科技部補助專題研究計畫成果報告
期末報告

利伯氏遺傳視神經病變發病之性別差異研究

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執 行 期 間 ：102 年 08 月 01 日至 103 年 07 月 31 日
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計 畫 主 持 人 ：王安國

計畫參與人員：學士級-專任助理人員：林珮蓁

處 理 方 式 ：
1.公開資訊：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢
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中 華 民 國 103 年 10 月 31 日

中 文 摘 要 ： 利伯氏遺傳視神經病變(LHON)是一種母系遺傳的粒線體突變，它會造成急性視力減退，好發於男性，佔 80%，視力常惡化至零點一以下，伴隨視野缺損，兩眼可同時發病。粒線體突變會傳給所有母系細胞，然而具有突變基因的男性發病率僅有 50%，女性更只有 10%，顯示其發病率低與好發於男性的特點。此一特點，目前無明確原因，推測可能源於神經保護機轉，若能瞭解此機轉可能有助於未來的治療，我們提出計畫來探討雌激素與其受器在利伯氏遺傳視神經病變致病機轉所扮演的角色，特別著重於其發病率與性別差異。雌激素與其衍生物已証明具有神經保護作用，我們期待經由瞭解它在利伯氏遺傳視神經病變的作用，能帶來治療的新方法。

我們利用螢光免疫染色，來檢查三種雌激素受器(ERalpha, ERbeta, GPR30)在人類與小鼠視網膜之表現，發現雌激素受器有明顯的物種差異。ERalpha 蛋白在人類與小鼠視網膜均強烈表現在視網膜節細胞核。ERbeta 蛋白也表現在視網膜節細胞，但是強度較弱，此外，它還被發現表現於內叢層，尤其是人類視網膜表現很強，可能與神經突觸傳遞訊息功能有關。GPR30 蛋白在小鼠視網膜表現在 Muller 細胞，但是，在人類視網膜上， 則表現於視網膜節細胞的胞質與軸漿。

此外，我們施行視神經壓迫傷手術，檢查小鼠視網膜在受傷後之雌激素受器表現變化。我們發現手術後一天，GPR30 蛋白在小鼠視網膜表現增高。三天後，三種雌激素受器表現均有上昇現象，顯示雌激素受器可能與神經保護作用有關。五天後可見所有細胞蛋白(ERalpha, ERbeta, GPR30 and BRN3)都逐漸減低，可能代表受傷後之神經細胞損失。由這些結果推測，GPR30 蛋白可能代表神經受損後的神經膠細胞反應，而 ERalpha 與 ERbeta 蛋白則代表神經受損後的神經節細胞反應，顯示這些雌激素受器在神經修復與神經保護都有一定的功能。

中文關鍵詞： 利伯氏遺傳視神經病變,雌激素受器

英 文 摘 要 ： Leber’s hereditary optic neuropathy (LHON) is a maternally transmitted disease caused by mitochondria DNA (mtDNA) mutation. It is characterized by acute and subacute visual loss predominantly affecting young man. The mtDNA mutation is transmitted to all the maternal lineages. However, only approximately 50% of men and 10% of women harboring a pathogenic mtDNA mutation develop optic neuropathy, reflecting

both the incomplete penetrance and its unexplained male prevalence that over 80% of patients are male. It is still the major unexplained issue in LHON, which may result from undefined neuroprotective mechanism potentially beneficial for future treatment. We propose this project to explore the role of estrogen and its receptor in the pathogenesis of LHON. Estrogen and its non-feminizing analogues have already proved its efficacy in the neuroprotection. We expect there will be even more therapeutic options in the future if the mechanism for LHON gender difference is well understood.

We have examined the expression of estrogen receptors (ERalpha, ERbeta, GPR30) in human and mouse retina. There is significant species difference of estrogen receptor expression in the retina. ERalpha protein is intensely expressed on the nucleus of retinal ganglion cells in both mouse and human retina. ERbeta protein is also expressed in the retinal ganglion cells, but of less intensity than ERalpha. It is also found in the inner plexiform layer implying its role in the synaptic transmission, especially in human retina. GPR30 seems to be expressed in the Muller cell of mouse retina. But, it exists in the cytoplasm and axonal plasma of the human retinal ganglion cells.

Following optic nerve injury, we examined the expressional change of estrogen receptor. At 1 day post-crush, there is an up-regulated expression of GPR30 protein. At 3 days, all the estrogen receptors were strongly expressed in response to the axonal injury. From 5 days afterwards, all the markers including ERalpha, ERbeta, GPR30 and BRN3 diminished gradually, which may reflect neuronal cell loss. GPR30 may represent the glial reaction to the optic nerve injury. ERalpha and ERbeta may reflect the retinal ganglion cell reaction following optic nerve injury in the mouse. All these estrogen receptor may react and play an important role in the neuro-repair and neuro-protection.

英文關鍵詞： Leber’s hereditary optic neuropathy, estrogen receptor

科技部補助專題研究計畫成果報告

（□期中進度報告/V期末報告）

利伯氏遺傳視神經病變發病之性別差異研究

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計畫編號：MOST 102-2629-B-075 -001 -

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計畫主持人：王安國

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中　華　民　國　103　年　10　月　31　日

目錄

一.中文摘要

利伯氏遺傳視神經病變(LHON)是一種母系遺傳的粒線體突變，它會造成急性視力減退，好發於男性，佔80%，視力常惡化至零點一以下，伴隨視野缺損，兩眼可同時發病。粒線體突變會傳給所有母系細胞，然而具有突變基因的男性發病率僅有50%，女性更只有10%，顯示其發病率低與好發於男性的特點。此一特點，目前無明確原因，推測可能源於神經保護機轉，若能瞭解此機轉可能有助於未來的治療，我們提出計畫來探討雌激素與其受器在利伯氏遺傳視神經病變致病機轉所扮演的角色,特別著重於其發病率與性別差異。雌激素與其衍生物已証明具有神經保護作用，我們期待經由瞭解它在利伯氏遺傳視神經病變的作用，能帶來治療的新方法。

我們利用螢光免疫染色，來檢查三種雌激素受器(ERalpha, ERbeta, GPR30)在人類與小鼠視網膜之表現，發現雌激素受器有明顯的物種差異。ERalpha蛋白在人類與小鼠視網膜均強烈表現在視網膜節細胞核。ERbeta蛋白也表現在視網膜節細胞，但是強度較弱，此外，它還被發現表現於內叢層，尤其是人類視網膜表現很強，可能與神經突觸傳遞訊息功能有關。GPR30蛋白在小鼠視網膜表現在Muller細胞，但是，在人類視網膜上，則表現於視網膜節細胞的胞質與軸漿。

此外，我們施行視神經壓迫傷手術，檢查小鼠視網膜在受傷後之雌激素受器表現變化。我們發現手術後一天，GPR30 蛋白在小鼠視網膜表現增高。三天後，三種雌激素受器表現均有上昇現象，顯示雌激素受器可能與神經保護作用有關。五天後可見所有細胞蛋白(ERalpha, ERbeta, GPR30 and BRN3)都逐漸減低，可能代表受傷後之神經細胞損失。由這些結果推測，GPR30 蛋白可能代表神經受損後的神經膠細胞反應，而 ERalpha 與 ERbeta 蛋白則代表神經受損後的神經節細胞反應，顯示這些雌激素受器在神經修復與神經保護都有一定的功能。

二. 英文摘要

Summary

Leber's hereditary optic neuropathy (LHON) is a maternally transmitted disease caused by mitochondria DNA (mtDNA) mutation. It is characterized by acute and subacute visual loss predominantly affecting young man. Vision usually deteriorates to the degree of less than 20/200, accompanied with a cecocentral scotoma. Both eyes are involved with or without intervals. The mtDNA mutation is transmitted to all the maternal lineages. However, only approximately 50% of men and 10% of women harboring a pathogenic mtDNA mutation develop optic neuropathy, reflecting both the incomplete penetrance and its unexplained male prevalence that over 80% of patients are male. It is still the major unexplained issue in LHON, which may result from undefined neuroprotective mechanism potentially beneficial for future treatment. We propose this project to explore the role of estrogen and its receptor in the pathogenesis of LHON especially focusing on the incomplete penetrance and male prevalence. Estrogen and its non-feminizing analogues have already proved its efficacy in the neuroprotection. We expect there will be even more therapeutic options in the future if the mechanism for LHON gender difference is well understood.

