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以基因體層面探討天麻水萃物之抗憂鬱功效

Transcriptomic Profile of the Anti-Depressant Effects of *Gastrodia elata* Bl.

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兩年的研究生涯終於到了尾聲。曾聽人說很多老師收到學生的碩士論文都只看「謝誌」頁，如果在謝誌頁中沒有找到自己的名字，就會直接把那本論文丟了。然而，這本論文的產生要感謝的人實在太多了。首先要感謝指導教授沈立言老師對於研究生的無比包容以及讓學生們放手一搏的信任，使得我學會了研究及待人處事的精隨。特別要感謝沈老師總在百忙之中能抽空與研究生討論，給予研究上的建議以及論文的指正，並關心學生們的進度。再來要感謝台灣大學生物科技研究所蔡孟勳老師在微陣列分析上的分析架構與本論文的細心指導及審閱，以及生技所的學長姐們，尤其是政瑩和逄昌學長，對於分子生物實驗技術上的耐心指導。感謝台灣大學心理系的賴文崧老師以及中國醫藥大學精神科醫生蘇冠賓主任給予本論文相當多的指正與建議，使得本論文更臻完善。特別感謝中央研究院的坤海和誼寧學長在微陣列分析的操作上給予無比耐心的指導。

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Abbreviations

ACTB: ß-actin

BDNF: Brain Derived Neurotrophic Factor

bw: body weight

CNS: central nervous system

$C_{t:}$: cycle threshold

ECT: electroconvulsive Treatment

FST: Forced Swimming Test

GABA: γ- AminoButyric Acid

GEB: *Gastrodia elata* bl.

KEGG : Kyoto Encyclopedia of Genes and Genomes

MAOI: monoamine oxidase inhibitor

MAP: microtubule associated proteins

MAPK: microtubule associated protein kinase

QRT-PCR: quantitative reverse transcriptase-polymerase chain reaction

SSRI: serotonin reuptake inhibitor

TRK: tyrosine kinase

WGE: water extraction of *Gastrodia elata* Bl.

Abstract

Depression has been a serious issue, as the annual worldwide reported cases of depression have been constantly increasing, according to the statistics published by WHO. In fact, WHO predicts that it will be the leading psychological disease by year 2020. However, antidepressants generally demonstrate serious side-effects, such as anxiety disorders, gastrointestinal problems, and sexual dysfunction in patients; therefore, it is important to find an effective way to prevent the occurrence of depression. In this research, *Gastrodia eleta* Bl., an Oriental herb that has been shown to demonstrate anti-depression effects, was investigated in the form of water extraction. In the present study, the water extract of *Gastrodia eleta* Bl. (WGE) was orally administered to Sprague-Dawley rats at the dose of 0.5 g/kg BW each day for 21 consecutive days. Forced swimming test (FST) was performed to induce depression in the rodent model, and results showed that the WGE group demonstrated shorter immobile time compared to the negative control group, indicating that FST has successfully induced depression-like behaviours in these rats. Total mRNA samples were then obtained from the frontal cortex, hippocampus, and striatum, after having sacrificed the rats after 21 days. In order to deduce a possible anti-depression pathway at the genomic level, cDNA microarray was performed to generate gene expression profiles of depression relevant brain regions: cortex, hippocampus, and striatum. To confirm the findings, real-time polymerase chain reaction (QRT-PCR) analysis of several neuroplasticity-related, differentially expressed genes, was performed. The microarray data showed that WGE altered axongenesis/neurogenesis, nervous system development, and dopamine secretion pathways in cortex and hippocampus, from the evidence that they yield the lowest *p*-values from all other pathway matches with the

KEGG database. However, there were no depression-related pathways shown in the results of the microarray data for the striatum. QRT-PCR results validated that genes involved in neurogenesis, such as *Map1b*, *RhoA*, *profilin-1*, and *CRMP2* were significantly altered ($p < 0.05$) in the cortex and hippocampus. Therefore, neuroplasticity might be the mechanism that WGE takes. The neuroplasticity effects of WGE in the rodent model of antidepressant action strengthen the case for further testing the results in the same context using other tools, and can serve as the basis of future antidepressant drugs, especially in the area of WGE demonstrating anti-depressant effects in the aspect of genomics.

Keywords: depression; forced swimming test; *Gastrodia elata* Bl.; microarray analysis; neuroplasticity

摘要

憂鬱症是精神疾病中盛行率極高的情感性疾病，並且根據 WHO 公佈的統計顯示，全球憂鬱症患者逐年攀升，並且將會在 2020 年成為精神疾病之首。由於目前抗憂鬱劑治療方式會帶給許多病人嚴重的副作用，例如焦慮，腸胃問題，以及性功能衰減。因此如何預防憂鬱症是個重要的課題。本研究應用中藥食療預防醫學探討天麻水萃物對大鼠情感相關腦組織之影響，如前額葉皮質，海馬迴，以及紋狀體。本研究以管餵的方式將 0.5 g/kg BW 劑量之天麻水萃物 (WGE) 給予 Sprangue-Dawley 大鼠，每日一次並為期連續 21 天，並進一步利用強迫游泳試驗 (FST) 來誘導憂鬱症。研究結果顯示，天麻水萃物試驗組 (WGE) 的大鼠在 FST 中比控制組 (NE) 的老鼠取得有顯著性差異較低的不活動時間，顯示 FST 有成功的誘導大鼠憂鬱症行為。在 21 天試驗期結束後，將大鼠犧牲，並取得前額葉皮質，海馬迴，紋狀體等憂鬱症相關之大腦區域組織，接著萃取 total mRNA. 進一步利用微陣列分析來探討可能的天麻抗憂鬱機制，並運用即時聚合酶鏈式反應來驗證實驗結果。微陣列分析結果顯示在大鼠前額葉皮質以及海馬迴中，axongenesis, neurogenesis, nervous system development, 以及多巴胺分泌 (dopamine secretion) 為最有可能的機制，根據與資料庫的資料比對後取得的 *p*-value 值大小排列，最有可能的機制為最小的數值。然而紋狀體的微陣列分析結果並沒有顯式與憂鬱症有關的機制。即時聚合酶鏈式反應實驗結果驗證了 WGE 確實有顯著性的提升與神經可塑性 (neuroplasticity) 相關的基因表現量， 如 *Map1b*, *RhoA*, *profilin-1*, 以及 *CRMP2* ($p < 0.05$). 因此，神經可塑性為天麻水萃物之可能的抗憂鬱機制。本研究結果可為未來同一試驗模式但不同體系之研究做為參考，並為未來抗憂鬱製藥的研究有所貢獻，尤其是中藥天麻在基因體學中之抗憂鬱研究領域。

關鍵字：憂鬱症，強迫游泳試驗，天麻水萃物，微陣列分析，神經可塑性

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1 Introduction

Depression is a prevalent psychiatric disease, and its prevalence is approximately 20% in females and 10% in males of the global population, according to the statistics of WHO using DALY (Disability-adjusted life year) as the indicator (WHO, 2008). In fact, it was estimated that depression will be the 2nd highest global burden of disease among all the illnesses in 2010, using DALY as the indicator (Thase, 2005). In addition, the suicide rate of depressed patients is 20.4 to 30 times higher than the normal people in the general world population (Terra, 2008). Also, it has been estimated that approximately 87.1% of the suicide victims in Eastern Taiwan suffered from depression (Cheng, 1995). Unfortunately, depression patients do not receive medical treatments aggressively. In Taiwan, 4 to 5% of the population has been diagnosed with depression, but many of them do not receive medical treatments and the number of patients receiving treatments is not increasing (Hwu et al., 1989).

The low rate of depression patients receiving medical treatment is because of the potential side effects of the medication, such as anorexia (Brambilla et al., 2005) or a decreased desire in sexual behavior (Clayton et al., 2006). In addition, electro-convulsive test, the most effective treatment for depression, can cause temporary anemia as its major adverse effect (Datto, 2000), although it does not lead to brain tissue damage (Agelink et al., 2001). Therefore, it is crucial to look for an alternative therapy to prevent the patient from developing depression. *Gastrodia elata* Bl. (GEB), also known as Tian Ma, has been discovered to have anti-depression effects. There is no evidence of GEB causing side-effects yet, neither has any previous research on GEB's antidepressant effects on screening for a particular mechanism at the genomic level.

Depression can be caused by several factors, including neurotransmitters, monoamines, oxidative stress, and neuroplasticity (Hayley et al., 2005). Since there could be many mechanisms that would lead to depression, a specific, possible mechanism was to be deduced for the effects of WGE on the depressed animals. Microarray is a powerful, high throughput screening tool as it can study the whole genome in the established gene bank simultaneously (22,523 genes from the gene bank of rats in this study), as opposed to traditional molecular biology tools such as Northern blotting that could only study one gene at a time.

In this research, the anti-depression effects of GEB were investigated at the genomics level by cDNA microarray analysis and real-time PCR on forced swimming induced Sprague-Dawley rats. Results showed that WGE does not cause significant adverse-effect, specifically weight loss, on the rats. The WGE group displayed a lower immobility time, indicating that depression was successfully induced in the rodents. Neuroplasticity, as one of the possible mechanisms by which WGE affects the depressed animals, was acquired by pathway analysis software and were validated by QRT-PCR. Genes involved in neurogenesis and neuroplasticity, such as *Map1b*, *RhoA*, *profilin-1*, and *CRMP2* were significantly altered ($p < 0.05$) in the cortex and hippocampus. Therefore, neuroplasticity might be our interested mechanism. In this thesis, a preliminary discovery of neuroplasticity has been achieved solely based on the microarray data, with a partial validation of QPCR, only on the neuroplasticity-related genes. A closer investigation involving tissue staining of neurons will be performed to validate neurogenesis being the mechanism.

2 Literature Review

2.1 What is depression?

Depression is a psychological disorder that greatly affects social behavior of an individual, such as work performance and social relationships. There are many forms of depression, including unipolar and bipolar.

2.2 Depression symptoms

The diagnosis of depression and other mood disorders is based on symptoms shown on patients in clinical studies. The common ways of classification are International Classification of Disease (ICD) and Diagnostic and Statistical Manual of Mental Disorders, 4th edition, DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV).

For example, symptoms of major depressive disorder (MDD), based on the DSM-IV system include the following:

- depressed mood (such as feelings of sadness or emptiness)
- reduced interest in activities that used to be enjoyed
- loss of appetite or significant weight changes

- sleep disturbances (either not being able to sleep well or sleeping too much)

- loss of energy or a significant reduction in energy level

- difficulty concentrating, holding a conversation, paying attention, or making decisions that used to be made fairly easily

- loss of values in life

- suicidal thoughts or intentions.

If the above symptoms appear almost everyday and almost throughout the day for at least two weeks continuously, then the person experiences MDD (NIMH, 2009; Diagnostic and Statistical Manual of Mental Disorders, 4th edition, DSM-IV).

2.3 Chemical treatments of depression

2.3.1 Antidepressants medication

Antidepressants function by blocking the reuptake of certain neurotransmitters (norepinephrine, serotonin, dopamine, etc.) back into the nerve ending and blocking some of the other neurotransmitter receptors. The most clinically relevant receptor blockade occurs at muscarinic (acetylcholine), histaminic (H1), alpha-1 adrenergic, dopaminergic (D2), and serotonergic (5-HT2A) receptors (Stahl, 2000; Richelson, 2003; Nierenberg, 1992).

Monoamine Oxidase Inhibitors (MAOIs):

Examples include moclobemide (Manerix), tranylcypromine (Parnate), phenelzine (Nardil), selegiline (Eldepryl). MAOIs could inhibit the degradation of noradrenaline and serotonin, thereby increasing their concentration and effect. Side-effects include orthostatic hypotension, hypertensive crises, dizziness, reflex tarchycardia, sexual dysfunction, hepatotoxicity, pyridoxine deficiency, weight gain, and sedation (Khawam et al., 2006).

Tricyclics:

Examples include Amitryptyline, Imipramine, and Doxepine. These medicines were introduced shortly after monoamine oxidase inhibitors, but are less commonly used compared to SSRIs, as tricyclics have higher toxicity. Tricyclics block reuptake of serotonin and norepinephrine. They are competitive antagonists at the muscarinic, histaminergic, and alpha 1 and 2 adrenergic receptors. Side-effects include dry mouth and cardiac effects (Khawam et al., 2006).

