科技部補助專題研究計畫報告

男性荷爾蒙及其接受器對男性憂鬱症的影響：NOD2及TOLLIP的角色(第3年)

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計 畫 主 持 人： 洪一永
共 同 主 持 人： 黃條來、康宏佑

計畫參與人員： 此計畫無其他參與人員

本研究具有政策應用參考價值：■否 □是，建議提供機關
（勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
本研究具影響公共利益之重大發現：□否 □是

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中 文 摘 要 ： 計畫成果包含了許多部分，包含男性荷爾蒙接受器差異對壓力承受程度的影響，不同位置系統的免疫調控反應在憂鬱症病人上的變化，其中發現健康男性PBMCs 相對於健康女性有顯著較高的TOLLIP and NOD2 mRNA表現，然而，在憂鬱症病人的比較上發現NOD2的差異消失了，此外， NOD2 and AR mRNAs 在憂鬱症男性表現量低於健康男性，透過細胞學研究發現男性荷爾蒙接受器調控NOD2 mRNA表現量，在男性荷爾蒙接受器對BDNF的影響中發現,男性荷爾蒙接受器的減少會使公鼠承受壓力不出現憂鬱行為的時間長度減少，而這樣的影響是透過減少海馬迴的BDNF所產生，男性荷爾蒙接受器在神經發展上或神經退化上皆有明顯的影響，

中 文 關 鍵 詞 ： 憂鬱症, 先天免疫, 發炎, 男性荷爾蒙接受器

英 文 摘 要 ： PBMCs from males had significantly higher TOLLIP and NOD2 mRNA levels than those from females. By contrast, mRNA for TOLLIP, but not NOD2, was higher in males than females in MDD. In addition, NOD2 and AR mRNAs were found to be lower in male MDD patients than in male healthy controls. Through cell experiments using mHippoE-14, we showed that inhibition of AR signaling with the antagonist flutamide suppressed NOD2 expression, whereas treatment AR signaling with the agonist dihydrotestosterone and antagonist flutamide could not increase NOD2 expression. In study about the effects of androgen receptor on BDNF in male mice, we noticed that in androgen receptor knockout mice, the tolerance to chronic mild stress decreased compared with wild type mice. The mechanism is associated with the effects of androgen receptor on BDNF. Androgen receptor has effects on both neurogeneration and neurodegeneration.

英 文 關 鍵 詞 ： major depressive disorder, innate immunity, inflammation, androgen receptor

Part I:

前言及文獻探討

Major depressive disorder (MDD) is a leading cause of disability in developed countries and is responsible for 7.4% of total disability-adjusted life years [1, 2]. However, some fundamental questions remain unanswered. One of the most striking unexplained observations is the sex-based difference in the prevalence of MDD, which is twice as common in women as men [3-5]. Sex differences in exposure to stressful life events, coping styles, and reactivity to stress have been previously invoked to explain this difference [6]. However, whether the immune system plays a role in this relationship is still unknown. Recent accumulating evidence indicates that activation of the inflammatory immune system influences neurochemical reactions and contributes to MDD [7-9]. Interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1) [9-12], and other inflammation-related proteins have been found in the plasma and cerebrospinal fluid of MDD patients as well as in postmortem samples [13]. Sex-based differences in cytokine expression have been recently reviewed [14]. Virus challenge in adult rats results in higher expression of genes encoding MYD88 (myeloid differentiation primary response gene 88), STAT3 (signal transducer and activator of transcription), JAK2 (Janus kinase 2), VISA (virus-induced signaling adaptor), JUN, IFNAR1 (interferon-α [IFN-α] and -β receptor subunit 1) and the interferon-induced GTP-binding protein, MX2, in female rats than in male rats [15]. Moreover, the production of IFN-α after exposure of peripheral blood mononuclear cells (PBMCs) to Toll-like receptor (TLR)-7 ligands is higher in females than in males [16]. A vaccination study also showed that the expression of TNF and other pro-inflammatory genes in PBMCs is also higher in women than in men [17]. From the perspective of inflammation, cytokines can partially explain why the prevalence of MDD is higher in females than in males. Recent studies have demonstrated a significant association between innate immune, especially TLR-4–mediated signaling, and depression [18-21]. In contrast to sex differences in cytokines, sex differences in innate immune responses in MDD have received less research attention [22]. It has been reported that male mice exhibit greater morbidity after lipopolysaccharide (LPS) injection than female mice [23]. However, there is no difference in the severity of sickness symptoms after LPS injection in humans, even though women exhibited a greater pro-inflammatory response than males [24]. Patients with MDD show higher TLR-4 expression in the prefrontal cortex [19] and higher TLR-4 signaling in PBMCs [21]. There are significant associations between MDD severity and anxiety, body

weight loss, and TLR-4 mRNA levels [25]. However, TLR-4 expression levels in adults were reported to be higher in males than in females [22], which argues against data relating to prevalence. In addition, our previous investigation of negative regulators of TLR signaling, including IRAK3 (IL-1 receptor-associated kinase 3), SOCS1 (suppressor of cytokine signaling 1), MyD88s (myeloid differentiation 88 short), TOLLIP (Toll-interacting protein), TNFAIP3 (TNFα–induced protein 3), ST2L (suppressor of tumorigenicity 2, full-length form), and SIGIRR (single immunoglobulin IL-1R–related receptor) showed that TNFAIP3 pathways play an important role in MDD [26]. However, no previous studies have reported sex differences in these regulators or their influence on sex-specific diseases.

