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Altered G-Protein Coupling in an mGluR6 Point Mutant Associated with Congenital Stationary Night Blindness[S]

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ABSTRACT

The highly specialized metabotropic glutamate receptor type 6 (mGluR6) is postsynaptically localized and expressed only in the dendrites of ON bipolar cells. Upon activation of mGluR6 by glutamate released from photoreceptors, a nonselective cation channel is inhibited, causing these cells to hyperpolarize. Mutations in this gene have been implicated in the development of congenital stationary night blindness type 1 (CSNB1). We investigated five known mGluR6 point mutants that lead to CSNB1 to determine the molecular mechanism of each phenotype. In agreement with other studies, four mutants demonstrated trafficking impairment. However, mGluR6 E775K (E781K in humans) suggested no trafficking or signaling deficiencies measured by our initial assays. Most importantly, our results indicate a switch in G-protein coupling, in which E775K loses G_o coupling but retains coupling to G_i, which may explain the phenotype.

The metabotropic glutamate receptors (mGluRs) are classified into three subfamilies based on their molecular identity, pharmacology, and G-protein coupling profile (Schoepp, 2001). Group III mGluRs (4, 6, 7, and 8) couple exclusively to $G_{i/o}$ proteins and negatively regulate adenylyl cyclase (Prézeau et al., 1994). mGluR6 is a highly specialized G-protein-coupled receptor that is exclusively expressed in the postsynaptic dendritic region of retinal ON bipolar cells (Nakajima et al., 1993; Vardi et al., 2000; Quraishi et al., 2007). Upon activation, mGluR6 initiates a signaling cascade that ultimately results in the inhibition of a nonselective cation channel, leading to hyperpolarization of the ON bipolar cells (Nawy, 1999; Masland, 2001). Recent studies suggest that the molecular identity of the regulated cation channel is probably transient receptor potential channel melastatin 1 (TRPM1) (Bellone et al., 2008; Nakajima et al., 2009; Shen et al., 2009), and a few reports suggest that regulation is mediated by $G\alpha_o$ rather than the $G\alpha_i$ subunits or by $G\beta\gamma$ (Nawy, 1999; Dhingra et al., 2000, 2004). Our laboratory and others have demonstrated strong coupling of mGluR6 to $G\alpha_o$ in reconstitution experiments (Tian and Kammermeier, 2006), and antibodies directed toward $G\alpha_o$ were able to block the effect of glutamate on the channel in retinal slice recordings (Nawy, 1999).

Genetic defects in the GRM6 gene, which encodes mGluR6, can lead to congenital stationary night blindness (CSNB), characterized by myopia and mild to severe impairment of night vision. In humans, mGluR6 signaling abnormalities, caused by autosomal recessive mutations, lead to complete loss of night vision, CSNB1 (Dryja et al., 2005; Zeitz et al., 2005, 2007), along with the inability to adapt to light-dark cycles and reduced responsiveness to light stimuli in animal models (Takao et al., 2000; Pinto et al., 2007). CSNB2, which is characterized by less severe phenotypes, resolves from mutations occurring elsewhere in the ON visual pathway (Chang et al., 2006; Zeitz et al., 2006). Genetic studies in humans with CSNB1 and deletion of the GMR6 gene in mouse models result in the absence of the electroretinogram b-wave (Dhingra et al., 2000, 2002; Dryja et al., 2005; Zeitz et al., 2005; Pinto et al., 2007), which reflects the depolarization of ON bipolar cells after exposure of photoreceptors to light. Interestingly, the absence of the b-wave was also observed in mice lacking $G\alpha_o$ in bipolar cells (Dhingra et al., 2000, 2002), which correlates well with the hypothesis that mGluR6 strongly couples to $G\alpha_o$ to facilitate the modulation of the cation channel.

To date, there are at least five mGluR6 point mutations that lead to CSNB in humans (P46L, G58R, G150S, C522Y, and E781K). Recent work suggests that each of these mu-

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; BBS, bungarotoxin binding site; CSNB, congenital stationary night blindness; SCG, superior cervical ganglion; HEK, human embryonic kidney; PTX, pertussis toxin; m3, muscarinic type 3 receptor; AM, acetoxymethyl ester; GFP, green fluorescent protein; i3, third intracellular loop; GLU, glutamate.

tants exhibits impaired plasma membrane expression in HEK293 cells (Zeitz et al., 2005). Based on the location of these mutations, we anticipated that these mutants would have impaired glutamate responses due to decreased affinity for glutamate or compromised G-protein coupling. Our results confirm that four of these molecules fail to express on the plasma membrane in neurons. The mGluR6 E775K (E781K in humans) expressed and functioned normally in our preliminary assays. However, calcium mobilization data suggest that E775K couples predominantly to $G\alpha_i$ rather than to $G\alpha_o$, which may explain the CSNB phenotype in humans.

