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之雌激素相關受器與性別差異研究

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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：林珮蓺

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中 華 民 國 104 年 10 月 16 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。

其發病率低與好發於男性的特點，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，雌激素與其衍生物已証明具有神經保護作用，我們提出此計畫來探討雌激素相關受器(Estrogen-related receptor, ERRs)在利伯氏遺傳視神經病變致病機轉與其發病率性別差異所扮演的角色，特別著重於神經保護與粒線體生物生成(mitochondria biogenesis)功能。我們利用螢光免疫染色，來檢查三種雌激素相關受器(ERRα, ERRβ and ERRγ)在小鼠視網膜的表現，發現ERRα和ERRγ蛋白在小鼠視網膜全層都有表現，特別在節細胞層(ganglion cell layer)與感光細胞內層(inner segment of photoreceptor)有較強表現，視網膜節細胞(retinal ganglion cell)與無軸細胞(amacrine cell)都會表現不等程度的ERRα和ERRγ蛋白。此外，ERRβ主要表現在從神經纖維層(nerve fiber layer)延伸到外叢層(outer plexiform layer)的視網膜內層，而其外的外層視網膜沒有表現此蛋白，ERRβ的表現與Muller細胞纖維範圍相符，顯示此蛋白主要表現在Muller細胞。

我們施行視神經壓迫傷手術(optic nerve crush)，檢查小鼠視網膜在受傷後之三種雌激素相關受器表現變化，發現三種雌激素相關受器表現有類似的變化趨勢，在受傷後，它們在節細胞層都逐漸增加表現量，第三天達到最高量，隨後漸漸降低，在第五到七天會低於對照組。然而，此三種雌激素相關受器的表現變化，在雄鼠與雌鼠的表現相似，並無性別差異。最後，我們採用西方墨點試驗，檢驗雌激素相關受器的表現變化，發現其變化趨勢與螢光免疫染色相同，這樣的變化趨勢，代表神經細胞對於軸突損傷所產生的細胞反應。

中 文 關 鍵 詞 ： 利伯氏遺傳視神經病變,雌激素相關受器

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. 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.

The incomplete penetrance and male prevalence is still the major unexplained issue in LHON. These low penetrance and male prevalence may result from undefined neuroprotective mechanism which may be beneficial for

future treatment. Estrogen and its non-feminizing analogues have already proved its efficacy in the neuroprotection. We propose to investigate the role of estrogen and esotrogen-related receptor (ERRs) family in the gender difference of LHON and its potential role in neuroprotection and mitochondrial biogenesis. We have examined the expression of estrogen-related receptors (ERRα, ERRβ and ERRγ) in mouse retina. ERRα and ERRγ proteins are both widely expressed over the whole retinal layers, with intense level over ganglion cell layer and photoreceptor inner segment. Both retinal ganglion cell and amacrine cell may express various amounts of ERRα and ERRγ proteins. ERRβ is expressed mainly over inner retina extending from nerve fiber layer to the outer plexiform layer, with the outer retina relatively spared. Its expression corresponds to the span of Muller cell processes.

Following optic nerve injury, we observe the expressional change of estrogen-related receptors (ERRα, ERRβ and ERRγ) in the adult mice retina in response to optic axonal injury. All three estrogen-related receptors showed a similar trend that their expression in the ganglion cell layer increased gradually and peaked around 3 days after injury, and then declined at 5 days to 7 days thereafter. Nevertheless, we did not observe any gender difference of estrogen-related receptors expression throughout the whole course. Western blot analysis showed a similar pattern of expressional change after optic nerve injury, which may reflect neuronal cell response to axonal injury.

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

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（□期中進度報告/V期末報告）

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計畫主持人：王安國

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中　華　民　國　104　年　10　月　16　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。

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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.

The incomplete penetrance and male prevalence is still the major unexplained issue in LHON. These low penetrance and male prevalence may result from undefined neuroprotective mechanism which may be beneficial for future treatment. Estrogen and its non-feminizing analogues have already proved its efficacy in the neuroprotection. We propose to investigate the role of estrogen and esotrogen-related receptor (ERRs) family in the gender difference of LHON and its potential role in neuroprotection and mitochondrial biogenesis. We have examined the expression of estrogen-related receptors (ERRα, ERRβ and ERRγ) in mouse retina. ERRα and ERRγ proteins are both widely expressed over the whole retinal layers, with intense level over ganglion cell layer and photoreceptor inner segment. Both retinal ganglion cell and amacrine cell may express various amounts of ERRα and ERRγ proteins. ERRβ is expressed mainly over inner retina extending from nerve fiber layer to the outer plexiform layer, with the outer retina relatively spared. Its expression corresponds to the span of Muller cell processes.

Following optic nerve injury, we observe the expressional change of estrogen-related receptors (ERRα, ERRβ and ERRγ) in the adult mice retina in response to optic axonal injury. All three estrogen-related receptors showed a similar trend that their expression in the ganglion cell layer increased gradually and peaked around 3 days after injury, and then declined at 5 days to 7 days thereafter. Nevertheless, we did not observe any gender difference of estrogen-related receptors expression throughout the whole course. Western blot analysis showed a similar pattern of expressional change after optic nerve injury, which may reflect neuronal cell response to axonal injury.