Selective Serotonin Reuptake Inhibitors (SSRIs):

Examples include fluoxetine (Prozac), sertraline (Zoloft), Paroxetine (Paxil), fluvoxamine (Luvox), citalopram (Celexa), and escitalopram (Lexapro). (Khawam et al., 2006) SSRIs have replaced tricyclic antidepressants as the first line of depression treatment and have been accounted for most of the antidepressant prescriptions in the United States (Khawam et al., 2006) These medicines could

selectively inhibit serotonin reuptake at the presynaptic nerve terminal, thereby increasing its concentration. They are generally well-tolerated; however, it has been reported that about 15% of patients cannot tolerate the side-effects and therefore have stopped taking the medication (Khawam et al., 2006). Side-effects include sexual dysfunction, gastrointestinal problems, central nervous system side-effects, and motor-muscle discoordination. (Khawam et al., 2006)

2.3.2 Electroconvulsive therapy

ECT is an extremely effective and fastest acting treatment in which seizures (lasting at least 15 seconds, 30-60 seconds optimally) are electrically induced through the electrodes applied on the anesthetized patient's temples (Potter and Rudorfer, 1993; Gomez, 2004). It is effective against both unipolar and bipolar depression, mania, and catatonia. ECT is usually recommended for severe depression after both psychotherapy and antidepressants medication have shown no effect, or for patients who cannot tolerate the side-effects of medication (Scott et al., 2005). ECT causes side-effects including anterograde and retrograde amnesia, cardiovascular problems, headache, nausea, confusion, and muscle pain (Datto, 2000; Nobler and Sackeim, 2008a; Nobler and Sackeim, 2008b). It is more recurrent than medication (Kellner and Bourgon, 1998). However, the efficacy of treatment has been shown that continuation of ECT is better than long-term medication treatment (Gomez, 2004; McClintock et al., 2011). If steps are taken to decrease potential risks, ECT is generally accepted to be relatively safe during all trimesters of pregnancy, particularly when compared to pharmacological treatments (Miller, 1994; Walker and Swartz, 1994; Ferrill et al., 1992).

2.3.3 Combined treatment

Depression is also very often treated by one or more methods at the same time. For example, psychotherapy can be combined with medication (Duncan and Miller, 2000), and medication can be combined with ECT (Kellner and Bourgon, 1998).

Class	Mechanism of Action	Drugs	Side-Effects
Serotonin reuptake inhibitors (SSRIs)	Serotonin reuptake inhibition at the synapse	fluoxetine (Prozac) sertraline (Zoloft) Paroxetine (Paxil) fluvoxamine (Luvox) citalopram (Celexa) escitalopram (Lexapro)	Sexual dysfunction Central nervous Nausea Insomnia Sedation
Monoamine oxidases inhibitors (MAOs)	Irreversibly inactivate the enzyme monoamine oxidase in the central nervous system, platelets, liver, and gastrointestinal tract	moclobemide (Manerix) tranylcypromine(Parnate) phenelzine (Nardil) selegiline (Eldepryl)	orthostatic hypotension hypertensive crises dizziness reflex tarchycardia sexual dysfunction hepatotoxicity pyridoxine deficiency weight gain sedation
Tricyclic	Norepinephrine Serotonin Muscarinic Histaminergic Alpha-1,2 adrenergic blockade	Amitryptyline Imipramine Doxepine	postural hypotension cardiac effects anticholinergic effects sexual dysfunction sedation weight gain
Selective serotonin and noradrenaline reuptake inhibitors (SNRIs)	serotonin and noradrenaline reuptake inhibition at the synapse	Venlafaxine (Effexor) milnacipran	Nausea dizziness insomnia somnolence dry mouth.
Noradrenaline reuptake inhibitors (Sambunaris et al., 1997)	Noradrenaline reuptake inhibition at the synapse	reboxetine	

Table 1. Summary of Side-effects of Different Classes of Antidepressants (Khawam et al., 2006).

2.4 Tian Ma (*Gastrodia elata* Bl.)

Gastrodia elata Bl. (GEB) is a Chinese herb that can be found in China, Taiwan, Japan, and Korea. GEB is one of the earliest and most important traditional herbs of the Oriental countries, used in folk medicine for treatment of various diseases for centuries. Its earliest applications were used as an anti-convulsant, analgesic, and sedative against vertigo. It has also been used for the treatment of inflammatory diseases, headache, epilepsy, paralysis, and tetanus (Kim et al., 2001). Its beneficial effects in central nervous system such as memory consolidation, anti-anxiety, and anti-depression have also been documented (Niu et al., 2004; Liu and Mori, 1992; Hsieh et al., 1997; Kim et al., 2003; Sun et al., 2004; Jung et al., 2006; Jung et al., 2007; Huang et al., 2007). Recently, it has been further reported to possess anti-atherosclerotic properties (Hwang et al., 2009; Lee et al., 2006), and anti-tumor activity (Heo et al., 2007).

The active components of GEB are mostly phenols, including the principal ones that have been investigated the most such as HBA (4-hydroxybenzylalcohol), HB (4-hydroxybenzyl aldehyde), and vanillin (4-hydroxy-3-methoxybenzaldehyde). The content of gastrodin in a GEB sample was determined to be 0.419%. In addition to these components, GEB is also comprised of other organic compounds including benzyl alcohol, bis-(4-hydroxyphenyl) methane, 4(4'-hydroxybenzyloxy) benzyl methylether, 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxy-3-methoybenzoic acid (Jang et al., 2009).

2.5 Neurobiology and genomics of depression: Neuroplasticity

Early studies of depression focused on changes in neurotransmitter concentrations and receptor levels (D'sa and Duman, 2002; Pittenger and Duman, 2008). However, the monoamine hypothesis does not address why the inhibition of serotonin and norepinephrine reuptake requires immediately, but therapeutic action of antidepressants requires chronic administration (D'sa and Duman, 2002; Pittenger and Duman, 2008). Therefore, the neuroplasticity hypothesis, a focus on regulation of key signaling pathways involved in cellular survival and plasticity, is most widely accepted today. Yet, it is still not fully understood as each class, or even each type but in the same class of antidepressant, yields to different results (D'sa and Duman, 2002; Pittenger and Duman, 2008).

Evidence linking stress, depression, and antidepressants suggest that depression may result from impairment of neurons to make appropriate adaptions and/or synaptic connections (D'sa and Duman, 2002; Pittenger and Duman, 2008). Functional imaging studies of prefrontal cortical, ventral striatal, and hippocampal volume is decreased in depression patients. (Sheline et al., 1996; Bremner et al., 2000). The emerging pictures from these clinical studies is that cellular loss and volume decrease is associated with depressive disorders (D'sa and Duman, 2002; Pittenger and Duman, 2008). Dendritic restructuring, decreased neurogenesis, decreased cell survival occurs in stress, and may provide a cellular basis for the impairments seen in the brains of patients with depression (Czech et al., 2001). The results of Czech et al. demonstrate that chronic, not acute treatment with the antidepressant tianeptine, reverses the stress- induced impairments such as changes

in metabolite concentrations, decreases in neurogenesis, and reduction in volume in the hippocampus (Czech et al., 2001). There is no evidence to date if stress reduces the total number of neurons in the hippocampus and if so, the cellular mechanisms by which this neuronal loss occurs. It appears that permanent damage may occur in the brain only after extreme conditions.

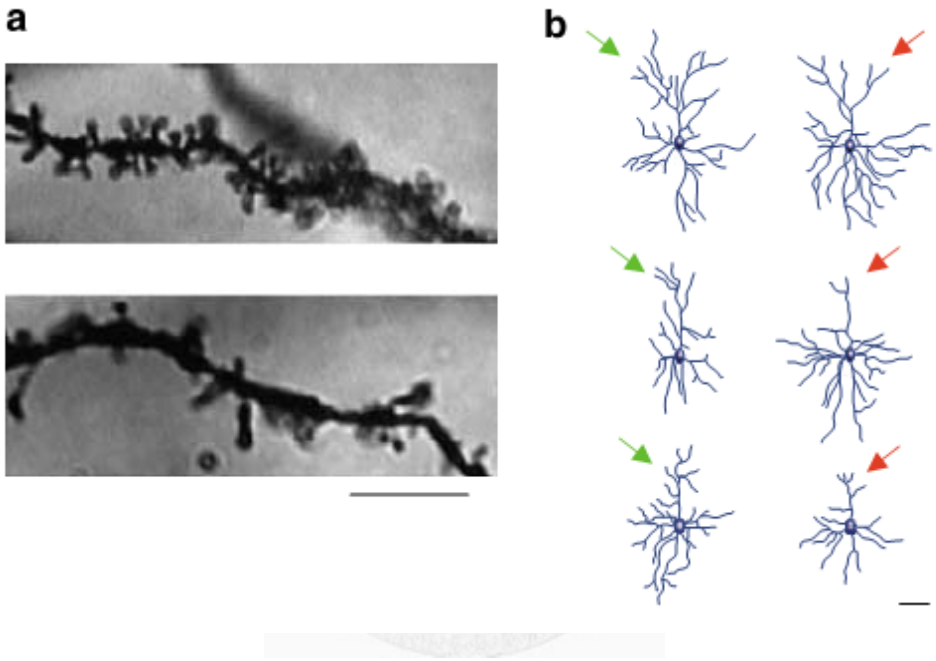


Figure 1 Image visualized by Golgi staining showing how stress alters neuroplasticity, which then affects the dendritic structure, length, and complexity. A) The number of dentritic spines is lower in the prefrontal cortex in the stressed rats (lower panel), compared to the controls (upper panel); scale bar = 5 micrometers. B) The length and complexity of the dendrites are reduced in the prefrontal cortex in the stressed rats (structures on the right and labeled by the red arrow), compared to the controls (structures on the left and labeled by the green arrow); scale bar = 50 micrometers.

(Pittenger and Duman, 2008)

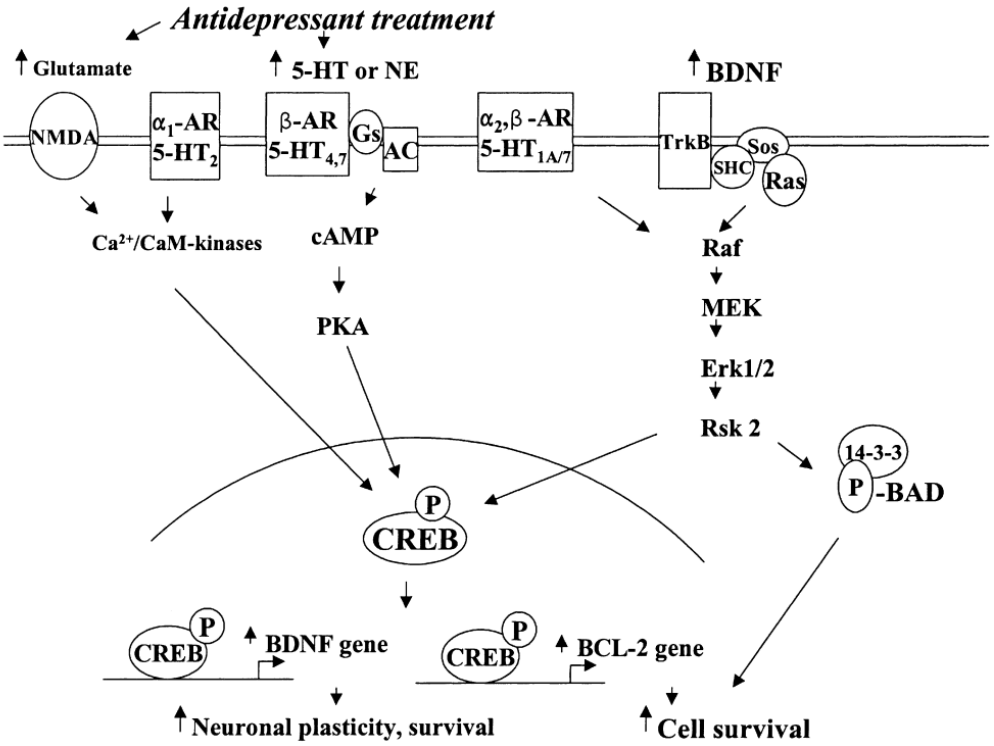


Figure 2 Schematic depicting the influence of antidepressant treatment on the cyclic adenosine monophosphate (cAMP)-cAMP response element-binding protein (CREB) and the neurotrophin-mitogen- activated protein kinase (MAPK) pathway in neuroplasticity and cell survival. Antidepressant treatment increases serotonin and norepinephrine concentrations at the synapse, and these molecules bind to the monoaminergic receptors on the cell membrane and activate the intracellular cAMP-CREB signaling cascade, increasing the mRNA expression and the secretion of BDNF and BCl-2, thereby increasing neuronal plasticity and cell survival. In addition, increased secretion of BDNF bind to the tyrosine kinase receptors (TrkB) and activate the Ras- MAPK signaling cascade, thus increasing neuronal plasticity and cell survival.