Materials and Methods

Superior Cervical Ganglion Neuron Isolation, cDNA Injection, and Molecular Methods. Detailed descriptions of the isolation and injection procedures have been described previously (Ikeda, 1997). In brief, the superior cervical ganglion (SCG) neurons were dissected from adult rats and incubated in Earle's balanced salt solution (Invitrogen, Carlsbad, CA) containing 0.6 mg/ml trypsin (Worthington Biochemicals, Freehold, NJ) and 0.8 mg/ml collagenase D (Roche Diagnostics, Indianapolis, IN) for 1 h at 35°C. Cells were centrifuged twice, transferred to minimum essential medium (Thermo Fisher Scientific, Waltham, MA), plated, and placed in an incubator at 37°C until cDNA injection. Injection of cDNA was performed with an Eppendorf 5247 microinjector and Injectman NI2 micromanipulator (Eppendorf North America, New York, NY) 4 to 6 h after cell isolation. Plasmids were stored at −20°C as a 1 μg/μl stock solution in buffer containing 10 mM Tris and 1 mM EDTA, pH 8. mGluR6 insert was subcloned into pCDNA3.1(−) (Invitrogen). All receptor cDNAs were injected at 0.1 μg/μl. Neurons were coinjected with "enhanced" green fluorescent protein cDNA (0.005 μg/μl, pEGFPN1; Clontech, Mountain View, CA) or other fluorescent marker if necessary for identification of injected cells. Point mutants (P40L, G52R, G144S, C516Y, and E775K) were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), and sequence was verified. Bungarotoxin binding site (BBS)-mGluR6 was constructed by subcloning the bungarotoxin binding site sequence (5′-TGGAGATACTACGAGAGCTCCCTGGAGCCCTACCCTGAC-3′) between the 22nd and 23rd residues of the original mGluR6 clone using the overlapping extension PCR method. G_q chimeras were also constructed using the QuikChange strategy. After injection, cells were incubated overnight at 37°C, and experiments were performed the next day.

Electrophysiology and Data Analysis. Patch-clamp recordings were made using 8250 glass (Garner Glass, Claremont, CA). Pipette resistances were 1 to 3 MΩ yielding uncompensated series resistances of 1 to 5 MΩ. Series resistance compensation of 70 to 80% was used in all recordings. Data were recorded using an Axopatch 200B patch-clamp amplifier from Molecular Devices (Sunnyvale, CA). Voltage protocol generation and data acquisition were performed using custom data acquisition software (generously donated by Stephen R. Ikeda, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD) on a Macintosh G3 computer with an Instrutech ITC18 data acquisition board (HEKA Elektronik, Lambrecht/Pfalz, Germany). Currents were sampled at 0.5 to 5 kHz low pass-filtered at 3 to 5 kHz using the 4-pole Bessel filter in the patch-clamp amplifier, digitized, and stored on the computer for later analysis. All patch-clamp experiments were performed at 21–24°C (room temperature). Data analysis was performed using Igor software (WaveMetrics, Lake Oswego, OR). For calcium current recordings in SCG, the external (bath) solution contained 145 mM tetraethylammonium methanesulfonate, 10 mM HEPES, 15 mM glucose, 10 mM $CaCl_2$, and 300 nM tetrodotoxin, pH 7.4, with osmolality of 320 mOsmol/kg. The internal (pipette) solution contained 120 mM *N*-methyl-D-glucamine methanesulfonate, 20 mM tetraethylammonium, 11 mM EGTA, 10 mM HEPES, 10 mM sucrose, 1 mM $CaCl_2$, 4 mM MgATP, 0.3 mM Na_2GTP, and 14 mM Tris-creatine phosphate, pH 7.2, with osmolality of 300 mOsmol/kg. Pertussis toxin (PTX) (List Biological Lab. Inc., Campbell, CA) was applied at 500 ng/ml media overnight at 37°C.

PTX-Insensitive Reconstitution Recordings. A good stoichiometric balance between the α and $\beta\gamma$ subunits was necessary to perform the reconstitution experiments. As described previously, the triple pulse protocol that produces the "pre" and the "post" currents is an effective way to detect an imbalance (Kammermeier et al., 2003). Excess of $G\alpha$ subunits will result in a "pre" current that is slightly higher than the "post" current due to α-GDP serving as a "sponge" for $G\beta\gamma$. By contrast, surplus of $G\beta\gamma$ will result in a "pre" current that is already inhibited (Herlitze et al., 1996; Ikeda, 1996; Ikeda and Dunlap, 1999). To overcome this obstacle, we only analyzed cells that had a post/pre ratio between 1 and 1.3. Ratios between these values are indicative a good stoichiometric G-protein subunit balance (Kammermeier et al., 2003). The α subunit plasmids were injected at 5 to 6 ng/μl and $G\beta_1$ and $G\gamma_2$ plasmids were injected at 8 to 10 ng/μl final concentration.

Confocal Microscopy. Injected SCG neurons (in glass coverslips) with the desired plasmid along with "enhanced" green fluorescent protein cDNA to confirm expression were incubated overnight at 37°C. Cells (16–18 h after injection) were transferred into a perfusion chamber and washed with PBS, followed by treatment with 1 μM α-bungarotoxin Alexa Fluor 647 conjugate (Invitrogen) for 1 min. Cells were washed again with PBS, and imaging was performed using an inverted Nikon microscope through a 40× oil immersion objective lens. Confocal images were obtained by using the EZ-C1 3.60 software program (Nikon, Melville, NY). Cells were excited at 488 and 633 nm, and signal was detected at 530 ± 30 and 688 ± 20 nm for GFP and Alexa Fluor 647, respectively.

