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# Specific Deficit of the ON Response in Visual Transmission by Targeted Disruption of the mGluR6 Gene

**Masayuki Masu,[1] Hideki Iwakabe,[1] Yoshiaki Tagawa,[1] Tomomitsu Miyoshi,[2] Masayuki Yamashita,[2] Yutaka Fukuda,[2] Hitoshi Sasaki,[3] Kano Hiroi,[4] Yasuhisa Nakamura,[5] Ryuichi Shigemoto,[6] Masahiko Takada,[6] Kenji Nakamura,[7] Kazuki Nakao,[7] Motoya Katsuki,[7] and Shigetada Nakanishi[1]**

[1]Institute for Immunology
[4]Department of Ophthalmology
[6]Department of Morphological Brain Science
Kyoto University Faculty of Medicine
Kyoto 606
Japan
[2]Department of Physiology
Osaka University Medical School
Osaka 565
Japan
[3]Department of Physiology
Hyogo College of Medicine
Hyogo 663
Japan
[5]Department of Anatomy
Tokyo Medical and Dental University
School of Medicine
Tokyo 113
Japan
[7]Medical Institute of Bioregulation
Kyushu University
Fukuoka 812
Japan

## Summary

**Taking advantage of the restricted expression of metabotropic glutamate receptor subtype 6 (mGluR6) in retinal ON bipolar cells, we generated knockout mice lacking mGluR6 expression. The homozygous mutant mice showed a loss of ON responses but unchanged OFF responses to light. The mutant mice displayed no obvious changes in retinal cell organization nor in the projection of optic fibers to the brain. Furthermore, the mGluR6-deficient mice showed visual behavioral responses to light stimulation as examined by shuttle box avoidance behavior experiments using light exposure as a conditioned stimulus. The results demonstrate that mGluR6 is essential in synaptic transmission to the ON bipolar cell and that the OFF response provides an important means for transmitting visual information.**

## Introduction

Visual information is segregated into parallel ON and OFF pathways at the level of retinal bipolar cells (Miller and Slaughter, 1986; Daw et al., 1990; Tessier-Lavigne, 1991; Schiller, 1992; DeVries and Baylor, 1993). This segregation is a key process in promoting the detection of weak contrasts and rapid changes in light intensity (Schiller, 1992). In the cone system, the ON and OFF bipolar cells form synapses with ON center and OFF center ganglion cells, respectively. The ON bipolar cell becomes depolarized in response to light, whereas the OFF bipolar cell is hyperpolarized upon light exposure. In the rod system, all bipolar cells represent the ON type and form synapses with AII amacrine cells. The amacrine cells in turn connect with the ON ganglion cells through gap junctions and with the OFF ganglion cells through inhibitory synapses. Thus, in both systems, the ON and OFF responses are evoked in response to the onset and termination of light, respectively.

ON bipolar cells have a specific glutamate receptor that activates cGMP phosphodiesterase through a G protein (Nawy and Jahr, 1990; Shiells and Falk, 1990). The activation of this receptor is thought to decrease intracellular cGMP concentrations and lead to the closure of cGMP-gated cation channels, thus hyperpolarizing the ON bipolar cells (Nawy and Jahr, 1990, 1991; Shiells and Falk, 1990; Wässle et al., 1991). We cloned and identified a novel metabotropic glutamate receptor (mGluR) coupled to a G protein, termed mGluR6, from the rat retina (Nakajima et al., 1993). This mGluR6 subtype, in accordance with the pharmacological properties of the putative mGluR in the ON bipolar cell, responds selectively to L-2-amino-4-phosphonobutyrate (L-AP4) (Nakajima et al., 1993). The expression of this mRNA is confined to the bipolar cell layer, and the localization of this receptor protein is restricted to the postsynaptic site of the ON bipolar cell (Nakajima et al., 1993; Nomura et al., 1994). Furthermore, this specific receptor distribution occurs in accordance with the formation of synaptic connections in the developing retina (Nomura et al., 1994). These data strongly suggested that mGluR6 is responsible for synaptic transmission from photoreceptors to ON bipolar cells. The mGluR family, however, consists of at least eight different subtypes (Nakanishi, 1994), and mGluR7, which is also sensitive to L-AP4, is expressed in the bipolar cell layer (Akazawa et al., 1994). It is thus important to provide direct evidence for the role of mGluR6 in the ON visual pathway.

The use of gene targeting was expected to be very advantageous and useful to study the function of mGluR6 in the visual system, because this receptor is restrictively expressed in ON bipolar cells. Thus, this investigation concerns the targeted disruption of the mGluR6 gene and the characterization of the visual system in the mGluR6-deficient mice. The questions addressed in this study are threefold. First, is mGluR6 essential for synaptic transmission in the ON pathway? Second, does the specific lack of the postsynaptic mGluR6 bring about any effect on retinal cellular organization and projections in the visual pathways? Third, how does the mutant mouse behave in response to light stimulation? We report here the electrophysiological, morphological, and behavioral characterization of the visual system of the mGluR6-deficient mutant mice.