(D'Sa and Duman, 2002)

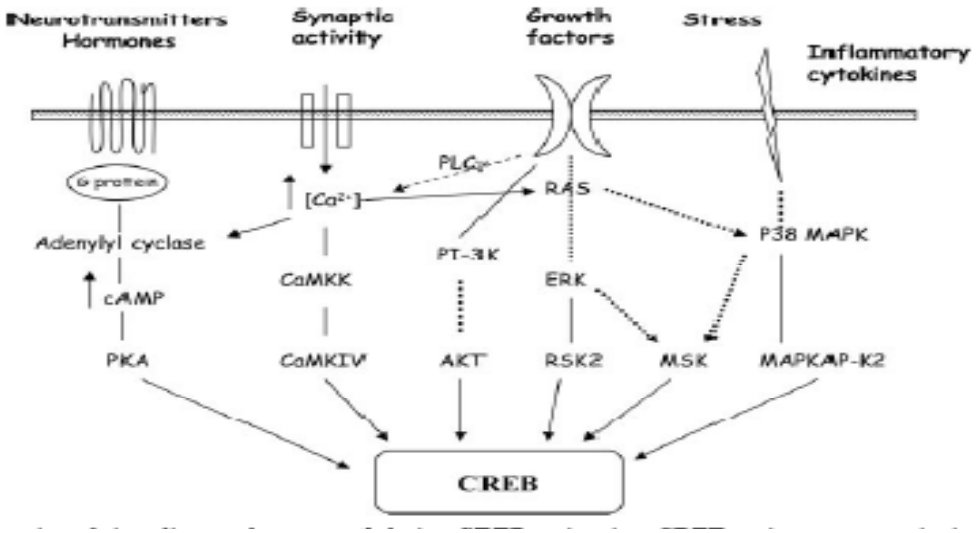


Figure 3 Schematic illustrating that not only can neurotrophic factors and neurotransmitters increase the gene expression and secretion of CREB, but also growth factors and inflammatory cytokines play an important role in the up-regulation of CREB.

(Tardito et al., 2006)

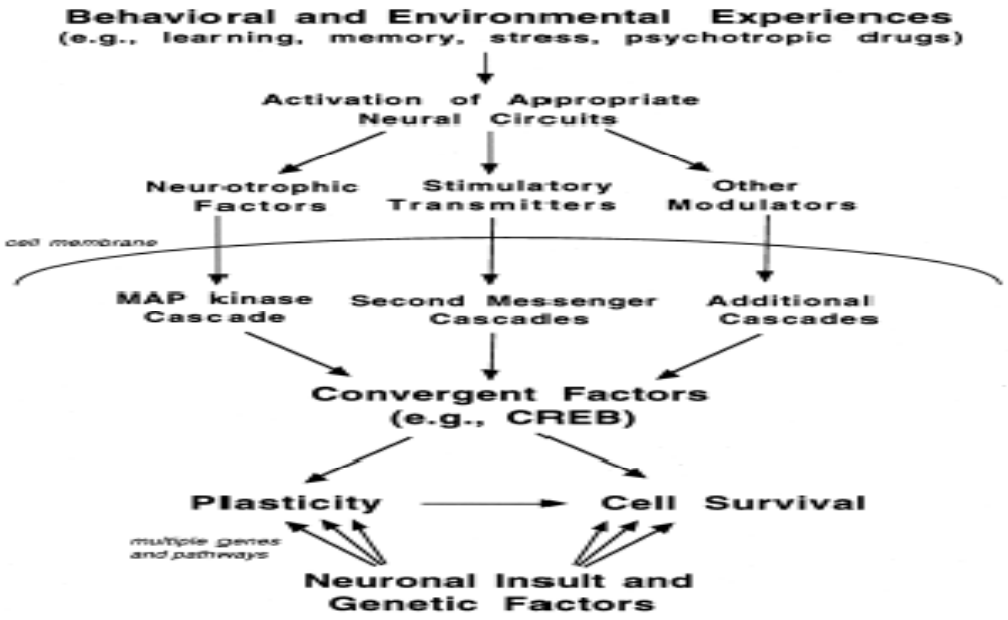


Figure 4 Summary of different types of stimulators that can increase the expression of CREB, thereby promoting neuroplasticity and cell survival.

(Duman et al., 2000; Hayley et al., 2005)

2.6 Neurobiology of depression: Overall

Depression can be caused by several mechanisms, including neurotransmitters regulation, HPA axis, apoptosis, inflammation, GABA, and neuroplasticity. Due to the limited scope this thesis, only the theory of the neurogenesis is discussed. Figure 5 summarizes the various mechanisms of pathology of depression.

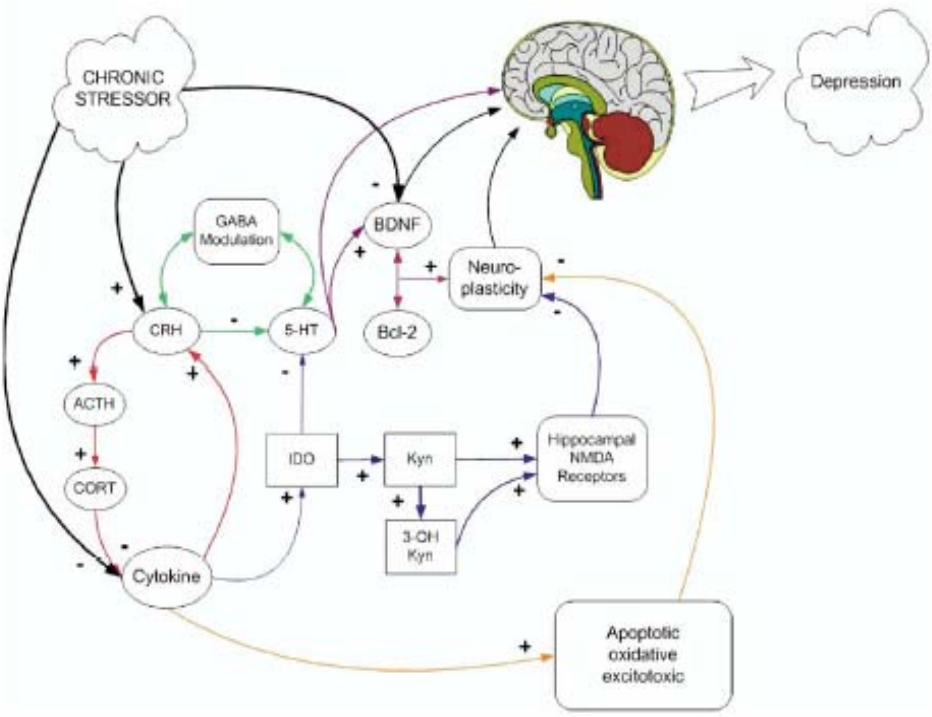


Figure 5 Summary of different potential routes depicting how chronic stressors could lead to depression by various mechanisms. Positive signs represent increasing the activity or secretion of the target, and vice versa for the negative signs. Chronic stressors could decrease cytokine activity, increase the production of CRH, and inhibit BDNF (black lines). CRH could reciprocally modulate GABA secretion, and decrease the production of 5-HT (green lines). The inhibition of 5-HT results in depression, and the increase in 5-HT secretion excites BDNF and Bcl-2 release, thereby achieving neuroplasticity (purple and pink lines). CRH, in addition, could excite the release of

ACTH and CORT, which consequently inhibit cytokine activity, therefore causing apoptosis of neurons in the brain, resulting in depression (orange lines). Furthermore, the inhibition of cytokine activity leads to higher IDO and Kyn activity, resulting in more NMDA receptors and they inhibit serotonin. The promotion of IDO inhibits serotonin production, and thus inhibiting neuroplasticity (purple lines). These pathways are not considered exclusive of one another.

(Hayley el al., 2005)

3 Purpose of Study

The purpose of this study is using the microarray technology to deduce a potential antidepressant pathway, focusing on the genomics level only. The details are as followed:

1. Apply the widely-adopted animal depression induction model (forced swimming test) to investigate the antidepressant effects of WGE in animal research, based on the difference in immobility time, measured by animal behavior analysis software.

2. Perform microarray experiments and data analysis to further investigate how WGE alters the gene expression in the rodent brain - specifically the depression related areas: the frontal cortex, the hippocampus, and the striatum - and to screen for a possible mechanism. The monitored target, total mRNA from these brain tissues, would be extracted by a standard handling protocol to ensure high quality of stability.

3. Perform QRT-PCR to validate the results of microarray data analysis to support the preliminary findings.

4 Experimental Framework

5 Materials and Methods

5.1 Materials

Animal treatment:

Gastrodia *elata* Bl.: Koda Pharmaceutical Co., Ltd., Taoyuan County, Taiwan

Fluoxetine (Prozac®): Eli Lilly and Company, Inc., Taipei, Taiwan

FST behaviour analysis software: Sunpoint Co., Ltd., Taoyuan County, Taiwan

RNA extraction:

Centrifuge: Hermle Z 300K

Trizol: 100 ml; Invitrogen, CA, USA

Bromochloropropane (BCP): 250 ml; Invitrogen, CA, USA

Isopropanol: 750 ml; Bioman Scientific Co., Ltd., Taipei, Taiwan

DEPC water: 250 ml; Bioman Scientific Co., Ltd., Taipei, Taiwan

Microarray analysis software:

Gene Spring: Agilent Technologies, CA, USA

Pathway Studio: Ariadne, CA, USA

QPCR:

Primers (refer to the list followed)

SYBR Green PCR Kit: Applied Biosystems, Foster City, CA, USA

ViiA 7 software: Applied Biosystems, Foster City, CA, USA

5.2 Methods

5.2.1 Sample preparation and dosage

The GEB sample was acquired from Koda Pharmaceutical Co., Ltd. (Taoyuan County, Taiwan), in the water extraction form (WGE), and the dosage used was 0.5 g/kg body weight (Chen, 2008). The dosage was chosen as it yielded the lowest concentrations of 5-hydroxyindoleacetic acid (5-HIAA), that were most significantly different from the negative control group, compared to other dosages, in the frontal cortex, and hippocampus (Chen, 2008). 5-HIAA is the metabolic product of 5-HT, which plays an important role in mood regulation.

5.2.2 Animals and treatments

Four-week-old male SD rats (BioLASCO Taiwan Co., Ltd., Yilan County, Taiwan) were randomized into four groups with three in each: the treatment group, the positive control group, the negative control group, and the normal control group. The rats were housed at the Animal House Facility at Institute of Food Science and Technology at a temperature of 22±2℃,with 12 hours dark/light cycle, and had access to unlimited supply of commercial rodent food (BioLASCO, Yilan County, Taiwan) and water *ad libitum*. After acclimation for two weeks, the animals were gavaged once daily for 21 days. The treatment group was orally administered with 0.5 g/kg bw of GEB dissolved in 10 ml/kg bw of ddH_2O. The positive control group was administered with 18 mg/kg bw of Fluoxetine (Prozac®) dissolved in equal volume of ddH_2O. As for the negative control and the normal

control groups, the rats were orally administered with equal volume of ddH_2O. During the adaptation weeks, the rats were not orally administered with any of the sample – only with water to adapt the rodents to the injection needle.

