodopsin and isorhodopsin, respectively.*

All visual pigments consist of retinal (vitamin A aldehyde) attached to a protein, opsin. The configuration of the retinal chromophore appears crucial for the function of these pigments, since visual excitation is believed to involve the isomerization of the chromophore from the 11-*cis* to the all-*trans* form (*1*). Rhodopsin, the most extensively studied visual pigment, consists of 11-*cis* retinal joined to the opsin found in vertebrate rods. When extracted into digitonin, rod opsin can also combine with 9-*cis* retinal to form isorhodopsin (*2*); however, substantial evidence indicates that isorhodopsin is virtually nonexistent in vivo under normal conditions (*1, 3*).

In both vertebrates and invertebrates, the role of the visual pigments in mediating visual excitation is well established. For example, the excitation spectra of visual systems agree quite well with the absorption spectra of the visual pigments present in the photoreceptors (*4*). In the vertebrate rod, the content of rhodopsin also appears to be closely related to the logarithm of visual sensitivity during dark adaptation. Three types of evidence support this view: (i) after strong light adaptation, the recovery of log sensitivity during the "slow," or photochemical, phase of dark adaptation proceeds in parallel with the regeneration of rhodopsin (*5–7*); (ii) if the regeneration of rhodopsin after strong light adaptation is prevented by isolation of the retina from the back of the eye, the retina does not recover its original, dark-adapted level of sensitivity (*8*); and (iii) rats deficient in vitamin A exhibit both a depressed level of rhodopsin and substantially decreased visual sensitivity (*9*). As yet, however, no one has induced the formation of rhodopsin in an isolated retina and observed an increase in visual sensitivity.

We report here that 11-*cis* retinal, externally applied to the light-adapted, isolated all-rod retina of the skate (*6, 7, 10*), promotes both the rapid formation of rhodopsin and a sharp increase in the sensitivity of the photoreceptors. In addition, we present evidence that externally applied 9-*cis* retinal leads to increased receptor sensitivity with the formation of isorhodopsin in the photoreceptors.

Eyes from dark-adapted skates (*Raja oscellata* or *R. erinacea*) were enucleated, hemisected, and drained of vitreous humor under dim red light. Rectangular pieces of eyecup measuring approximately 3 mm by 5 mm were trimmed from the tapetal region of the eye and soaked in a skate Ringer solution containing 75 m*M* sodium L-aspartate (*11*). The L-aspartate suppresses the activity of neurons in the retina proximal to the photoreceptors, thereby isolating the receptor response (*12*). For electrophysiological recording, the retina was isolated from the eyecup, mounted upon moistened filter paper with the photoreceptor layer up, and positioned in a shielded cage under a gentle stream of moist oxygen. All adapting exposures (bleaches) and test flashes were carried out with light spectrally shaped by passage through a Kodak Wratten 58 (green) filter and through Schott-Jena KG-1 and KG-3 heat filters. The light was attenuated by neutral density filters and focused to give full-field illumination of the retina from above. The photoreceptor potential was recorded extracellularly across the retina (*7*). The recording electrode, placed at the surface of the receptor layer, was a glass pipette containing a wick of glass fibers and filled with aspartate Ringer solution; the reference electrode, a grounded loop of chlorided silver wire, was positioned beneath the preparation. Responses elicited by 0.2-second test flashes were amplified (bandpass of 0.1 to 1000 hertz), displayed on an oscilloscope, and recorded on a pen oscillograph. A photoreceptor response of amplitude 3 μv was used as a criterion to determine threshold intensities of the test flash; photoreceptor sensitivity was defined as the reciprocal of the measured threshold. Spectrophotometry was performed with an apparatus previously described (*13*); for measurements of transmissivity before and after the application of retinal, the isolated retina was mounted (with the photoreceptor layer up) on a piece of Vitallium mesh (Howmet Corp.), which lay in contact with moistened filter paper in a clear plastic dish.

Figure 1 describes the results of two typical electrophysiological experiments. In the first experiment (upper curve), we exposed a dark-adapted isolated retina to intense illumination which bleached more than 90 percent of the rhodopsin initially present (*14*). Threshold measurements made after the offset of the adapting light showed that the sensitivity of the photoreceptors had been reduced by the adapting illumination to a stable value, approximately 4 log units less than the dark-adapted value (*15*). Aliquots of all-*trans* retinal or 11-*cis* retinal suspended in ethanol-aspartate Ringer solution were then applied dropwise to the upper surface of the retina at the times indicated by the arrows in Fig. 1. Repeated threshold determinations showed that the sensitivity of the photoreceptors was unaffected by the