We have examined the expression of estrogen receptors (ERalpha, ERbeta, GPR30) in human and mouse retina. There is significant species difference of estrogen receptor expression in the retina. ERalpha protein is intensely expressed on the nucleus of retinal ganglion cells in both mouse and human retina. ERbeta protein is also expressed in the retinal ganglion cells, but of less intensity than ERalpha. It is also found in the inner plexiform layer implying its role in the synaptic transmission, especially in human retina. GPR30 seems to be expressed in the Muller cell of mouse retina. But, it exists in the cytoplasm and axonal plasma of the human retinal ganglion cells.

Following optic nerve injury, we examined the expressional change of estrogen receptor. At 1 day post-crush, there is an up-regulated expression of GPR30 protein in the mouse retina. At 3 days, all the estrogen receptors were strongly expressed in response to the axonal injury, which indicate estrogen receptor does play an important role in the neuro-protection. From 5 days afterwards, all the markers including ERalpha, ERbeta, GPR30 and BRN3 diminished gradually, which may reflect neuronal cell loss following axonal injury. GPR30 may represent the glial reaction to the optic nerve injury. ERalpha and ERbeta may reflect the retinal ganglion cell reaction following optic nerve injury in the mouse. All these estrogen receptor may react and play an important role in the neuro-repair and neuro-protection.

三. 報告內容

3.1 緣由與目的

A.Background

Leber's hereditary optic neuropathy (LHON)[1]

LHON is a maternally transmitted disease characterized by acute and subacute visual loss predominantly affecting young man.[1-3] It usually onsets between 15 to 35 years of age, with a male predominance.[1,4] The course of visual loss is usually acute or subacute. The optic disc became hyperemic and associated with peripapillary telangiectasia. The retinal nerve fiber layers are swollen. Over months, the disc edema subsided and became pallor and atrophic. Vision deteriorated to the degree of less than 20/200, commonly accompanied with a cecocentral scotoma.[1] Both eyes are involved with or without intervals.[1]

Primary mutations of mtDNA in LHON[1]

Three primary mitochondrial DNA (mtDNA) mutations underlie the main pathogenesis of LHON. The first association of a mtDNA mutation with LHON was reported by Wallace and colleagues in 1988.[5] They found a mtDNA mutation at nucleotide 11778, which encodes the NADH dehydrogenase subunit 4 of Complex I of the respiratory chain. In 1991 Huoponen at al[6] and Howell et al identified the second mtDNA mutation at nucleotide 3460, which encodes the subunit 1 of NADH dehydrogenase.[7] The third mtDNA mutation at nucleotide 14484 was found in 1992,[8] which converts methionine to valine in subunit 6 of NADH dehydrogenase.

These three mutations, 11778, 3460 and 14484, of mtDNA are considered as primary mutations, since each alone can cause LHON. The percentage for 11778 mutation is 50-70%, for 3460 mutation is 8-25% and for 14484 mutation is 10-15% in Caucasian.[3] Our previous study showed that the percentage in Chinese is 83%, 0% and 17% respectively.[9]

Gender prevalence of LHON[1]

Since 1988 when the first primary mutation was associated with LHON, there were intense studies on the clinical and molecular aspects of this disorder. Nevertheless, the pathogenesis of LHON is still unclear especially in the area of gender prevalence and penetrance.[1]

The mtDNA mutation is transmitted to all the maternal lineages. However, only approximately 50% of men and 10% of women harboring a pathogenic mtDNA mutation develop optic neuropathy, reflecting both the incomplete penetrance and the gender prevalence difference.[1] LHON is well known for its male prevalence that over 80% of

patients are male.[1,2] This male predominance exists in different mutation groups with a male to female ratio of 3:1, 4–6:1 and 8:1 in patients harboring 3460, 11778 and 14484 mutations, respectively.[1,4] The reason for this male predominance remains unknown.

Penetrance of LHON[1]

The penetrance in LHON is incomplete and variable that a positive family history was found in 50% of patients with 11778 mutation, 71% with 3460 mutation and 100% with 14484 mutation.[1,10] The penetrance of LHON is variable even with the same mutation in homoplasmic fashion within the same family in different pedigree branch.[1,11] All these features can not be explained by a single point mutation of mtDNA alone. Thus, genetic and epigenetic factors have been presumed to be involved in the penetrance of the LHON. Prior investigated genetic modifiers include heteroplasmy, [12-15] secondary mtDNA mutations,[16-17] mtDNA haplogroup,[18-20] X-linked modifying gene or susceptibility locus,[21-23] and other nuclear genes.[24] Tobacco and alcohol consumption were considered as epigenetic factors which may affect the penetrance as well.[25-26]

Estrogen and its receptors

Estrogens are a family of cholesterol-derived steroid hormones that may regulate growth and differentiation in various tissues including reproductive system and central nervous system.[27,28] It is well known to have proliferative effect on the breast and uterus, and being involved in the pathogenesis of the breast and endometrial cancer.[29] Its action was mediated through estrogen receptors (ERs). Estrogens enter the nucleus of the target cell and then fuse with ERs to make an estrogen-ER complex, which in turn may react with the estrogen response element (ERE) of the target genes. Then the transcription of these estrogen-dependent genes will be activated.

Two major estrogen receptors, ERalpha and ERbeta, exist on various tissues. ERalpha, encoded by ESR1 gene on chromosome 6q25, is the major receptor in the adult uterus and responsible for the proliferative reaction in uterus.[30,31] It seems to be the major receptor mediating estrogen reaction. ERbeta, encoded by ESR2 gene on chromosome 14q22-24, has been cloned later and its mRNA has been found in different areas of the brain and other organs.[31-33] The specific functions of the ESR1 and ESR2 are still under investigation that similar or distinct effects, which may even being opposite, have been reported.[31,34,35] In addition, these two ERs may have different biologic functions, as indicated by their distinct expression patterns and different phenotypes of two knockout animals.[36-38]

Recently, a third possible estrogen receptor, GPR30, has been found. It is a g-protein coupled receptor, which may react with estrogen. GPR30 was found to be expressed in various tissues including breast cancer and brain. The exact function of this receptor remains to be elucidated.[39,40]

The expression of ERs in the retina

Estrogen receptor protein was detected in bovine and rat retinas by western blot and immunohistochemical staining in 1998.[41] Estrogen receptor mRNA exists in the retina and retinal pigment epithelium of rat, rabbit and human eyes.[42] With western blot and immunohistochemistry, ERalpha protein was found in the retina and retinal pigment epithelium of young female eyes but not from men and postmenopausal women.[43] ERalpha immunostaining was observed across neuroretina, in most nuclei of outer and inner nuclear layer, and some nuclei of ganglion cell layer.[43] Munaut C et al reported that ERalpha and ERbeta proteins were detected in the human retina by western blot analysis.[44] ERalpha immunostaining was positive across the human retina, with more intensity over ganglion cell layer.[44] ERbeta immunostaining was observed in the ganglion cell layer and choroid, which appears to be similar in tissues of both sexes.[44] ERalpha and ERbeta mRNA expression was detected by RT-PCR in ocular tissues from patients of both sexes. The expression of ERbeta mRNA was relatively constant between different donors, but there was more variation with ERalpha mRNA.[44] Thus, the expression of ERaplha in retina seems to be variable or undetermined yet, which may need further clarification. In addition, none of these studies explore the expression of ERs in the optic nerve head. Besides, the expression of GPR30 in human retina has not been investigated yet.