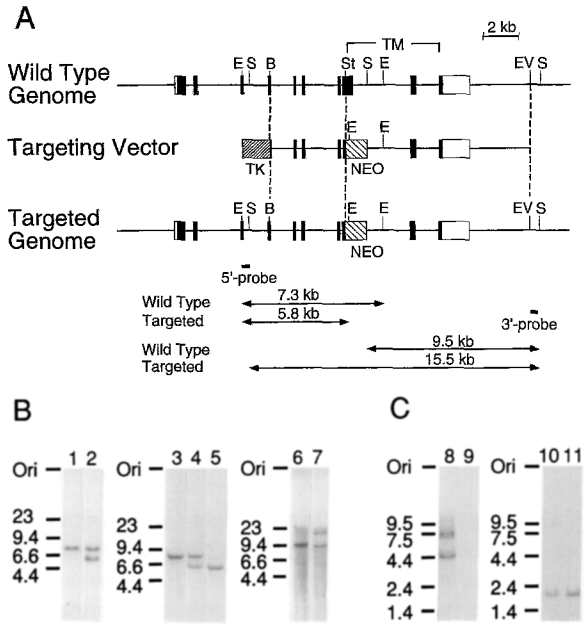



Figure 1. Targeted Disruption of the mGluR6 Gene

(A) Exons of the mGluR6 gene are shown by boxes in which the protein-encoding regions are indicated by closed boxes. Homologous recombination resulted in replacement of the 1.2 kb fragment encoding a part of the mGluR6 transmembrane (TM) region with the neomycin resistance gene (NEO). The herpes simplex virus thymidine kinase gene (TK) was attached to the 5′ end of the targeting vector for negative selection. Restriction sites indicated are as follows: BamHI (B), EcoRI (E), EcoRV (EV), SacI (S), and StuI (St). The locations of the 5′- and 3′-flanking probes are shown. The 5′- and 3′-flanking probes generated the 7.3 kb EcoRI and the 9.5 kb SacI fragments from the wild-type mGluR6 gene, respectively, and the 5.8 kb EcoRI and the 15.5 kb SacI fragments from the properly disrupted gene, respectively.

(B) Southern blot analyses of genomic DNAs from ES cell clones with the wild-type mGluR6 gene (lanes 1 and 6) and those with the properly disrupted mGluR6 gene (lanes 2 and 7) and from tails of the wild-type (lane 3), heterozygous (lane 4), and homozygous mutant mice (lane 5) are shown. EcoR1-digested DNAs and SacI-digested DNAs were hybridized with the 5′-flanking probe (lanes 1–5) and the 3′-flanking probe (lanes 6 and 7), respectively. Positions of size markers are shown on the left.

(C) Northern blot analysis of retinal RNAs from wild-type and homozygous mutant mice. Two species (7.5 and 4.4 kilonucleotides) of mGluR6 mRNA in wild-type mice (lane 8) are absent in mutant mice (lane 9). The same filter was rehybridized with the human elongation factor-1α cDNA probe (lanes 10 and 11).

