日本財団 The Nippon Foundation 助成事業

財団法人　日中医学協会

2010年度共同研究等助成金報告書－在留中国人研究者－

2011年　03　月　03　日

財団法人　日中医学協会　御中

貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：**研究報告書**

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１．助成金額：　600，000　円

２．研究テーマ

医療への応用に最適化されたチャネルロドプシンの開発

３．成果の概要

Channelrhodopsins (ChRs, including ChR1 and ChR2) function as light gated cation channels. In recent years, ChR2 mediated optogenetic stimulation is widely applied to fire neuronal spiking. However, ChR2 preferred to short wavelength light (470 nm) with rapid desensitization in the photocurrent To modify the properties of ChRs and identify the molecular structures which affect the photocurrent properties, we exchanged G segment (containing the seventh helix) of ChR1 with the counterpart of ChR2 and examined the effect of exchanging segment between ChR1 and ChR2 on the photocurrent properties using whole cell recording. This G to g substitution between ChRs resulted in a shift in the wavelength sensitivity. The mutations of Ile291Leu and Asp292Glu in the seventh helix caused marked red shift in the wavelength sensitivity respectively. These new findings indicated that the residues close to the retinal binding position in the $G^N(g^N)$, especially Asp and Ile/Leu, play important role in the determination of the wavelength sensitivity of ChRs. More red shifted I291LD292E may be applied to activate neurons in the deep brain.

４．研究業績

（１）学会における発表　　㊙・有（学会名・演題）

－　日中医学協会助成事業　－

医療への応用に最適化されたチャネルロドプシンの開発

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要旨

Channelrhodopsins (ChRs, including ChR1 and ChR2) function as light gated cation channels. In recent years, ChR2 mediated optogenetic stimulation is widely applied to fire neuronal spiking. However, ChR2 preferred to short wavelength light (470 nm) with rapid desensitization in the photocurrent To modify the properties of ChRs and identify the molecular structures which affect the photocurrent properties, we exchanged G segment (containing the seventh helix) of ChR1 with the counterpart of ChR2 and examined the effect of exchanging segment between ChR1 and ChR2 on the photocurrent properties using whole cell recording. This G to g substitution between ChRs resulted in a shift in the wavelength sensitivity. The mutations of Ile291Leu and Asp292Glu in the seventh helix caused marked red shift in the wavelength sensitivity respectively. These new findings indicated that the residues close to the retinal binding position in the $G^N(g^N)$, especially Asp and Ile/Leu, play important role in the determination of the wavelength sensitivity of ChRs. More red shifted I291LD292E may be used to excite neurons in the deep brain.

Key words: channelrhodopsin; optogenetics; mutation; wavelength sensitivity

緒言：

Channelrhodopsins are seven transmembrane helix opsins, are involved in the photomovement of *Chlamydomonas Reinhardtii*. It is reported that ChR1 and ChR2 function as light gated cation channels (Nagel G et al, 2003). Light illumination on ChR expressing mammalian cells evoked inward photocurrents. In recent years, with precise temporal and neuronal population control, ChRs, especially, ChR2 mediated optogenetic stimulation is becoming an effective and popular tool to fire specific neuronal spiking (Bamann C, et al, 2010; Hegemann P and Möglich A, 2011).

Although sharing high similarities in the residue sequence, the ChR2 photocurrent was maximally activated at about 470 nm with rapidly desensitization while ChR1 was maximally activated at 505 nm with negligible desensitization. To modify the properties of ChRs and identify the molecular structures which differentiate the photocurrent properties, we examined the effect of exchanging the G (g) segment containing the seventh transmembrane helix between ChR1 and ChR2 on the photocurrent properties using whole cell recording, and found that the substitution of seventh helix from ChR1 to ChR2 resulted in a significant shift in the wavelength sensitivity. This new findings indicated that that the residues close to the retinal binding position in the G (g) segment, especially Asp and Ile/Leu, play important role in the determination of the wavelength sensitivity of ChRs.

対象と方法：

ChR variants were created by PCR and KOD plus mutagenesis Kit (Toyobo). These ChR variants were transfected into HEK293 cells using Effectene transfection reagent (Qiagen, Tokyo, Japan). The photocurrent of each ChR variant was measured under whole cell voltage clamp. All the data in the text are analyzed with Clampfit software (Axon Instrument) and presented as means ± S.E. (number). Statistical analysis was performed using Mann-Whitney U test.