treatment with all-*trans* retinal; however, on the subsequent dropwise application of 11-*cis* retinal, there occurred a striking increase in the sensitivity of the photoreceptors. Once initiated, this increase in sensitivity proceeded rapidly over a period of about 25 minutes and appeared to continue at a lower rate thereafter. By the end of this experiment, the receptor sensitivity was approximately 2.7 log units greater than before the treatment with 11-*cis* retinal (*16*). Typically, the increase in receptor sensitivity was accompanied by a substantial recovery of amplitude of the receptor potential. In the second experiment (lower curve), another dark-adapted isolated retina was exposed to light which bleached approximately 40 percent of the rhodopsin. After the offset of the adapting light, the receptor sensitivity attained a stable value 2.1 log units less than the dark-adapted level. We then applied 9-*cis* retinal, suspended in ethanol-aspartate Ringer solution, to the upper surface of the retina in the standard fashion; within about 25 minutes, there again occurred a sharp increase in the sensitivity of the photoreceptors. This sensitization of about 1.6 log units returned the receptor threshold to within 0.5 log unit of the initial, dark-adapted value (*17*).

Figure 2 describes the spectral characteristics of light-sensitive material formed when exhaustively bleached isolated retinas were treated with 11-*cis* or 9-*cis* retinal. Application of 11-*cis* retinal led to the formation in the dark of a material that, like rhodopsin, absorbed maximally at about 500 nm and was apparently unreactive with hydroxylamine (*2, 18*). The formation of this material (Fig. 2 inset) followed closely the increase of receptor sensitivity, measured electrophysiologically. Intense illumination of the treated preparation in the presence of hydroxylamine eliminated this material, and the resulting difference spectrum (open circles) closely matched that of skate rhodopsin (filled squares). These data provide strong evidence that the material formed on treatment with 11-*cis* retinal was rhodopsin. By contrast, the difference spectrum of light-sensitive material formed in the presence of 9-*cis* retinal (filled circles) was maximal at about 489 nm, and closely resembled the spectrum of isorhodopsin (*2, 3, 19*).

In summary, our electrophysiological experiments have shown that 11-*cis* retinal, applied externally to partially bleached photoreceptors of the isolated skate retina, induces a large and rapid increase in the sensitivity of the receptors. The sensitizing activity depends critically on the isomeric form of retinal applied, since the all-*trans* isomer lacks the ability to promote the sensitization. To our knowledge, these findings constitute the first demonstration of an electrophysiological effect exerted by externally applied 11-*cis* retinal upon functionally active photoreceptors. Our spectrophotometric results further indicate that the application of 11-*cis* retinal to a bleached isolated skate retina causes the formation of rhodopsin in the photoreceptors simultaneously with the increase in sensitivity of the receptors. Accordingly, we suggest that externally applied 11-*cis* retinal induces within strongly light-adapted photoreceptors a process that closely resembles photochemical dark adaptation. Of particular interest are our results with 9-*cis* retinal, which suggest

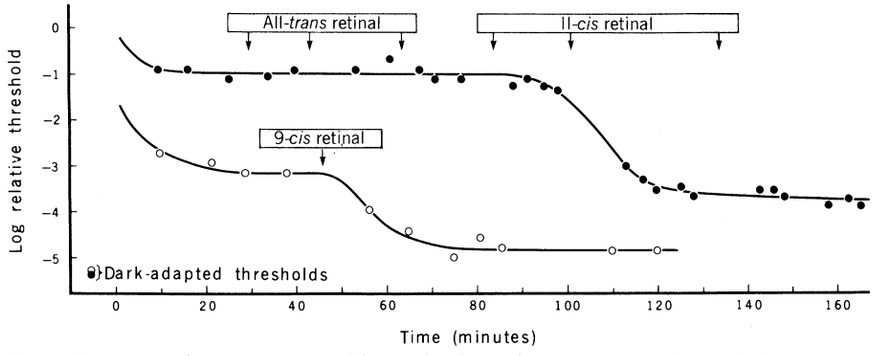

Fig. 1. Treatment of partially bleached isolated retinas with all-*trans*, 11-*cis*, and 9-*cis* retinal. In each case, the bleaching light was extinguished at time zero. In one experiment (●) all-*trans* retinal and 11-*cis* retinal were separately suspended in ethanol-aspartate Ringer solution (1 : 100 by volume) at a concentration of 1.5 μmole/ml. At the times indicated by arrows, the following volumes of these suspensions were gently delivered dropwise to the upper surface of the retina with the use of disposable 1-ml plastic syringes fitted with 25-gauge hypodermic needles: 0.12, 0.15, and 0.09 ml (sequentially) of all-*trans* retinal and 0.11, 0.15, and 0.11 ml (sequentially) of 11-*cis* retinal. In another experiment (○), 9-*cis* retinal was suspended in ethanol-aspartate Ringer solution (3 : 100 by volume) at a concentration of 3.1 μmole/ml. At the indicated time, the retina was treated with 0.09 ml of this suspension in a manner similar to that described above.