研究目的

Accordingly, we here sought to investigate sex differences in negative regulators of TLR signal pathways in patients with MDD and human volunteers and assess the effects of androgen and androgen receptor (AR) signaling on these regulators

研究方法

Our aim was to examine sex-specific differences in negative regulators of TLR signaling in healthy controls and patients with MDD. Design, setting, and participants This study was embedded in our previous work, an observational study that investigated negative regulators of TLR signaling in MDD [26]. Institutional Review Board approval was obtained from the hospital ethics committee (101-5012A3, 103-5114B, and 103-6984A3). Patients and healthy controls were enrolled in the study after receiving verbal and written information about the study and providing written consent. Patients with MDD were screened by two psychiatrists before entering the study. A Structured Clinical Interview for DSM-IV Axis I Disorders as well as a detailed evaluation of current psychiatric symptoms and previous medical treatments were performed during screening. Patients with other major psychotic disorders, substance dependence (including alcohol), severe systemic physical illness, including metabolic syndrome, obesity (body mass index [BMI] > 34 kg/m2) or inflammatory disease, or those who received immune-modulating drugs, were excluded from the study. All patients were examined for blood pressure and received chest X-rays, electrocardiographic examinations, and routine blood tests to exclude possible chronic systemic physical illness. None reported taking any antidepressants for at least1 week before entering the study. Healthy controls with neither a personal history nor a first-degree relative with a psychiatric disorder were recruited

from the community. The same psychiatrist who performed screens of MDD patients assessed the healthy control group using the Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition). MDD patients were free from antidepressant treatment at the time of the study.After screening, blood samples were collected. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis Venous blood (10 mL) samples were taken after fasting for 9 hours. PBMCs were isolated from venous blood samples by Ficoll-Paque (GE, #17-5442-02) density gradient centrifugation. Immediately after collection, samples were stored at 80°C until they were assayed. The protocol used for mRNA analyses was the same as that used in our previous study (Hung et al, 2016). qRT-PCR was performed using primers listed in Table 1. Expression levels of target genes in each sample relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as an endogenous control, were calculated based on the threshold cycle, CT, where the difference in CT (-ΔCT) representing relative expression in clinical samples was defined as CT GAPDH – CTsample. The 2−ΔΔCT method was used to calculate relative changes in expression of target genes for cell assays, where ΔΔCT = ΔCTtreatmentgroup – ΔCTcontrol group.

Cell culture

The mHippoE-14 embryonic mouse hippocampus cell line was obtained from CEDARLANE and maintained in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies/GIBCO, Cat# 12100-046) containing 10% charcoal-stripped serum (CS-FBS). All media contained 1.5 μg/mL penicillin/streptomycin/neomycin, and cells were incubated at 37°C in a humidified 5% CO2 atmosphere. In dihydrotestosterone (DHT) experiments, cells were seeded in 6-well plates and treated with 10 nM DHT for 24 hours. In flutamide experiments, cells were seeded in 6-well plates and treated with 10 μM flutamide for 48 hours.

Statistical analysis

All results are presented as means ± standard deviation. mRNA levels shown in Tables and Figure 1a are presented as -ΔCT; mRNA levels in Figure 1b and c are presented as 2−ΔΔCT. Independent t-tests were used to compare differences in age and BMI in Table 2, Figure 1a and 1b. Analysis of covariance (ANCOVA) with age and BMI adjustment was used for all factors in Tables 3, 4and Figure 1c and 1d. Two-tailed t-test was used to compare differences in cell line models (Figure 1e). All statistical analyses were performed using Statistical Product and Service Solutions (SPSS), version 22. For each test, P-values < 0.05 were considered significant.

結果與討論

Demographic data

A total of 153 subjects were included in the study, of which 100 were patients with MDD (79 females, 21 males) and 53 were healthy controls (38 females, 15 males). As shown in Table 2, there was no difference in age between males and females (male vs. female: 44.86 ± 8.66 vs. 45.91 ± 10.33 years [P = 0.67] for MDD, and 39.80 ± 15.73 vs. 37.54 ± 10.48 years [P = 0.26] for healthy controls), BMI (male vs. female: 22.63 ± 3.76 vs. 24.39 ± 4.76 [P = 0.08] for MDD and 23.72 ± 3.35 vs. 22.43 ± 2.88 [P = 0.17] for healthy controls), or HAMD score among MDD patients (male vs. female: 28.55 ± 6.65 vs. 26.65 ± 4.63 [P = 0.24]).

NOD2 mRNA levels are higher in males than in females in healthy controls but not in MDD patients

To investigate sex-related differences in negative regulators of TLR signaling, we evaluated the mRNA expression of SOCS1, TOLLIP, SIGIRR, MyD88s, NOD2, TNFAIP3, ST2L, and IRAK3. There were no differences in SOCS1, SIGIRR, MyD88s, TNFAIP3, ST2L, or IRAK3 mRNA levels between males and females in either group. TOLLIP mRNA levels, expressed as -ΔCT, were higher in males than in females in both the MDD group (male vs. female: -9.57 ± 2.06 vs. -10.85 ± 1.84 [F = 6.203, p = 0.015]) and healthy control group (male vs. female: -8.66 ± 1.75 vs. -10.45 ± 2.44, [F = 7.175, p = 0.010]). By contrast, NOD2 mRNA levels were higher in males than in females in the healthy control group (male vs. female: -5.21 ± 1.27 vs. -6.16 ± 1.77 [F = 4.099, p = 0.048]), but not in the MDD group (male vs. female: -5.96 ± 1.02 vs. -6.43 ± 1.41 [F = 2.337, p = 0.13])(Tables 3 and 4).

NOD2 and AR mRNA levels are lower in male MDD patients than in healthy controls

To investigate the association between negative regulators of the TLR pathway and MDD in males, we compared mRNA levels of IL-6, AR, and negative regulators in male healthy controls and MDD patients. This analysis showed that IL-6 mRNA expression was significantly higher in male MDD patients than in healthy controls (-9.35 ± 2.08 vs. -7.35 ± 2.76 [F = 5.352, p = 0.027]), whereas MYyD88s, NOD2, and AR mRNA expression were lower in the MDD group than in healthy group (healthy controls vs. MDD: MYyD88s, -5.98 ± 0.91 vs. -6.60 ± 0.76 [F =6.115, p = 0.019]; NOD2, -5.21 ± 1.27 vs. -5.96 ± 1.02 [F =5.256, p = 0.029]; AR, - 10.81 ± 1.04 vs. -11.69 ± 1.37 [F = 5.449, p = 0.026]) (Figure 1a.). These results suggest an important role of the AR and NOD2 in depression.