Digital Imaging of Intracellular Ca^{2+} in Muscarinic Type 3 Stable HEK293 Cells. Muscarinic type 3 (m3) HEK293 cells, obtained from Dr. Trevor Shuttleworth (University of Rochester, Rochester, NY) were loaded with 2 mM Ca^{2+}-sensitive dye fura-2 AM at 37°C for 15 to 20 min. Transfected cells were loaded with fura-2 AM and then transferred into a perfusion chamber. Cells were perfused in HEPES-buffered physiological saline containing 137 mM NaCl, 0.56 mM $MgCl_2$, 4.7 mM KCl, 1 mM Na_2HPO_4, 10 mM HEPES, 5.5 mM glucose, and 1.26 mM $CaCl_2$, pH 7.4. Imaging was performed using an inverted Nikon microscope (Nikon, Tokyo, Japan) through a 40× oil immersion objective lens (numerical aperture, 1.3). Fura-2 AM loaded cells were excited alternately with light at 340 and 380 nm by using a monochromator-based illumination system (TILL Photonics, Pleasanton, CA), and the emission at 510 nm was captured by using a digital frame transfer charge-coupled device camera. Images were captured every 2 s with an exposure of 40 ms and 4 × 4 binning. Analysis was performed by TILL Vision software. Any signal below 0.1 ratio was designated as background noise. The receptors and the G_q chimeras were transfected at 1 μg/35-mm dish, β_1 and γ_2 plasmids were transfected at 0.5 μg/35 mm dish, and finally dSRed-nuc was transfected at 0.2 to 0.3 μg/35-mm dish (for the supplementary experiment) and cherry for the Ca^{2+} mobilization experiments.

Results

Modulation N-Type ($Ca_{V2.2}$) Calcium Channels in SCGs by mGluR6 and Five CSNB Point Mutants. Our initial hypothesis regarding the CSNB point mutants evolved from the idea that these receptors have a reduced or diminished ability to respond to glutamate. Thus, we examined modulation of $Ca_{V2.2}$ channels in SCG neurons by each point mutant to examine receptor activity. The triple-pulse voltage protocol, shown in Fig. 1A (inset), was used to assess channel activity and consists of two test pulses to +10 mV from a −80

mV holding potential, separated by a strong depolarizing step to +80 mV. The +80-mV step partially reverses the well characterized, voltage-dependent, G$\beta\gamma$-mediated inhibition (Elmslie et al., 1990; Ikeda, 1991, 1996).

To determine the effects of five CSNB point mutations on mGluR6 signaling, the corresponding mutants were made in the rat mGluR6 sequence, and each was expressed in SCG neurons. Figure 1A shows a typical time course of an SCG neuron expressing mGluR6, illustrating the inhibition of calcium currents during glutamate application. No effect was observed in uninjected or GFP-only injected cells (data not shown).

In Fig. 1B, the glutamate concentration-response curve for the wild-type and each point mutant is shown. Four of the mutants, P40L, G52R, G144S, and C516Y, showed no response to glutamate. By contrast, cells expressing mGluR6 E775K showed responses similar to those of the wild-type mGluR6. We noticed a slight rightward shift in the EC_{50} values of mGluR6 E775K concentration-response curve (EC_{50} for mGluR6 was 8 μM and 20 μM GLU for the mutant). However, at 100 μM glutamate applications, the responses were identical (45 $\pm$ 5 and 44 $\pm$ 2% current inhibition for mGluR6 and E775K, respectively). These data suggest that four of the mGluR6 CSNB point mutants are nonfunctional. By contrast, mGluR6 E775K seems to function similarly to the wild type.

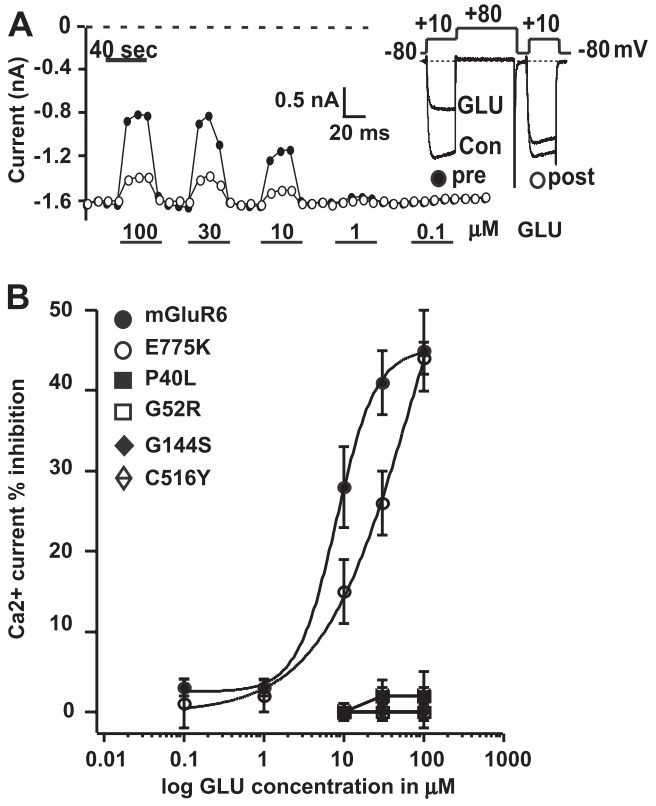

Fig. 1. Inhibition of calcium channels by mGluR6 and CSNB point mutants. A, a representative time course and current trace (inset) of mGluR6-mediated inhibition of the native calcium currents in SCG neurons at indicated agonist (GLU) concentrations. B, concentration-response curve of mGluR6 and CSNB point mutants. The wild-type and the mutants are represented by the indicated symbols. The data are represented as the average calcium current percentage of inhibition $\pm$ S.E.M. for mGluR6 (n = 11), P40L (n = 3), G52R (n = 6), G144S (n = 4), C516Y (n = 7), and E775K 100 μM (n = 23), 30 μM (n = 12), 10 μM (n = 9), and 1 and 0.1 μM GLU (n = 4).