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]

Three primary mitochondrial DNA (mtDNA) mutations underlie the main pathogenesis of LHON. The first association of a mtDNA 11778 mutation with LHON was reported by Wallace and colleagues in 1988.[5] These three primary mutation of mtDNA 11778, 14484 and 3460 encodes the NADH dehydrogenase subunit 4, subunit 6 and subunit 1 of Complex I of the respiratory chain respectively.[5-8]

Male prevalence of LHON[1]

Despite intense studies on the clinical and molecular aspects of LHON from 1988, the pathogenesis 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.[1,9] The penetrance of LHON is variable even with the same mutation in homoplasmic fashion within the same family in different pedigree branch.[1,10] 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,[11-14] secondary mtDNA mutations,[15-16] mtDNA haplogroup,[17-19] X-linked modifying gene or susceptibility locus,[20-22] and other nuclear genes.[23] Tobacco and alcohol consumption were considered as epigenetic factors which may affect the penetrance as well.[24-25]

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.[26-28] 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.[29,30] The third estrogen receptor, GPR30, has been found recently with its exact function remaining to be elucidated.[31,32]

Estrogen and Neuroprotection

Estrogen has been shown to have neurotrophic and neuroprotective effect in various tissues.[33] In retinal pigment epithelium cells, 17-beta estradiol protects the cells from oxidative stress through the ERbeta-dependent mechanism. The cytoprotection of estrogen for retinal neurons occurs by reduction of ROS production, induction of cellular antioxidant genes, and preservation of mitochondrial function.[34-37]

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.[38] They have provided a possible metabolic basis for the unexplained male predominance in LHON. It indicates that estrogen do have a neuroprotective effect on LHON. However, the exact mechanism of how the estrogen influence gender difference in LHON remains unknown.

Estrogen-Related Receptor alpha (ERRalpha)[39]

The esotrogen-related receptor (ERR) family is consisted of three major family members (alpha, beta, and gamma).[39-41] They possess the typical structure of nuclear receptors, which contains a zinc finger DNA binding domain and a conserved C-terminal domain with a putative ligand binding domain and interaction surfaces for coactivators and corepressors.[39] These ERR members have a significant homology in DNA binding domain to the ERalpha, and with lesser similarity of the N- and C- terminal.[42] Estrogen has also been shown to stimulate the ERRalpha gene expression, [42] which was found to bind many

different ER response elements (EREs).[43-45] In addition, it could interact with similar coactivators of ERalpha.[44,45] Thus, ERRalpha gene may act as a downstream target of ERalpha.[42]

ERRalpha and Neuroprotection

PGC-1alpha (Peroxisome proliferator activated receptor Gamma Coactivator 1-alpha) is a master regulator for many cellular functions including mitochondrial biogenesis, metabolism, suppression of ROS, and stress response.[46-48] PGC-1alpha may promote the SIRT3 expression through ERRalpha receptor, which binds to and induces SIRT3 promoter.[49] Up-regulated SIRT3 may in turn deacetylate and activate mitochondrial enzymes involved in fatty acid beta-oxidation, amino acid metabolism, electron transport chain, and antioxidant defenses.[48] On the other hand, SIRT3 may also up-regulate the expression of PGC-1alpha by a positive feedback system mediated by AMP-activated kinase.[50-52] In addition to the increase of mitochondrial energy metabolism, SIRT3 prevents apoptosis by lowering reactive oxygen species and decreasing the formation of mitochondrial permeability transition pore.[48] Both the antioxidant effect and the anti-apoptotic effect mediated by SIRT3 suggest a neuroprotective role of ERRalpha which may improve neuronal survival and reduce aging effect in cell.[48,53]

The pathogenesis of male prevalence in LHON is still unclear yet.[1] We hypothesize that estrogen may play a role in this gender difference through neuroprotective function with unclear mechanism. In the current project, we propose to investigate the role of ERRs, a possible downstream target of estrogen, in the pathogenesis of gender difference in LHON. In addition, we will also investigate its potential role in neuroprotection and mitochondrial biogenesis.

3.2 研究方法與過程

Material and Methods

Animal and surgery

Adult Balb/c mice will be kept in the animal facility of our hospital. Institute guideline will be followed on handling of animals. Mice will be given food and water ad libitum. Adult mice that are at least two months old and body weight over 25 g, will be intraperitonially anesthetized with a combination of Zoletil 50 (0.1 mg/ 20 g body weight) and Rompun (0.023 mg/20 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 (day 1, day 3, day 5 and day 7) using cervical dislocation under anesthesia.

Immunohistochemistry

(a) Preparation and Fixation

Adult mice 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℃. Incubate the eyeballs with 30% sucrose/PBS overnight, then embed in OCT, and stored at –80℃ till use. Sections were cut at 12 μm thick and collected on SuperFrost slides (Fisher Scientific, PA).

(b) Immunostaining

Sections are fixed with 2% paraformaldeyhyde for 20 min, and then pretreated with 0.3 % H_2O_2 in PBS for 15 min at room temperature. They are then incubated with 0.2% Trypsin in PBS for 5 min, and then with 0.3 % Triton X-100 in PBS for 5 min at room temperature. Sections are blocked with 5% BSA in PBS with 0.3 % Triton X-100 for 60 min at room temperature. Sections are incubated in primary antibodies, diluted in 5% BSA/PBS, for overnight at 4℃. They are then incubated with matched secondary antibodies for 2 hours at room temperature. Each step is preceded by washes in 1xPBS. Sections are then incubated with4',6-diamidino-2-phenyindole (DAPI, 0.16 g/ml) for 15 min at room temperature for nuclear staining, and washed with 1xPBS for three times. The sections are mounted with antifade medium (Citifluoro, Canada) and stored at 4℃.

For estrogen-related receptors, primary antibodies include: anti-ERRα antibody (Santa Cruz), anti-ERRβ antibody (abcam), anti-ERRγ ntibody (Santa Cruz).

For retinal-specific markers, Anti-Brn3 (Santa Cruz), Anti-Brn3a (millipore) , Anti-CHAT (abcam), Anti-GFAP (millipore) , Anti-Glutamine synthetase (GS) (millipore) .

For western blot internal control, anti-GAPDH (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).

Western Blot

Tissue samples will be loaded with an equal volume of 2X loading buffer (125 μM Tris HCl, pH 6.8; 4% SDS; 20% glycerol and 10% 2-mercaptoethanol), boiled for 5 min and resolved by SDS-polyacrylamide gel (10%)electrophoresis.