In response to neuronal injury, the expression of ERs may increase as a part of the neuroprotective effect of estrogen. Vanoye-Carlo described that significant increase of ERs was found in hippocampus after kainic acid insult. They also observed a relocation of ERbeta from the cytoplasm to the nucleus of neuronal cells.[45]

Estrogen and neuroprotection

Estrogen has been shown to have neurotrophic and neuroprotective effect in various tissues.[46] In retinal pigment epithelium cells, 17-beta estradiol protects the cells from oxidative stress through the ERbeta-dependent mechanism. The cytoprotection occurs by reduction of ROS production, induction of cellular antioxidant genes, and preservation of mitochondrial function.[47] It may also help to reduce retinal damage in a rat model of ischemia-reperfusion injury. The retinal injury in the inner retina was significantly reduced with recovery of b-wave amplitude through the inhibitory effect of 17-beta estradiol on leukocyte accumulation.[48] In a retinal ganglion cell line (RGC-5), 17-beta estradiol and its analogues may protect the cells from glutamate-induced cytotoxicity through mechanisms independent of estrogen receptors.[49] In addition to its effect on the ganglion cell body, it has been shown that 17-beta estradiol significantly prevented TNF-induced axonal loss in the optic nerve.[50]

Estrogen and LHON

Giordano C. et al. tried to explore the reasons for the higher prevalence of LHON in males and they proposed the potential compensatory effect of estrogen on mutant cell metabolism may underlie the gender prevalence.[51] In their study, cybrid cell lines were constructed using enucleated cells from LHON patients (11778, 3460, 14484) as mitochondria donors and the osteosarcoma (143B.TK$^-$)-cell line as acceptor rho0 cell line. They found that LHON cybrids in galactose medium presented overproduction of ROS, which resulted in decreased mitochondrial membrane potential and increased apoptotic rate compared with control cybrids. Treatment with 17-beta estradiol may significantly rescue these pathologic conditions through estrogen receptors by activation of antioxidant enzyme superoxide dismutase 2.[51] They have provided a possible metabolic basis for the unexplained male predominance in LHON. However, the osteosarcoma cell line is derived from a 13-year-old female patients' tumor and therefore the LHON cybrids and control cybrids were all female cells with various mitochondrias. The increased apoptosis in the LHON cybrids which could be rescued by 17-beta estradiol indicates that estrogen do have a neuroprotective effect on LHON. On the other hand, it does not prove there is a gender difference of injury susceptibility nor was gender difference of estrogen level or estrogen receptor sensitivity.

B. Purpose

The pathogenesis of LHON and the reason for male prevalence are not known yet. We try to explore the role of estrogen in the pathogenesis of LHON especially focusing on the incomplete penetrance and male prevalence. We will examine the expression of estrogen receptors (ERalpha, ERbeta, GPR30) in the human retina and optic nerve. Double staining technique will be used to verify the exact cell types in the sections. In addition, we will try to investigate the expressional change of estrogen receptor in a stressed retina. We plan to conduct optic nerve crush injury in adult mice and examine the expressional change with immunohistochemistry.

3.2 研究方法與過程

Material and Methods

Human Tissues

All experiments will be performed according to institutional protocol guidelines.

Experiments involving human tissues will be conformed to the guidelines set forth in the Declaration of Helsinki for the use of human tissue in research, and will be approved by the Institutional Review Board of the Taipei Veterans General Hospital for the use of human tissue in research work.

Animal and surgery

Adult Balb/c mice (male and female) were kept in the animal facility of our hospital. Institute guideline was followed on handling of animals. Mice were given food and water ad libitum. Adult mice that were at least two months old and body weight over 25 g, were intraperitonially anesthetized with a combination of ketamine (1 mg/10 g body weight) and xylazine (0.2 mg/10 g body weight). Surgery is done under sterile conditions using a stereomicroscope. A conjunctival incision is made over the dorsal aspect of one eye, which is then gently rotated downward in the orbit. The orbital muscles are teased and deflected aside to expose the optic nerve at its exit from the globe, which is then crushed twice with jewelers forceps near the back of the eye (within 0.5 mm). Care is taken not to damage the ophthalmic artery and retrobulbar sinus. The eye is then rotated back into position and rinsed with sterile saline and covered with 0.3% garamycin ointment. The mice are kept warm in cage till recovery. Mice are sacrificed at different time points using cervical dislocation under anesthesia.

Immunohistochemistry

(a) Preparation and Fixation

For human tissue sections, the posterior segments of donated eyeballs were dissected carefully at the limbal region to make a small hole for fixative penetration. These posterior segments were fixed in 4% paraformladehyde/PBS solution at 4^{o}C overnight. The eyeballs were transferred to 30% sucrose/PBS overnight. In the next morning, eyeballs were divided into small pieces, submerged in OCT compound, and frozen on dry ice. Sections were cut at 12 μm thick and collected on SuperFrost slides (Fisher Scientific, PA, USA). These sections were stored at –20^{o}C until use.

For mice tissue sections, eyes were enucleated and maintained in Hank's solution. Posterior eyecups were fixed with 4% paraformadehyde in 0.1M phosphate buffer (pH 7.25) for 1 hr at 4^{o}C. Incubate the eyeballs with 30% sucrose/PBS overnight, then embed in OCT, and stored at －70^{o}C till use. Sections were cut at 12 μm thick and collected on SuperFrost slides (Fisher Scientific, PA). The sections were fixed with 4% paraformaldehyde solution for one hour at room temperature. Then the sections were washed with PBS/0.1% Tween (PBST) for 15 min, followed by PBS for 15 min twice.

(b) Immunostaining

Sections are fixed with 4% paraformaldeyhyde for 20 min, and then pretreated with 0.1% trypsin for 10 min at room temperature. They were then incubated with 0.2% Triton X-100 in PBS for 10 min, and then with 5% BSA in PBS for 60 min at room temperature. Sections were incubated in primary antibodies, diluted in 5% BSA/PBS, for overnight at 4^{o}C. They were then incubated with matched secondary antibodies for 2 hours at room temperature. Each step was preceded by washes in 1xPBS. Sections were then incubated with 4', 6-diamidino-2-phenyindole (DAPI, 0.16 μg/ml) for 15 min at room temperature for nuclear staining, and washed with 1xPBS for three times. The sections were mounted with antifade medium (Citifluoro, Canada) and stored at 4^{o}C. For estrogen receptors, primary antibodies include: anti-ERα antibody (Santa Cruz), anti-ERβ antibody (Santa Cruz), anti-GPR30 antibody (Santa Cruz).

(c) Image acquisition

Sections and coverslips will be analyzed with a Nikon Diaphot inverted microscope (E300, Nikon, Tokyo, Japan) equipped with a MicroMax cool CCD (Princeton Instrument, Trenton, NJ, USA). Image acquisition is performed using MetaMorph software (Universal Image Corporation, Downingtown, PA, USA) with individual filter sets for each channel and are assembled with Adobe PhotoShop (Adobe Systems, CA).