Four CSNB Point Mutants Lack Plasma Membrane Expression. To determine why the nonfunctional mutant receptors lacked responses, the plasma membrane expression of each was examined. Zeitz et al. (2007) reported that all of the CSNB point mutants are retained in the endoplasmic reticulum when overexpressed in HEK293 cells. To evaluate plasma membrane expression in SCG neurons, wild-type mGluR6 and each CSNB mutant were tagged with a N-terminal, extracellular BBS (Sekine-Aizawa and Huganir, 2004). The plasma membrane expression of each receptor was then examined by application of a fluorescently tagged α-bungarotoxin to SCG neurons expressing each receptor. Figure 2 illustrates representative neurons with the indicated plasmid along with phospho-enhanced green fluorescent protein. Cells expressing GFP alone showed no membrane fluorescence after treatment with α-bungarotoxin-Alexa Fluor 647 (shown in red). Furthermore, no detectable membrane labeling was evident in cells expressing P40L, G52R, G144S, or C516Y. However, cells expressing the wild-type or mGluR6 E775K mutant demonstrated comparable surface membrane labeling (Fig. 2). To verify that the bungarotoxin binding site tag did not interfere with receptor function, electrophysiological experiments were performed by monitoring calcium-channel modulation by 100 μM glutamate in cells expressing mGluR6 or BBS-mGluR6. Similar responses were observed in SCG neurons expressing each receptor (Fig. 2). Calcium currents were inhibited 43 $\pm$ 4 (n = 14) and 48 $\pm$ 5% (n = 8) in cells expressing mGluR6 and BBS-mGluR6, respectively.

E775K Does Not Activate Gα_o. Although most of our labeling data correlated with the previous report (Zeitz et al., 2007), one CSNB mutant, mGluR6 E775K, was able to traffic to the plasma membrane and function similarly to the wild-type mGluR6 in SCG neurons. Because wild-type mGluR6 seems to act in the retina through Gα_{oA} stimulation (Dhingra et al., 2002), it is possible that the E775K mutant may produce CSNB due to an inability to activate Gα_{oA} rather than lack of expression or due to trafficking defects. Because the E775K residue resides in the third intracellular loop (i3), a change in the G-protein coupling seemed possible (Francesconi and Duvoisin, 1998). Therefore, the G-protein coupling of mGluR6 E775K was examined more closely.

As with wild-type mGluR6, E775K responses were abol-

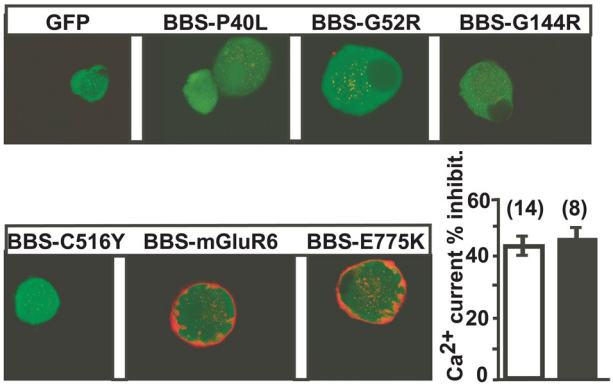

Fig. 2. Surface membrane labeling of mGluR6 and CSNB point mutants in SCG neurons. BBS tagged wild-type and point mutants were coinjected with GFP (green) and treated with α-bungarotoxin Alexa Fluor 647 conjugate (red). Average calcium current inhibition by glutamate for the wild-type (□) and BBS-tagged mGluR6 (■) in SCGs is shown in the bar graph. The number of cells is indicated in parentheses.

ished by overnight PTX treatment, indicating that the mutant coupled exclusively to $G_{i/o}$ proteins (see Fig. 3). Calcium currents were inhibited 45 ± 4% (n = 6) by 100 μM GLU and 6 ± 1% (n = 5) with overnight PTX treatment when mGluR6 was expressed in SCG neurons. Similar responses were obtained (42 ± 3%, n = 11, and 3 ± 1%, n = 7) when mGluR6 E775K was expressed.

Next, the possibility that the E775K mutation changes from a predominantly G_o-coupled to a predominantly G_i-coupled receptor was tested. Therefore, coupling of mGluR6 E775K to specific G-proteins was examined by reconstitution using PTX-insensitive $G_{i/o}$ protein mutations of the cysteine residue near the end of the C terminus (351/2 position, the site for ADP ribosylation) to glycine, rendering the G-proteins insensitive to PTX, as described previously (Bahia et al., 1998). SCG neurons expressing either mGluR6 wild-type or E775K were treated with PTX to inactivate endogenously expressed $G\alpha_{i/o}$ proteins, and calcium current modulation was examined when $G\beta_1$ and $G\gamma_2$ were coexpressed along with each $G\alpha_{i/o}$CG PTX-insensitive protein (Fig. 3).

Consistent with previous results (Tian and Kammermeier, 2006), reconstitution with $G\alpha_{oA}$CG resulted in a strong coupling to mGluR6 wild-type (32 ± 1% inhibition, n = 7). However, no coupling was detected with E775K mutant (2 ± 1% inhibition n = 6). Surprisingly, no coupling was observed with G_{i1}CG, G_{i2}CG, or G_{i3}CG either (3 ± 2, 3 ± 2, and 4 ± 2% inhibition, respectively) (Fig. 4), despite a strong PTX-sensitive signal when mGluR6 E775K was expressed alone. Thus, although the glutamic acid-lysine mutation seemed to abolish signaling through $G\alpha_{oA}$, it is possible that the reconstitution assay is not sufficiently sensitive, or that the cysteine-glycine mutation may selectively disrupt coupling to the mutant receptor. Therefore, an alternative approach to investigate mGluR6 E775K signaling was necessary.