Gels will be electro-blotted onto PVDF membranes (Millipore) in 25 mM Tris HCl, pH 8.3 containing 192 mM glycine, 20% methanol. Membranes will be blocked in PBS/0.1% tween (PBST) containing 5% fat-free dry milk (Carnation) for 1 hr. Primary antibody of anti-ERRalpha antibody, anti-ERRbeta antibody and anti-ERRgamma antibody will be incubated overnight in PBST with 5% dry milk. with Anti-GAPDH as internal control. After washing, HRP-conjugated secondary antibody will be incubated for 1 hr. Immunoreactive bands will be detected by enhanced chemiluminescence (ECL, Amersham) followed by exposure to Hyperfilm MP (Amersham).

3.3 研究結果

A. ERR Expression in Normal Mouse Retina

To verify the localization of estrogen-related receptors in the normal mouse retina, immunostaing was performed using anti- estrogen-related receptors (ERRα, ERRβ and ERRγ) antibody. The result showed that ERRα protein is expressed on the retinal ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer and inner segment of photoreceptor (Fig 1A). ERRβ protein is expressed on the nerve fiber layer, retinal ganglion cell layer, inner plexiform layer, inner nuclear layer and outer plexiform layer (Fig 1D). ERRγ protein is expressed on the nerve fiber layer, retinal ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer and inner segment of photoreceptor (Fig 1G). Both ERRα and ERRγ proteins are expressed over the whole retina, with high level of expression over ganglion cell layer and inner segment of photoreceptor. On the other hand, ERRβ is expressed over inner retina extending from nerve fiber layer to the outer plexiform layer, which corresponds to the profile of Muller cell processes.

To further investigate if retinal ganglion cells (RGCs) express estrogen-related receptors (ERRα, ERRβ and ERRγ), we have labeled adult mice retina with anti-Brn3 antibody, which is immune marker for RGCs. The results showed that ERRα and ERRγ proteins are expressed on both Brn3-positve and Brn3-negative cells in the ganglion cell layer. It implies that both RGCs and amacrine cells may express ERRα and ERRγ proteins. Meanwhile, the expressional level of ERRα and ERRγ proteins vary within these Brn3-positve cells. In addition, not all Brn3-postive cells express ERRα and ERRγ protein (Fig 1, C-C1, I-I1). ERRβ protein is uniquely expressed on slender cell processes in the inner retina, extending around retinal ganglion cells (Fig 1, F-F1).

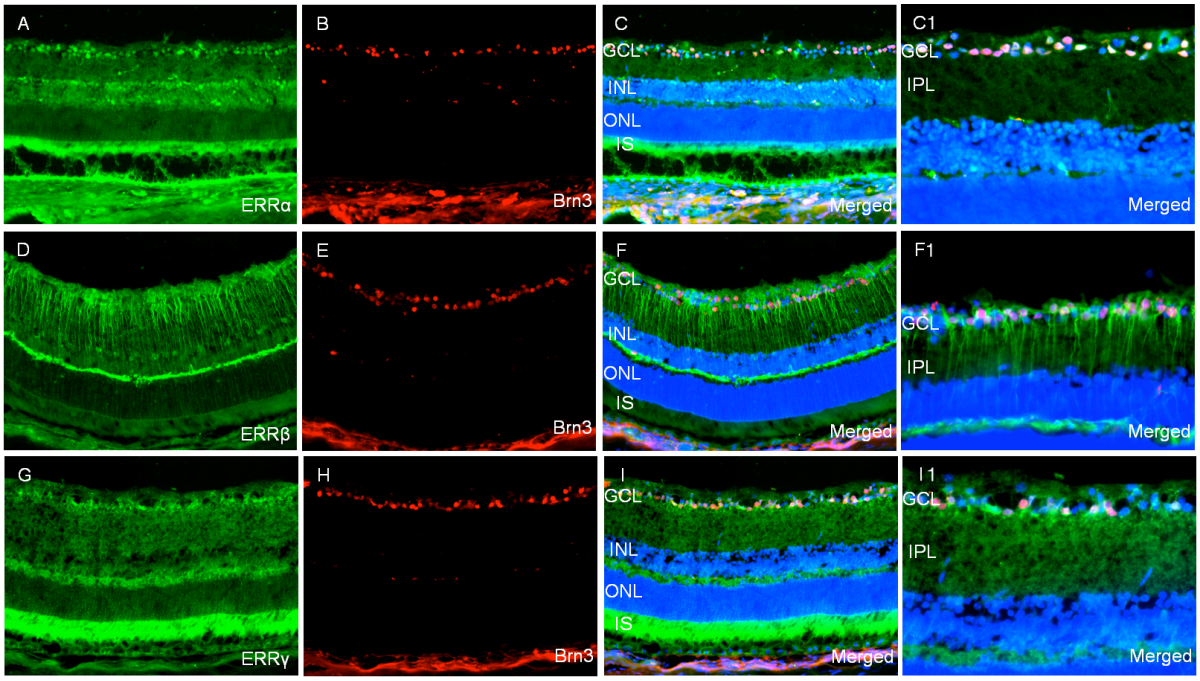

Figure 1. ERRs expression in adult mouse retina. Normal adult mouse were immunostained with anti-ERRα antibody (green, A-C), ant-ERRβ antibody (green, D-F) and anti-ERRγ antibody (green, G-I). Retinal ganglion cells are marked with Brn3 (red). DAPI (blue) was used as a nucleus marker. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

To characterize displaced amacrine cells in the ganglion cell layer, we have labeled adult mice retina with anti-ChAT antibody, which is immune marker for amacrine cells. It is expressed on the amacrine cells of the inner nuclear layer and also the displaced amacrine cells in the ganglion cell layer (Fig 2, B,E,H). These amacrine cells exist on both sides of inner plexiform layer, also called as starburst amacrine cells. In addition, ChAT protein is also expressed as parallel lines in the inner plexiform layer, which implies the synapse connecting retinal ganglion cell and bipolar cells (Fig 2).