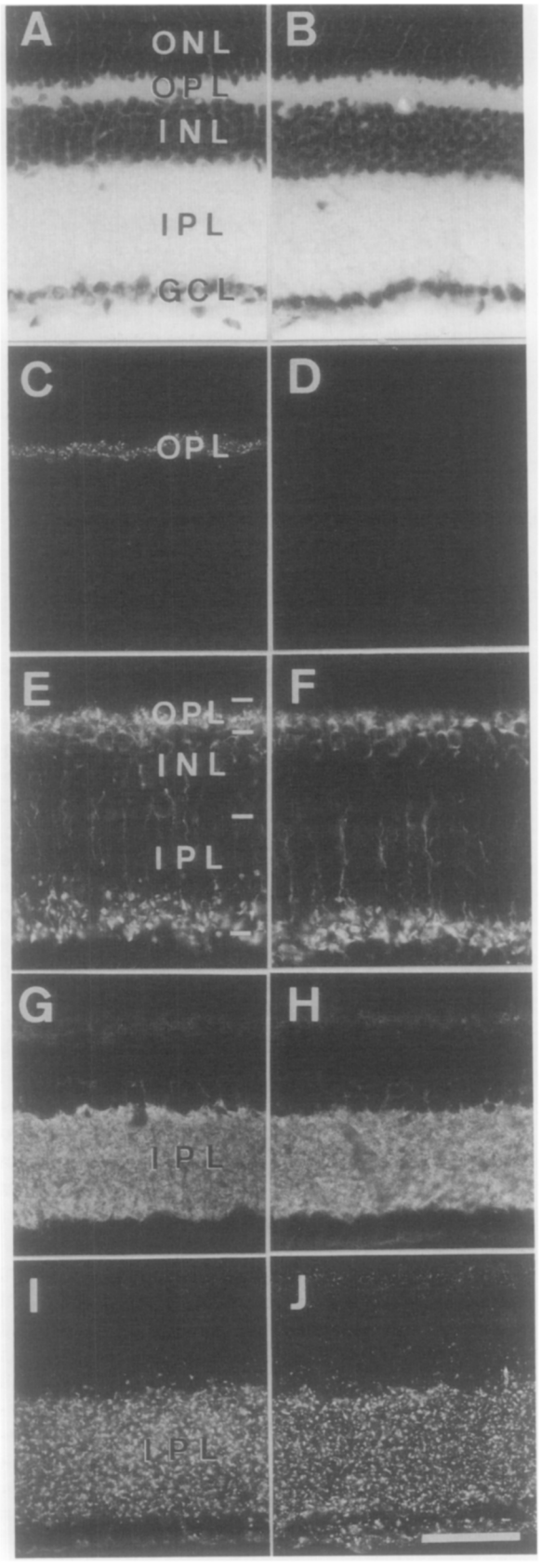
## Results

### Generation of mGluR6-Deficient Mice

We cloned and mapped the mouse mGluR6 gene and disrupted it in the embryonic stem (ES) cell line derived from the 129/SvJ mouse strain (Wurst and Joyner, 1993). The targeting vector included the neomycin resistance gene for positive selection and the herpes simplex virus thymidine kinase gene for negative selection. We incorporated 13.6 kb of the mGluR6 genomic sequence and replaced an internal 1.2 kb StuI–SacI fragment encoding a part of the mGluR6 transmembrane segments with the neomycin resistance gene (Figure 1A). The targeting vector was electroporated into ES cells, which were then selected using G418 and the pyrimidine analog GANC. Three targeted clones were identified from 88 G418- and GANC-resistant clones by Southern blotting with 5′-flanking and 3′-flanking probes (Figure 1B). This blotting analysis as well as that with the neomycin gene probe showed that there were no gross rearrangements, deletions, or secondary random integrations of the targeting sequence. Chimeric mice were generated by injecting ES cells from these cell lines into C57BL/6J blastocysts and then implanting the blastocysts into the uteri of pseudopregnant recipients. Heterozygous animals were identified by Southern blotting and were bred with each other to obtain homozygous animals. Southern blot analysis of genomic DNAs of the homozygous mice showed the properly targeted structure of the mGluR6 gene (Figure 1B). The genotypes of 443 offspring from heterozygote matings were 25.5% (113/443) homozygous mutant, 23.7% wild type (105/443), and 50.8% (225/443) heterozygous mutant. There was no apparent survival disadvantage of homozygous mutant mice nor was there any obvious abnormality in their appearance and behavior as compared with their wild-type or heterozygous littermates. Northern blot analysis confirmed that mGluR6 mRNA is absent in the retina of the homozygous mutant mice (Figure 1C).

### Cellular Organization and Optic Nerve Projection of mGluR6-Deficient Mice

The retina is well organized with distinctly ordered layers (Wässle and Boycott, 1991; Kolb, 1994). Different types of retinal cells can also be identified by immunostaining with antibodies of marker proteins: the amacrine and ganglion cells are selectively immunostained with antibodies against HPC-1/syntaxin and Thy-1, respectively (Barnstable and Dräger, 1984; Barnstable et al., 1985), while the antibody against protein kinase C (PKC) specifically labels rod bipolar cells and a certain type of amacrine cells (Negishi et al., 1988; Greferath et al., 1990). We addressed histologically and immunocytologically the key question as to whether the absence of mGluR6 in the bipolar cell affects the cellular organization of the retina. Hematoxylin and eosin staining revealed the retinae of homozygous mutant mice to be normally organized in an orderly layered anatomical arrangement (Figures 2A and 2B). In the mutant animal, punctate and intense immunoreactivity for mGluR6 observed in the wild-type animal was completely absent (Figures 2C and 2D). In contrast, the dendrites, cell bodies, and axons of rod bipolar cells were immunostained with the PKC antibody in the mutant mice. This immunostaining pattern, including the cell shapes and the axonal terminations of rod bipolar cells in the inner third of the inner plexiform layer, was comparable to that observed in wild-type animals (Figures 2E and 2F). No significant reduction of PKC-positive cells in number was observed. Furthermore, ganglion cells and amacrine cells in the mutant mice appeared normal when they were analyzed with the Thy-1 and HPC-1 antibodies, respectively (Figures 2G–2J). Thus, there are no gross changes in the overall cellular arrangement despite the absence of mGluR6 in the homozygous mutant mouse retina. We also attempted immunoelectron microscopic analysis of synaptic forma-




tions of rod bipolar cells with the PKC antibody but failed this attempt even in the wild-type mice for some unknown reason. Thus, the ultimate conclusion of intact synaptic connections of rod bipolar cells in the mGluR6-deficient mouse awaits further ultrastructural investigation.

With the aid of an anterograde neuronal tract tracing technique using wheat germ agglutinin–conjugated horseradish peroxidase (WGA–HRP), we next examined the terminal distribution of optic fibers in subcortical visual centers in wild-type and homozygous mutant mice. In both animals, optic nerve fibers projected into the superior colliculus, the lateral geniculate nucleus, and pretectal regions (Hayhow et al., 1962; Schiller, 1984) (Figure 3). Furthermore, the projections in both animals were found mainly on the contralateral side of the WGA–HRP injection and only sparsely on the ipsilateral side, and there was no obvious difference in the overall pattern of these projections between the wild-type and homozygous mutant mice. The results demonstrate that the ganglion cell fiber projection has no gross changes in the homozygous mutant mouse.

### Impairment of ON Responses in mGluR6-Deficient Mice

To examine the function of mGluR6 in visual synaptic transmission, we first compared the electroretinogram (ERG) patterns between the wild-type and homozygous mutant mice. The ERG response to a light flash can be divided into three major components: a-, b-, and c-waves (Steinberg et al., 1991). Although each wave represents a mass response derived from complex multicellular components in the retina, the b-wave principally arises from depolarization of ON bipolar cells (Knapp and Schiller, 1984; Stockton and Slaughter, 1989), while the a- and c-waves mainly originate in photoreceptors and the pigment epithelium/Müller cells, respectively (Steinberg et al., 1991). When the ERG was recorded from wild-type mice under dark-adapted conditions, typical responses composed of a-, b-, and c-waves were observed (Figure 4A). In mutant mice, the b-wave was fully abolished and

Figure 2. Histological and Immunohistochemical Analyses of the Retinae of the Wild-Type and mGluR6-Deficient Homozygous Mutant Mice

(A and B) Vertical sections of the adult mouse retinae from wild-type (A) and mutant mice (B) were stained with hematoxylin and eosin. Different layers consist of the following cells and cellular connections: ONL, the outer nuclear layer comprised of photoreceptors; INL, the inner nuclear layer consisting of bipolar, horizontal, and amacrine cells; GCL, the ganglion cell layer containing mostly ganglion cells; OPL, the outer plexiform layer in which photoreceptor, bipolar, and horizontal cells make synaptic connections; IPL, the inner plexiform layer in which bipolar, amacrine, and ganglion cells make synaptic contacts. Well-organized and layered structures of the retinae are preserved in the mutant mouse.