E775K Couples Predominantly to G_i Proteins. As an alternative strategy to investigate mGluR6 E775K signaling, Ca^{2+} mobilization was examined in m3 HEK293 stable cell lines expressing $G_qG_{i/o}$ chimeric proteins in which the extreme C-terminal amino acids were exchanged to allow activation by mGluR6 (Blahos et al., 1998). G_q chimeras have been widely used to study $G_{i/o}$-coupled G-protein-coupled receptor signaling (Kowal et al., 2002, 2003; Zhang et al., 2003; Walker et al., 2005).

To this end, $G_qG_{oA(9)}$ and $G_qG_{i1/2(9)}$ chimeras were generated (the last nine residues for G_{i1} and G_{i2} are identical) and used to perform Ca^{2+} fura-2 AM imaging experiments in m3 HEK293 stable cell lines. M3 receptors couple well to native G_q, which would serve as an internal control when 300 nM carbachol was applied. These cells, were heterologously transfected with each receptor (mGluR6 or E775K), the G_q chimera, and $G\beta_1\gamma_2$ constructs, along with cherry (a red fluorescent protein), to identify expressing cells.

Figure 5A shows representative Ca^{2+} traces illustrating Ca^{2+} signals when each agonist was applied. In cells transfected with the receptor alone, no signal was detected upon glutamate application (mGluR6 151 cells analyzed; E775K 149 cells). These results suggested that neither mGluR6 nor mGluR6 E775K could induce an intracellular Ca^{2+} signal via natively expressing G proteins. When the wild-type receptor was coexpressed with $G_qG_{oA(9)}$ and $G\beta_1\gamma_2$, a strong Ca^{2+} response was detected in 31 of 208 cells analyzed. By contrast, only 1 of 82 cells expressing mGluR6 E775K produced a detectable response, and this response was weak (Fig. 5, A and B). These results correlated well with the electrophysiological data (Fig. 3). When $G_qG_{i1/2(9)}$ was expressed, some signaling was detected with mGluR6 (3 of 88 cells). However, this signal was weaker and less frequent than when $G_qG_{oA(9)}$ was expressed. Interestingly, mGluR6 E775K was able to signal in response to glutamate in 15 of 460 $G_qG_{i1/2(9)}$-expressing cells (Fig. 5, A and B). These data confirm that mGluR6 couples strongly to $G\alpha_o$ and weakly to $G\alpha_i$ while demonstrating that E775K mutant receptor predominantly activates $G\alpha_i$. Furthermore, because this mutation causes CSNB in humans, effectively resulting in loss of function, these data support the conclusion that mGluR6 in retinal ON bipolar cells produces its primary signal via $G\alpha_o$ activation rather then via $G\alpha_i$ or $G\beta\gamma$.

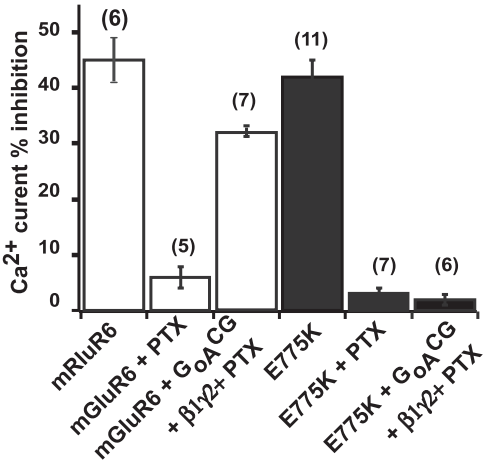

Fig. 3. Reconstitution of coupling to mGluR6 (□) and mGluR6 E775K mutant (■) using PTX-insensitive G_{oA}CG. Average calcium current inhibition (± S.E.M.) in SCG neurons expressing mGluR6 or E775K alone, after overnight PTX treatment, and in combination with PTX-insensitive G_{oA}CG (and $G\beta_1$ and $G\gamma_2$) are shown. The number of cells is indicated in parentheses.

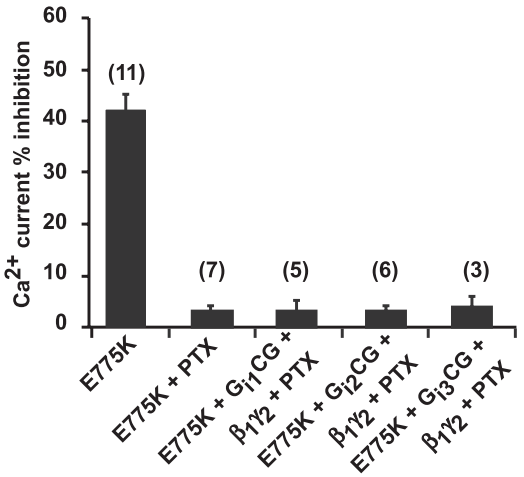

Fig. 4. Reconstitution of coupling to mGluR6 E775K using PTX-insensitive G_{i1}CG, G_{i2}CG, or G_{i3}CG. The average calcium current inhibition (± S.E.M.) in SCG neurons expressing E775K alone or in combination with the indicated PTX-insensitive $G\alpha$ protein (plus β_1 and γ_2) are shown. The number of cells is indicated in parentheses.