The results showed that ERRα and ERRγ proteins are expressed on both ChAT-positve and ChAT-negative cells in the ganglion cell layer. It implies that both amacrine cells and RGCs may express ERRα and ERRγ proteins. Meanwhile, ERRα and ERRγ proteins are expressed with different levels in these ChAT-positve cells, with some expressing high level and others none (Fig 2, C-C1, I-I1). ERRβ protein seems to be not expressed on the ChAT-positive cells (Fig 2, F-F1).

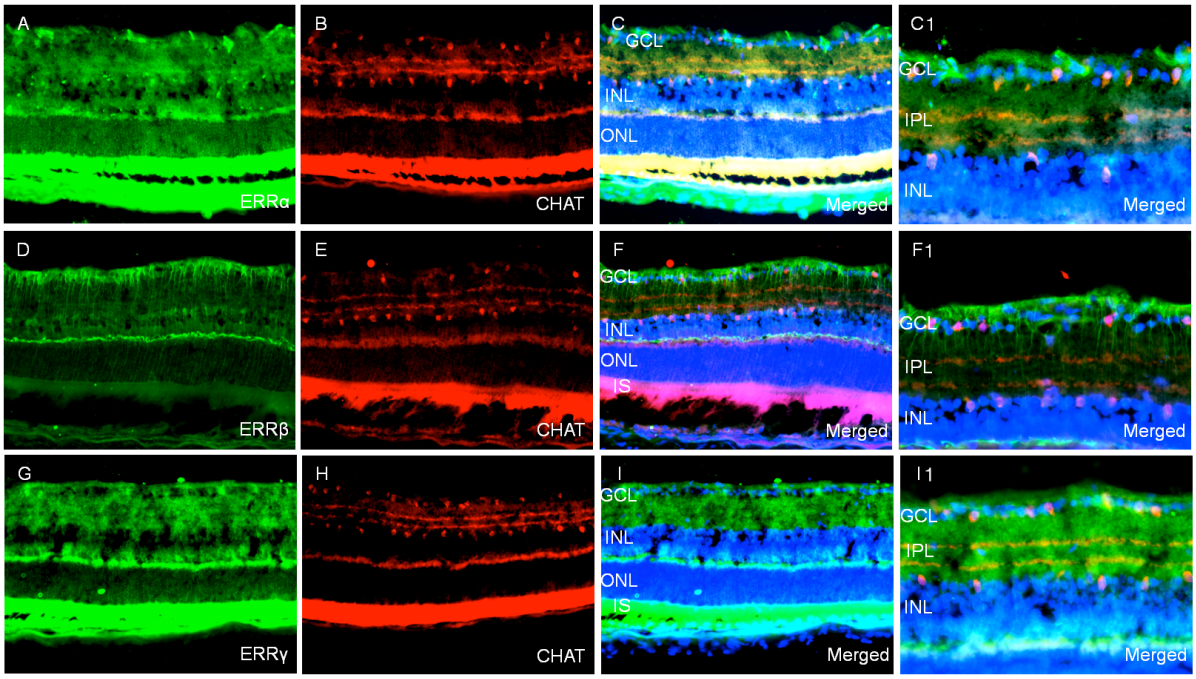

Figure 2. ERRs expression in adult mouse retina. Normal adult mouse were immunostained with anti-ERRα antibody (green, A-C), ant-ERRβ antibody (green, D-F) and anti-ERRγ antibody (green, G-I). Starburst amacrine cells are marked with anti-ChAT antibody (red). DAPI (blue) was used as a nucleus marker. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

From the above data, we learn that ERRβ protein is present in the nerve fiber layer, retinal ganglion cell layer, inner plexiform layer, inner nuclear layer and outer plexiform layer. It is uniquely expressed over inner retina extending from nerve fiber layer to the outer plexiform layer, which corresponds to the profile of Muller cell processes. This specific expressional pattern indicates ERRβ protein may be expressed exclusively in the retinal Muller cells. We have used two immune markers, GFAP and glutamine synthetase (GS), for double staining technique. GFAP is a general glial cell marker, while glutamine synthetase is a specific marker for Muller cells.

The results showed that in the ganglion cell layer, some ERRβ-positive cells are GFAP immune-reactive. These GFAP-positive retinal astrocytes exist in the inner part of ganglion cell layer, with their GFAP-positive cell processes extending horizontally over the nerve fiber layer (Fig 3, B,E). ERRβ protein is co-localized with GFAP protein on these retinal astrocytes, but not on the Muller cells. To further investigate, we co-stained glutamine synthetase (GS) with ERRβ protein. The results showed that ERRβ-positive cells are glutamine synthetase immune-reactive (Fig 3). Thus, Muller cell also express ERRβ protein on their cell processes.