3.3 研究結果

A. Expression of estrogen receptor in mouse retina

To verify the localization of estrogen receptors in the normal mouse retina, immunostaing was performed using anti- estrogen receptors (ERalpha, ERbeta, GPR30) antiserum. The result showed that ERalpha protein is expressed in the ganglion cell layer, inner nuclear layer, outer plexiform layer and inner segment of photoreceptor. ERbeta protein is expressed in the ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, inner segment of photoreceptor and retinal pigment epithelium. GPR30 protein is expressed on the nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer and outer plexiform layer. In addition, we found that GPR30 protein is expressed in a fascicular pattern which transverse the entire retinal thickness and mimic Muller cell morphology.

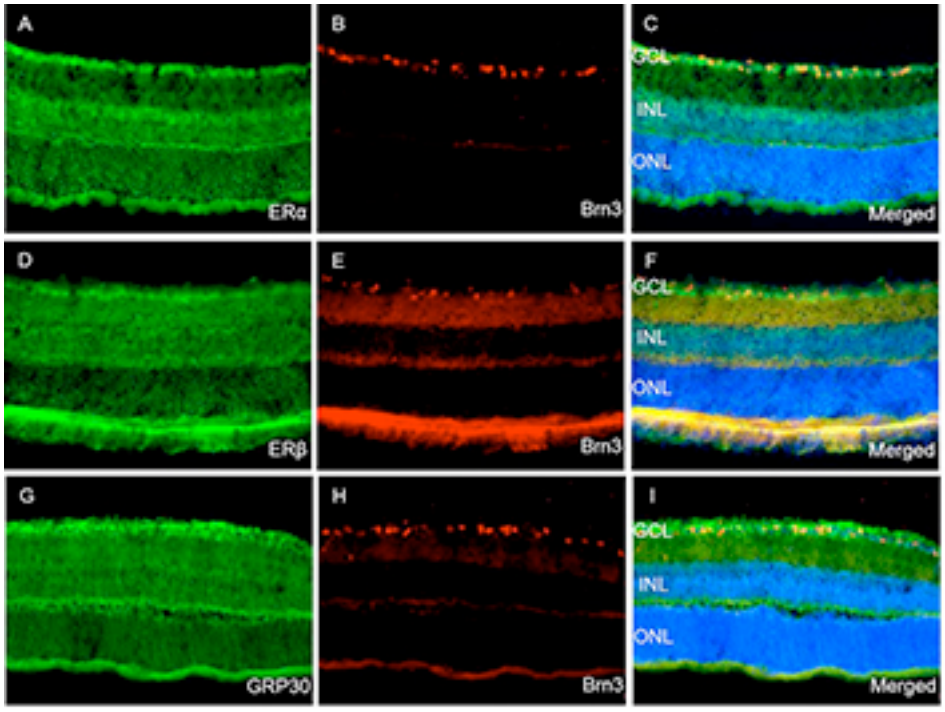

Figure 1. Expression of estrogen receptors (ERalpha, ERbeta, GPR30) in mouse retina. Normal adult mouse were stained with estrogen receptor antibodies: anti-ERalpha antibody (green, A, C), ant-ERbeta antibody (green, D, F) and anti-GPR30 antibody (green, G, I). Brn3 (red) was used as the marker of retinal ganglion cell (RGC). DAPI (blue) was used as a nucleus marker. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

To further investigate the cell type of retina expressing estrogen receptors (ERalpha, ERbeta, GPR30) in the ganglion cell layer, we further characterized these neurons with immune marker such as anti-Brn3 antibody, which is the marker for retinal ganglion cell. The

results showed that ERalpha and ERbeta protein was universally expressed on every cell in the ganglion cell layer, and was co-localized with DAPI stain. However, BRN3 protein was expressed on some cells in the ganglion cells, and was co-localized with ERalpha and ERbeta protein respectively (Figure 2, A-F). On the other hand, GPR30 protein was expressed as cellular processes around these neuronal cells in the ganglion cell layer, similar to the foot process of Muller cells.

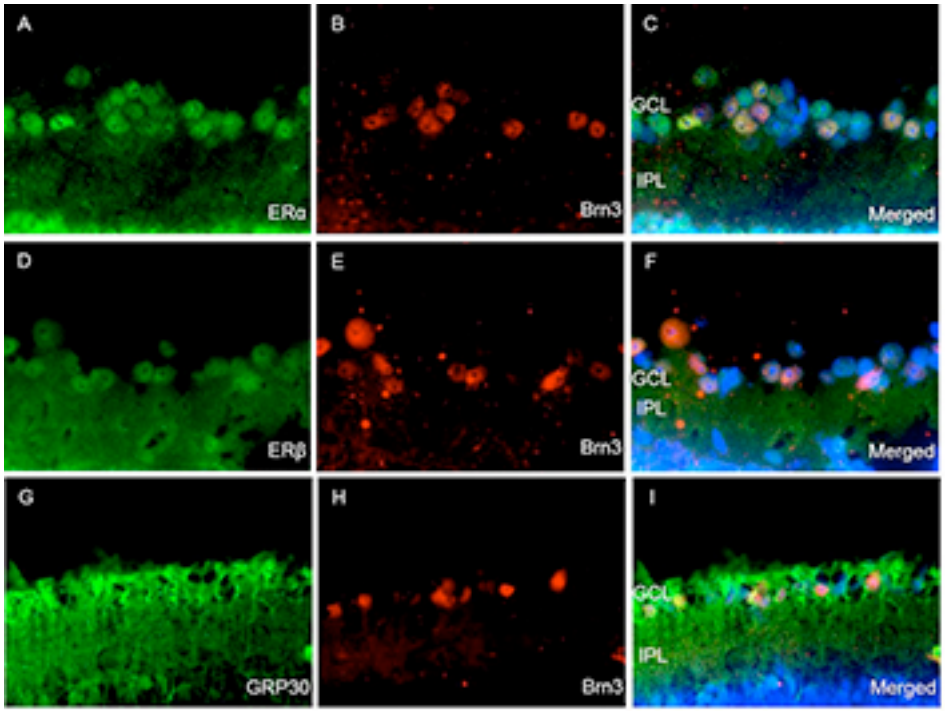

Figure 2. Expression of estrogen receptors in mouse ganglion cell layer (GCL). At high magnification, the expression of estrogen receptors (ERalpha, ERbeta and GPR30) was examined in the GCL. ERalpha and ERbeta were present in the nuclear portion of each neuron in the GCL, and GPR30 was expressed surrounding these neurons. On the other hand, not all neurons in GCL express the BRN3 protein. anti-ERalpha antibody (green, A, C); ant-ERbeta antibody (green, D, F); anti-GPR30 antibody (green, G, I); Anti-Brn3 (red); DAPI (blue); GCL: ganglion cell layer; IPL: inner plexiform layer.

B. Expressional change of estrogen receptor protein in mouse retina following optic nerve crush injury.

We also try to investigate the expressional change of estrogen receptors in a stressed retina. We used optic nerve crush injury in adult mice as a model of traumatic optic neuropathy. Immunohistochemical staining was performed to measure the expressional change of estrogen receptors in the retinal ganglion cell layer. The results showed that

GPR30 protein is up-regulated in the retina from day 1 after crush injury.