Discussion

We report here that a disease causing mGluR6 mutant (E775K) functions and expresses similar to the wild-type receptor in SCG neurons. Further investigation of the signaling cascade revealed that mGluR6 E775K is incapable of activating $G\alpha_o$ and primarily couples to $G\alpha_i$ proteins. Overall, the data presented here suggest that mGluR6 primarily functions through $G\alpha_o$ proteins in retinal ON bipolar cells and an inability of the receptor to activate $G\alpha_o$ results in loss of function, leading to CSNB.

Three of the CSNB mGluR6 point mutants examined here, P40L, G52R, and G144S, are located in the Venus flytrap domain near the agonist binding site (Bessis et al., 2000; Rosemond et al., 2002). The Cys516 residue is located in the cysteine-rich region of the receptor, which separates the Venus flytrap domain from the seven-transmembrane region and is believed to be important in the intramolecular signal transmission (Rondard et al., 2006). Therefore, the mechanism of loss of function for each seemed apparent. Surprisingly, each of these mutants lacked function not because of a predicted deficit in ligand binding or intramolecular signal transduction but because of a loss of plasma membrane expression. These results are consistent with those of Zeitz et al. (2007) in HEK293 cells and support the notion that mGluR6 trafficking is tightly regulated such that even small structural perturbations can severely alter receptor trafficking.

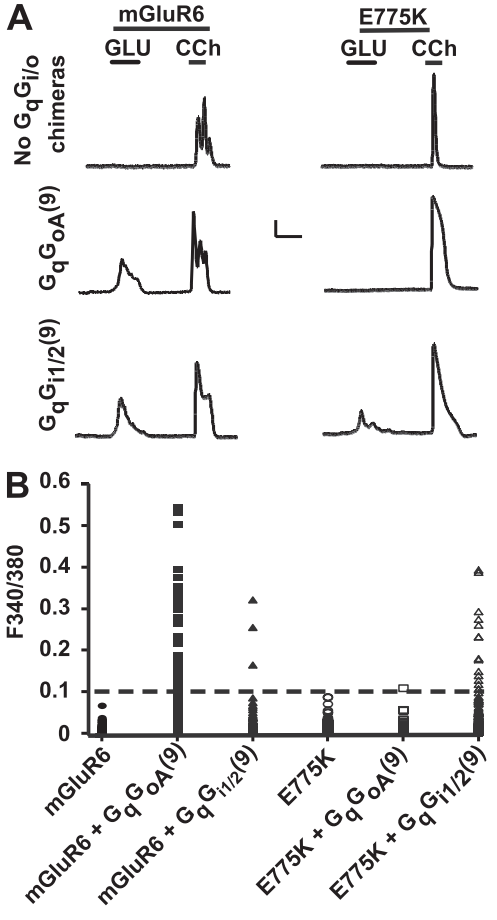

Fig. 5. Reconstitution of mGluR6 and mGluR6 E775K coupling in m3 stable HEK293 cells. A, representative traces from cells expressing mGluR6 (left) and mGluR6 E775K (right) alone or in combination with the indicated chimera with $G\beta_1$ and $G\gamma_2$. The scale bars represent 2 ratio units and 60 s. B, scatter plots showing the fluorescence intensity ratios (F_{340}/F_{380}) of mGluR6 or E775K alone or in combination with the indicated chimera (including $G\beta_1$ and $G\gamma_2$). The number of analyzed cells is indicated under *E775K Couples Predominantly to G_i Proteins.*

The Glu775 residue is located in the third intracellular loop of the receptor. The second (i2) and the third (i3) of mGluRs participate in G-protein contact and activation. It is interesting that the i3 loop of all mGluRs contains a conserved region, and single point mutations in this region have been known to impair receptor/G-protein coupling (Francesconi and Duvoisin, 1998).

According to the functional data presented here, E775K produced responses similar to the wild-type receptor at 100 μM GLU with only a slight shift in potency (Fig. 1). Membrane surface labeling indicated that the mutant receptor is present on the plasma membrane in SCGs after nuclear cDNA injection (Fig. 2) and in HEK293 cells when transfected (Supplemental Fig. 1). However, the observation that an mGluR6 point mutation that resulted in a nearly normal functioning receptor was puzzling.

Wild-type mGluR6 couples to G_o proteins in ON bipolar cells (Nawy, 1999) and in reconstitution studies in SCG neurons (Tian and Kammermeier, 2006). Furthermore, the primary signal transduction induced by mGluR6 in retinal ON bipolar cells, the inhibition of a nonselective cation conductance, presumably transient receptor potential channel melastatin 1 (Shen et al., 2009), seems to require the activation of $G\alpha_o$ because disruption of neither $G\alpha_i$ nor $G\beta\gamma$ can occlude this effect (Nawy, 1999; Dhingra et al., 2002). Therefore, a change in G-protein coupling preference of mGluR6 from $G\alpha_o$ to $G\alpha_i$ could result in CSNB even if the mutant receptor is otherwise functional. This hypothesis was tested for mGluR6 E775K with two strategies: first, by reconstitution of signaling using PTX-insensitive mutant $G\alpha$ proteins in SCG neurons, and second, by examining intracellular calcium signaling with fura-2 AM in HEK293 cells expressing $G\alpha_qG\alpha_{i/o}$ chimeric proteins.

Unfortunately, reconstitution of signaling with the mutant receptor in SCG neurons with PTX-insensitive G proteins could not produce a complete answer. The data suggested a loss of E775K coupling to $G_{oA}CG$. However, when the G_iCG mutants were used, no coupling was observed. These negative results may have resulted from a weak expression of the G-protein mutant in SCGs, low sensitivity of the assay (channel modulation), or the possibility that mGluR6 E775K coupled weakly to more than one G_i protein but not robustly to any one in isolation.