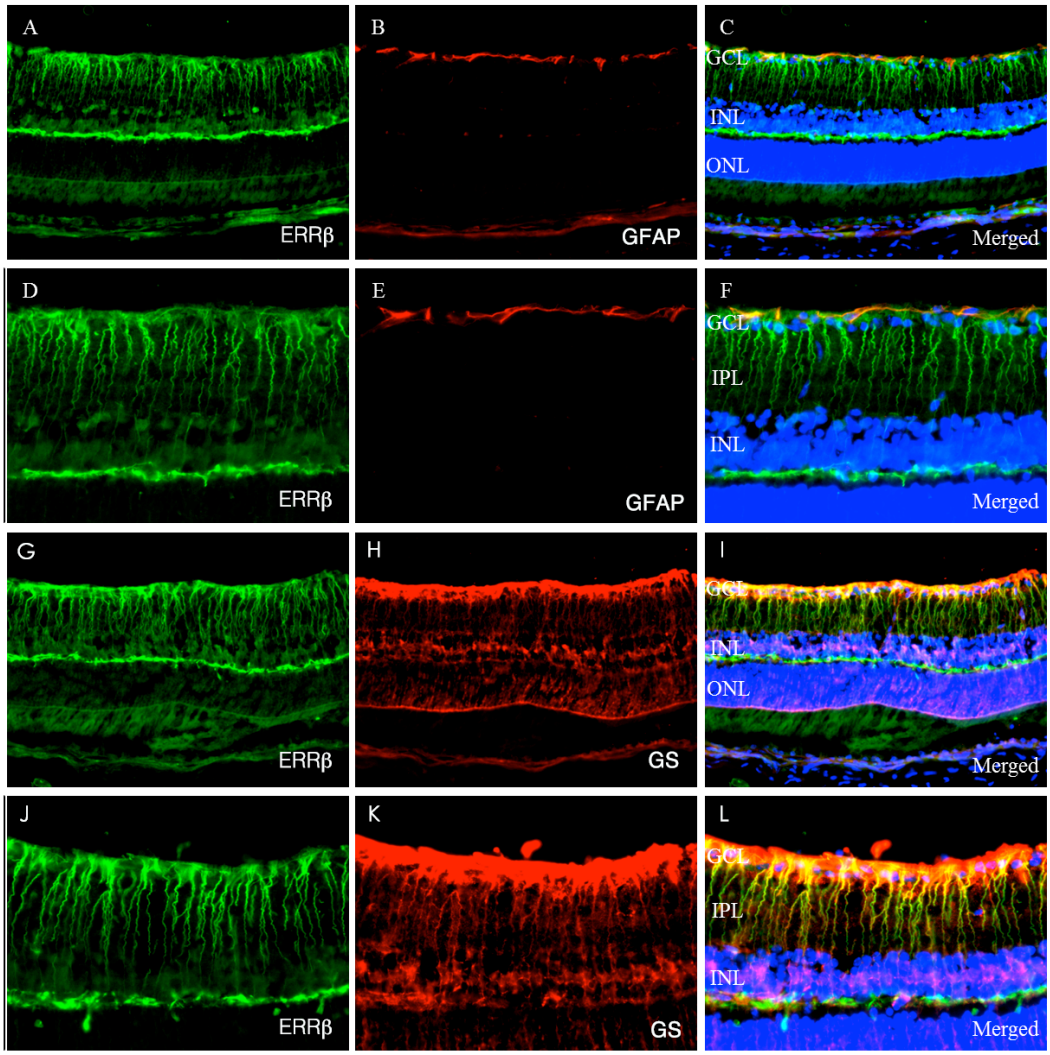

Figure 3. ERRβ expression in the retinal glia cells. Normal adult mouse were immunostained with ant-ERRβ antibody (green) , GFAP (red, B,E) and anti-glutamine synthetase (red, H,K). GFAP is marker fro glia cell. Glutamine synthetase is marker for Muller cells. DAPI (blue) is used as a nucleus marker. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer; IPL: inner plexiform layer.

B. Gender Effect on ERRs Expressional Change after Optic Nerve Crush

In this project, we try to investigate the expressional change of estrogen-related receptors (ERRα, ERRβ and ERRγ) in a stressed retina. We plan to conduct optic nerve crush injury in adult male and female mouse and examine the gender difference of expressional change with immunohistochemistry. The immunostaining was performed to measure the amount of estrogen-related receptors expression in the retinal ganglion cell layer. The results showed that the expression of ERRα, ERRβ and ERRγ increased in retinal ganglion cell layer at 3 days after optic nerve crush when compared with control group. However, the expression of ERRα, ERRβ, ERRγ and Brn3 (RGC marker) decreased gradually thereafter at 5 days and

7 days after crush. But, there is no significant gender difference of ERRα, ERRβ, ERRγ expression between male and female mice from 1 day to 7 days after optic nerve crush (Fig 4-6).