(C–J) Sections of wild-type (C, E, G, and I) and mutant mouse (D, F, H, and J) retinae were immunostained with the mGluR6 antibody (C and D), the PKC antibody (E and F), the HPC-1 antibody (G and H), and the Thy-1 antibody (I and J). Punctate mGluR6 immunoreactivity seen in the OPL of the wild-type mouse retina is absent in the mutant mouse retina, whereas no gross changes in the immunostained patterns of PKC, HPC-1, and Thy-1 are observed in the mutant mouse retinae. Scale bar, 50 μm.

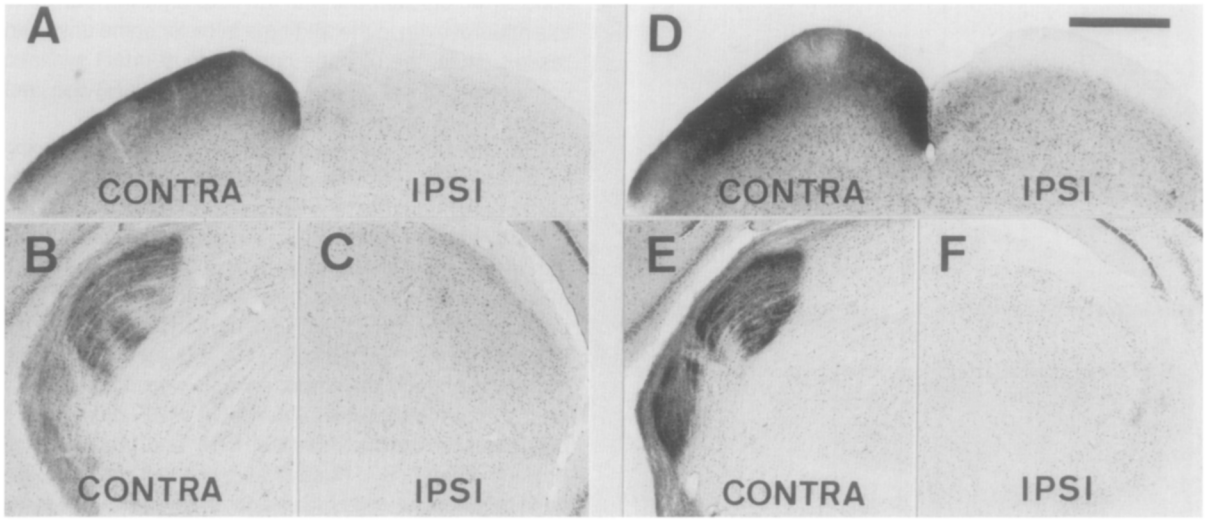



Figure 3. Distribution of the Optic Fiber Projections in the Superior Colliculus and Lateral Geniculate Nucleus

Two days after injection of WGA–HRP into the right eyeball, brain sections were subjected to the tetramethylbenzidine reaction and counterstained with neutral red. The superior colliculus of the wild-type (A) and mutant mice (D) and the lateral geniculate nucleus of the wild-type (B and C) and mutant mice (E and F) are indicated; contra and ipsi, contralateral and ipsilateral sides to the WGA–HRP injection, respectively. Scale bar, 500 μm.

a broad, negative deflection was instead observed due to the absence of a negating positive b-wave component (Figure 4B). This negative deflection was followed by a slow positive deflection of the c-wave comparable to that in the wild-type animal. As the b-wave has a lower threshold for light intensities than the a-wave (Steinberg et al., 1991), we monitored the appearance of the a- and b-waves as a function of illumination intensities. In the wild type, the b-wave first appeared at lower intensities and the a-wave gradually increased at higher intensities (Figure 4C). Oscillatory potentials also overlay the b-wave at the higher intensities. In homozygous mutant mice, the b-wave never appeared by increasing illumination intensities and thus did not negate the negative-going a-wave deflection (Figure 4D). The ERG analysis thus indicates that the mutation in the mGluR6 gene results in an impairment of synaptic transmission in ON bipolar cells. We also tried to measure the d-wave that represents OFF responses, but could detect no clear d-wave in the wild-type mouse.

To examine more directly the effects of the mGluR6 deficiency on ON and OFF responses in visual transmission, we recorded light-evoked field potentials from the superior colliculus, a major synaptic target of the retinal ganglion cells (Knapp and Schiller, 1984; Fukuda and Iwama, 1978; Sasaki et al., 1993). In wild-type mice, a strong light flash produced a large negative potential followed by an oscillatory response (Figure 5A). When the duration of illumination was elongated in a stepwise manner, the two responses were more clearly separated; the early response was induced with a nearly constant latency after the light onset, whereas the late oscillatory response was evoked with an almost constant latency from the end of the flash (Figure 5A). This observation indicates that the early and the late signals represent summated postsynaptic ON and OFF responses, respectively, in the superior colliculus (Sasaki et al., 1993). The analysis of ON and OFF responses in the homozygous mutant mice clearly showed that the ON response was abolished, whereas the OFF response invariably appeared with a constant latency after cessation of the light stimulus (Figure 5B). In the wild-type mice, the peak ON and OFF responses were observed at approximately 800–1100 μm in depth from the cortical surface in ten penetrations of three different animals. Similarly, the peak OFF response was recorded in this range of depths in recordings from eleven penetrations of three mutant mice. These recording sites of both the wild-type and homozygous mutant mice were confirmed by histological examination to reside in the superficial layers of the superior colliculus. In contrast, no obvious ON response was observed, even when different depths (100–2000 μm from the cortical surface) were systematically surveyed for every mutant mouse.