結果：

1. Effect of the substitution of G segment of ChR1 with g segment of ChR2 on the wavelength sensitivity

Previously, the N terminal regions of ChR1 (residues 1-345) and ChR2 (residues 1-315) were divided into seven segments (namely, A, B, C, D, E, F, G in ChR1 and a, b, c, d, e, f, g in ChR2) containing the corresponding transmembrane helix as much as possible(Wang H et al, 2009). We replaced the G segment of ChR1 with the counterpart of ChR2, and obtained ChR-ABCDEFg. Compared with ChR1, the maximal sensitivity of ChR-ABCDEFg photocurrent was 20 nm blue-shifted (Fig.1 A). However, the F to f segment replaced chimera, ChR-ABCDEfG didn't show any wavelength shift (Fig.1 B), almost being identical to that of ChR1. Chimera ChR-ABCDEfg showed similar wavelength sensitivity to that of ChR-ABCDEFg (Wang H et al, 2009). These results revealed the importance of G (g) in the determination of wavelength sensitivity.

2. Effect of exchanging the differential residue from g^N to G^N on the wavelength sensitivity

G segment was divided into two parts: G^N subsegment and G^C subsegment (Fig.2A.). Correspondingly, g segment of ChR2 was subdivided into g^N and g^C (Fig.2A.). ChR-ABCDEfG^{N}g^C and ChR-ABCDEfg^{N}G^C were generated. The maximal sensitivity of ChR-ABCDEfG^{N}g^C was almost equal to that of ChR-ABCDEfG whereas the maximum of ChR-ABCDEfg^{N}G^C was clearly 20 nm blue shifted (Fig.2B and C). These results reflected that the G^N (g^N) subsegment determined the wavelength sensitivity. Our previous results indicated that compared with wild ChRs, ChR-ABCDEfg photocurrent was large in the amplitude with enhanced membrane expression. In order to investigate the effect of differential residues between G^N and g^N, We used ChR-ABCDEfg as a backbone to generate residue-exchanged mutants. We substituted each differential residue in g^N with the counterpart of G^N and found that only the wavelength sensitivity of ChRABCDEfg-I291L was partially red-shifted and its G/B ratio was significantly enhanced (Fig.3). The reverse mutant, ChR(ABCDEfG)-L291I showed a blue shift in wavelength sensitivity (Fig.3C). Taken together, these results indicated that Ile^{291} in the g^N act as an more important role in the wavelength shift.

3. Effect of the mutation of Asp to Glu in helix G^N (g^N) on the wavelength sensitivity.

Asp^{292} in g^N of ChR-ABCDEfg is a unique negative charged residue in the vicinity region of retinal chromophore (Fig 4A). Considering the narrow space around chromophore, this charged residue might affect on the wavelength sensitivity through exerting an influence on to the microenvironment around Schiff base. To test this possibility, the Asp^{292} was substituted with Glu. ChR-ABCDEfg, D292E caused the maximal sensitivity shifted from 480 nm to 500 nm (Fig.4B). Double mutation, ChRABCDEfg-I291L/D292E was generated. As shown in, this double mutation induced a more 20 nm red shift (Fig.4C). These results demonstrated the importance of Ile291 and Asp292 in the determination of the wavelength sensitivity.

考察：

In this study, we identified that the g^N to G^N replacement caused a noticeable red-shift and the exchange of Ile291 to Leu in g^N induced a pronounced red-shift. It is noted that the retinal chromophore binds to Lys in G (g) segment. Probably the minor structural change from Ile to Leu affected the microenvironment in the neighboring region of retinal Schiff base and changed the wavelength sensitivity. It is reported that Asp212 to Asn in bR resulted in a red shift of light adapted spectrum (Needleman R, et al. 1991). Consistently, the equal site in ChR1, mutation of Asp292 to Glu produced a similar red-shift. The double mutation, I291L/D292E, unexpectedly led to a more red-shift in the wavelength sensitivity. Up to now, there is no other report about the effect of residue replacement around chromophore binding region in g/G segment (helix) on the wavelength sensitivity or other properties. Probably the tiny change in the structure and properties of Ile 291 exerted a strong impact onto the neighbor, Asp292, and further disrupted the surrounding environment of retinal Schiff base. More red shifted I291LD292E may be used to excite

neurons in the deep brain.

In conclusion, the present study demonstrates that the residues close to the retinal binding position in the $G^N(g^N)$, especially Asp and Ile/Leu, play important role in the determination of the wavelength sensitivity of ChRs.

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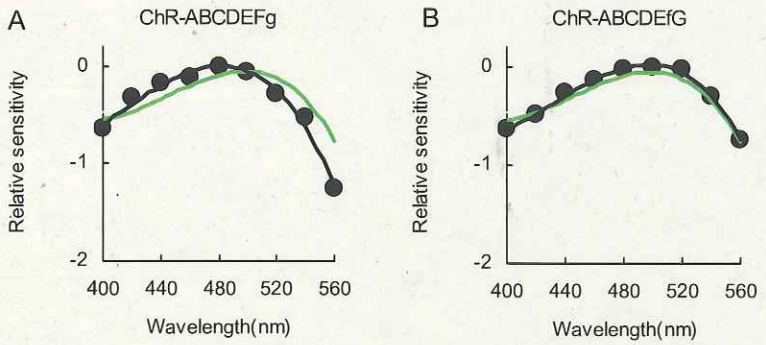

Figure .1. The effect of exchanging F and G segment of ChR1 with f and g of ChR2 on the wavelength sensitivity.