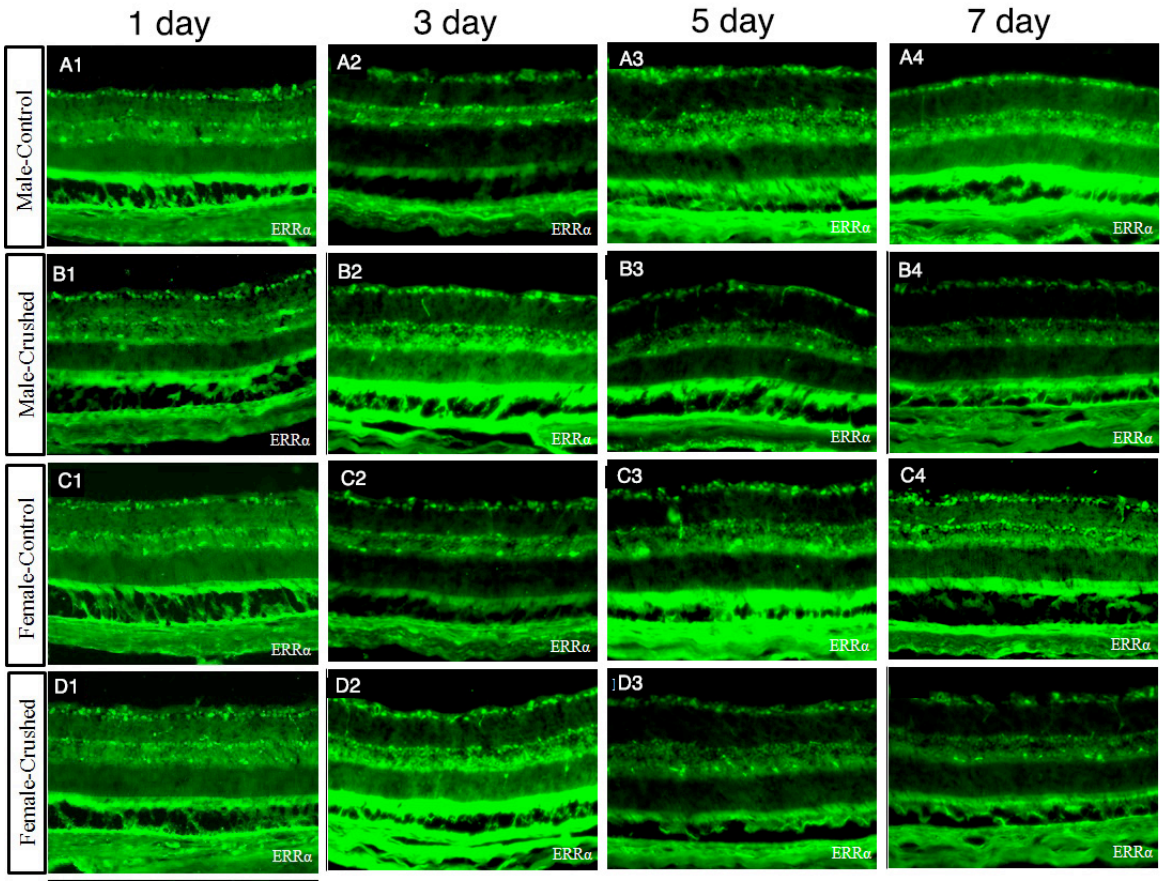

Figure 4. Gender Effect on ERRα Expressional Change after Optic Nerve Crush Optic nerve crush injury was performed in left eye of adult mice, with right eye spared as control. Both control and injured retina were labeled with anti-ERRα antibody (green). There is no significant difference between the crushed and control retina at 1 day after optic nerve injury (A1-D1). The expression of ERRα increased significantly in the crushed retina at 3 days post crush, as compared with control (A2-D2). The pattern of increased expression was similar among male and female mice. It decreased gradually in the crushed retina thereafter. ERRα expression in crushed retina became less as compared with control retina at 5 days and 7 days postcrush, with no gender difference (A3-D4).

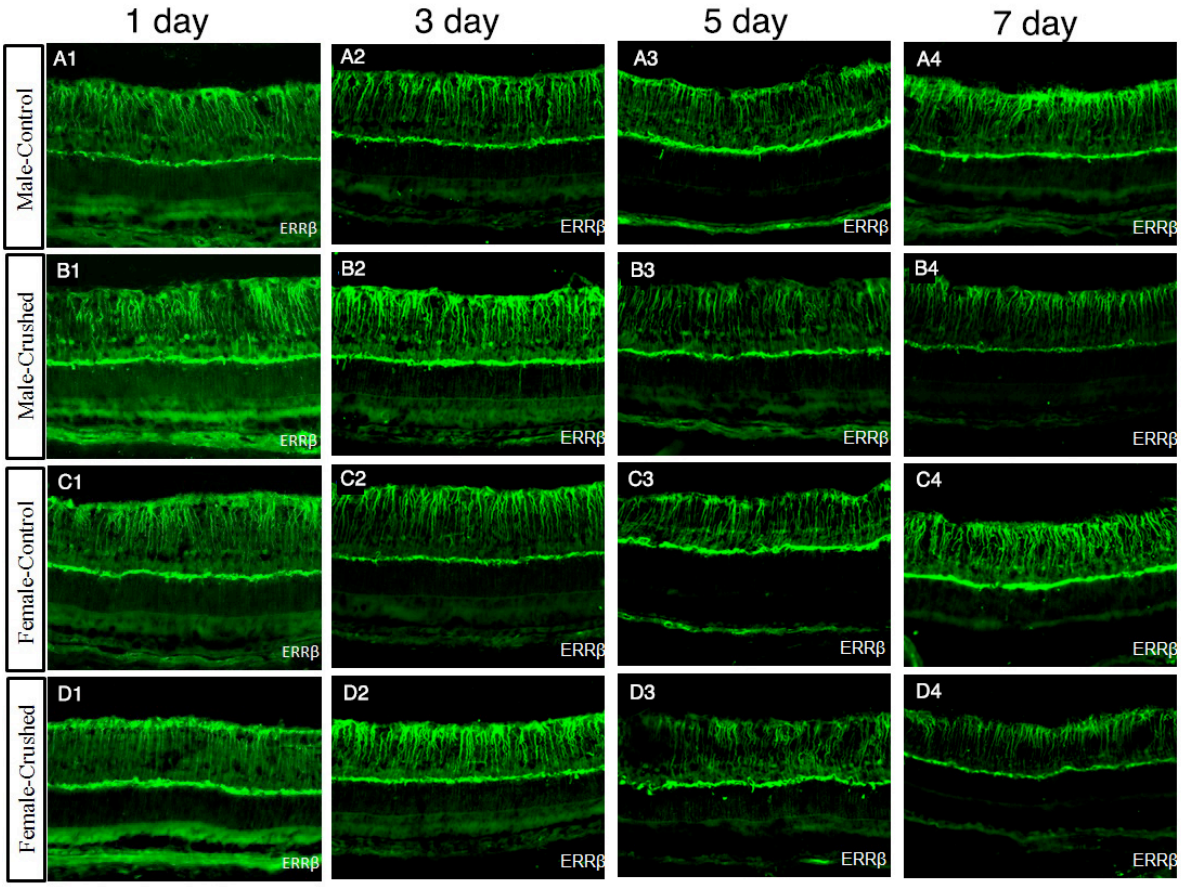

Figure 5. Gender Effect on ERRβ Expressional Change after Optic Nerve Crush Optic nerve crush injury was performed in left eye of adult mice, with right eye spared as control. Both control and injured retina were labeled with anti-ERRβ antibody (green). There is no significant difference between the crushed and control retina at 1 day after optic nerve injury (A1-D1). The expression of ERRβ increased significantly in the crushed retina at 3 days post crush, as compared with control (A2-D2). The pattern of increased expression was similar among male and female mice. It decreased gradually in the crushed retina thereafter. ERRβ expression in crushed retina became less as compared with control retina at 5 days and 7 days postcrush, with no gender difference (A3-D4).