To this end, a more sensitive assay, Ca^{2+}-imaging experiments with fura-2 AM, was used. We constructed $G_qG\alpha_{i/o}$ chimeras in which the last nine residues were swapped with that of the G_{oA}, G_{i1}, and G_{i2} (the last nine residues of G_{i1} and G_{i2} are identical, so this construct was called $G_qG_{i1/2}$). Similar constructs have been used to study mGluR2 and mGluR4 signaling in Chinese hamster ovary cells (Kowal et al., 2003). In accordance with our electrophysiological data, mGluR6 was able to strongly couple to G_qG_{oA} with less robust coupling to $G_qG_{i1/2}$. However, mGluR6 E775K failed to couple to G_qG_{oA} but elicited substantial Ca^{2+} signals when expressed with $G_qG_{i1/2}$. It is important to note that during these experiments, several plasmid constructs were required (receptor, chimera, $G\beta_1$, $G\gamma_2$, and cherry), so the probability of getting all of the components in any one cell is low. Therefore, the low number of responding cells in each experiment was expected.

In conclusion, we demonstrate here an explanation for the CSNB phenotype (for E781K mutant in humans) as an alternative to those proposed previously. These results indicate that mGluR6 E775K functions and expresses like the wild-type receptor, but its ability to activate $G\alpha_{oA}$ has been diminished. The mutant receptor retains the capability to activate G_i proteins in heterologous and reconstitution assays, but that may not be sufficient to produce effective signaling in retinal ON bipolar cells.

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References

Bahia DS, Wise A, Fanelli F, Lee M, Rees S, and Milligan G (1998) Hydrophobicity of residue 351 of the G protein Gi1 alpha determines the extent of activation by the alpha 2A-adrenoceptor. *Biochemistry* **37:**11555–11562.

Bellone RR, Brooks SA, Sandmeyer L, Murphy BA, Forsyth G, Archer S, Bailey E, and Grahn B (2008) Differential gene expression of TRPM1, the potential cause of congenital stationary night blindness and coat spotting patterns (LP) in the Appaloosa horse (*Equus caballus*). *Genetics* **179:**1861–1870.

Bessis AS, Bertrand HO, Galvez T, De Colle C, Pin JP, and Acher F (2000) Three-dimensional model of the extracellular domain of the type 4a metabotropic glutamate receptor: new insights into the activation process. *Protein Sci* **9:**2200–2209.

Blahos J 2nd, Mary S, Perroy J, de Colle C, Brabet I, Bockaert J, and Pin JP (1998) Extreme C terminus of G protein α-subunits contains a site that discriminates between Gi-coupled metabotropic glutamate receptors. *J Biol Chem* **273:**25765–25769.

Chang B, Heckenlively JR, Bayley PR, Brecha NC, Davisson MT, Hawes NL, Hirano AA, Hurd RE, Ikeda A, Johnson BA, et al. (2006) The nob2 mouse, a null mutation in Cacna1f: anatomical and functional abnormalities in the outer retina and their consequences on ganglion cell visual responses. *Vis Neurosci* **23:**11–24.

Dhingra A, Faurobert E, Dascal N, Sterling P, and Vardi N (2004) A retinal-specific regulator of G-protein signaling interacts with Galpha(o) and accelerates an expressed metabotropic glutamate receptor 6 cascade. *J Neurosci* **24:**5684–5693.

Dhingra A, Jiang M, Wang TL, Lyubarsky A, Savchenko A, Bar-Yehuda T, Sterling P, Birnbaumer L, and Vardi N (2002) Light response of retinal ON bipolar cells requires a specific splice variant of Galpha(o). *J Neurosci* **22:**4878–4884.

Dhingra A, Lyubarsky A, Jiang M, Pugh EN Jr, Birnbaumer L, Sterling P, and Vardi N (2000) The light response of ON bipolar neurons requires G[alpha]o. *J Neurosci* **20:**9053–9058.

Dryja TP, McGee TL, Berson EL, Fishman GA, Sandberg MA, Alexander KR, Derlacki DJ, and Rajagopalan AS (2005) Night blindness and abnormal cone electroretinogram ON responses in patients with mutations in the GRM6 gene encoding mGluR6. *Proc Natl Acad Sci U S A* **102:**4884–4889.

Elmslie KS, Zhou W, and Jones SW (1990) LHRH and GTP-gamma-S modify calcium current activation in bullfrog sympathetic neurons. *Neuron* **5:**75–80.

Francesconi A and Duvoisin RM (1998) Role of the second and third intracellular loops of metabotropic glutamate receptors in mediating dual signal transduction activation. *J Biol Chem* **273:**5615–5624.

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, and Catterall WA (1996) Modulation of Ca^{2+} channels by G-protein bg subunits. *Nature* **380:**258–262.

Ikeda SR (1991) Double-pulse calcium channel current facilitation in adult rat sympathetic neurones. *J Physiol* **439:**181–214.

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. *Nature* **380:**255–258.

Ikeda SR (1997) Heterologous expression of receptors and signaling proteins in adult mammalian sympathetic neurons by microinjection, in *Methods in Molecular Biology* (Challis RAJ ed) pp 191–202, Humana Press, Inc., Totowa, NJ.

Ikeda SR and Dunlap K (1999) Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. *Adv Second Messenger Phosphoprotein Res* **33:**131–151.

Kammermeier PJ, Davis MI, and Ikeda SR (2003) Specificity of metabotropic glutamate receptor 2 coupling to G proteins. *Mol Pharmacol* **63:**183–191.