NOD2 mRNA expression is regulated by androgen/AR signalings

To further clarify the effect of androgen/AR signaling on TOLLIP and NOD2, we treated the mHippoE-14 hippocampal cell line with the AR agonist

dihydrotestosterone (DHT) or antagonist flutamide for 48 hours. Blocking AR signaling with flutamide for 48 hours significantly inhibited both TOLLIP and NOD2 mRNA expression. However, DHT could not increase NOD2 level when AR was inhibited by flutamide. These results suggest an important role of the AR in regulating NOD2 expression.

Discussion

In this work, the findings of higher IL-6 mRNA level in patients with MDD compared with healthy control in both gender correspond to previous clinical studies in male [27] and in female [28]. The lower AR mRNA level in male MDD patients is also consistent with previous post mortem study which reported that the amount of AR mRNA in the paraventricular nucleus of the hypothalamus was decreased by ~2.7-fold in the depressed patients as compared to the controls [29]. A number of studies have investigated sex differences in immune and psychiatric diseases. Women have stronger immune responses than men in terms of the percentage of total lymphocytes mobilized and cytotoxic lymphocytes [30], and are more likely to experience feelings of social isolation and depression following LPS injection than males, an association that is correlated with cytokine levels [31]. Changes in Th1 cytokines following antidepressant treatment of MDD patients show a trend towards differences according to sex [32]. However, sex-related differences in negative regulators and their association with MDD. have not been previously reported. In the current study, we found that TOLLIP and NOD2 mRNA levels were higher in males than in females in healthy controls, whereas only TOLLIP mRNA levels were higher in males in the MDD group. Considering that previous studies have shown that the association between an enhanced immune response and the prevalence of depression is stronger in females than in males [3-5][7-9], the reported elevated expression and activity of TLR in males represents a contradictory outcome [33]. Our data is the first that suggest some possible explanations to resolve this conflict. Dual roles of NOD2 in TLR4-mediated signal transduction [34] reflect its different effects on behavior. The association of NOD2 with major depression was the subject of a recent investigation, which showed that anxiety levels, Water avoidance stress-induced recognition memory deficits, as well as corticosterone levels were elevated in Nod1/2-double-knockout mice [35]. In contrast, a NOD2 agonist and LPS were shown to synergize with each other to worsen mouse sickness behavior [36]. Few studies have reported sex differences in NOD2 expression level. However, a mutation in the NOD2 gene has been implicated as a possible cause of Crohn's disease, which is more prevalent in females than males [37].

There is also virtually no consideration of the relationship between androgen and NOD2 in the literature, with only a single report noting that NWD1 (NACHT and WD repeat domain-containing protein 1), another member of the NLR family, modulates AR signaling in prostate tumorigenesis [38]. These results are the first to point out the interaction. Differences in TOLLIP expression between males and females seem controversy. Female rats have been reported to express higher levels of TOLLIP mRNA in colonizing microglia than males [39]. In prostate cancer cells, both estrogen and testosterone can cause DNA methylation in the TOLLIP gene [40]. TOLLIP has previously been shown to interact with ARIP3 (AR-interacting protein 3) [41]. Although it is not clear why these differences were observed, especially on TOLLIP and NOD2 between male and female, one of the possible explanations is the effect of androgen. In our cell line, androgen could increase mRNA expression of TOLLIP and NOD2 both but only NOD2 could be decreased in the presence of flutamide. The change of NOD2 is associated with AR but the interaction of TOLLIP and androgen/AR is not simply through direct interaction. Therefore, the specific mechanism responsible for the higher expression of TOLLIP in males compared with females needs further clarification.There are several limitations to this study. First, numerous confounding factors, including other hormones, lifestyle and environment, may influence the observed sex differences. Thus, interactions among antidepressants, cytokines, and negative regulators will require further controlled studies. Second, increases in mRNA expression were not reflected in increases in protein expression. Third, the sample size was relatively low, especially for males. Further analyses using larger sample sizes and well-designed experiments are needed to confirm these results. Besides, a more comprehensive approach using an animal model can confirm these findings. Conclusion In conclusion, we provide a possible explanation for the conflict between the concept that depression is more prevalent in females and the observed higher expression level of TLR in males than in females. Our data further suggest that, in conjunction with current therapeutic regimens, modulating the expression of NOD2 in males or TOLLIP in females to rebalance TLR-mediated inflammatory signaling may provide a potential approach for MDD management.

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Part II

前言、

憂鬱症的盛行率在女性相對於男性是比較高的，但憂鬱症男性自殺死亡率則是女性兩倍以上。男性憂鬱症比女性更不容易識別，男性更可能以非典型的憂鬱症狀如易怒，藥物濫用，和激動來表現。因此，男性憂鬱症需要更多的研究來了解其成因及相關可能的治療方法，以降低死亡風險。

憂鬱症與發炎反應在男女性別差異

細胞激素的分泌是有性別差異的，刺激第七型類鐸接受器(Toll-like receptor (TLR)-7) 相較於男性，女性會產生比較多的 IFN-α，而女性在疫苗注射的研究中也被發現會產生比較多的 tumor necrotic factor (TNF)。然而，男女性別差異在內生性免疫的領域就相對比較少被提及。過去曾報導，公的小鼠在注射 lipopolysaccharide (LPS) 後，出現比較多的生病行為[19]。但在人體上的研究卻說明了女性產生比較多的發炎反應。過去許多研究認為憂鬱症的病人不管是在額葉或是在周邊血液單核細胞中，TLR-4 的表現量是比健康對照組高的，而憂鬱症的症狀是與 TLR-4 核糖核酸表現量有相關性。然而比較難以解釋的，卻是過去的報告認為男性的 TLR-4 表現量高於女性，這樣的結果與盛行率調查不同，也無法解釋為何女性在壓力下細胞激素分泌較多。去年已經報告男性與女性在內生性免疫的負向回饋機制上的差異，今年更近一步分析微小核醣核酸在內生性免疫的負向調控上的男女差異。