5.2.3 Weight change and food intake

Loss of appetite followed by loss of weight change would be a common adverse-effect of antidepressants. In this research, the two observable and accessible factors weight change and food intake were used as the indicators in evaluation of antidepressant effect of WGE. The rodents' food intake were weighed and recorded at a daily basis, and their weights were weighed once per week.

5.2.4 Forced swimming test

Forced swimming test, the widely adopted behavioural test for evaluating antidepressant effects, was performed on the 22nd and the 23rd day to induce depression (Porsolt et al., 1977a, b). All of the 9 rats (3 from the treatment group, 3 from the negative control, and 3 from the positive control) were subjected to FST individually on the 22nd day, each with 15 minute duration, in a glass cylinder 3/4 filled with room-temperature tap water (22±1℃). After 24 hours, on the 23rd day, the rats were once again subjected to FST for 5 minutes to induce depression and subsequently sacrificed. The entire FST for all the rats in both regimes were video recorded individually and analyzed by behavioural analysis software for immobility times (Sunpoint Co., Ltd., Taoyuan County, Taiwan). The definition of immobility was defined as complete immobility, with no struggling with the fore or

back limbs in any degree to minimize the factor of immobility due to fatigue.

5.2.5 Tissue dissection

The rats were anesthetized with CO_2, and were sacrificed by decapitation. Next, the tissues were dissected on a metal surface on ice into the frontal cortex, striatum, and hippocampus, and the brain was separated based on the dissecting method proposed by Glowinski (Glowinski and Iversen, 1966). The samples were then snap frozen with liquid nitrogen and were stored at -80℃. These tissues were selected as studies have shown that the neurotransmitters level change in these regions after depression and antidepressant treatment (Zhong et al., 2008; Chen, 2008).

5.2.6 RNA extraction, purification, and quality control

Total RNA was purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and was quantified using Beckman DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) to ensure that the A260/A280 ratio of each sample was greater than 1.8. Then, Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to evaluate the integrity of the RNA in each sample. Samples with RNA integrity number (RIN) greater than 8 were used for microarray analysis.

5.2.7 Microarray

Two chips of Illumina RatRef-12 Expression BeadChip acquired from Gene Tai (Taipei, Taiwan) were used, which could efficiently generate genome-wide expression profiles for twelve samples in parallel on a single BeadChip. Each chip consisted of 22,503 oligonucleotide probes, and each probe consisted of 50-mer oligonucleiodes arranged in the sense orientation. The content was primarily based on RefSeq content (Release 16).

5-2-8 Microarray data analysis

Normalization: The data acquired from two rat-ref 12 chips were quantile normalized using Gene Spring. Normalization is a process that removes systematic effects and brings data from different microarray chips onto a common scale, as cDNA microarray experiments can inevitably generate systematic variations (Smyth and Speed, 2003). In microarray raw data, there is slight variation between results for two different chips, and even within the control and treatment arrays there is some variation in the average intensity. The reason is that there are slight variations during production of the arrays and that one array might have got slightly more RNA sample due to different levels of hybridization (Quackenbush, 2002). Therefore, it is important to apply normalization to reduce these systematic errors before further analysis.

Quantile normalization: It is a technique for making two distributions identical in statistical properties. The values of both a test distribution and a reference distribution are sorted so that they are composed of the same length. The highest entry in the test distribution then takes the value of the highest entry in the reference distribution, the next highest entry in the reference distribution, and so on, until the test distribution matches the reference distribution (Bolstad et al., 2003).

Fold change analysis: Fold change analysis was performed on the normalized data for all twenty-four samples was performed fold change analysis using Gene Spring (Agilent Technologies, CA, USA). The WGE group, and the normal control group, respectively, was compared to the NE group for cortex, hippocampus, and striatum

regions respectively. All genes that were significantly expressed in both trials (two separate biochips), determined by absolute fold change values greater than 1.5, were selected and classified in gene lists for further investigation.

Gene clustering: The genes with significant expression level were selected by the fold change analysis method and the gene list corresponding to each brain region tissue (including expression intensities of each gene) was imported into the genomics analysis software, Gene Spring. The expression intensity of each significant gene was represented by different intensities of red and green, with the highest intensity of red being most up-regulated, and the highest intensity of green being most down-regulated. The expression intensities of each gene between different experimental groups were placed side by side, as represented by heatmaps.

Pathway analysis: The genes with significant expression level were selected by the fold change analysis method and the gene list corresponding to each brain region tissue was imported into the pathway analysis software, Pathway Studio (Ariadne, CA, USA). The genes on each list were investigated to the one neighbouring level, and pathways were deduced and listed from the lowest p-values to the highest p-values, corresponding to the order of decreasing probabilities. The algorithm in the database was based on KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis pathway. The concept of the algorithm can be found in the manual of Gene Set Analysis Toolkit (http://bioinfo.vanderbilt.edu/webgestalt/) (Zhang et al., 2005).

5-2-9 QPCR

The SYBR Green PCR kit was used (Applied Biosystems, Foster City, CA, USA) for labeling the probes and ViiA 7 software (Applied Biosystems, Foster City, CA, USA) was used to generate the amplified curve. 18S ribosomal RNA was used as the internal control. Delta C_t (cycle threshold) was acquired by the subtraction of average Ct of 18S from the average C_t of gene of interest. The relative gene expression level was determined by delta delta C_t method.

Table 2 lists the forward and reverse DNA sequences of the primers used in QPCR

Primer Name	Sequences (5' to 3')	Base Pair (mer)	Primer Name	Sequences (5' to 3')	Base pair(mer)
rat GAPDH-F	GGT TAC CAG GGC TGC CTT CT	20	rat GAPDH-R	TTG AAC TTG CCG TGG GTA GAG	21
ROBO1-F	CCA GCC CCC ACC TCT GAT A	19	ROBO1-R	CCC TTT AGA GAC GAT CAG GTC TGA	24
ROBO2-F	AAC ATG GCA CAT TCA AAG CAA TAC T	25	ROBO2-R	TCA TCA GCA TCC TCA TCT GGA A	22
ROBO3-F	GCA GTC CAC AGC CAC TCT TAC C	22	ROBO3-R	GAT ACG CTG AGA GGA GAA CTT GGT	24
ROBO4-F	TCC TAG GCA TTG CTG TGT GTA TCT AC	26	ROBO4-R	TCC TAG GCA TTG CTG TGT GTA TCT AC	23
CRMP2-F	CTG AAT TCG CTC	21	CRMP2-R	CTT GCT GGG ATC	20

	ACC CAA GTC			TGC ATG GT	
Ywhaq-F	GGT ATC TTG CTG AAG TCG CTT GT	23	Ywhaq-R	GGA TCG GAT GTG TAG GTT GCA	21
Ywhaz-F	CTG CTG GTG ATG ACA AGA AAG G	22	Ywhaz-R	TGA GGG CCA GAC CCA GTC T	19
Ywhab-F	GCG CCT TTG CTC CCT ACA G	19	Ywhab-R	GCT GCG GCC ATA TCA TCA TA	20
Ywhae-F	GGT TTT GCT GTA ACG TGT GTT TG	23	Ywhae-R	AGA GGG AGG AGT CGG CAA GA	20
SLIT-ROBO-1-F	CCT GTT GTC TCC GGT GAA CTG	21	SLIT-ROBO-1-R	ATC TGC ATG AAC CGC ATA ATG A	22
SLIT-ROBO-2-F	GCT GGC GTC CTC AAG CTT T	19	SLIT-ROBO-2-R	GAT ATG CAC AGC TCT CTC TTG CA	23
SLIT-ROBO-3-F	TCT TTG CTT TCC TCA ATC ACC TT	23	SLIT-ROBO-3-R	ATA CAG GGT CCT GCC CAT CA	20
Slit 1-F	GAG CTG AAC GGT ATC AAA TCC AT	23	Slit 1-R	GGC ATC GGG TGC AAT CTC	18
Slit 2-F	TCC TGC CCC ATA TCA CTG TTC	21	Slit 2-R	GCT TTC GGT TGC GTG AAA AG	20
Slit 3-F	TCC GAC AGC TGA CAC TAA TTG AC	23	Slit 3-R	ATG GAC CGG GAT GCA TCT C	19
Rhoa-F	TGG ACG	20	Rhoa-R	TGT CGA	23

	GGA AGC AGG TAG AG			TGG AAA AAC ACA Sequences (5' to 3') TCA GT	
Rock1-F	CTG CGG GTA CGA AGG TAT CG	20	Rock1-R	CCA ATC CAT CCA GCA AAC AA	20
Rock2-F	GCC CGA TCA TCC CCT AGA AC	20	Rock2-R	GTG GAT GGA AGA ATA TGA TCA CCT T	15
Cdk5-F	GGC CCG AGC CTT TGG TAT	18	Cdk5-R	TTG GCC AGC TCT GCA AAG AT	20
Cdc42-F	TCC GCC CTC ACA CAG AAA G	19	Cdc42-R	TGC GTT CAT AGC AGC ACA CA	20
MAPT-F	CAG GGA AGG CAA AGA TTT GG	20	MAPT-R	ACA CAC GGA CTC CCA TCC A	19
MAP1a-F	ACA GTG ACC TTT CCA CGG AAG A	22	MAP1a-R	AAT CTG ACT GTG AGG AGC CTG AGT	24
MAP1b-F	ACC CTA CTG TGG GTG CTG GTA	21	MAP1b-R	TCT GTC GGC GGA ATC TTA TGT	21
MAP2-F	AGG CTC CCA GTG CGT TTA AA	20	MAP2-R	CCT GCG AGG AGG GAG GAT	18
Profilin1-F	CCC CCA CCT TCA ATG TCA CT	20	Profilin1-R	GCA GGT GAG AGG CCA TTT CA	20
Csk-F	GCA GCT CGA GCA CAT CAG AA	20	Csk-R	CAG CAG GGC ACC AAA TCT GT	20
Abl1-F	AGG GCC TGG GCG AAA G	16	Abl1-R	GCG CTG AAC AGG TTG GTC	20

				TT	
Rasgrp1-F	CGC TGT AAA GAC TGT GGG ATG A	22	Rasgrp1-R	TTG GCA CCA CAG AGC TGA TG	20
Rassf5-F	GCA CCC TCA CCC CAA CCT	18	Rassf5-R	AGT GCT TCT CCC GGC TGT TA	20
Rasl-11b-F	TCT CCA TCA CCG ACC ACA AG	20	Rasl-11b-R	GCA AGA GGT CGG CTT TGT TG	20
Rgs4-F	TTT TCT GCT TTG AGG TAA GAG CTT GT	26	Rgs4-R	CCC GTC CTC CTC TTT TCT CTT C	22
Gpr88-F	TAG GGC GCG AGG TGT GAA	18	Gpr88-R	CCG CCC ACG ATT CTT CTT C	19

5-2-10 Statistical analysis

Each value is presented as means ± SD. For the animal body weight change and food intake data, Student t-test was used. The data of the FST were also analyzed by Student t-test. The results of microarray analysis were normalized and analyzed by fold change analysis. The QPCR data were analyzed by Student t-test. The threshold $p < 0.05$ was considered statistically significant for all analyses, using the software SAS.

6 Results

Weight change and food intake

Both the amount of food intake and the weight change were significantly lower ($p < 0.05$) in the PC group in weeks 1, 2, and 3, as compared to the NE group (Figures 6 and 7).

Forced swimming test

The parameter "immobility time" was used as the indicator for "despair" – one of the symptoms of human depression. The treatment group (WGE group, 0.5 g/kg bw WGE) demonstrated significantly shorter ($p < 0.05$) immobility time than the negative control group (NE). Likewise, the positive control group (PC group, 18.0 mg/kg bw fluoxetine) displayed significantly shorter immobility time than the negative control group (NE) (Figure 8).