Kowal D, Nawoschik S, Ochalski R, and Dunlop J (2003) Functional calcium coupling with the human metabotropic glutamate receptor subtypes 2 and 4 by stable co-expression with a calcium pathway facilitating G-protein chimera in Chinese hamster ovary cells. *Biochem Pharmacol* **66:**785–790.

Kowal D, Zhang J, Nawoschik S, Ochalski R, Vlattas A, Shan Q, Schechter L, and Dunlop J (2002) The C-terminus of Gi family G-proteins as a determinant of 5-HT_{1A} receptor coupling. *Biochem Biophys Res Commun* **294:**655–659.

Masland RH (2001) The fundamental plan of the retina. *Nat Neurosci* **4:**877–886.

Nakajima Y, Iwakabe H, Akazawa C, Nawa H, Shigemoto R, Mizuno N, and Nakanishi S (1993) Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4- phosphonobutyrate. *J Biol Chem* **268:**11868–11873.

Nakajima Y, Moriyama M, Hattori M, Minato N, and Nakanishi S (2009) Isolation of ON bipolar cell genes via hrGFP-coupled cell enrichment using the mGluR6 promoter. *J Biochem* **145:**811–818.

Nawy S (1999) The metabotropic receptor mGluR6 may signal through G(o), but not phosphodiesterase, in retinal bipolar cells. *J Neurosci* **19:**2938–2944.

Pinto LH, Vitaterna MH, Shimomura K, Siepka SM, Balannik V, McDearmon EL, Omura C, Lumayag S, Invergo BM, Glawe B, et al. (2007) Generation, identification and functional characterization of the nob4 mutation of Grm6 in the mouse. *Vis Neurosci* **24:**111–123.

Prézeau L, Carrette J, Helpap B, Curry K, Pin JP, and Bockaert J (1994) Pharmacological characterization of metabotropic glutamate receptors in several types of brain cells in primary cultures. *Mol Pharmacol* **45:**570–577.

Quraishi S, Gayet J, Morgans CW, and Duvoisin RM (2007) Distribution of group-III metabotropic glutamate receptors in the retina. *J Comp Neurol* **501:**931–943.

Rondard P, Liu J, Huang S, Malhaire F, Vol C, Pinault A, Labesse G, and Pin JP (2006) Coupling of agonist binding to effector domain activation in metabotropic glutamate-like receptors. *J Biol Chem* **281:**24653–24661.

Rosemond E, Peltekova V, Naples M, Thøgersen H, and Hampson DR (2002) Molecular determinants of high affinity binding to group III metabotropic glutamate receptors. *J Biol Chem* **277:**7333–7340.

Schoepp DD (2001) Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. *J Pharmacol Exp Ther* **299:**12–20.

Sekine-Aizawa Y and Huganir RL (2004) Imaging of receptor trafficking by using alpha-bungarotoxin-binding-site-tagged receptors. *Proc Natl Acad Sci U S A* **101:**17114–17119.

Shen Y, Heimel JA, Kamermans M, Peachey NS, Gregg RG, and Nawy S (2009) A transient receptor potential-like channel mediates synaptic transmission in rod bipolar cells. *J Neurosci* **29:**6088–6093.

Takao M, Morigiwa K, Sasaki H, Miyoshi T, Shima T, Nakanishi S, Nagai K, and Fukuda Y (2000) Impaired behavioral suppression by light in metabotropic glutamate receptor subtype 6-deficient mice. *Neuroscience* **97:**779–787.

Tian L and Kammermeier PJ (2006) G protein coupling profile of mGluR6 and expression of G alpha proteins in retinal ON bipolar cells. *Vis Neurosci* **23:**909–916.

Vardi N, Duvoisin R, Wu G, and Sterling P (2000) Localization of mGluR6 to dendrites of ON bipolar cells in primate retina. *J Comp Neurol* **423:**402–412.

Walker MW, Jones KA, Tamm J, Zhong H, Smith KE, Gerald C, Vaysse P, and Branchek TA (2005) Use of *Caenorhabditis elegans* G{alpha}q chimeras to detect G-protein-coupled receptor signals. *J Biomol Screen* **10:**127–136.

Zeitz C, Forster U, Neidhardt J, Feil S, Kälin S, Leifert D, Flor PJ, and Berger W (2007) Night blindness-associated mutations in the ligand-binding, cysteine-rich, and intracellular domains of the metabotropic glutamate receptor 6 abolish protein trafficking. *Hum Mutat* **28:**771–780.

Zeitz C, Kloeckener-Gruissem B, Forster U, Kohl S, Magyar I, Wissinger B, Mátyás G, Borruat FX, Schorderet DF, Zrenner E, et al. (2006) Mutations in CABP4, the gene encoding the Ca2+-binding protein 4, cause autosomal recessive night blindness. *Am J Hum Genet* **79:**657–667.

Zeitz C, van Genderen M, Neidhardt J, Luhmann UF, Hoeben F, Forster U, Wycisk K, Mátyás G, Hoyng CB, Riemslag F, et al. (2005) Mutations in GRM6 cause autosomal recessive congenital stationary night blindness with a distinctive scotopic 15-Hz flicker electroretinogram. *Invest Ophthalmol Vis Sci* **46:**4328–4335.

Zhang JY, Nawoschik S, Kowal D, Smith D, Spangler T, Ochalski R, Schechter L, and Dunlop J (2003) Characterization of the 5-HT6 receptor coupled to Ca^{2+} signaling using an enabling chimeric G-protein. *Eur J Pharmacol* **472:**33–38.

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