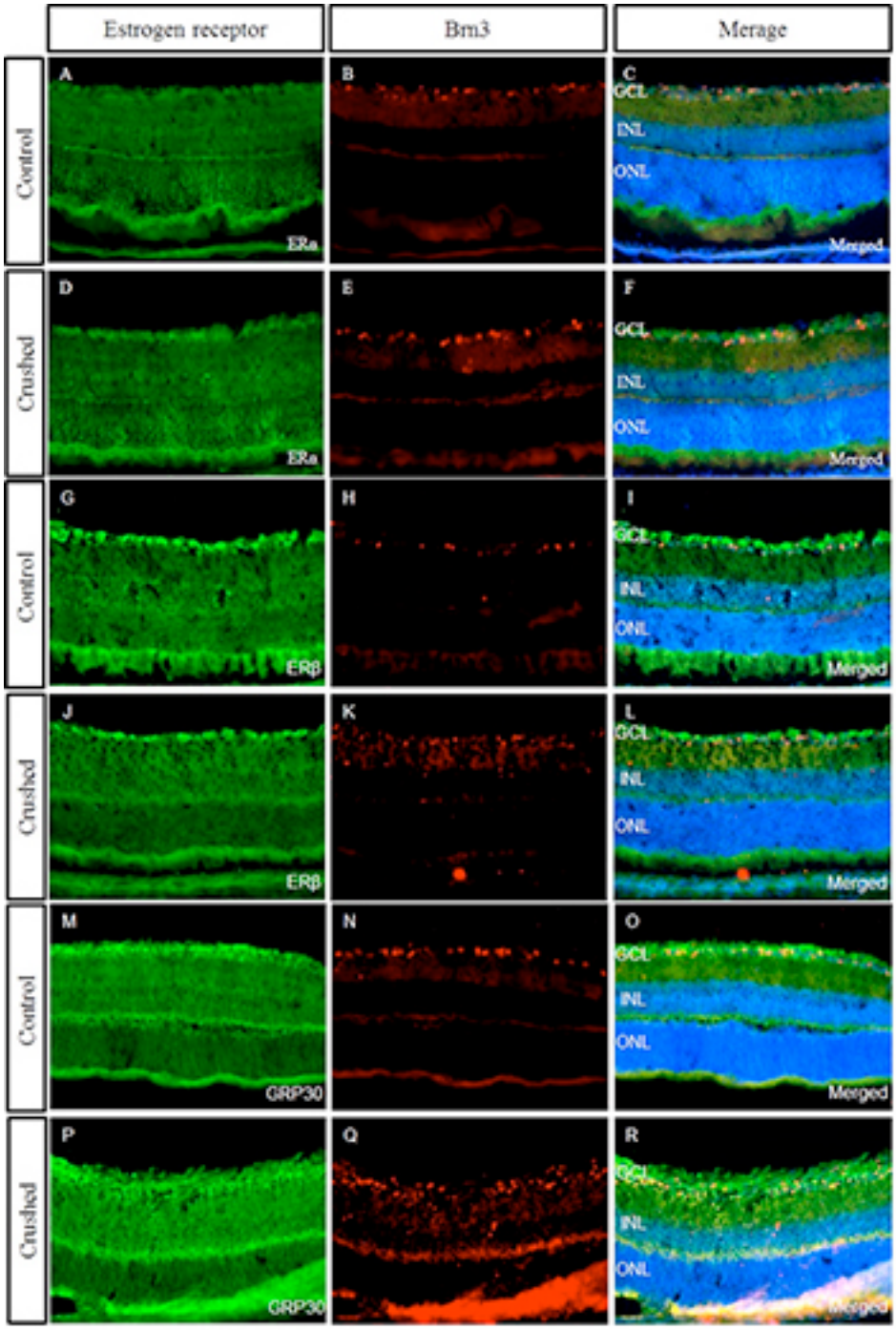

Figure 3. The expression of estrogen receptors in adult mouse retina 1 day after optic nerve injury. Expression of GPR30 protein is increased over the crushed retina at day 1. anti-ERalpha antibody (green, A,C, D, F); ant-ERbeta antibody (green, G, I, J, L); anti-GPR30 antibody (green, M, O, P, R); Anti-Brn3 (red); DAPI (blue); GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

At day 3 after optic nerve crush, we found that ERalpha, ERbeta and GPR 30 protein increased their expression in retinal ganglion cell layer as compared with control group.

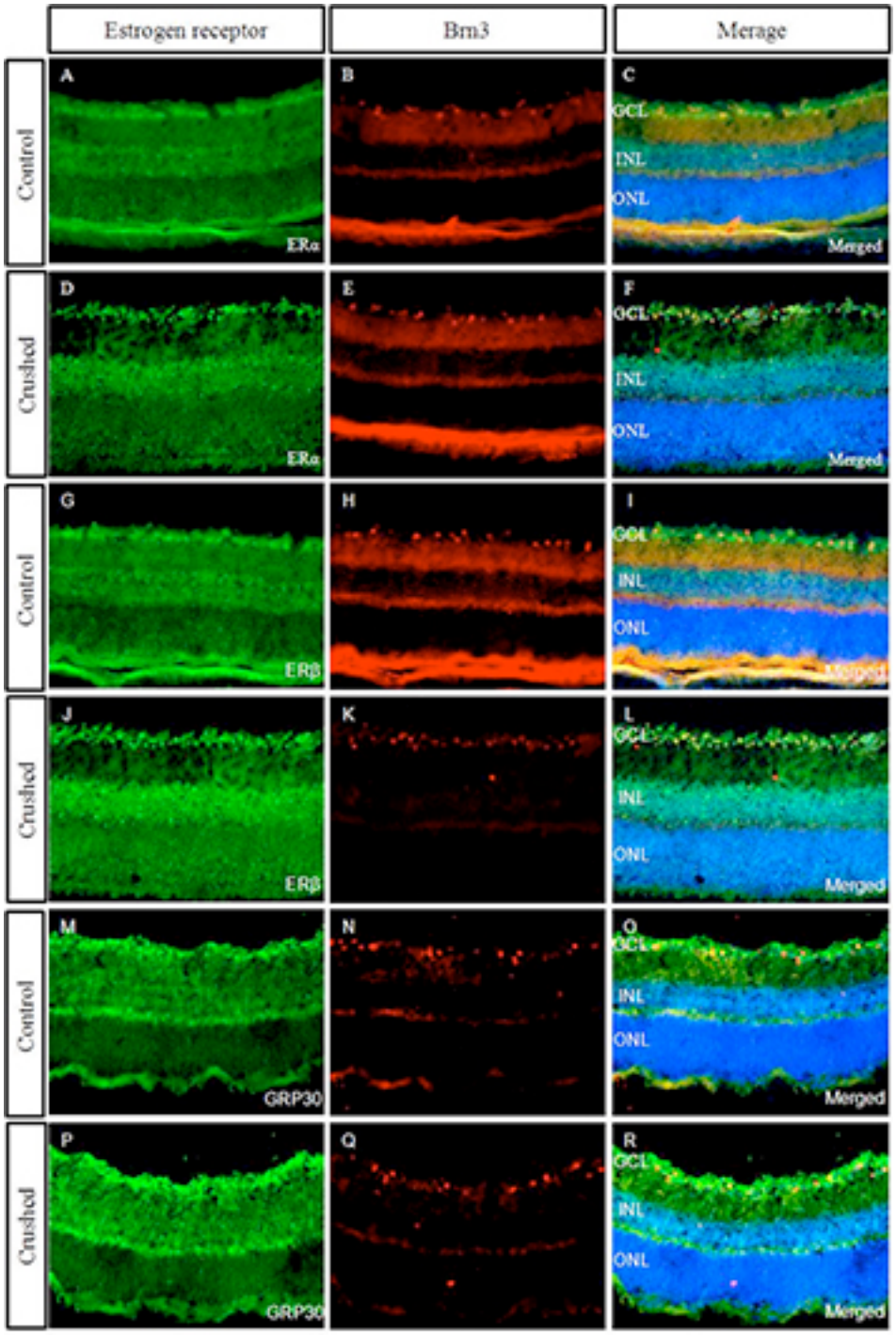

Figure 4. The expression of estrogen receptors in adult mouse retina 3 days after optic nerve injury. The expression of ERalpha, ERbeta and GPR30 increased in retinal ganglion cell layer at day 3. anti-ERalpha antibody (green, A,C, D, F); ant-ERbeta antibody (green, G, I, J, L); anti-GPR30 antibody (green, M, O, P, R); Anti-Brn3 (red); DAPI (blue); GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

However, the expression of ERalpha, ERbeta, GPR30 and Brn3 decreased significantly in retinal ganglion cell layer after optic nerve crush from day 5 to day 7.