Microarray analysis overview, array design, principal component analysis

To investigate the pathways of WGE at the gene expression level, the overall change of gene expression was analyzed by oligonucleotide microarray, and Illumina rat ref-12 chip was chosen, and two such chips were used for data analysis. From the Principal Component Analysis graph (Figure 10), the resulting signal intensities from the two trials were very spread out, as a result of biological variances. Therefore, no statistical measures were applied onto the analysis of the results; rather, fold change analysis was applied onto each individual trial. Two biological repeats were performed (one on each

chip), as there was no necessity to apply statistical measures. Genes that were expressed with intensities higher than the threshold (fc ≥ 1.5) in both trials were further investigated (n=2) to minimize the possibilities of having false-positive results. The data acquired by microarray experiments was normalized using *Gene Spring* V.3, and the pathways were deduced by *Pathway Studio*. Among the 22,523 probes that we analyzed from the database, WGE altered the expression level of 200 transcripts.

Data normalization

Quantile normalization was chosen because it would yield the lowest variation for animal studies (Bolstad et al., 2003). After normalization, we can see that the intensity distribution for each sample array is about the same, as the median of each intensity distribution is lined up at almost the same level (Figure 9).

Fold change analysis

Fold change analysis was applied to select the significant genes, and the threshold was set at 1.5. Genes that were significant from the results of the first chip is represented by the red circle, and results from the second chip are represented by the blue one. The overlapped circle in the centre represents genes that were significant from both biological repeats 1 and 2. Because of wide biological variances, the number of significant genes in both biological repeats may vary greatly up to ten-fold. In the frontal cortex, the numbers of genes acquired from both repeats were 31 up-regulated and 15 down-regulated (Figure 11). As for the hippocampus, the numbers of up-regulated and down-regulated genes were 68 and 36, respectively (Figure 14). Finally, there were 42 genes up-regulated and 18 genes down-regulated in striatum

(Figure 17).

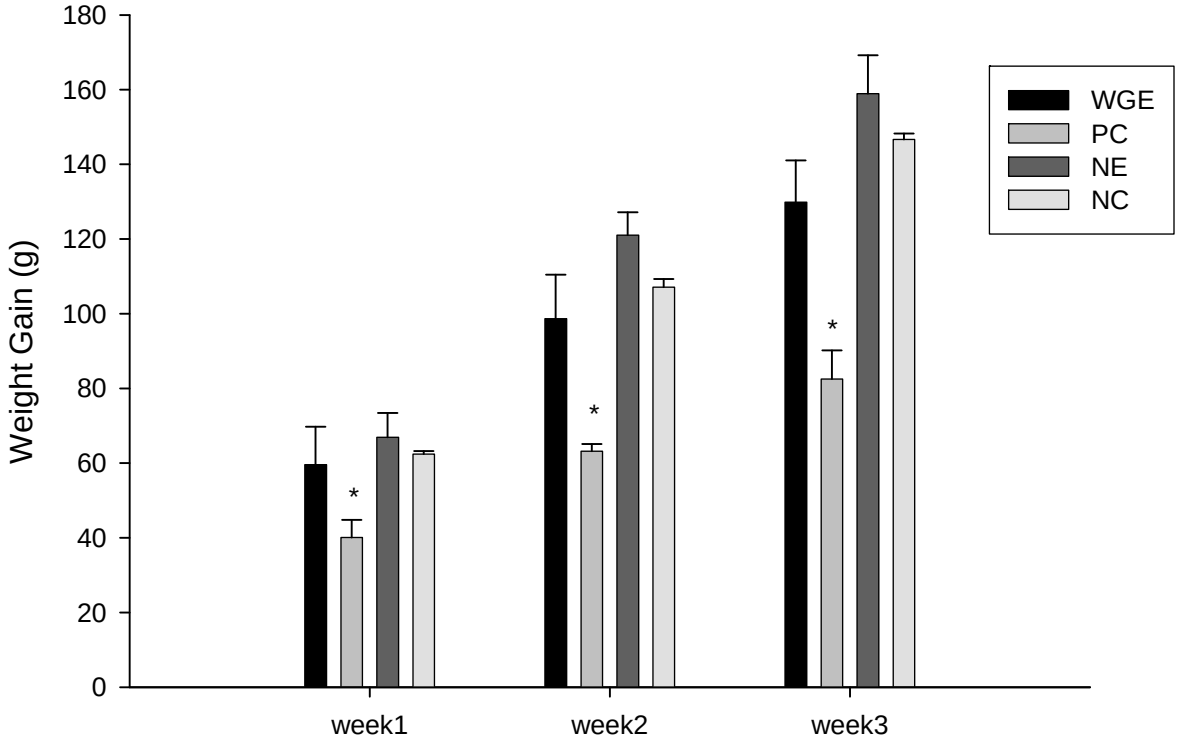


Figure 6 shows the effects of different treatments on weight gain weighed once per week. The fluoxetine group displays significantly slower weight gain compared to the saline water group, in all three weeks. Data are represented as mean +/- SD (n=3) and analyzed statistically using Student t-test. Bars with asteroids represent the week in which the PC group shows significant difference compared to the control group in weight gain ($p < 0.05$). WGE: *Gastrodia elata* Bl.; PC: fluoxetine; NE: saline water; NC: saline water, without forced swimming test induction.

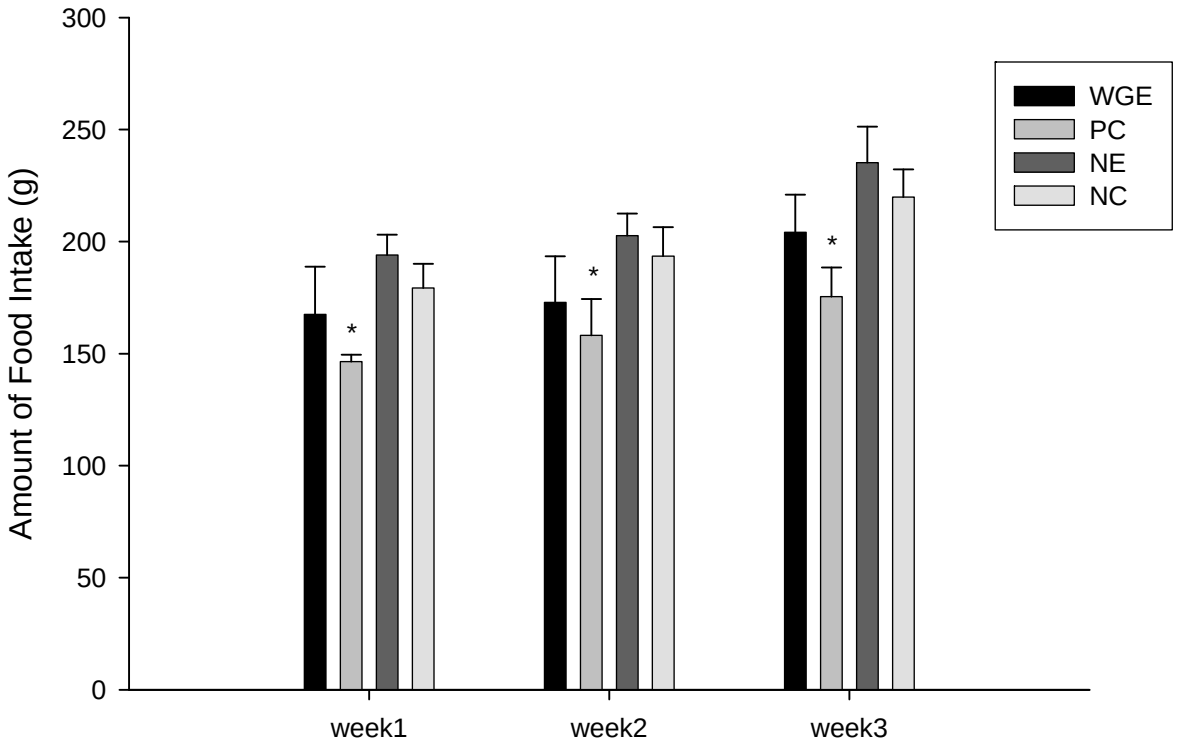


Figure 7 shows the effects of different treatments on food intake. The fluoxetine group displays significantly lower food intake compared to the saline water group, in all three weeks. Data are represented as mean +/- SD (n=3) and analyzed statistically using Student t-test. Bars with asteroids represent the week in which the PC group shows significant difference compared to the control group in weight gain ($p < 0.05$). WGE: *Gastrodia elata* Bl.; PC: fluoxetine; NE: saline water; NC: saline water, without forced swimming test induction.

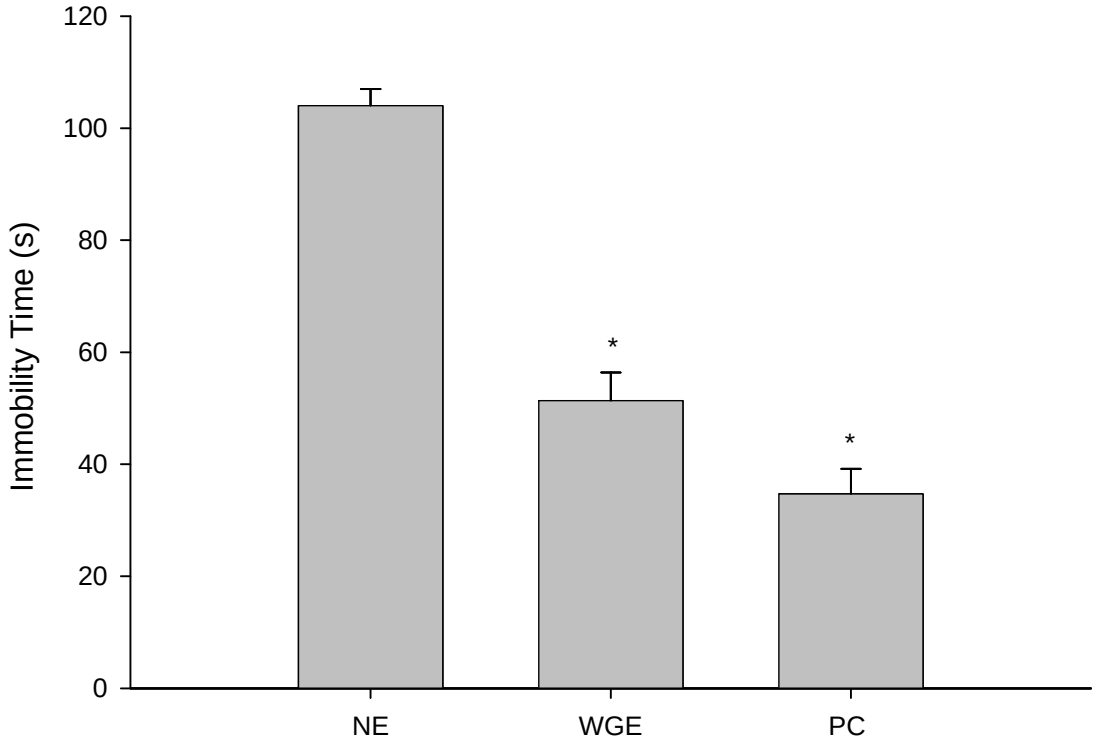


Figure 8 shows the effects of WGE and fluoxetine on the immobility time of different groups of rats in FST. Immobility time is defined as complete immobility. Data are represented as mean +/- SD (n=3) and analyzed statistically using Student t-test. Bars with asteroids show that the WGE and PC groups demonstrate significantly lower immobility times compared to the control ($p < 0.05$).