To obtain further evidence for the lack of ON responses in the mutant mice, we adopted another protocol to separate ON and OFF responses. We repeated alternate light and dark cycles (each for 2 s) and recorded field potentials evoked by onset and termination of light in the superior colliculus. Because both ON and OFF responses attenuated within 1 s after onset and termination of illumination, respectively (Figure 5A), the 2 s interval of ON–OFF light cycle clearly separated ON and OFF responses recorded in the wild-type animal (Figure 5C). When this protocol was applied to the homozygous mutant mice, the ON response was abolished but the OFF response remained unchanged (Figure 5D). Our study of both the ERG and superior colliculus recordings thus demonstrates that the lack of mGluR6 expression in the bipolar cell results in a loss of the ON function but has no obvious effect on the OFF function in visual transmission.

Both rod bipolar cells and ON-type cone bipolar cells are thought to use an mGluR for depolarization in response to light. To confirm that mGluR6 is responsible for ON-type

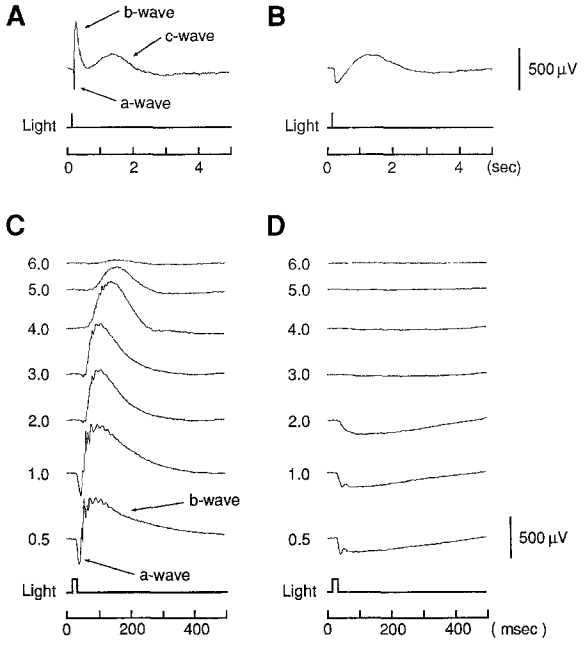



Figure 4. ERGs of the Wild-Type and Mutant Mice

(A and B) ERGs in response to diffuse, strong flash stimuli were recorded in the wild-type mouse (A) and mutant mouse (B) under dark-adapted conditions. Four responses were averaged. a-wave, b-wave, and c-wave are seen in the wild type, but b-wave is completely abolished in the mutant animal.

(C and D) ERGs in response to diffuse stimuli of different illumination intensities were recorded. Numbers on the left indicate the values of the neutral density (ND) filter in log units. The maximum luminance (ND filter 0) was 35,000 lux at the corneal surface of the animal. With increasing light intensity in the wild-type mouse (C), the amplitude of the b-wave becomes larger and its latency is shortened. The a-wave and the oscillatory potentials overlying the b-wave appear with stronger stimuli. In the mutant mouse (D), the b-wave is not seen in any stimulus intensity, whereas the a-wave and c-wave (data not shown) are normal.

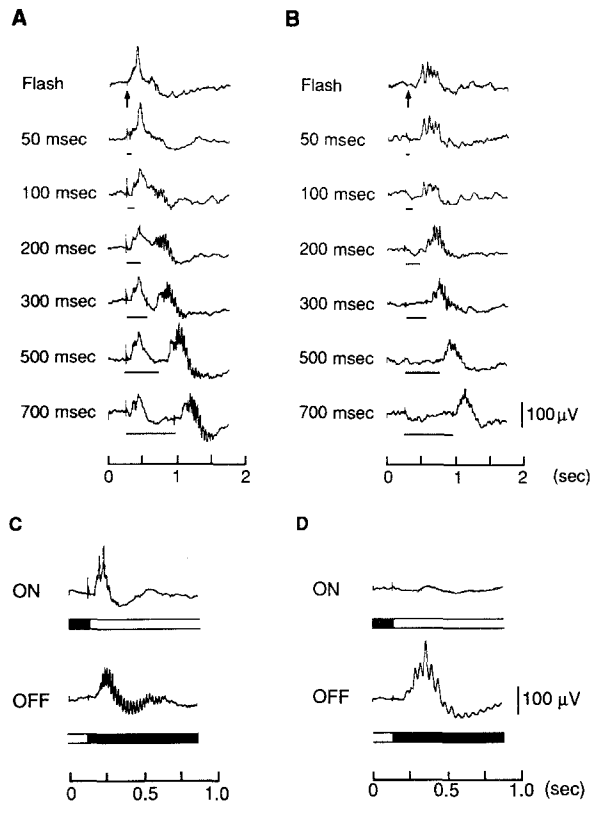



Figure 5. Electrophysiological Responses to Light in the Superior Colliculus of the Wild-Type and the Mutant Mice

At least three animals were examined, and their representative electrophysiological patterns are indicated. Strong and diffuse light was provided to the left eye of the wild-type (A and C) and mutant mice (B and D), and 16–32 responses of the light-evoked potentials recorded from the right superior colliculus were averaged.

(A and B) Light was applied by a flash (as indicated by an arrow), or the duration of light stimuli was increased from 50–700 ms (as indicated by bars). In the wild-type mouse, ON responses appear with a fixed latency from the onset of the stimuli, and oscillatory OFF responses become manifest by increasing the duration of light stimuli. In this protocol, ON responses are not seen in the mutant mice, but OFF responses remain unchanged.