研究目的、

1. 了解比較男性憂鬱症患者內生性免疫負向調控上的微小核醣核酸(microRNA)的表現量，並同時以臨床調查評估其對憂鬱症症狀表現的影響。透過整合長庚研究計畫所收入的女性病患及健康對照組，正好可分四組比較。並透過分組了解憂鬱症狀與微小核糖核酸的相關性。

文獻探討、

內生性免疫相關微小核醣核酸在憂鬱症病人的狀態

過去也曾報導負向調控 TLR4 信號的 microRNA 與發炎疾病有關。miR-21 通過針對 PDCD4 來負調控 TLR4，被認為在實驗性自身免疫性腦脊髓炎模型中是重要的。miR-146a 和 miR-125 在調節天然免疫和炎症方面被認為與類風濕性關節炎有關(Lee et al., 2016)。 Lopez 也報導了來自全血的 miR-146a 與抗憂鬱藥的反應有關 [(Lopez et al., 2017a). 。白血球中負調節 TLR4 信號的的 microRNA 可能在重度憂鬱症中有重要作用。

越來越多的證據表明，microRNA 在精神疾病 (Issler and Chen, 2015) ，尤其是憂鬱 (Lopez et al., 2017b)中起著重要的作用。篩選的周邊血液（包括 PBMC，全血，血清和血漿）的樣品都曾經被用以鑑定與憂鬱症有關的可能的 microRNA。然而僅血漿（miR-34b-5p，miR-34c-5p，miR-107，miR-381 ）(Sun et al., 2016)和 CSF (miR-16)(Song

et al., 2015)報導了 mircroRNA 的臨床嚴重程度與表達之間的關係。。 microRNA 對憂鬱症相關的途徑分析曾有報導，但沒有研究指出 microRNA 對憂鬱症發炎反應，特別是 TLR4 信號通路負調控的影響。

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研究方法

RT PCR

以 RT-PCR 分析前述 microRNA 的表現量。使用 Biozol® Total RNA extraction reagent (Bioflux, Inilab, Madrid, Spain)由檢體中萃取出 Total RNA，經過 DNase 處理後，使用 (Epicentre, Tech. Corp., Madison, Wisconsin)反轉錄得到 cDNA，接著以 ABI PRISM 7500 Real Time Cycler (Applied Biosystems, Madrid, Spain)進行定量 PCR，得到 Ct 值，並以 U6 為參考基因計算各基因的相對表現量。

Assay ID	Assay Name	Mature microRNA Sequence
002406	hsa-let-7e	UGAGGUAGGAGGUUGUAUAGUU
000583	hsa-miR-9	UCUUUGGUUAUCUAGCUGUAUGA
000397	hsa-mir-21-5P	UGAGGUAGUAGGUUGUAUGGUU
002198	hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA

000449	hsa-miR-125b	UCCCUGAGACCCUAACUUGUGA
002278	hsa-miR-145	GUCCAGUUUUCCCAGGAAUCCCU
000468	hsa-miR-146a	UGAGAACUGAAUUCCAUGGGUU
002623	hsa-miR-155	UUAAUGCUAAUCGUGAUAGGGGU
002287	hsa-miR-155*	CUCCUACAUAUUAGCAUUAACA
002285	hsa-miR-186	CAAAGAAUUCUCCUUUUGGGCU
000498	hsa-miR-199a	CCCAGUGUUCAGACUACCUGUUC
000508	hsa-miR-204	UUCCCUUUGUCAUCCUAUGCCU
002295	hsa-miR-223	UGUCAGUUUGUCAAAUACCCCA
002340	hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
001516	hsa-miR-425-5p	AAUGACACGAUCACUCCCGUUGA
001973		U6

Cell culture

本研究預計採用 THP-1、SIM-A9、mHippoE 等細胞株，探討 microRNA 的生物性影響與相關的機制。THP-1 細胞株購自 ATCC，培養於含有 10% FBS 的 RPMI 1640 培養基中。SIM-A9 細胞株同樣購自 ATCC，以 DMEM/F12 培養基培養之，並添加所需的 5% HS 與 10% FBS。mHippoE 細胞株購自 CEDARLANE，使用添加 10% FBS 的 DMEM 培養基 所有的細胞培養基均添加 100 U/ml penicillin and 100 μg/ml streptomycin.。細胞培養於 5% CO2、37°C 細胞培養箱中。

SiRNA Transient Transfection

抑制 microRNA 表現的 siRNA 購自 Dharmacon。細胞生長於 6-well plate，分別以 DharmaFECT 轉染入 si-RNA 或 si-non-target，48 小時後進行後續分析。

結果與討論

3.1. Demographic and clinical characteristics

共招募了 84 名患有憂鬱症的患者，其中包括 20 名男性和 64 名女性。其中，69 名患者接受抗抑鬱藥治療 4 週，並返回進行隨訪檢查。對照組年齡（41.88±9.03 歲）略低於 MDD 患者（45.20±11.00 歲）和治療後（45.56±10.46 年）（（1）vs（3），（2 vs（3），表 1）。治療前健康對照組和 MDD 患者的 BMI 相似，憂鬱症組吸煙率明顯較高，HAMD-17 評分在（8.91±5.08）治療後顯著低於治療前（24.16±5.48）。患者分為緩解組和非緩解組，治療前年齡，性別，BMI，吸煙和 HAMD 評分無差異（（4）vs（5），表 1）。

Table 1. Demographic and clinical characteristics of patients with major depressive disorder and healthy controls