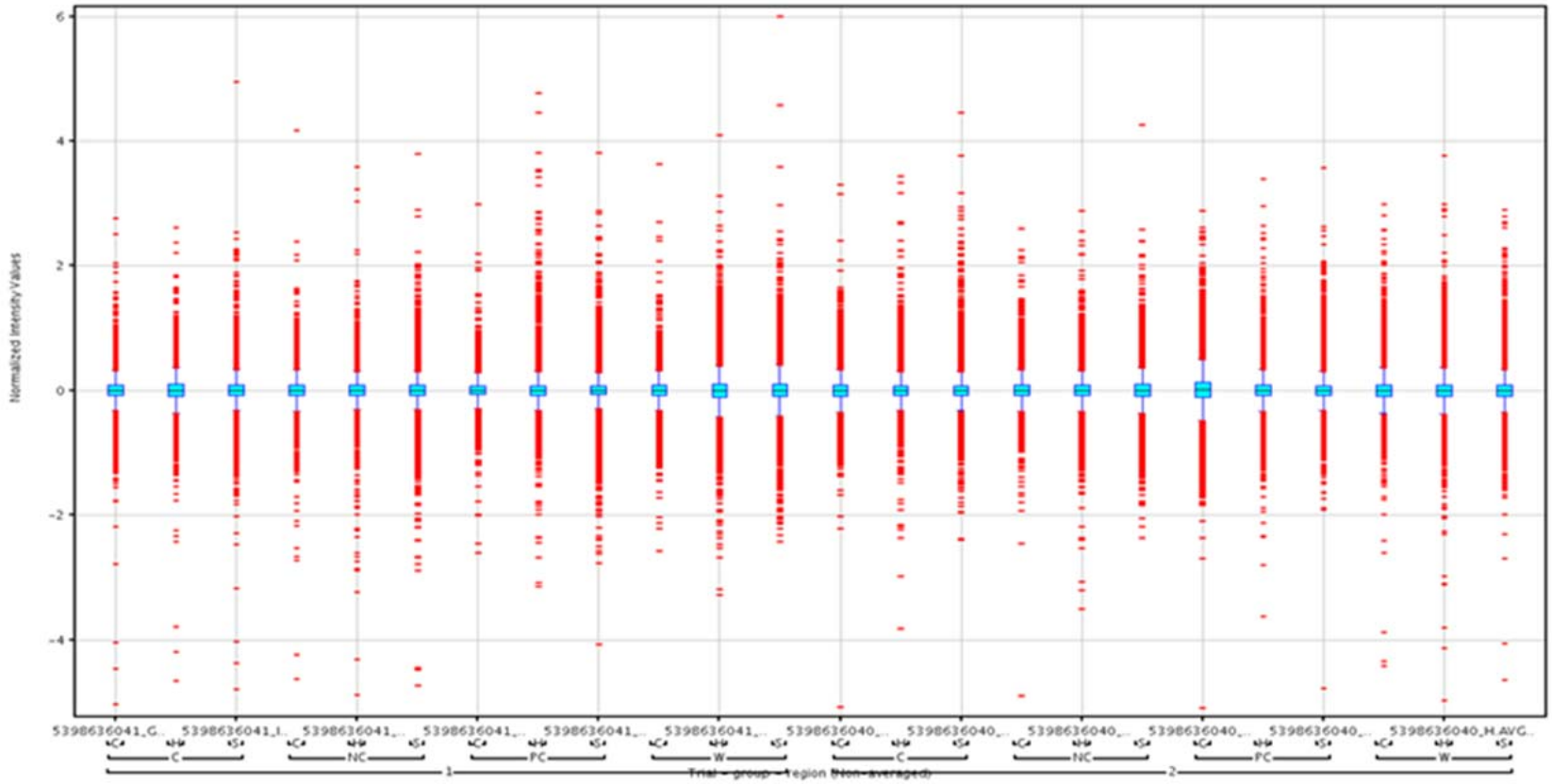


Figure 9 shows the box plot distribution of normalized microarray results after processing the raw data of all 24 samples from both chips 1 and 2 by the quantile algorithm. The figure is created by *Gene Spring*™.

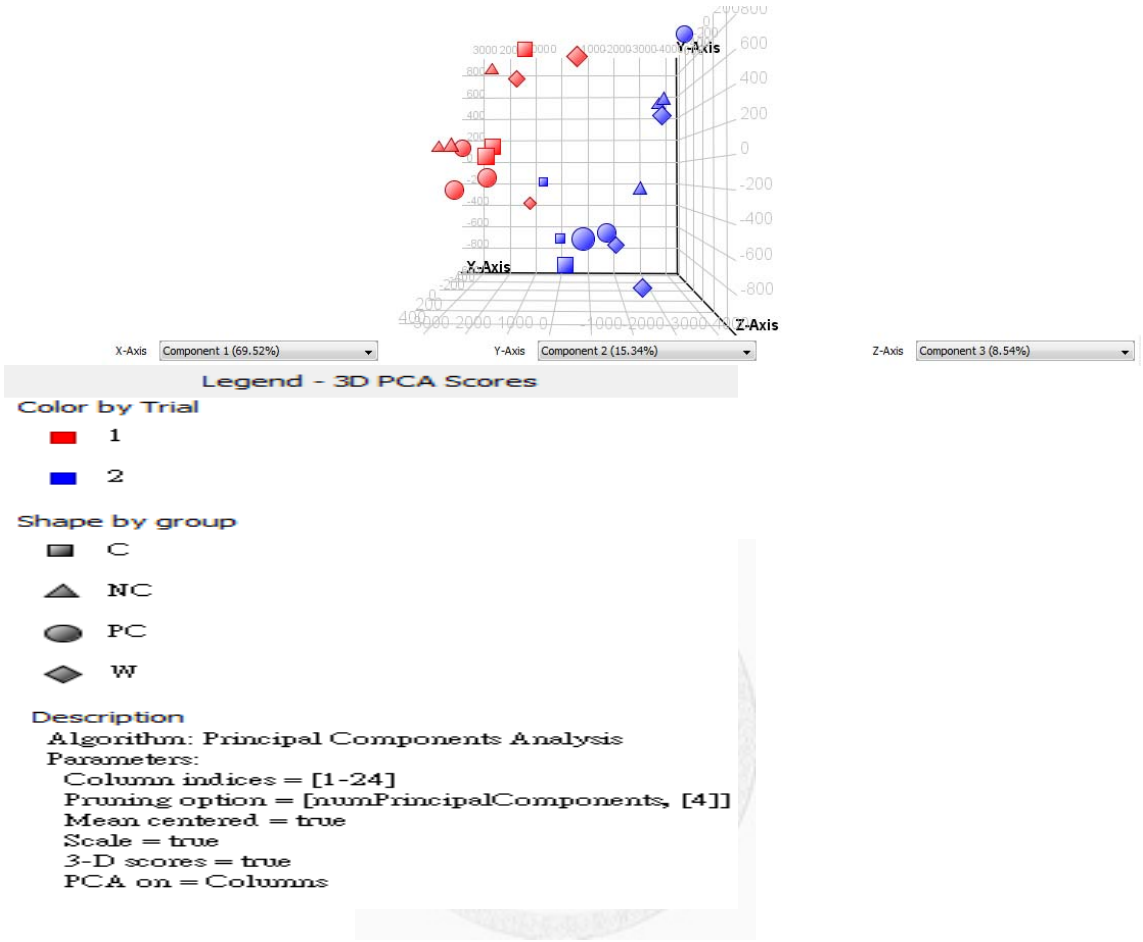


Figure 10 shows the principal analysis diagram created by *Gene Spring*™. It displays the variations between two biological repeats are significantly different based on principal component analysis scores. It is presumed that the differences are due to batch differences and biological variations. Red represents samples from trial/chip 1, and blue represents samples from trial/chip 2. Squares, triangles, circles, and diamonds represent the negative control, normal control, positive control, and treatment group, respectively.

(A)

(B)

Figure 11 shows the Venn diagram created by *Gene Spring*™ that displays the numbers of genes altered by WGE in the frontal cortex in both up and down regulations. The pink region represents the genes acquired from chip 1, and the teal region represents the genes acquired from chip 2. The overlapped region represents the genes altered in both chips (fold change > 1.5). (A) 31 genes are up-regulated. (B) 15 genes are down-regulated.

Table 3 shows the list compiled by *Gene Spring*™ displaying the genes that have been altered in expression level upon treatment of WGE

In the frontal cortex (fold change > 1.5, WGE vs. negative control)

Entrez gene ID	Gene name	Full name	Fold change	Function
24173	Adra1b	adrenergic, alpha-1B-, receptor	2.49	couples with G alpha(h) (transglutaminase II) to activate phospholipase C; facilitates Ca2+ release and capacitative Ca^{2+} entry
24211	Atp1a1	ATPase, Na^+/K^+ transporting, alpha 1 polypeptide	1.79	mediates Na^+ and K^+ transport; may play a role in regulation of blood pressure
171051	Cabp1	calcium binding protein 1	1.93	component of calcium-mediated signal transduction; may be involved with the dendritic cytoskeleton
84347	Cacng2	calcium channel, voltage-dependent, gamma subunit 2	1.60	subunit of voltage-dependent calcium channel; involved in influencing channel properties
25305	cort	Cortistatin	1.82	inhibits growth hormone secretion; may act as a neuropeptide to mediate signaling via a somatostatin receptor subtype
24373	Fst	Follistatin	1.89	binds activin and inhibits activin-mediated signaling pathways
25136	Gfra2	GDNF family receptor alpha 2	2.30	stimulates c-Ret tyrosine phosphorylation by mediating a crosslink with GDNF of c-Ret
64317	Gpx3	glutathione peroxidase 3	1.53	belongs to the glutathione peroxidase family, which functions in the detoxification of hydrogen peroxide. This gene is highly expressed in the kidney where it may play a role in protecting the kidney from oxidative damage.
59324	Kcnk1	potassium channel, subfamily K, member 1	1.56	a putative potassium channel with two P domains
85327	Lin7a	lin-7 homolog a	1.71	may mediate the formation of cell-cell junctions
24604	Npy	neuropeptide Y	1.71	peptide hormone; involved in regulating food intake

29480	Rgs4	regulator of G-protein signaling 4	2.10	regulates G-protein coupled receptor signaling by accelerating the inactivation of active G-proteins; may regulate dopamine D2 receptor signaling
Entrez gene ID	Gene name	Full name	Fold change	Function
291061	Riok1	RIO kinase 1	1.51	This gene encodes a member of the RIO family of atypical serine protein kinases. A similar protein in humans is a component of the protein arginine methyltransferase 5 complex that specifically recruits the RNA-binding protein nucleolin as a methylation substrate
362993	Rnd1	Rho family GTPase 1	1.58	Uncharacterized
64347	Sncg	synuclein, gamma (breast cancer-specific protein 1)	2.11	may play a role in the neurofilament network
116470	Stx1a	syntaxin 1A (brain)	2.01	plays a role in neurotransmitter release
25567	Tnr	tenascin R	1.75	an extracellular matrix protein; involved in regulating neurite outgrowth
362708	Trim54	tripartite motif-containing 54	2.81	May bind and stabilize microtubules during myotubes formation
81822	Actb	actin, beta	-1.88	mRNA expression increases following axon injury; may play a role in acceleration of axonal outgrowth
25669	Cpe	carboxypeptidase E	-1.81	may play a role in post-translational processing of peptide hormones including preproopiomelanocortin
29707	Gabra5	gamma-aminobutyric acid (GABA) A receptor, alpha 5	-1.71	subunit of the GABAA receptor; may be part of distinct GABAA receptors within the olfactory bulb
500040	Tes	testis derived transcript	-1.74	Scaffold protein that may play a role in cell adhesion, cell spreading and in the reorganization of the actin cytoskeleton. Plays a role in the regulation of cell proliferation. May act as a tumor suppressor
54320	Pdpn	Podoplanin	-1.52	membrane glycoprotein that may function in lung development
117017	Polr2g	polymerase (RNA) II (DNA directed) polypeptide G	-1.62	human homolog is a subunit of RNA polymerase II, which catalyzes DNA dependent synthesis of mRNA in transcription; binds the human von Hippel-Lindau (VHL) tumor suppressor

				protein
360480	Litfl	LITAF-like protein	-1.78	Belongs to the LITAF family.
Entrez gene ID	Gene name	Full name	Fold change	Function
314313	Znf183	ring finger protein 113A2	-1.89	contains RING and CCCH-type zinc finger domains
303073	*Cyfip2* (predicted)	cytoplasmic FMR1 interacting protein 2	1.55	Uncharacterized
365748	*Bhlhb5* (predicted)	basic helix-loop-helix family, member e22	-2.49	Uncharacterized
314399	RGD1359144 (predicted)	chromosome 14 open reading frame 130 (similiar)	-2.07	Uncharacterized
298591	Igsf21	immunoglobin superfamily, member 21	1.66	Uncharacterized
313783	Olfm2	olfactomedin 2	1.63	Uncharacterized
363869	Ubl3	ubiquitin-like 3	1.67	Uncharacterized
500304	RGD1561270 (predicted)	Zinc finger protein 248 (similar)	1.50	Uncharacterized
292539	RGD1562905 (predicted)	60S ribosomal protein L17 (L23) (similar)	14.11	Uncharacterized
290790	RGD1565014 (predicted)	Low-density lipoprotein receptor-related protein 4 precursor (LDLR dan) (similiar)	2.24	Uncharacterized
500717	RGD1566401 (predicted)	GTL2 (similiar, imprinted maternally expressed untranslated)	3.31	Uncharacterized
500297	RGD1560607 (predicted)	env precursor (similiar)	1.68	Uncharacterized
360435	LOC360435 (predicted)	neurotrimin (hypothetical gene)	1.71	Uncharacterized
367923	LOC367923 (predicted)	60S ribosomal protein L23a (similar)	1.59	Uncharacterized
362484	Plekhf2	pleckstrin homology domain containing,	2.03	Uncharacterized