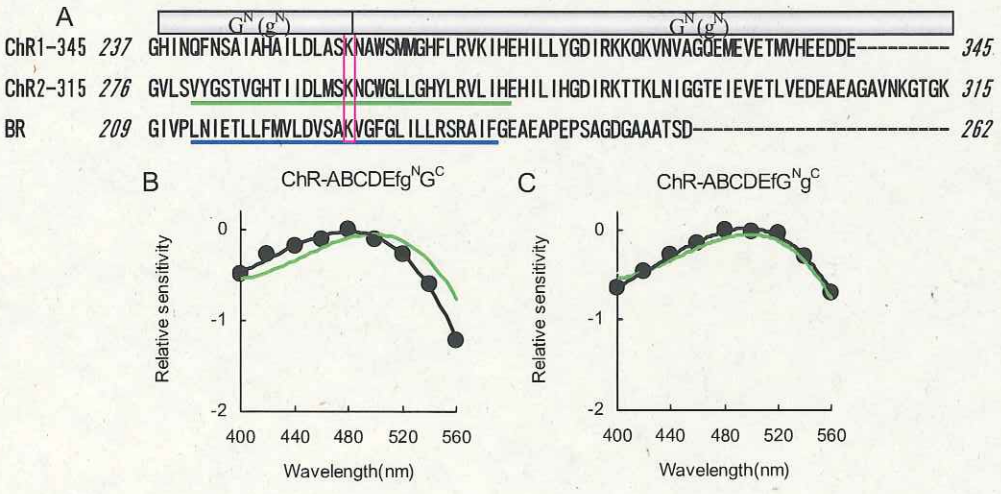

Figure .2. The effect of exchanging G^N and G^C with g^N and g^C on the wavelength sensitivity.

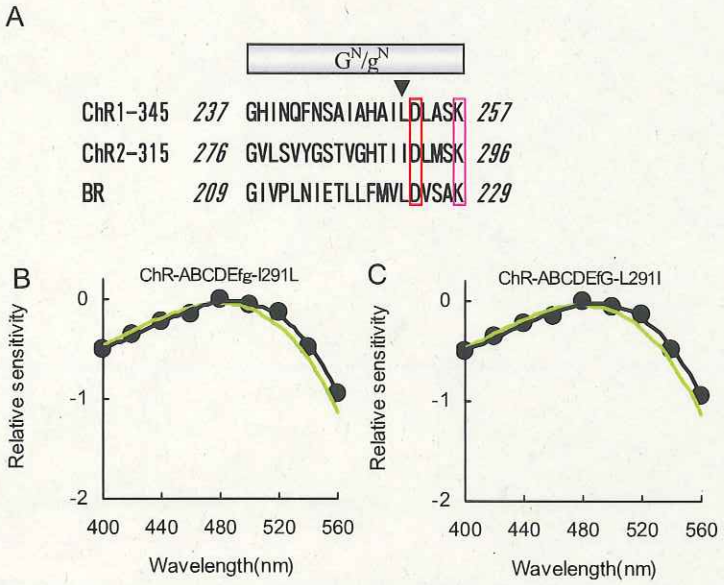

Figure.3. The effect of the mutation ChR-ABCDEfg-I291L (black) and ChR-ABCDEfG-L291I (black)on the wavelength sensitivity.

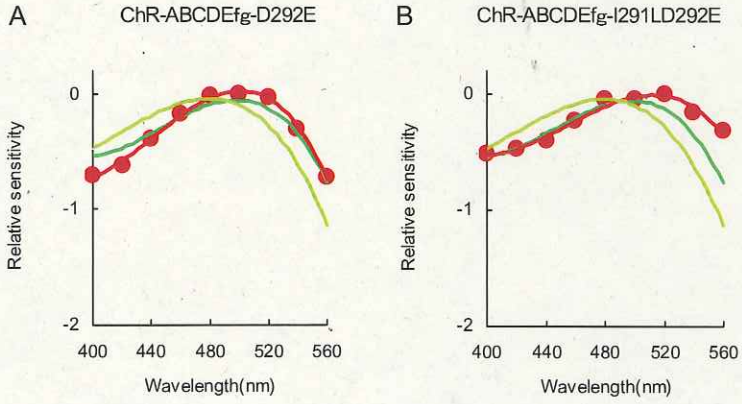

Figure.4. The effect of mutation ChR-ABCDEfg-D292E (red) and the ChR-ABCDEfg-I291LD292E(red) on the wavelength sensitivity.

作成日：2011 年 3 月 3 日