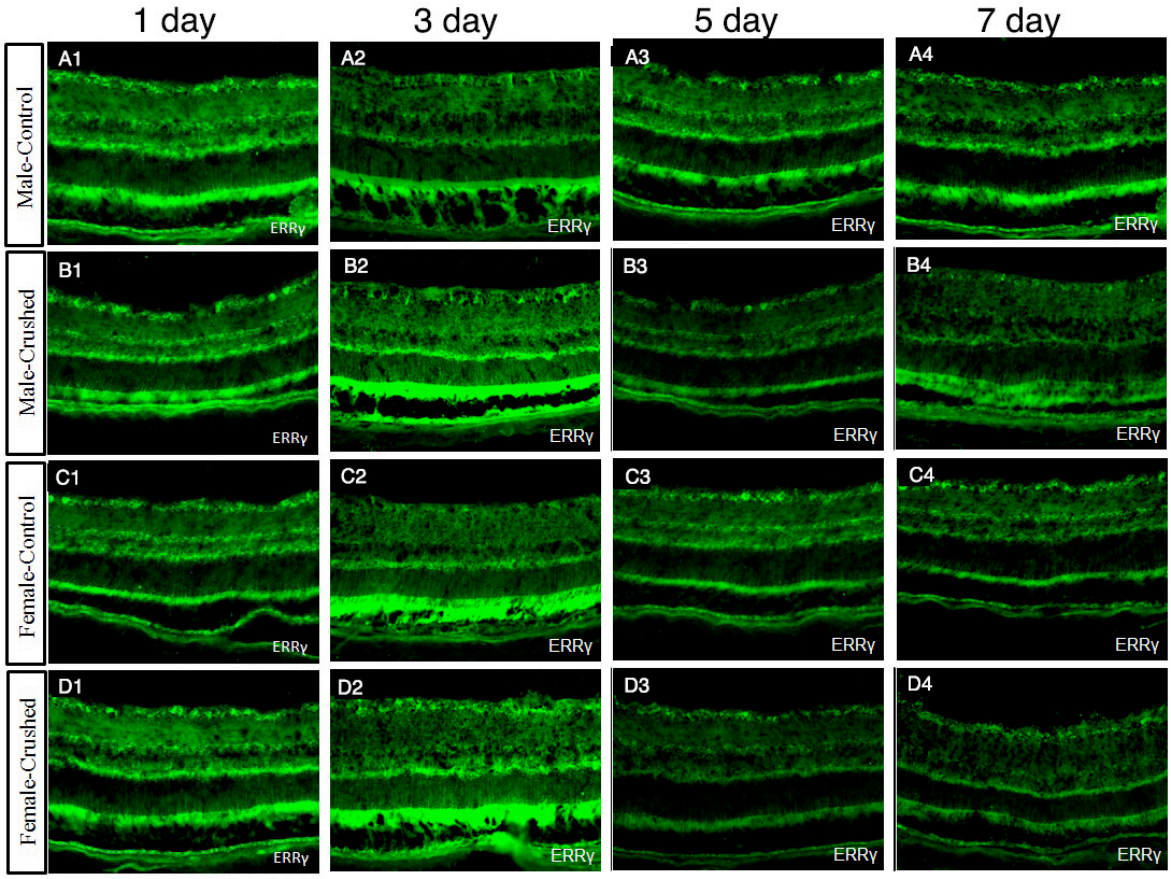

Figure 6. Gender Effect on ERRγ Expressional Change after Optic Nerve Crush Optic nerve crush injury was performed in left eye of adult mice, with right eye spared as control. Both control and injured retina were labeled with anti-ERRγ antibody (green). There is no significant difference between the crushed and control retina at 1 day after optic nerve injury (A1-D1). The expression of ERRγ increased significantly in the crushed retina at 3 days post crush, as compared with control (A2-D2). The pattern of increased expression was similar among male and female mice. It decreased gradually in the crushed retina thereafter. ERRγ expression in crushed retina became less as compared with control retina at 5 days and 7 days postcrush, with no gender difference (A3-D4).

C. ERRs Expressional Change after Optic Nerve Crush by Western Blot

We try to investigate the expression amount of estrogen-related receptors (ERRα, ERRβ and ERRγ) by western blot. Retinal estrogen-related receptors (ERRα, ERRβ and ERRγ) proteins show an increase at 3 days after optic nerve crush, and decrease at 5 days or 7 days after optic nerve crush, as compared with control retina. Interestingly, western blot analysis has a similar pattern of temporal change as those of immunostaining result (Fig 7).

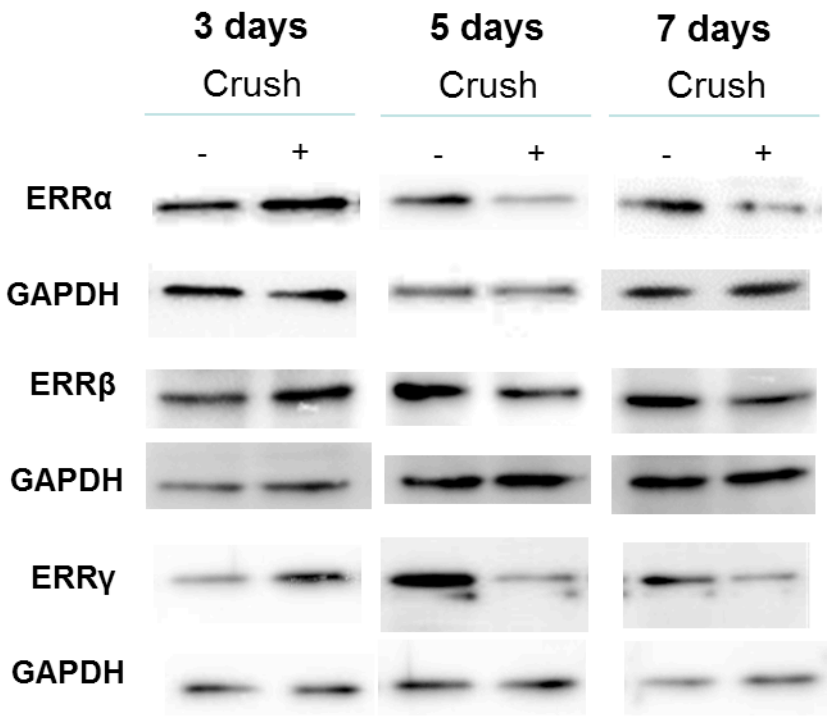

Fig 7. Western Blot of estrogen-related receptors (ERRα, ERRβ and ERRγ) Western blot analysis of retinal protein was performed with GAPDH as internal control. Crushed retina has an increased amount of ERRs at 3 days after crush, which decreased at 5 day and 7 days in comparison with control retina.