	(predicted)	family F (with FYVE domain) member 2		
Entrez gene ID	Gene name	Full name	Fold change	Function
288513	RGD1305593 (predicted)	RIKEN cDNA 1810042K04 (similiar)	1.80	Uncharacterized
497920	LOC497920 (predicted)	RGD1563967 (similiar)	-2.01	Uncharacterized
501223	LOC501223 (predicted)	RGD1563967 (similiar)	-1.56	Uncharacterized
498405	RGD1560273 (predicted)	RGD1560273 (similiar)	-1.50	Uncharacterized
291580	RGD1312005 (predicted)	Uncharacterized	-1.70	Uncharacterized

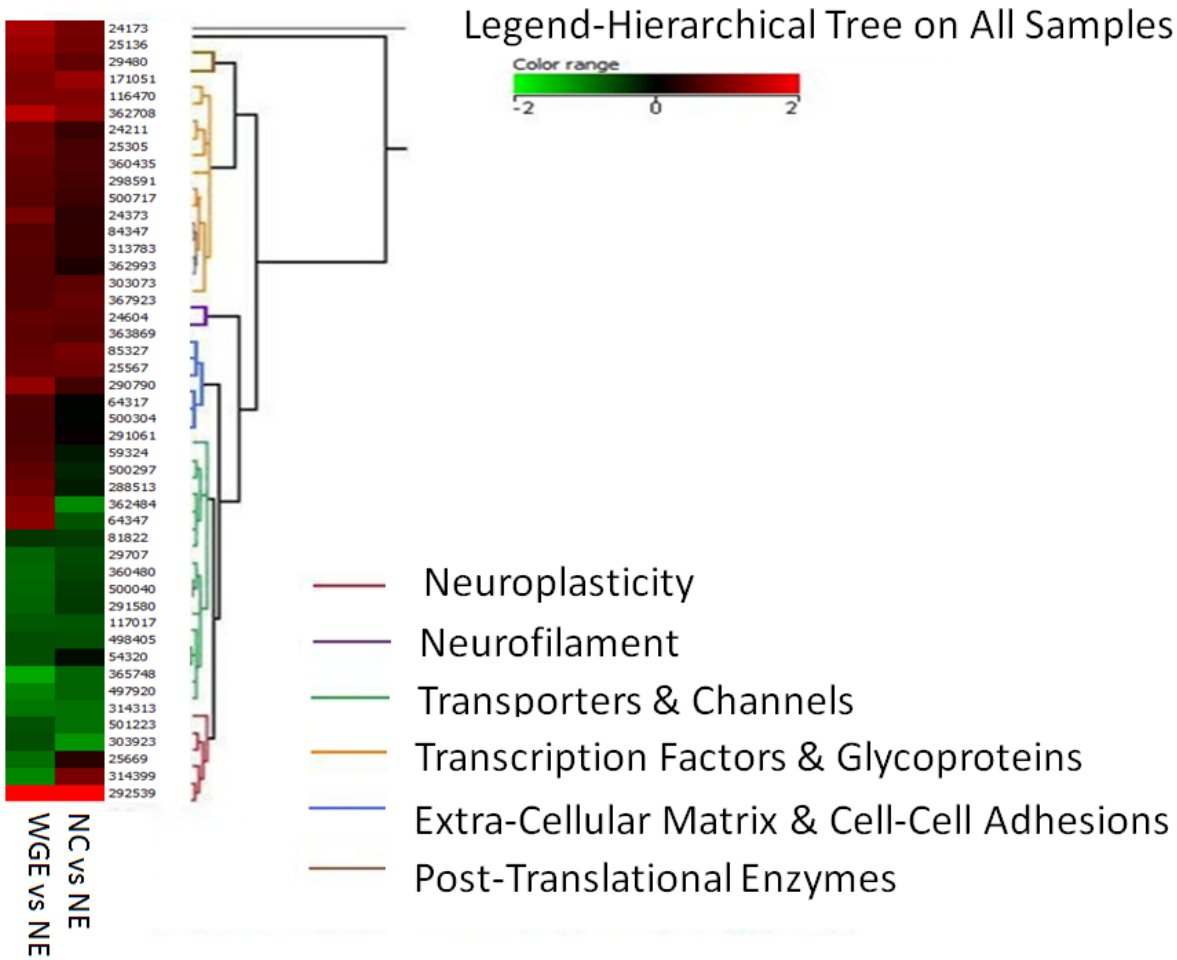
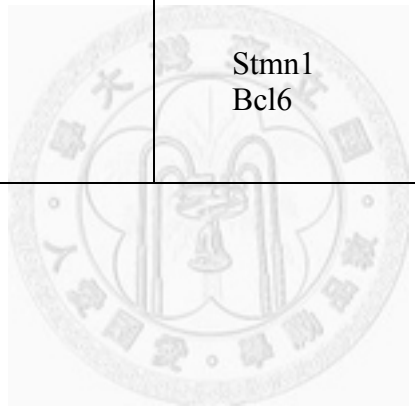

Legend-Hierarchical Tree on All Samples
Color range
-2
0
2
24173
25136
29480
171051
116470
362708
24211
25305
360435
298591
500717
24373
84347
313783
362993
303073
367923
24604
363869
85327
25567
290790
64317
500304
291061
59324
500297
288513
362484
64347
81822
29707
360480
500040
291580
117017
498405
54320
365748
497920
314313
501223
303923
25669
314399
292539
NC vs NE
WGE vs NE
Neuroplasticity
Neurofilament
Transporters & Channels
Transcription Factors & Glycoproteins
Extra-Cellular Matrix & Cell-Cell Adhesions
Post-Translational Enzymes

Figure 12 shows the heatmap of the 46 genes that have been altered in expression level upon treatment of WGE in frontal cortex analyzed by hierarchical clustering analysis. The genes were classified in 6 main groups based on gene ontology: neural plasticity, red; neurofilament, purple; transporters & channels, green; transcription factors & glycoproteins, orange; extra-cellular matrix & cell-cell adhesions, blue; post-translational enzyme, brown. As the legend shows, red means up-regulation, and green means down-regulation. The figure is created by *Gene Spring*™.

Table 4 shows the possible KEGG pathways of genes regulated by WGE in frontal cortex, arranged in the order of increasing *p*-value. Data is generated by *Pathway Studio*™.

Rank	Pathway name	Overlapping entities	*P*-value
1	Axon development	Sncg Actb	0.00143310
2	Negative regulation of microtuble depolymerization	Trim54	0.00189955

3	Inactivation of MAPK activity	Rgs4	0.00189955
4	Regulation of dopamine secretion	Sncg	0.0104909
5	Positive regulation of cell motion	Stmn1 Bcl6	0.0205670



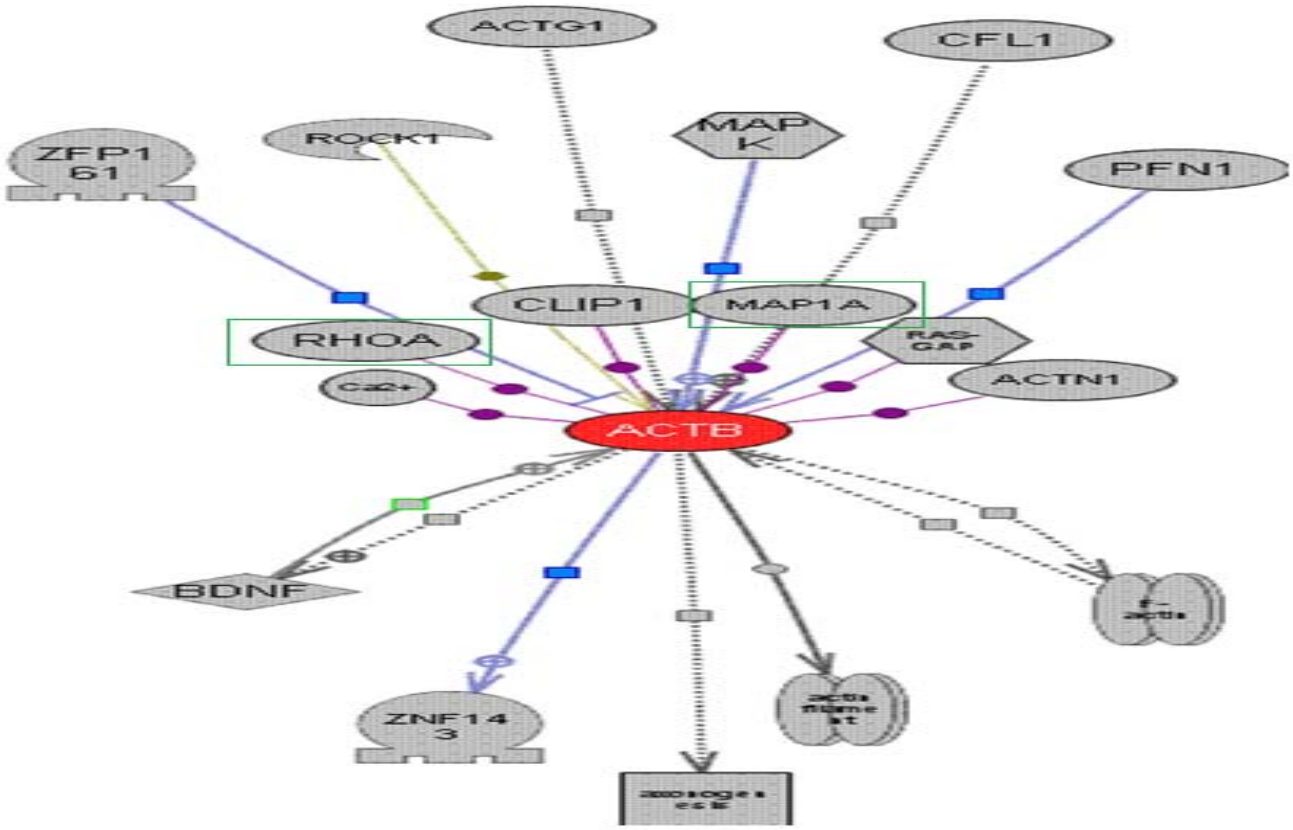


Figure 13 shows the gene network created by *Pathway Studio*™ showing *Actb* and the related genes altered by WGE in the frontal cortex. After filtering out the unrelated genes, a gene network analysis was constructed, with *Actb* located in the centre of the network. The gene was selected because it is a regulator of actin production, an important component for axon/dendrite growth. Boxes highlight other regulators of neurogenesis that were screened out by network analysis, which were one gene neightbouring (eiher upstream or downstream) to the central gene in the network.

(A)

(B)

Figure 14 shows the Venn diagram created by *Gene Spring*™, which displays the numbers of genes altered by WGE in the hippocampus in up and down regulations. The pink region represents the genes acquired from chip 1, and the teal region represents the genes acquired from chip 2. The overlapped region represents the genes altered in both chips (fold change > 1.5). (A) 68 genes are up-regulated (B) 36 genes are down-regulated.

Table 5 shows the list showing the genes that have been altered in expression level upon treatment of WGE in the hippocampus (fold change > 1.5, WGE vs. negative control).