(C and D) Alternate light and dark stimuli with each cycle of 2 s were provided. In the mutant mouse, only OFF responses are seen. Closed and open boxes indicate dark and light periods, respectively.

synaptic transmission in the cone system, we measured ERGs in the mGluR6-deficient mice under light-adapted conditions. The wild-type mice showed a small but clear b-wave, whereas no such response was seen in mutant mice (data not shown). However, because the rod bipolar cells highly dominate the population of mouse retinal bipolar cells, we could not be completely certain that the b-wave observed in the wild type represents an ON response of the cone bipolar cells rather than that of the dominant rod bipolar cells. To separate the cone responses from the rod responses, we also attempted to measure the flicker (30 Hz) ERG response characteristic of the cone system (Dodt, 1951) but failed to detect clear flicker responses even in the wild-type mouse. Although this ambiguity remains to be solved in ERG experiments, we could not detect any ON responses even after exposure to high intensity light in recordings from the superior colliculus of mGluR6-deficient mice. It is therefore most likely that mGluR6 is responsible for glutamatergic transmission from photoreceptors to ON bipolar cells in both the cone and rod systems.

## Behavioral Analysis of Visual Responses of mGluR6-Deficient Mice

Careful observation of homozygous mutant mice showed no abnormal behaviors under both light and dark conditions. To examine accurately whether the mGluR6-deficient mice retain or lose visual functions in response to light stimulation, we performed shuttle box avoidance learning analysis in conjunction with light exposure (Burešová and Bureš, 1976). The animal was placed in a shuttle box whose floor was divided into two compartments. Each trial was initiated by turning on light as a conditioned stimulus (CS), and 10 s later, a floor shock was applied to the animal as an unconditioned stimulus (US). In the early stage of training with 100 trials per day, the mouse escaped in direct response to foot shock, but as the training

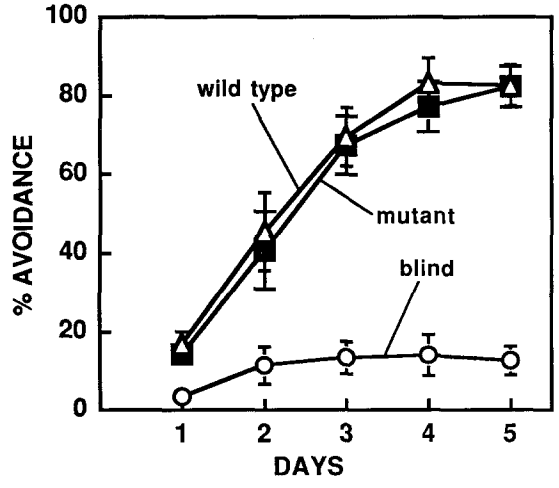



Figure 6. Visual Behavioral Responses of the Wild-Type, mGluR6-Deficient, and Blind Mice

Behavioral responses to light stimuli were examined by shuttle box avoidance learning analysis in conjunction with light exposure as a conditioned stimulus. The ordinate indicates the percentage of successful avoidance (mean ± SEM, n = 8 for both wild-type and mutant mice and n = 5 for blind mice) during training by a massed trial (100 trials per day) procedure. No significant difference in visual behavioral responses and general basal activity (data not shown) is observed between wild-type and mutant mice, whereas significant difference (Scheffe's F test, $P < 0.05$) is seen between blind mice and wild-type/mGluR6-deficient mutant mice.

proceeded, the animal learned to associate the CS and US and crossed into the other compartment within 10 s of light onset without receiving a foot shock (Figure 6). Figure 6 illustrates mean percentages of avoidance learning acquisition by the wild-type and mutant mice over five consecutive training days. The results indicate that there is no significant difference in avoidance learning acquisition in response to light exposure between the wild-type and mutant mice. In control experiments, the blind mice showed no avoidance behavior, indicating that visual perception is essential for this avoidance learning. This analysis thus reveals that the mGluR6-deficient mouse is capable of perceiving visual inputs. Because the ON response is absent in electrophysiological recordings of the homozygous mouse, this finding was unexpected. However, the behavioral paradigm used in this analysis includes various visual cues, such as detection of shapes derived from the experimental apparatus, and is thus more complex than a simple detection of light increment. Thus, taken into consideration our observation of unaltered OFF responses in the mGluR6-deficient mouse, this behavioral analysis strongly suggests that OFF responses also serve as an important means for transmitting visual information.

## Discussion

In this investigation, targeted disruption of the mGluR6 gene was undertaken to examine the role of mGluR6 in the ON pathway by taking advantage of the restricted expression of the mGluR6 gene in ON bipolar cells. Disruption of the mGluR6 gene results in a complete loss of mRNA and immunoreactivity for mGluR6 in the bipolar cells. The homozygous mutant mice develop normally and show no apparent behavioral abnormality. ERG analysis and recordings from the superior colliculus have indicated that the mGluR6 deficiency abolishes ON responses without significant change of OFF responses in visual transmission. The present investigation thus provides compelling evidence that mGluR6 of the bipolar cell is essential for synaptic transmission in the ON pathway.

The ionotropic glutamate receptors evoke rapid excitatory postsynaptic potentials in response to glutamate transmitter and are thus responsible for rapid excitation in many neuronal cells (Nakanishi and Masu, 1994). mGluRs, on the other hand, are thought to modulate glutamatergic or other neurotransmission by coupling to intracellular signal transduction pathways (Schoepp and Conn, 1993; Hayashi et al., 1993). This investigation thus provides a novel example of a specific mGluR subtype directly mediating synaptic transmission. Because photoreceptors hyperpolarize in response to light, the important role of mGluR6 in the visual system is a sign inversion of signals in which a hyperpolarization of the presynaptic photoreceptor cell is converted to a depolarization in the postsynaptic bipolar cell. This depolarization then results in excitation of the subsequent visual transmission. In addition, mGluR6 is postulated to amplify signal transmission at the bipolar cell level by coupling to the cGMP cascade reminiscent of that in phototransduction (Nawy and Jahr, 1990; Shiells and Falk, 1990). mGluR6 is thus important not only in amplifying small graded presynaptic potentials to evoke action potentials in the postsynaptic cells (Ashmore and Falk, 1980), but also in segregating visual inputs into the two distinct parallel pathways.