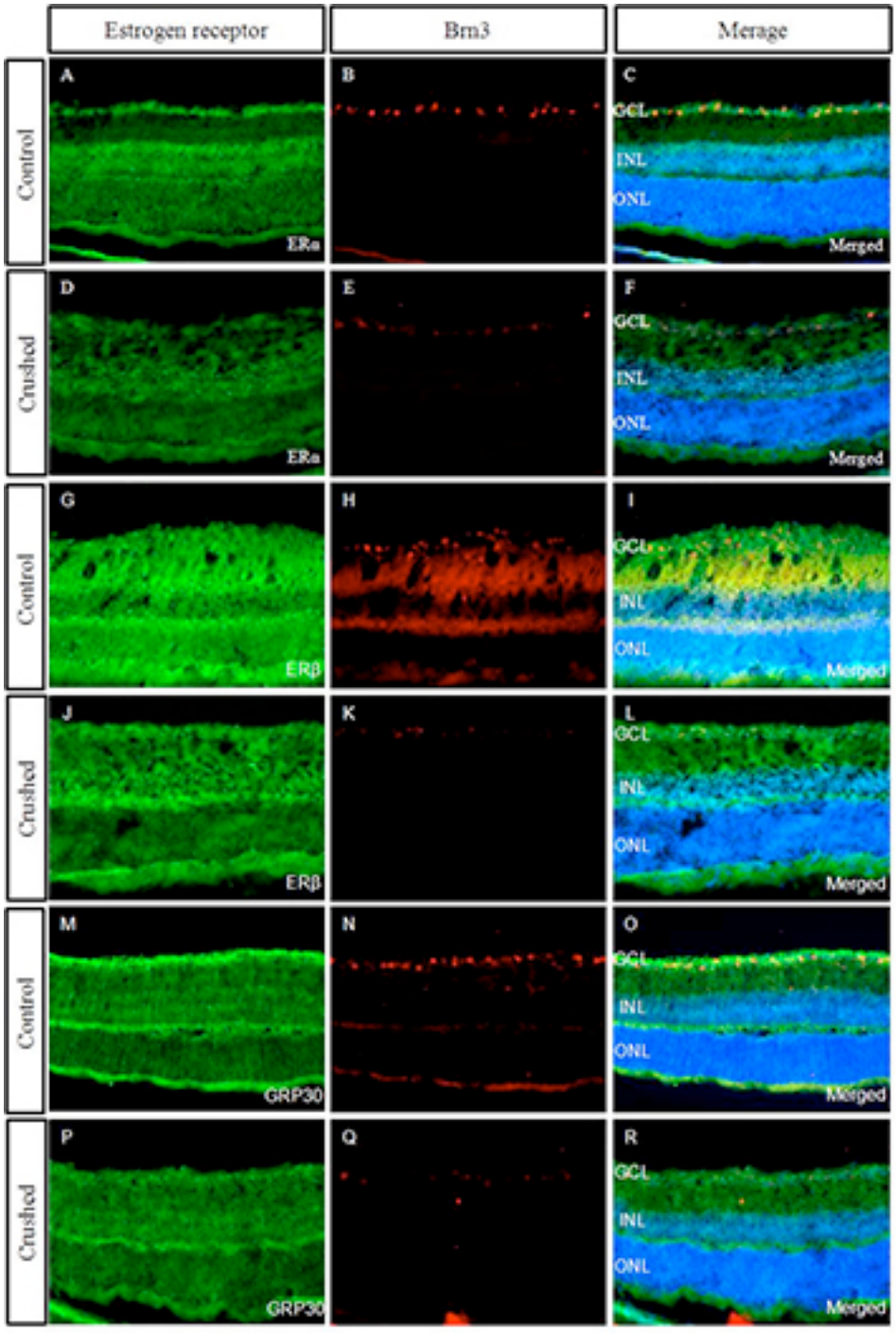

Figure 5. The expression of estrogen receptors in adult mouse retina 5 days after optic nerve injury. The expression of ERalpha, ERbeta, and GPR30 in retinal ganglion cell layer decreased significantly at 5 days following optic nerve crush. anti-ERalpha antibody (green, A,C, D, F); ant-ERbeta antibody (green, G, I, J, L); anti-GPR30 antibody (green, M, O, P, R); Anti-Brn3 (red); DAPI (blue); GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