	(1) MDD before treatment (n = 84)	**(2) MDD after treatment (n = 69)**	**(3) Healthy controls (n = 43)**	***p*-value**		**(4) MDD Remission (n = 31)**	**(5) MDD Non-remission (n = 38)**	***p*-value**
				(1) vs (3)	(2) vs (3)			(4) vs (5)
Age (years)	45.20 ± 11.00	45.56 ± 10.46	41.88 ± 9.03	*p* = 0.086	*p* = 0.059	45.39 ± 9.85	45.71 ± 11.06	*p* = 0.899
Sex (M/F)	20/64	16/63	6/37	*p* = 0.251	*p* = 0.793	8/23	4/34	*p* = 0.098
BMI (kg/m2)	24.61 ± 4.26	24.82 ± 4.19	23.91 ± 3.25	*p* = 0.259	*p* = 0.195	24.55 ± 4.17	25.05 ± 4.23	*p* = 0.624
Smoking (yes/no)	30/54	22/47	5/38	*p*= 0.005 *	*p*= 0.015 *	13/18	9/29	*p* = 0.087
HAMD-17	24.16 ± 5.48	8.91 ± 5.08	-	-	-	22.90 ± 5.17	25.45 ± 5.69	*p* = 0.056

Results reported as mean ± SD or as number
Age and BMI were compared by Student's t tests.
Sex and smoking were compared by the chi-square test
* $P < 0.05$

3.2. Levels of microRNAs and effect of antidepressants in PBMCs

為了探索 TLR4 信號傳導的細胞內 microRNA 調節因子的表現量，從憂鬱症患者和健康對照中分離 PBMC，以及它們的 microRNA 表現量，包括 let-7e，miR-21-5p，miR-145，miR-223，miR-146a 和 miR-155。在調整年齡，性別，吸煙和 BMI 後，從急性期 MDD 患者分離的 PBMC 中 let-7e，miR-21-5p，miR-146a 和 miR-155 的水平顯著低於健康對照組（1） vs（3），表 2）。憂鬱症患者 IL-6 mRNA 的表達高於健康對照組。然而，miR-145 和 miR-223 的水平在這兩組中沒有顯著差異。

為了評估抗抑鬱治療和 TLR4 信號傳導的 microRNA 調節劑之間的關聯，從獲自用抗抑鬱藥治療 4 週的 69 名患者獲得的 PBMC 中分離 RNA。與基線相比，治療 4 週後，PBMC 中 let-7e，miR-223，miR-146a 和 miR-155 的表現量顯著增加，IL-6 顯著下調（（1）vs（2），表 2）。

Table 2. Expression in PBMCs of individual microRNAs regulating TLR signaling in patients with major depressive disorder and healthy controls

	(1) MDD before treatment (n = 84)	**(2) MDD after treatment (n = 69)**	**(3) Healthy Controls (n = 43)**	**vs (3)** F and *p*-value	**vs (2)** *p*-value
let-7e	-4.09±1.76	-3.62±1.40	-3.41±1.16	F= 4.605 *p* = 0.034*	*p* = 0.002*
miR-21-5p	-6.00±2.11	- 5.54±2.12	-5.28±1.18	F= 4.097 *p* = 0.045*	*p* = 0.062
miR-223	2.95±1.56	3.36±1.49	3.41±0.77	F= 2.906 *p* = 0.091	*p* = 0.002*
miR-145	-5.75±1.43	-5.61±1.50	-5.17±1.25	F= 3.748 *p* = 0.055	*p* = 0.111
miR-146a	-1.88±2.06	-1.63±2.06	-0.60±0.85	F= 15.374 *p* = 0.000*	*p* = 0.038*
miR-155	-3.23±1.78	-2.90±1.43	-2.25±0.77	F= 11.386 *p* = 0.001*	*p* = 0.004*
IL-6	-9.50±1.80	-9.73±1.70	-10.13±1.48	F= 5.113 *p* = 0.026*	*p* = 0.025*

Results reported as mean ± SD

(1) vs (3) is compared by ANCOVA after adjustment for age, sex, smoking and BMI

(1) vs (2) is compared by paired t-tests.

* $p < 0.05$

3.3. Levels of microRNAs and effects of antidepressants in monocytes

還在從 PBMC 分離的單核細胞製劑中評估細胞內 microRNA 調節劑的表現量。與從 PBMC 獲得的結果類似，來自 MDD 患者的單核細胞中的 miR-146a 和 miR-155 表現量顯著低於來自健康對照的單核細胞（（1）vs（3），表 3）。
還在單核細胞中研究了抗抑鬱治療對 TLR4 信號傳導的 microRNA 調節劑表現的影響。與 PBMC 中的發現類似，在抗憂鬱治療後，單核細胞中的 let-7e，miR-146a 和 miR-155 表現量增加（（1）vs（2），表 3）。此外，單核細胞中 miR-145 表現量增加，而 miR-21-5p 表現量降低。

Table 3. Expression in monocytes of individual microRNAs regulating TLR signaling in patient with major depressive disorder and healthy controls

	(1) MDD before treatment n = 47	(2) MDD after treatment n = 33	(3) Healthy controls n = 33	vs (3) F and *p*-value	vs (2) *p*-value
let-7e	-2.52±0.79	-1.70±0.82	-2.10±0.92	F=3.088 *p* =0.083	*p* = 0.001*
miR-21-5p	-3.78±1.38	-5.16±1.22	-3.53±0.75	F=0.394 *p* =0.532	*p* = 0.022*
miR-223	5.15±0.77	5.44±0.78	5.14±0.80	F=0.128 *p* =0.722	*p* = 0.054
miR-145	-6.17±1.42	-5.44±0.97	-5.58±0.93	F=2.932 *p* =0.091	*p* = 0.006*
miR-146a	-2.71±1.55	-2.27±0.95	-1.48±1.23	F=12.320 *p* =0.001*	*p* = 0.034*
miR-155	-2.57±1.17	-2.11±0.685	-1.72±0.95	F=10.208 *p* =0.002*	*p* = 0.025*