The appearance of mGluR6 protein at the bipolar postsynaptic site is consistent with the formation of synapses onto bipolar cells in the developing retina (Nomura et al., 1994). The present study has shown that in spite of the absence of mGluR6, no obvious change occurs in the organization of the retina nor in the optic nerve fiber projections to visual centers. The retinal cellular organization thus seems to be formed and maintained independent of neural activity involving mGluR6-mediated synaptic transmission, and this finding strongly suggests that the specific localization of mGluR6 is encoded by a general program of bipolar cell differentiation (Nomura et al., 1994). In the absence of mGluR6 in the ON bipolar cells, it is expected that the mGluR6-coupled phosphodiesterase remains inactive and may thus persistently depolarize ON bipolar cells through the accumulation of cGMP. This sustained excitation of the bipolar cells, however, could be avoided by desensitization of subsequent intracellular effectors or by other mechanisms. Intracellular signaling changes in the mGluR6-deficient ON bipolar cells awaits further investigation.

In spite of the lack of light-induced ON responses in the mGluR6-deficient mice, we could find no significant difference in the visual behavioral responses between wild-type and mutant mice as assessed by the shuttle box avoidance learning tasks. This finding was initially unexpected, but could be interpreted by the fact that the visual

functions analyzed in this experimental paradigm include not only simple detection of light stimulation but also various perceptual functions in vision, such as the detection of shapes inevitably derived from the experimental apparatus and movement perception due to the animal movements. It is thus most likely that the animal can perceive visual inputs through the OFF pathway and can learn to avoid noxious events. This interpretation is very much consistent with a series of physiological studies using L-AP4. L-AP4 has been used as a useful tool to abolish ON responses selectively without knowledge of the molecular entity of the L-AP-sensitive receptors (Slaughter and Miller, 1981). L-AP4 is now thought to activate mGluR6 and eventually block the ON pathway. The lack of mGluR6 and the persistent activation of mGluR6 thus both result in a defect of function in the ON pathway. Interestingly, blockade of ON responses by L-AP4 application has been shown to exert no effect on the orientation and direction selectivities in the lateral geniculate nucleus and the visual cortex (Schiller, 1982; Knapp and Mistler, 1983; Horton and Sherk, 1984; Sherk and Horton, 1984). Furthermore, injection of L-AP4 into monkey retina results in a pronounced loss of contrast sensitivity, but has no obvious effects on other perceptual functions, such as the perception of shapes and colors (Schiller et al., 1986). Thus, more subtle behavioral experiments may be required for detecting possible visual deficits of the mGluR6-deficient mice, such as those testing absolute threshold detection and a low level of contrast discrimination. Although this question awaits further investigation, the present study demonstrates that mGluR6 is essential in ON synaptic transmission and also leads to the important conclusion that OFF transmission provides an indispensable means for transmitting information to visual centers.

## Experimental Procedures

### Targeting Vector Construction and Transfection of ES Cells

The mouse mGluR6 gene was isolated from a mouse genomic library prepared from 129/SvJ mice DNA (Stratagene) by hybridization with the 665 bp DraII fragment of the rat mGluR6 cDNA used as a probe (Nakajima et al., 1993). The targeting vector consisted of the 13.6 kb genomic sequence in which the 1.2 kb StuI–SacI fragment encoding a part of the transmembrane region of mGluR6 was replaced with the 1.2 kb neomycin gene derived from pMC1neopolyA. A 1.9 kb herpes simplex virus thymidine kinase gene fragment was attached to the 5′ end of the mGluR6–neomycin fragment for negative selection. CCE ES cells (a gift of Dr. E. Robertson) obtained from an inbred mouse line 129/SvJ were cultured essentially as previously described (Robertson, 1987). ES cells ($3.7 \times 10^7$–$4.5 \times 10^7$) were transfected with 50 μg of the linearized targeting vector DNA by electroporation with a setting of 500 μF capacitance, 270 V/1.8 mm (BTX Inc, ECM 6000). G418 (250 μg/ml; Sigma) and GANC (5 μM; a gift of Nihon Syntex) were added to the medium for selection 24–48 hr after the transfection. ES cell lines with targeted disruption of the mGluR6 gene were identified by Southern blot analysis of EcoRI- or SacI-digested genomic DNA; the probes used were the 5′- and 3′-flanking regions and the neomycin gene. Three clones from a total of 88 G418 and GANC doubly-resistant colonies contained the desired targeted allele.

### Generation of mGluR6-Deficient Mice

Chimeric mice were generated by injecting the mGluR6-disrupted ES cells into C57BL/6J blastocysts and then by implanting them into the uteri of pseudopregnant Jcl MCH:ICR recipients (CLEA Japan Co.). Twenty-nine agouti coat color chimeric male mice were obtained, and the germline transmission was examined by mating them to BDF1 females. The agouti coat color offspring were analyzed by Southern blot hybridization. Sixteen of them were germline chimeras, which produced a total of 183 offspring and transmitted the disrupted mGluR6 allele to 46% (85/183) of the agouti offspring. Heterozygous animals were interbred to generate homozygous mice lacking the functional mGluR6 gene. Homozygous mutant mice and their wild-type littermates of the F2 generation (11–16 weeks old for morphological and electrophysiological analysis and 15–20 weeks old for behavioral analysis) were used for all experiments. RNA blot analysis was carried out by hybridization of total retinal RNA (5 μg) with the 665 bp DraII fragment of the rat mGluR6 cDNA and the 1.8 kb BamHI fragment of the human elongation factor-1α cDNA.