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.[1,54] 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 propose this project to explore the role of estrogen-related receptors (ERRα, ERRβ and ERRγ) in the pathogenesis of LHON, not only for the scientific interest but also for the possible therapeutic chance. The low penetrance in female LHON 11778 patients may results from some protective mechanism of estrogen and ERRs. 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-related receptors (ERRα, ERRβ and ERRγ) in mouse retina. Expression of ERRα protein is found in the retinal ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer and inner segment of photoreceptor. ERRβ protein is mainly expressed in the inner two third of mice retina, over the nerve fiber layer, retinal ganglion cell layer, inner plexiform layer, inner nuclear layer and outer plexiform layer. ERRγ protein expression is distributed over the nerve fiber layer, retinal ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer and inner segment of photoreceptor. The expression patterns of ERRα and ERRγ proteins are similar that they are widely expressed over the whole retinal layers, with intense level over ganglion cell layer and photoreceptor inner segment. On the other hand, ERRβ is expressed over inner retina with outer retina spared. It extends from nerve fiber layer to the outer plexiform layer, corresponding to the Muller cell processes. Double staining has shown that ERRβ expression is co-localized with Muller cell marker glutamine synthetase.

With anti-Brn3 antibody (marker for RGCs) and anti-ChAT antibody (marker for amacrine cell), we have found that both RGCs and amacrine cells may express ERRα and ERRγ proteins. Meanwhile, the expressional level of ERRα and ERRγ proteins vary widely among these Brn3-positve or ChAT-positive cells. On the other hand, ERRβ protein seems to be not expressed on the Brn3-positive or ChAT-positive cells

We try to investigate the expressional change of estrogen-related receptors (ERRα, ERRβ and ERRγ) in a stressed retina after optic nerve crush. Gender difference in the expressional change of estrogen-related receptors was assessed as well. We found the expression of three estrogen-related receptors show a similar trend that its expression in the ganglion cell layer increased gradually and peaked at 3 days after optic nerve crush, and then decreased at 5 days and 7 days after crush injury. Nevertheless, we did not observe gender difference of estrogen-related receptors expression throughout the whole course. Western blot analysis showed a similar pattern of expressional change after optic nerve injury, which may reflect neuronal cell response to 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-related receptors (ERRα, ERRβ and ERRγ) in the pathogenesis of LHON especially focusing on gender difference.

We have examined the expression of estrogen-related receptors (ERRα, ERRβ and ERRγ) in mouse retina. ERRα and ERRγ proteins are both widely expressed over the whole retinal layers, with intense level over ganglion cell layer and photoreceptor inner segment. ERRβ is expressed mainly over inner retina, with the outer retina relatively spared. Its expression corresponds to the span of Muller cell processes, and was co-localized with Muller cell marker glutamine synthetase.

Following optic nerve injury, we observe the expressional change of estrogen-related receptors (ERRα, ERRβ and ERRγ) in the injured retina. These three estrogen-related receptors showed a similar trend that their expression in the ganglion cell layer increased gradually and peaked around 3 days after injury, and then declined at 5 days and 7 days thereafter. We did not observe gender difference of estrogen-related receptors expression throughout the whole course. Western blot analysis showed a similar pattern of expressional change after optic nerve injury, which may reflect neuronal cell response to axonal injury.

科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/16

科技部補助計畫	計畫名稱: 利伯氏遺傳視神經病變之雌激素相關受器與性別差異研究
	計畫主持人: 王安國
	計畫編號: 103-2629-B-075-001- 學門領域: 性別主流科技計畫

無研發成果推廣資料

103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：**103-2629-B-075-001-
計畫名稱：利伯氏遺傳視神經病變之雌激素相關受器與性別差異研究	

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	1	0	0%	篇	論文撰稿中
		研究報告/技術報告	0	0	100%		
		研討會論文	1	0	0%		論文撰稿中
		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（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%		
國外	論文著作	期刊論文	1	0	0%	篇	論文撰稿中
		研究報告/技術報告	0	0	100%		
		研討會論文	1	0	0%		論文撰稿中
		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。）	已完成動物實驗，利用螢光免疫染色與西方墨點試驗，檢查三種雌激素相關受器(ERRα, ERRβ and ERRγ)，在小鼠視網膜的表現，並利用視神經壓迫傷手術(optic nerve crush)，檢查小鼠視網膜在受傷後之三種雌激素相關受器表現變化，此外，特別著重於雄鼠與雌鼠的表現差異性，我們的論文在撰稿中。

	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）
   已完成動物實驗，利用螢光免疫染色與西方墨點試驗，檢查三種雌激素相關受器(ERRα, ERRβ and ERRγ)，在小鼠視網膜的表現，並利用視神經壓迫傷手術(optic nerve crush)，檢查小鼠視網膜在受傷後之三種雌激素相關受器表現變化，此外，特別著重於雄鼠與雌鼠的表現差異性，我們的論文在撰稿中。

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。
   　　其發病率低與好發於男性的特點，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，雌激素與其衍生物已証明具有神經保護作用，我們提出此計畫來探討雌激素相關受器(Estrogen-related receptor, ERRs)在視神經受損所扮演的角色，特別著重於其性別差異，若能瞭解此機轉可能有助於未來的治療，。