Results reported as mean ± SD

(1) vs (3) compared by ANCOVA after adjustment for age, sex, smoking and BMI

(1) vs (2) compared by paired t test

* $p < 0.05$

3.4. The association between microRNA expressions and clinical findings

為進一步研究 microRNA 變化與治療反應之間的關係，將患者分為緩解組和非緩解組。這兩組之間的基線沒有差異（（1）vs（2），表 4）。那些在治療後達到緩解的患者顯示 PBMC 中 let-7e，miR-223，miR-145 和 miR-155 的水平顯著增加（（1）vs（3），表 4）。然而，非緩解組未顯示這些微小 RNA 中的任何變化（（2）vs（3），表 4）。
在接受 4 週抗憂鬱治療的 69 名患者中，21 名患者接受 SSRIs，而 32 名患者接受 5-羥色胺 - 去甲腎上腺素再攝取抑製劑（SNRIs）。 SSRIs 治療顯著增加了 PBMC 中 let-7e 和 miR-155 的水平，而 SNRIs 治療組沒有效果。 SSRI 還增加 miR-223 和 miR-145 表達水平（補充表 2）。

為了探討抑鬱嚴重程度與負調控 microRNA 表達之間的關係，採用多元線性回歸分析，結果顯示 let-7e，miR-146a 與 HAMD-17 評分呈負相關，而 miR-155 呈正相關。 HAMD-17 評分（表 5）。

Table 4. Expression in PMBCs of microRNAs negatively regulating TLR signaling before and after treatment with antidepressants in patients experiencing remission (n = 31) and non-remission (n = 38)

	Before antidepressant treatment		After antidepressant treatment		*p*-value		
	(1) Remission	(2) Non-remission	(3) Remission	(4) Non-remission	vs (2)	vs (3)	(2) vs (4)
let-7e	-4.38±1.80	-4.16±1.80	-3.56±1.21	-3.70±1.54	F = 0.332 *p* = 0.567	*p* = 0.002*	*p* = 0.115
miR-21-5p	-6.16±2.02	-6.03±2.20	-5.44±2.42	-5.62±1.87	F = 0.040 *p* = 0.842	*p* = 0.135	*p* = 0.296
miR-223	3.00±1.69	2.77±1.72	3.64±1.64	3.10±1.34	F = 0.197 *p* = 0.659	*p* = 0.001*	*p* = 0.177
miR-145	-6.05±1.40	-5.82±1.46	-5.44±1.42	-5.75±1.58	F = 0.252 *p* = 0.618	*p* = 0.020*	*p* = 0.820
miR-146a	-1.90±2.27	-2.05±2.16	-0.92±1.00	-1.73±2.19	F = 0.036 *p* = 0.849	*p* = 0.107	*p* =0.202
miR-155	-3.32±1.58	-3.38±2.11	-2.85±1.31	-2.98±1.55	F = 0.000 *p* = 0.992	*p* = 0.001*	*p* = 0.115

Results reported as mean ± SD

(1) vs (2) is compared by ANCOVA after adjustment for age, sex, smoking and BMI

(1) vs (3) and (2) vs (4) are compared by paired t test

* $p < 0.05$

Table 5. Correlations between HAMD-17 scores and expression of microRNA in monocytes as determined by multiple linear regression analysis

Independent factors	HAMD-17 score		
	standardized coefficients	t	*p*-value
let-7e	-0.793	-2.946	$p = 0.006$*
miR-21-5p	0.004	0.012	$p = 0.990$
miR-223	0.316	0.793	$p = 0.434$
miR-145	-0.027	-0.114	$p = 0.910$
miR-146a	-1.111	-3.500	$p = 0.002$*
miR-155	1.001	2.886	$p = 0.007$*

Part III

前言及文獻探討

Major depressive disorder (MDD) is highly associated with marked personal, social and economic morbidity and the susceptibility of MDD is affected by the interactions of multiple functional genetic variants and environmental factors. In the U.S., MDD has the greatest impact of all diseases on disability; in Europe, it is the third leading cause of disability (Alonso *et al*, 2004). In adults, the prevalence of MDD is higher in female than in male (Gu *et al*, 2013). Among older people, more men than women reported depressive symptoms, and the largest proportion was found among men in the age group 75-80 years (Djukanovic *et al*, 2014). Under low plasma testosterone levels, men often exhibit depressive symptoms, and testosterone replacement has been shown to improve these symptoms (Burris *et al*, 1992; Wang *et al*, 1996). These evidences strongly suggest that testosterone may have protective effects on adult men against MDD.

The biological actions of androgens are mediated by a ligand-dependent nuclear transcription factor, the androgen receptor (AR). An inverse relationship between AR transcriptional activity and the number of polyglutamine repeats in the AR transcriptional activation domain is well documented (Beilin *et al*, 2000; Chamberlain *et al*, 1994). Previous study examined the relationship between the length of polyglutamine repeats and MDD and found that the length of CAG repeats in AR gene could predict the severity of negative affect in young men (Sankar and Hampson, 2012). Other studies showed that complete androgen blockade with combined leuprolide and flutamide, an AR antagonist, to treat prostate cancer significantly increased the rate of development of depression (Cherrier *et al*, 2009; Lee *et al*, 2015). Recent studies reported that depression was observed in 36% of patients with complete androgen insensitivity syndrome due to loss of AR activity (Fliegner *et al*, 2014; Mueller *et al*, 2014). Together, these studies suggest that loss of AR activity may be associated with increasing the risk of MDD.

Exposure to stress in mouse depression models has been shown to decrease the expression of Brain-derived neurotrophic factor (BDNF), which is also decreased in the serum of patients with major depression (Bus *et al*, 2015; Huang *et al*, 2008) and in hippocampus from suicide victims (Autry and Monteggia, 2012; Castren and Rantamaki, 2010; Castren *et al*, 2007). In the light of the associations between the BDNF Val66Met SNP and depression in men (Licinio and Wong, 2010; Verhagen *et al*, 2010), conditional BDNF knockout mice in which the BDNF gene was deleted selectively in forebrain displayed gender differences in depression-related behaviors (Monteggia *et al*, 2007). Testosterone treatment has been shown to be effective on spine maturation in sublayers of dendritic spines in hippocampus CA1 area of

gonadectomized male mice via regulation of BDNF (Li *et al*, 2012a). However, the molecular mechanisms of androgen/AR-mediated BDNF-TrkB signaling in stress-induced depression in male remain to be investigated.