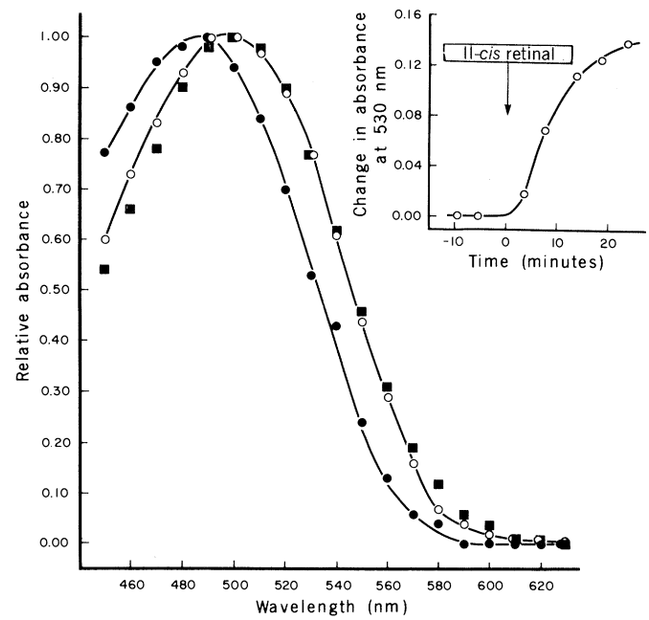

Fig. 2. Normalized difference spectra obtained in the presence of hydroxylamine. In one experiment (○), a dark-adapted isolated retina was exhaustively bleached with Wratten 58-filtered light; the 5-minute bleaching exposure eliminated at least 97 percent of the rhodopsin originally present. Eighteen minutes after the offset of the bleaching light, 11-*cis* retinal (1.7 μmole), suspended in 0.11 ml of ethanol-aspartate Ringer solution (1 : 5 by volume), was gently applied dropwise to the retina. Shown in the inset is the time course of changes in absorbance at 530 nm following the application of the 11-*cis* retinal. Approximately 26 minutes after the application of the 11-*cis* retinal, the preparation received 0.10 ml of 0.70*M* hydroxylamine (*p*H 6.0), applied dropwise to the upper surface of the retina. Beginning 12 minutes after the treatment with hydroxylamine, the retina was subjected to a second 5-minute bleaching exposure; the open circles represent normalized differences in absorbance which resulted from the second intense illumination. In a second experiment (●), a dark-adapted isolated retina was similarly treated, except that 9-*cis* retinal (1.7 μmole), suspended in 0.11 ml of ethanol-aspartate Ringer solution (1 : 5 by volume), was applied to the retina after the first bleaching exposure; as above, the filled circles represent normalized differences in absorbance which resulted from a second 5-minute bleach carried out in the presence of hydroxylamine. In a third experiment (■), a dark-adapted isolated retina was treated with hydroxylamine and subsequently bleached for 5 minutes; the filled squares represent the normalized difference spectrum obtained as a result of this single exposure. The peak absolute values of absorbance for the difference spectra illustrated are (○) 0.161 (at 500 nm), (●) 0.094 (at 490 nm), and (■) 0.285 (at 500 nm).

that isorhodopsin, although not normally present in vivo in significant quantity, possesses physiological activity. Thus, the mechanism in the photoreceptor that relates sensitivity and the content of visual pigment appears to "accept" isorhodopsin, as well as rhodopsin.

DAVID R. PEPPERBERG, MARK LURIE*
PAUL K. BROWN, JOHN E. DOWLING
Biological Laboratories, Harvard University, Cambridge, Massachusetts

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15. A decrease in visual sensitivity of more than 4 log units upon bleaching about 90 percent of the rhodopsin in the skate eye is consistent with earlier studies, which have compared b-wave sensitivity with levels of rhodopsin during dark adaptation (*6*).
16. The sensitizing effect of 11-*cis* retinal did not depend on prior treatment of the partially bleached isolated retina with all-*trans* retinal, since in other experiments (not illustrated) the application of 11-*cis* retinal alone was observed to promote a substantial increase in receptor sensitivity.
17. In most experiments, complete recovery of sensitivity did not occur. Usually, as here, more exhaustively bleached or older preparations displayed a higher final threshold. In the range of bleaching that we examined (about 34 to 97 percent of the rhodopsin bleached), increases in receptor sensitivity induced by 11-*cis* or 9-*cis* retinal did not appear to depend critically on the time of application of the retinal.
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* Present address: Division of Ophthalmology, Stanford University Medical Center, Stanford, California 94305