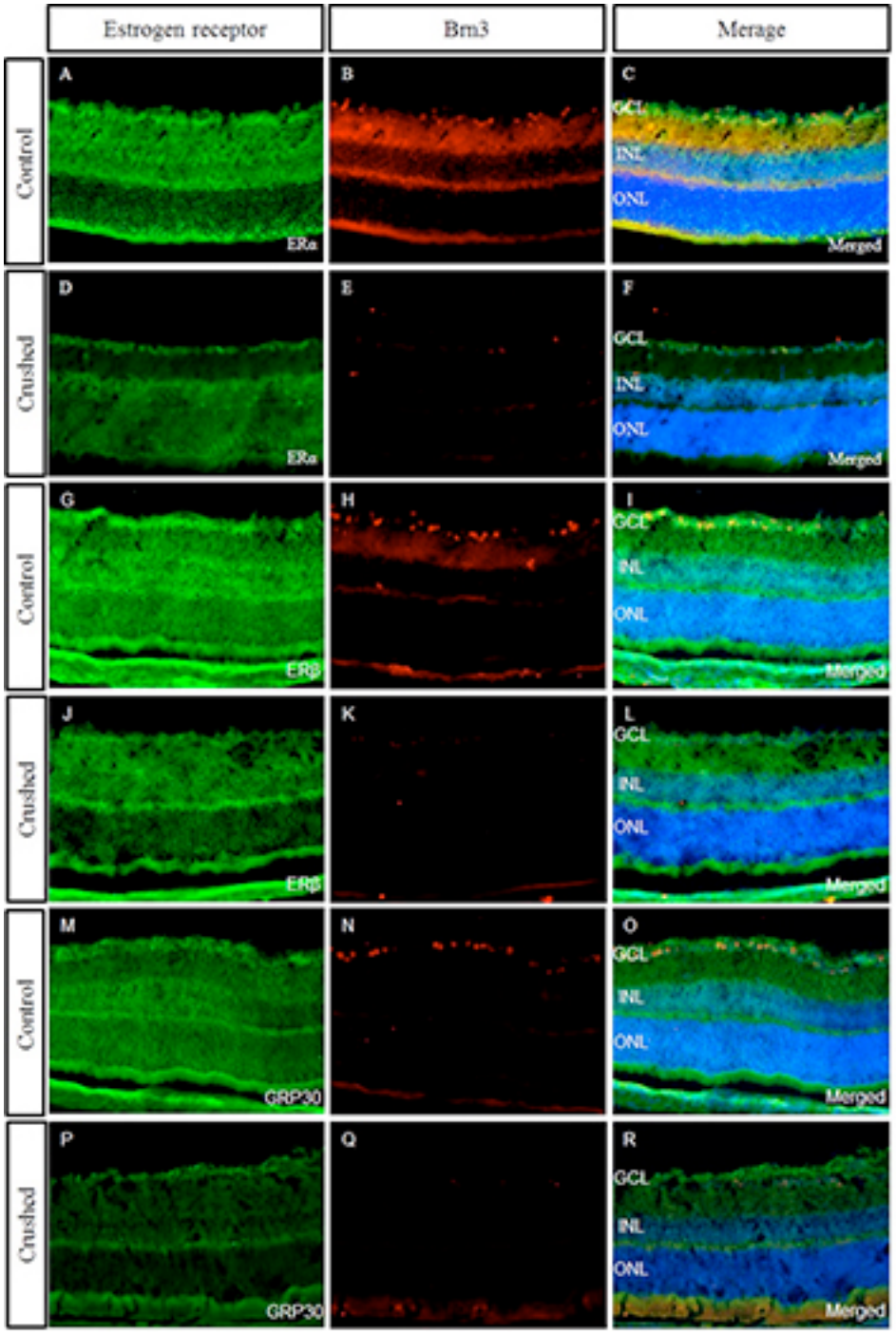

Figure 6. The expression of estrogen receptors in adult mouse retina 7 days after optic nerve injury. The results showed that the expression of ERalpha, ERbeta, and GPR30 decreased significantly in retinal ganglion cell layer at day 7 after optic nerve crush. anti-ERalpha antibody (green, A,C, D, F); ant-ERbeta antibody (green, G, I, J, L); anti-GPR30 antibody (green, M, O, P, R); Anti-Brn3 (red); DAPI (blue); GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

C. The expression pattern of estrogen receptors in the human retina.

In the prior work, we have identified that estrogen receptors is present in mouse retinal ganglion cell layer. We attempted to verify the expression of estrogen receptors in the normal human retina. We found that the result was different from those in mouse retina. ERalpha protein is expressed in the nerve fiber layer, ganglion cell layer, inner nuclear layer, outer plexiform layer and outer nuclear layer. ERbeta protein is faintly expressed in the nerve fiber layer and the ganglion cell layer, and intensely over inner plexiform layer and outer plexiform layer. GPR30 protein is intensively expressed on the nerve fiber layer and inner segment of photoreceptor, and faintly expressed over ganglion cell layer, inner nuclear layer and outer nuclear layer.

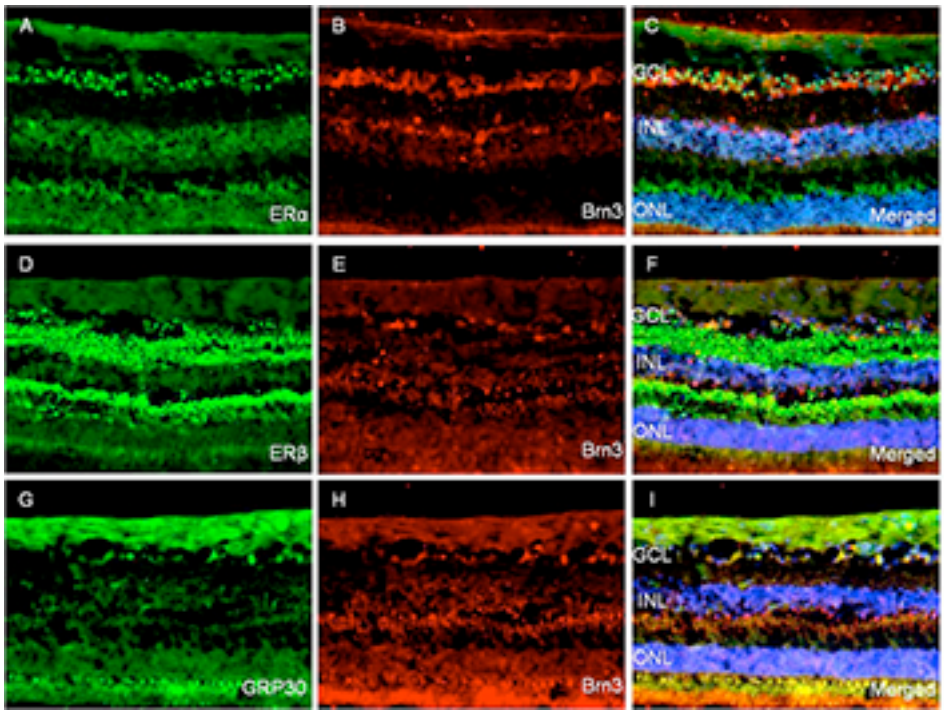

Figure 7. Immunofluorescent staining of estrogen receptors in human retina. Normal human were stained with anti-ERalpha antibody (green, A, C), ant-ERbeta antibody (green, D, F) and anti-GPR30 antibody (green, G, I). Brn3 (red); DAPI (blue); GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

To further characterize the cell types expressing estrogen receptors (ERalpha, ERbeta, GPR30) in the ganglion cell layer, we used anti-Brn3 antibody as a marker for retinal ganglion cells. The results showed that Brn3 signal is expressed in the cytoplasm of retinal ganglion cells, as a ring surrounding the nucleus. It exist in a large part of the neurons in the retinal ganglion cell layer, however, not every cell in GCL. ERbeta and GPR30 signals are

expressed in the cytoplasm of almost all retinal neurons in GCL. On the other hand, ERalpha signal is expressed in the nucleus of every retinal neuron in the GCL (Figure 8).

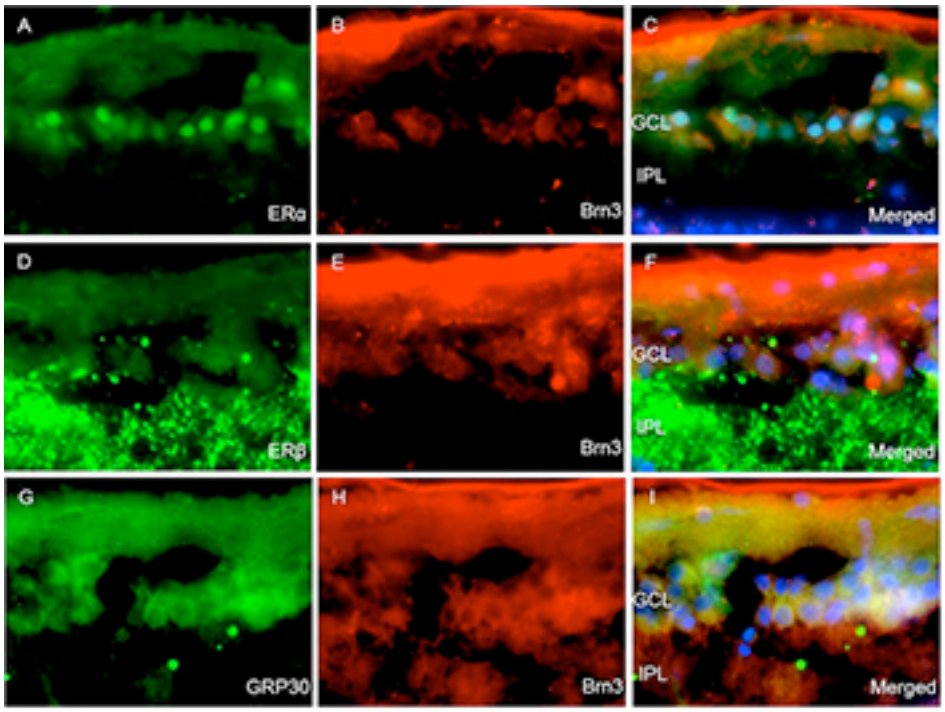

Figure 8. Expression of estrogen receptors in human ganglion cell layer (GCL). At high magnification, the estrogen receptors (ERbeta and GPR30) co-localized with Brn3 protein in the cytoplasm of retina ganglion cell. anti-ERalpha antibody (green, A, C), ant-ERbeta antibody (green, D, F); anti-GPR30 antibody (green, G, I); Anti-Brn3 (red); DAPI (blue); GCL: ganglion cell layer; IPL: inner plexiform layer.