研究目的

To explore the roles of AR in regulation of stress-induced depressive symptoms, we applied the chronic mild stress (CMS) to androgen receptor knockout (ARKO) mice and investigated whether the loss of AR function affected BDNF-TrkB axis, which increases the susceptibility to stress-induced depressive symptoms in mice.

研究方法

Animals

All animal studies were handled in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication), as adopted. All procedures for testing and handling were approved by the Committee on Animal Resources of Kaohsiung Chang Gung Memorial Hospital. We used Cre–Lox strategy to generate WT and ARKO mice and the floxed AR/ AR mice were established as previously described (Yeh *et al*, 2002). WT and ARKO male mice were established by mating floxed AR/AR female mice with Actb Cre $^{+/+}$ mice. The floxed AR/Y-Actb Cre $^{+/-}$ mice were used as ARKO and AR/Y-ActbCre$^{+/-}$ were used as WT control. All mice were group-housed in our colony at Kaohsiung Chang Gung Memorial Hospital with a 12/12 light/dark cycle, lights on at 0700. For the behavioral experiments, adult male mice (age = 10 weeks) were used in all tests and comparisons were made to age-matched littermate control cohorts. All testing were carried out in a behavioral suite adjacent to the holding room in the mouse facility at Kaohsiung Chang Gung Memorial Hospital. For behavioral tests requiring video-tracking, a video was acquired at 4 fps with 240 × 240 pixel spatial resolution using ethovision XT (Noldus).

CMS protocol

In the CMS paradigm the animals were exposed for 2 weeks to several stressors. The stressor schedule followed in this study (Table 1) was adapted from a previous protocol for mice (Schweizer *et al*, 2009) and rats (Herrera-Perez *et al*, 2008). The males in the unstressed groups were under handling and storage conditions comparable to the stressed animals.

Sucrose preference test and forced swimming test

The protocol of sucrose preference test with 1% sucrose solution and forced swimming test was adapted from previous study (Schweizer *et al*, 2009).

Open field test

The protocol of open field test was adapted from previous study(Jung *et al*, 2014). The total distance travelled and the percentage of time spent in the central zone were measured as indicative of activity and anxiety. Measurement of various parameters was electronically done for 10 min with ethovision XT (Noldus).

Nissl Staining, and Immunohistochemistry

Nissl Staining, BDNF and AR staining were performed on 5 μm paraffin brain sections. The sections were deparaffinized in xylene, and then hydrated in 100-70% alcohol. 0.1% cresyl violet solution was used for Nissl stain. Other sections were incubated with the anti-BDNF antibody (5 μg/mL, AB1534SP, Millipore) or anti-AR antibody (1 : 50, sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz) for 24 hour at 4º. The sections were then washed three times with 0.2% Triton X-100 in phosphate-buffered saline, and then incubated with Dako REAL EnVision/HRP, Rabbit/Mouse (ENV) for 30 minutes at room temperature, followed by a color reaction using Dako REAL DAB+ chromogen for 1 minute for BDNF and AR.

In situ hybridization (ISH) for BDNF

1. In situ probe generation: Probe sequences were as follows: CAGTTGGCCTTTGGATACCGGGACTTTCTCTAGGACTGTGACCGTCCC
2. Coronal sections (5 μm) were mounted on poly-l-lysine-coated slides and allowed to air-dry for 16 hours at 45°C, and were then deparaffinized and rehydrated. The BioTnA Biospot Kit protocol was applied to the sections. The ISH is completed after applying DAB Solution to the slides for 1 minute and the slides washed for 2 min under running tap water.

Laser capture microdissection, RNA Isolation, RT-PCR and amplification

LCM was performed with Veritas Automated Laser Capture Microdissection (LCM) System (Arcturus). A total of 10-15 series of sections were dissected for each sample. The RNA of Microdissected cells were extracted using the PicoPure RNA isolation kit (Life Technologies). The isolated RNAs were reverse transcribed using QuantiTect Rev. Transcription Kit (Qiagen) following the manufacturer's instructions. The cDNA was then pre-amplified using the TaqMan PreAmp Master Mix (Life Technologies) according to the manual of the manufacturer. The primer pool used for pre-amplification consisted of 10 TaqMan Assays (Life Technologies), including BDNF transcript variants I, IIA, IIB, IIC, III, IV, VI, IXA, total BDNF, and HPRT.

Quantitative Real-Time PCR

Pre-amplified cDNA (1:20 dilution) were added to master mix consisting of TaqMan® Fast Universal PCR Master Mix (Life Technologies), distilled water and the respective TaqMan Gene Expression Assay. RT-PCR primers were used to amplify total BDNF (Mm04230607_s1), BDNF transcript I (Mm01334047_m1), BDNF transcript IIA (Mm01334046_m1), BDNF transcript IIB (Mm01334045_m1), BDNF transcript IIC (Mm01334044_m1), BDNF transcript III (Mm01334043_m1), BDNF transcript IV (Mm00432069_m1), BDNF transcript VI (Mm01334042_m1), BDNF transcript IXA (Mm04230616_s1) and HPRT (Mm00446968_m1). RT-PCR was performed with a 7500 Fast Real-Time PCR System (Life Technologies).

Fluoxetine and 7,8-dihydroxyflavone (7,8-DHF) treatments for ARKO mice

Fluoxetine (Sigma, Taufkirchen, Germany) was dissolved in sterile phosphate-buffered saline (PBS) and administrated i.p. daily at 10 mg/kg of body weight (Couillard-Despres *et al*, 2009) for 14 days (figure 5a). 7,8-DHF (Abcam, USA) was dissolved in dimethyl sulfoxide (DMSO) and administrated i.p. twice per day at 5 mg/kg and 20 mg/kg of body weight for 14 days (figure 5a).