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Habituation of Reflexes in *Aplysia*: Contribution of the Peripheral and Central Nervous Systems

Abstract. *We studied the contribution of the* Aplysia *peripheral nervous system, in the siphon and gill, to habituation of the gill withdrawal reflex. After removal of one central ganglion, the parietovisceral, repeated stimulation of the siphon caused habituation of the reflex as it had with the ganglion intact, showing that there is a peripheral pathway between the siphon and gill with competence to mediate habituation. Repeated electrical stimulation of two efferent nerves to the gill, after removal of the parietovisceral ganglion, resulted in habituation of withdrawal movements, which shows that the terminals of the ganglion neurons in the gill are a site of habituation. Also, stimulation of one nerve dishabituates the withdrawal movements elicited by the other. These results identify two sites of habituation in the gill in addition to sites in the parietovisceral ganglion.*

The peripheral nervous system in *Aplysia*, a marine gastropod, is competent to mediate habituation and dishabituation of the siphon or gill responses to direct tactile stimulation in the absence of the central nervous system (*1, 2*). In contrast, central neurons have been thought to mediate habituation of the reflex withdrawal of the gill in response to tactile stimulation of the siphon (*3, 4*). The demonstrated competence of the peripheral nervous system to mediate habituation (*1, 2*) suggests that, in addition to central sites, peripheral sites in the reflex pathway for gill withdrawal in response to siphon stimulation are also contributing to habituation. We tested this hypothesis by (i) observing if the amplitude of the gill withdrawal changed with repeated electrical stimulation of the nerves to the gill after removal of the central nervous system and (ii) observing habituation of the gill withdrawal to tactile stimulation of the siphon before and after central nervous system removal. The results show that: the peripheral nervous system is competent to mediate habituation of the gill withdrawal reflex; the terminations of central motor neurons in the gill are sites of habituation; and the central nervous system exerts excitatory and inhibitory influences on the peripheral system.

We used 50 *Aplysia californica* (100 to 500 g; Pacific Biomarine, Venice, Calif.) kept in artificial seawater (Instant Ocean) at 15° to 16°C. The preparation consisting of the parietovisceral ganglion (PVG or abdominal ganglion), the gill, mantle, and siphon was pinned out in a chamber containing 500 or 1500 ml of seawater at 15°C. The PVG, the only part of the central nervous system remaining, innervates the gill, mantle, and siphon by the intact branchial, ctenidial, and siphon nerves (Fig. 1A). A punctate stimulus was applied to the siphon by a stiff wire with a plastic sleeve at the tip which had a total diameter of 1.5 mm. The wire was attached to a solenoid which was activated by square-wave pulses. By varying the voltage and the duration of the pulse to the solenoid the force of the tactile stimulus could be varied in discrete steps from 200 mg to 20 g (*5*). This stimulus applied to the siphon produced a reflex withdrawal of the gill (Fig. 1A). The reflex withdrawal is the same as that studied previously [figure 1A in (*4*); figure 1A in (*6*); and figure 3C in (*7*)]. The amplitude of the reflex was measured by recording the tension (Grass FT.03 force transducer) developed by the gill (Fig. 1A). Microelectrodes, filled with 0.6*M* K_2SO_4, were used for intracellular recordings. Suction electrodes were used to electrically stimulate specific nerves after PVG removal.

We first determined the effect of repeated electrical stimulation of the branchial and ctenidial nerves, which are motor pathways from the PVG to the gill, on gill movements after the PVG was removed (Fig. 1B). Others reported that habituation of gill movements does not occur with repeated electrical stimulation (*3*). Single pulses (0.5 to 0.7 msec in duration, about 25 volts), were applied to the nerves at the rate of one every 30 seconds. This interstimulus interval is commonly used in habituation paradigms (*1–4*). Repeated branchial nerve stimulation caused the amplitude of the gill movement to decrease (Fig. 1B). In 13 out of 18 preparations the response amplitude decreased (Fig. 1B, curve a) at a rate comparable to habituation elicited by tactile siphon stimulation when the PVG is present (*6–8*). In the other five cases the response amplitude increased initially and then decreased (curve c; see legend). A train of pulses applied to the ctenidial nerve, interposed between two trials of branchial nerve stimulation, resulted in dishabituation (curve a). The recovery, after rest, of the habituated withdrawal had the same time course as that from habituation elicited by tactile siphon stimulation (*4*). With repeated ctenidial nerve stimulation, the amplitude of the gill movement was more variable, decreased more slowly (curve b), and often resulted in sensitization-like responses in some preparations (curve d). Branchial nerve stimulation interposed between two stimuli ap-