### Histological Analysis

Immunohistochemistry of mGluR6 and other retinal marker proteins was carried out with frozen sections (10 μm thickness) of eyes enucleated from paraformaldehyde-fixed animals under deep anesthesia (Nomura et al., 1994). The antibodies used were as follows: the affinity-purified rabbit polyclonal antibody against the C-terminal sequence of the rat mGluR6 (Nomura et al., 1994); the affinity-purified rabbit polyclonal antibody against the rat HPC-1 (Inoue et al., 1992; a gift of Dr. Akagawa); the mouse monoclonal antibody against PKC (Amersham, clone MC5, RPN. 536); the rat monoclonal antibody against mouse Thy-1.2 (Pharmingen). The secondary antibodies used were as follows: for mGluR6 and HPC-1, the fluorescein isothiocyanate (FITC)-conjugated goat antibody against rabbit IgG (CAPPEL); for PKC, the FITC-conjugated goat antibody against mouse IgG (CAPPEL); for Thy 1.2, biotinylated goat antibody against rat IgG (Cedarlane), followed by further incubation with avidin-labeled fluorescein (Vector). For hematoxylin-eosin staining, 10% formalin and 1% glutaraldehyde in phosphate-buffered saline (PBS) was used as a fixative.

### Tracer Labeling

Two μl of 1% wheat germ agglutinin conjugated to horseradish peroxidase (WGA–HRP, Type VI, Sigma) dissolved in 50 mM Tris–HCl (pH 7.5) was injected into the vitreous chamber of the right eye of anesthetized mice. Mice were perfused with 10% formalin in PBS, 2 days after injection. The removed brain was cut serially into frontal frozen sections of 40 μm thickness on a cryostat after cryoprotection. These sections were treated with tetramethylbenzidine (Mesulam, 1978), mounted onto gelatin-coated slides, and counterstained with neutral red.

### ERG

Mice were anesthetized by intraperitoneal injection of a mixture of urethane (1 g/kg), xylazine (2.5 mg/kg), and ketamine (20 mg/kg). The pupils were dilated with 0.5% phenylephrine hydrochloride and 0.5% tropicamide. A carbon fiber electrode was placed on the corneal surface, and a reference electrode was attached subcutaneously in the nose. The scotopic and photopic ERGs were recorded after dark adaptation for more than 30 min and after light adaptation (300 lux) for more than 10 min, respectively. The luminance of an unattenuated light stimulus was 35,000 lux on the surface of the eye, and different neutral density filters (Kodak, number 96) were used to serially reduce the stimulus intensities. Responses were amplified with a bandpass frequency setting of 0.5–1000 Hz (Nihon Kohden, AVB-21). Four responses were averaged with an averager (Nihon Kohden, QC111J).

### Electrophysiological Recording

Electrophysiological recordings of the superior colliculus were performed as described previously (Sasaki et al., 1993). Mice were anesthetized by intraperitoneal injection of urethane (1.2 mg/kg), and atropine sulfate (50 mg) was used to counteract vagotonic effects of the anesthetic. The trachea was cannulated to prevent clogging by salivary secretion, but no artificial ventilation was performed. Recordings were performed from the right superior colliculus with glass microelectrodes filled with 4% pontamine sky blue in 0.5 M sodium acetate (5–6 MΩ). The microelectrode was stereotaxically advanced through the cerebral cortex, and the recording depth was estimated by reading a scale on the manipulator. Agar (4%) was used to prevent the brain surface from drying and to reduce pulsation. Diffuse light stimuli were provided to the left eye by a Xenon stroboscope (San-ei Instrument, 3G22) or a

slide projector (Kodak) with a halogen lamp as a light source. The duration of light stimuli from the projector were varied by an electric stimulator that controlled a shutter (Vincent Associates Inc., UniBlitz Model 100–2B). Responses (16–32) were recorded and averaged using an averager (Nihon Kohden, DAT-1100). At the end of the experiment, the recording sites were marked by injection of dye for histological analysis.

### Shuttle Box Learning

Shuttle box avoidance learning analysis was carried out essentially as described (Burešová and Bureš, 1976). The shuttle box consisted of a plastic box with a 22 × 10 cm floor made of stainless steel rods through which ~0.5 mA of scrambled foot shock was administered. The floor space was divided into two equal compartments by a 1.0 cm hurdle over which the mouse jumped to escape or avoid foot shock. The room was dimly illuminated by a 7 W midget lamp (1.2 cd/m$^2$). Each trial was started by turning on a 60 W lamp (200 cd/m$^2$), and the intertrial intervals (ITI) were randomized at 30 s, 45 s, and 60 s. The mouse could avoid foot shock if it jumped into the safety compartment within 10 s after light exposure. Foot shock was continued until the mouse escaped into the safe compartment, or for 20 s in the case of unsuccessful escape. The number of crossings of the mouse between the two compartments during the interval of each trial was recorded to estimate the general basal activity. The training was fully automated using a module for timing, counting, and foot shock. For each mouse, a 1 day habituation was followed by daily training sessions consisting of 100 trials performed for 5 days. For statistical analysis, a mixed type ANOVA was performed for the avoidance scores, and a post-hoc test (Scheffe's F test) was carried out to determine statistical differences in avoidance learning acquisition.

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