3.4 研究討論

LHON is the major hereditary optic neuropathy in Taiwan.[1] It has a minimum point prevalence for mtDNA LHON mutation of 11.82 per 100,000 subjects and the minimum point prevalence of visual failure due to LHON of 3.22 per 100,000 subjects in adults under 65 years of age at north-east England.[1,52] It may cause bilateral blindness in a young adult and cause severe disability. Thus, it is of utmost importance to understand this disease. Though it is not difficult to diagnose since the development of molecular diagnosis, there has been few treatment available for this disease.

The incomplete penetrance and male prevalence is still the major unexplained issue in LHON. We proposed this project to explore the role of estrogen and its receptor in the pathogenesis of LHON, not only for the scientific interest but also for the possible therapeutic chance. The low penetrance in male LHON 11778 patients may results from some protective mechanism which may be useful in the future treatment. The even lower penetrance in female LHON 11778 patients reflect the gender difference. If the underlying mechanism is well investigated, it could also benefit the future treatment. Estrogen and its non-feminizing analogues have already proved its efficacy in the neuroprotection. We expect there will be even more therapeutic options in the future if the mechanism for LHON gender difference is well understood.

We have examined the expression of estrogen receptors (ERalpha, ERbeta, GPR30) in human and mouse retina. There is significant species difference of estrogen receptor expression in the retina. ERalpha protein is intensely expressed on the nucleus of retinal ganglion cells in both mouse and human retina. It is also highly expressed on the human nerve fiber layer, which represent the optic axons of the retinal ganglion cells. ERbeta protein is expressed in the retinal ganglion cells as well, but of less intensity than ERalpha. It is expressed over the inner plexiform layer (IPL) of mouse retina, and intensely over the IPL of human retina, which may imply its role in the synaptic transmission between retinal ganglion cell and bipolar cell. GPR30 protein is absolutely different in these two species that it is expressed on the nerve fiber layer and inner segment of photoreceptor in the human retina. On the other hand, it seems to be expressed on the Muller cells in mouse retina.

In mouse retina, we found ERalpha and ERbeta is expressed over retinal neurons in the ganglion cell layer, which may include both the retinal ganglion cells and displaced amacrine cells. On the other hand, GPR30 seems to be expressed on the end feet of Muller cell process, extending around the retinal ganglion cells. In human retina, ERalpha is also significantly expressed on the retinal neurons in the ganglion cell layers. However, ERbeta seems to be

present on the inner plexiform layer, which may play a role in the synaptic transmission in human retina. GPR30 protein is highly expressed in the human nerve fiber layer, and it is also found in the cytoplasm of retinal neurons in the ganglion cell layer. Thus, GPR30 may exist in the cytoplasm and axonal plasma of the human retinal ganglion cells.

Following optic nerve injury, we try to observe the changing pattern of estrogen receptor. At earliest stage of 1-day post crush, we found there is an up-regulated expression of BPR30 protein in the mouse retina. At 3 days after crush injury, all these estrogen receptors were strongly expressed in response to the axonal injury, which indicate estrogen receptor does play a role in the neuro-protection. Nevertheless, the exact mechanism for this neuro-protection needs further investigation. From 5 days after optic nerve injury, all the markers including ERalpha, ERbeta, GPR30 and BRN3 decreased gradually, which may reflect neuronal cell loss following axonal injury.

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五. 計畫成果自評

Leber's hereditary optic neuropathy (LHON) is characterized by acute and subacute visual loss predominantly affecting young man. It is a maternally transmitted disease caused by mitochondria DNA (mtDNA) mutation, which is transmitted to all the maternal lineages. However, there is an unexplained male prevalence in LHON that over 80% of LHON patients are male. These low penetrance and male prevalence may result from undefined neuroprotective mechanism which may be beneficial for future treatment. We propose this project to explore the role of estrogen and its receptor in the pathogenesis of LHON especially focusing on gender difference.

We have examined the expression of estrogen receptors (ERalpha, ERbeta, GPR30) in human and mouse retina. There is significant species difference of estrogen receptor expression in the retina. ERalpha protein is intensely expressed on the nucleus of retinal ganglion cells in both mouse and human retina. ERbeta protein is also expressed in the retinal ganglion cells, but of less intensity than ERalpha. It is also found in the inner plexiform layer (IPL), which may imply its role in the synaptic transmission especially in human retina. GPR30 seems to be expressed in the Muller cell of mouse retina. But it exists in the cytoplasm and axonal plasma of the human retinal ganglion cells.

Following optic nerve injury, we observed the changing pattern of estrogen receptor. At 1 day post-crush, we found there is an up-regulated expression of GPR30 protein in the mouse retina. At 3 days after crush injury, all estrogen receptors were strongly expressed in response to the axonal injury, which indicate estrogen receptor does play a role in the neuro-protection. From 5 days afterwards, all the markers including ERalpha, ERbeta, GPR30 and BRN3 diminished gradually, which may reflect neuronal cell loss following axonal injury.

科技部補助計畫衍生研發成果推廣資料表

日期:2014/10/31

科技部補助計畫	計畫名稱: 利伯氏遺傳視神經病變發病之性別差異研究
	計畫主持人: 王安國
	計畫編號: 102-2629-B-075-001- 學門領域: 性別主流科技計畫

無研發成果推廣資料

102 年度專題研究計畫研究成果彙整表

計畫主持人：王安國	計畫編號：102-2629-B-075-001-
計畫名稱：利伯氏遺傳視神經病變發病之性別差異研究	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	1	1	100%		科技部報告書
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	1	100%		研究助理學習實驗技術，執行計劃
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	研究成果, 已完成計劃報告書, 研究論文撰寫中.

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

利伯氏遺傳視神經病變(LHON)是一種母系遺傳的粒線體突變，它會造成急性視力減退，好發於男性，佔 80%，視力常惡化至零點一以下，伴隨視野缺損，兩眼可同時發病。粒線體突變會傳給所有母系細胞，然而具有突變基因的男性發病率僅有 50%，女性更只有 10%，顯示其發病率低與好發於男性的特點。此一特點，目前無明確原因，推測可能源於神經保護機轉，若能瞭解此機轉可能有助於未來的治療，我們提出計畫來探討雌激素與其受器在利伯氏遺傳視神經病變致病機轉所扮演的角色，特別著重於其發病率與性別差異。雌激素與其衍生物已証明具有神經保護作用，我們期待經由瞭解它在利伯氏遺傳視神經病變的作用，能帶來治療的新方法。