Statistical and data analysis

All sections used for immunofluorescence, immunohistochemistry staining and ISH were microphotographed together in order to diminish the difference as much as possible. Data related to AR were analyzed with the IN Cell Analyzer 2200 Imaging System. Data related to the BDNF immunohistochemistry and in situ hybridization were analyzed with optical intensity staining using Image-Pro Plus software. The cell density of Nissl-stained cell, BDNF protein immunoreactive cells and BDNF RNA positive cells were counted by experienced staff. Independent T test was used to analyze the difference in sucrose preference and immunofluorescent intensity in WT mice received CMS for 6 weeks. The animal which were divided into four groups and received CMS for 2 weeks were analyzed by one-way analysis of variance (ANOVA) with LSD post hoc analysis. For all comparisons, $P < 0.05$ or $P < 0.01$ was used as a criterion for statistical significance.

結果與討論

AR expression pattern altered in the CA1 region of mouse hippocampus in response to CMS

Previously, we have demonstrated that AR proteins acquire ER stress chaperones to protect cells against the physiological fluctuations or stress disturbance *in vitro* (Yang *et al*, 2013) and recent study showed that recurrent depression shrinks the hippocampus (Schmaal *et al*, 2015) - an area of the brain

where AR is highly expressed (Kerr *et al*, 1995). Therefore, we further extend our cellular observation to investigate the effects of CMS on differential AR expression in the mouse hippocampus *in vivo*. We exposed a group of mice to 6 weeks of CMS to establish an animal model of depression (Figure 1a). The depressive symptomatology of the mice and the expression patterns of AR from these mice were compared to a control group of non-stressed mice (Figure 1b and 1c). In agreement with previous findings (Barnum *et al*, 2012), depressive-like behaviors such as sucrose preference were decreased by 20% in CMS-exposed-mice compared with control mice (week 5, 6, $P < 0.05$; Figure 1b). Interestingly, we found that CMS-exposed mice exhibited an increase in the AR protein expression in the CA1 region of the hippocampus (Figure 1d and 1f), but not in the CA3 region (Figure 1e and 1f).

Loss of AR accelerated the CMS-mediated depressive-like behavior in mice

Next, we sought to determine whether loss of AR might affect CMS-mediated depressive-like behavior in mice. Before 2-weeks CMS procedure, sucrose preference in the WT mice was similar to the ARKO mice (Figure 2b). While there was no difference between the WT and ARKO mice without CMS procedure, the ARKO mice had an early onset of depressive–like behavior with significantly decreased in the sucrose preference test compared to WT mice after 2-weeks CMS procedure (Figure 2b). In the forced swimming test, the immobilization duration was significantly longer in the ARKO mice that underwent CMS than in the ARKO without CMS exposure (Figure 2c). Comparing the level of activity and anxiety between the ARKO and WT mice, there was no statistical difference in both distance and duration of the open field test (Figure 2d and e).

Loss of AR decreases the expression of BDNF IV and VI transcripts in the CA1 region of mouse hippocampus in response to CMS

BDNF has been demonstrated to be significantly decreased in the hippocampus CA1 area of CMS-treated mice (Taliaz *et al*, 2011). By using immunohistochemistry, we examined the protein expression of BDNF in the hippocampus from WT and ARKO mice treated with or without CMS. We found that the density of BDNF immunoreactive cells in the CA1 area (Figure 3g, h and u) but not in CA3 area (Figure 3k, l and v) of the hippocampus of CMS-treated ARKO mice was significantly decreased. There was no change of the density of BDNF immunoreactive cells after CMS in WT mice in CA1 or CA3 (Figure 3e, f, I, j, u and v). In addition, there is no difference between the 4 groups in the cell density from the CA1 region of the hippocampus by using Nissl staining (Figure 3q, r, s, t and w).

By using in situ hybridization, we also examined BDNF mRNA expression and

localization in the hippocampus and found that the cell density of BDNF mRNA positive cells in the CA1 area was significantly decreased after the CMS procedure (Figure 4g, h and i). However, there was no difference between in WT mice with or without CMS exposure (Figure 4e, f and i). To further understand to possible mechanism by which AR acts on the BDNF gene regulation, the neuronal cells in the CA1 region of the hippocampus from WT and ARKO mice were first collected by laser-capture microdissection and mRNA levels from different exons of the BDNF gene were analyzed by real-time PCR. After screening all the BDNF mRNA transcripts, significantly decreased mRNA levels of BDNF exon IV and VI were identified in ARKO mice exposed to CMS (Figure 4j).

7,8-DHF attenuates depressive-like behavior in ARKO mice with CMS exposure

To dissect whether the BDNF/TrkB signaling pathways might be the downstream targets of androgen/AR signaling pathways, fluoxetine, an antidepressant which has been shown to increase BDNF (Nibuya *et al*, 1996), low-dose (5 mg/kg) and high-dose (20 mg/kg) of 7, 8-DHF, a TrkB receptor agonist, were applied to ARKO mice with CMS (figure 5a). We found that only high-dose of 7, 8-DHF and fluoxetine both attenuated depressive-like behavior (figure 5b), suggesting that AR may be the upstream of BDNF/TrkB signaling pathways.

106年度專題研究計畫成果彙整表

計畫主持人：洪一永	**計畫編號：**106-2629-B-182A-001-MY3
計畫名稱：男性荷爾蒙及其接受器對男性憂鬱症的影響：NOD2及TOLLIP的角色	

成果項目			量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文	0	篇	
		研討會論文	0		
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
國外	學術性論文	期刊論文	5	篇	1. Neuroimmunomodulation. 2019;26(3):153-158. 2. Cells. 2019 Jun 27;8(7):647. 3. Cells. 2019 Sep 2;8(9):1021. 4. Neuroimmunomodulation. 2018;25(1):42-48. 5. Neuroimmunomodulation. 2017;24(4-5):271-275.
		研討會論文	0		
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	0		
		博士級研究人員	0		
		專任人員	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士級研究人員	0		
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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。）					