Entrez gene ID	Gene name	Full name	Fold change	Function
286939	Clmp	CXADR-like membrane protein	1.92	Human homolog plays a role in cell-cell adhesion
360970	Cabp7	Cabp7 calcium binding protein 7	8.77	Negatively regulates Golgi-to-plasma membrane trafficking by interacting with PI4KB and inhibiting its activity.
24253	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	2.18	Transcription factor that binds to CCAATT motif on DNA and may facilitate IL-6 induced transcriptional activation
114490	Cited2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	1.59	A transcription factor
25669	Trh	thyrotropin releasing hormone	3.17	Mediates processes of vasopressin (AVP) and oxytocin (OT) biosynthesis and releases at the hypothalamo-neurohypophysial level
366196	Crls1	cardiolipin synthase 1	1.50	Catalyzes the reversible phosphatidyl group transfer from one phosphatidylglycerol molecule to another to form cardiolipin (CL) (diphosphatidylglycerol) and glycerol.
317399	Ddx21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	1.85	Can unwind double-stranded RNA (helicase) and can fold or introduce a secondary structure to a single-stranded RNA (foldase). Functions as cofactor for JUN-activated transcription
116663	Dusp6	dual specificity phosphatase 6	1.94	Protein tyrosine phosphatase involved in regulation of mitogen-activated protein kinase (MAPK)
25283	Gclc	glutamate-cysteine ligase, catalytic subunit	1.61	Catalyzes the first step in the synthesis of glutathione
191576	Tecr	trans-2,3-enoyl-CoA reductase	1.63	Synaptic glycoprotein expressed in many types of neurons
300443	Lppr2	lipid phosphate phosphatase-related protein type 2	1.60	Phosphatidate phosphatase activity
81682	Lum	lumican	1.79	Member of leucine-rich proteoglycan family; may play a role in immature and transient fibrosis of acute pancreatitis

Entrez gene ID	Gene name	Full name	Fold change	Function
362911	Mal2	mal, T-cell differentiation protein 2	2.77	Human homolog is a member of the MAL family of proteins, involved in transcytosis and targeting membrane proteins to the apical surface of the cell
29456	Map 1b	microtubule association protein 1b	1.77	Binds microtubules and may act as microtubule cross-linkers
29458	Neurod 1	neurogenic differntiation 1	3.44	A transcription factor; involved in neuronal differentiation
299757	Nts	neurotensin	8.57	Gene encodes a precursor protein for both peptides; neurotensin may be involved in smooth muscle contraction and possibly fat metabolism
24660	Pmp22	peripheral myelin protein 22	2.02	Mediates Schwann cell growth and peripheral myelin compaction; human homolog gene duplication causes Charcot-Marie-Tooth 1A (CMT1A) neuropathy
29434	Rasgrp1	RAS guanyl releasing protein 1	1.69	Putative guanine nucleotide exchange factor with Ca^{2+} and diacylglycerol binding domains; may activate Rap protein mediated inhibition of Ras signaling pathways
500137	Neurod 6	neurogenic differentiation 6	6.00	A transcription factor; involved in neuronal differentiation
314721	Anks1b	ankyrin repeat and sterile alpha motif domain containing 1B	1.68	Isoform 2 may participate in the regulation of nucleoplasmic coilin protein interactions in neuronal and transformed cells. Isoform 3 can regulate global protein synthesis by altering nucleolar numbers.
81763	Rpl5	ribosomal protein L5	1.72	Required for rRNA maturation and formation of the 60S ribosomal subunits
140941	Siah1a	seven in absentia 1A	1.95	Synaptophysin binding protein and function as E3 ubiquitin-protein ligases to regulate the ubiquitination and degradation of synaptophysin
64201	Slc25a11	solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	1.80	Reduced glutathione (GSH) transporter; involved in mitochondrial transport of GSH and 2-oxoglutarate
29484	Slc3a1	solute carrier family 3, member 1	2.55	Carrier protein involved with the transport of cystine and other amino acids across the membrane

Entrez gene ID	Gene name	Full name	Fold change	Function
64347	Sncg	synuclein, gamma (breast cancer-specific protein 1)	4.76	May play a role in the neurofilament network
113938	Snurf	SNRPN upstream reading frame	1.63	Produced from a bicistronic transcript along with SNRPN; each cistron may play a role in the imprinted Prader-Willi syndrome
171442	Svop	SV2 related protein	1.66	localized to synaptic vesicles and microvesicles in brain and endocrine cells; may play a role in synaptic vesicle transport
311621	Tomm34	translocase of outer mitochondrial membrane 34	1.75	Plays a role in the import of cytosolically synthesized preproteins into mitochondria. Binds the mature portion of precursor proteins. Interacts with cellular components, and possesses weak ATPase activity. May be a chaperone-like protein that helps to keep newly synthesized precursors in an unfolded import compatible state
64619	zfp238	zinc finger protein 238	4.69	Sequence-specific transcriptional repressor
303836	LOC303826 (predicted)	ribosomal protein L27a (similar)	2.31	Belongs to the ribosomal protein L15P family
497698	*Ykt6* (predicted)	YKT6 v-SNARE homolog (similiar)	2.01	Vesicular soluble NSF attachment protein receptor (v-SNARE) mediating vesicle docking and fusion to a specific acceptor cellular compartment. Functions in endoplasmic reticulum to Golgi transport; as part of a SNARE complex composed of GOSR1, GOSR2 and STX5. Functions in early/recycling endosome to TGN transport; as part of a SNARE complex composed of BET1L, GOSR1 and STX5. Has a S-palmitoyl transferase activity.
497708	*Fgfr1* (predicted)	Fibroblast growth factor receptor 1 (similiar)	1.58	Proportion of transmembrane and soluble extracellular isoforms may modulate fibroblast growth factor signaling; plays a role in regulation of neuronal cell proliferation
497785	*Wipf3* (predicted)	WAS/WASL interacting protein family, member 3 (hypothetical)	3.35	Binds SH3 domains; may play a role in MAP kinase mediated signal transduction

Entrez gene ID	Gene name	Full name	Fold change	Function
140590	Bmpr2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	-2.60	Human homolog is a receptor for bone morpogenetic proteins and plays a role in bone development
25590	Chrna4	cholinergic receptor, nicotinic, alpha 4	-2.06	Nicotinic acetylcholine receptor subunit responsible for binding calcium sensor protein visinin-like protein-1 (Vilip1), which modulates acetylcholine receptor expression and sensitivity
25305	Cort	cortistatin	-3.25	Inhibits growth hormone secretion; may act as a neuropeptide to mediate signaling via a somatostatin receptor subtype
25278	Cox6a2	cytochrome c oxidase subunit VIa polypeptide 2	-3.27	Heart isoform of subunit VIa of cytochrome c oxidase
500892	Cyp11b1	cytochrome P450, family 11, subfamily b, polypeptide 1	-4.40	Catalyzes the conversion of 11-deoxycorticosterone to corticosterone
63847	Fxyd6	FXYD domain-containing ion transport regulator 6	-1.99	Ion transport regulator
64443	Gpr88	G-protein coupled receptor 88	-7.05	Orphan G-protein coupled receptor
24415	Grm2	glutamate receptor, metabotropic 2	-2.19	Mediates synaptic transmission; may be involved in brain microvascular function
54259	Inpp5d	inositol polyphosphate-5-phosphatase D	-3.13	Phosphatase involved in the inhibitory action of the mast cell function-associated antigen
50693	Itih3	inter-alpha trypsin inhibitor, heavy chain 3	-1.65	Heavy chain peptide component of pre-alpha inhibitor, a serum protein formed by covalent linkage of the chondroitin sulfate chain of bikunin to the heavy chain carboxyl-terminal residue
84410	Klf5	Kruppel-like factor 5	-3.65	Transcription factor involved in positive regulation of cellular proliferation

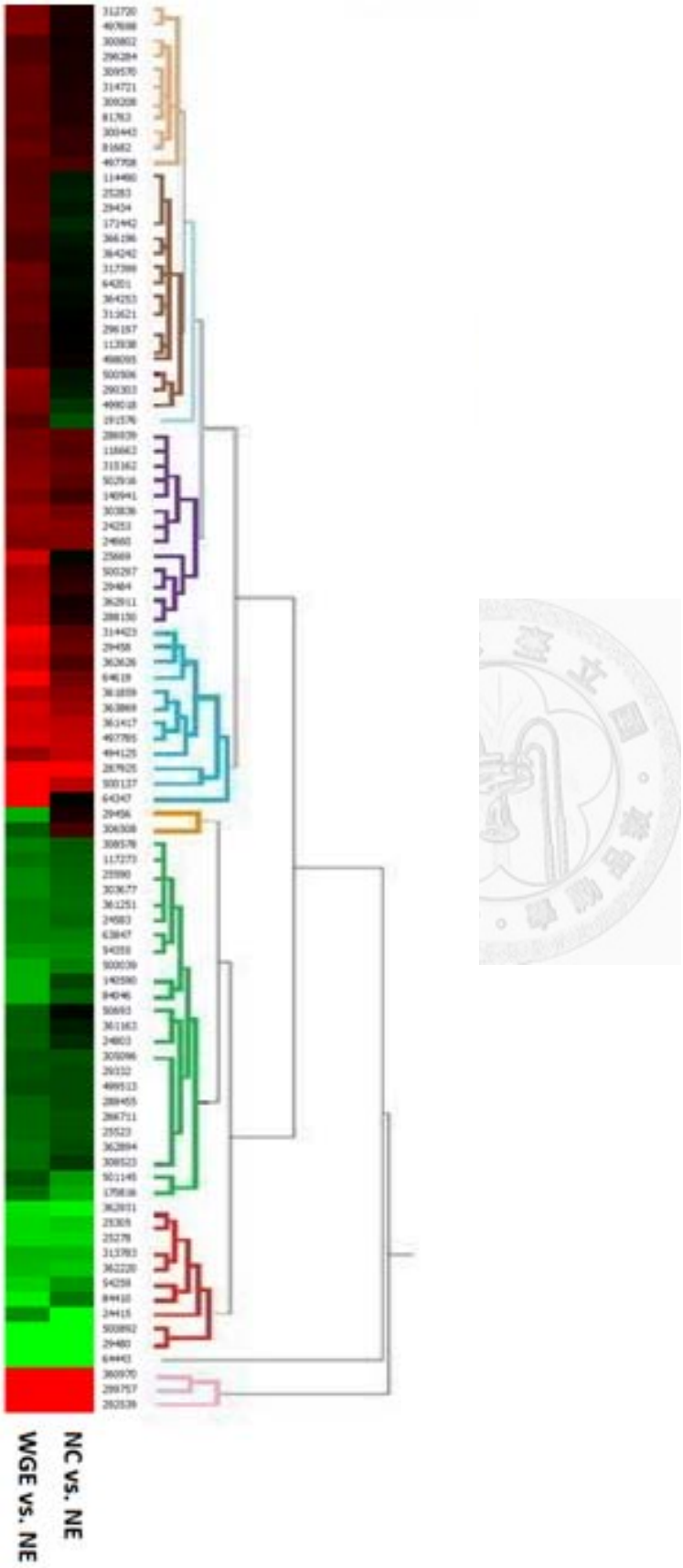
Entrez gene ID	Gene name	Full name	Fold change	Function
266711	Lzts1	leucine zipper, putative tumor suppressor 1	-1.71	Transcription factor; may play a role cell growth; may be involved with the function of postsynaptic site of excitatory synapses
24583	Myh3	myosin, heavy chain 3, skeletal muscle, embryonic	-1.98	Heavy chain of myosin; involved in muscle contraction
170816	Olr59	olfactory receptor 59	-1.79	Olfactory receptors interact with odorant molecules in the nose, to initiate a neuronal response that triggers the perception of a smell.
25523	Prkg2	protein kinase, cGMP-dependent, type II	-1.73	May regulate renin release and renal ion transport
54355	Rassf5	Ras association (RalGDS/AF-6) domain family member 5	-2.18	Interacts with guanine nucleotide exchange factor Mss4
29480	Rgs4	regulator of G-protein signaling 4	-4.16	regulates G-protein coupled receptor signaling by accelerating the inactivation of active G-proteins; may regulate dopamine D2 receptor signaling
117273	Rhoa	ras homolog gene family, member A	-1.85	Regulates a signal transduction pathway linking plasma membrane receptors to the assembly of focal adhesions and actin stress fibers. May be an activator of PLCE1. Activated by ARHGEF2, which promotes the exchange of GDP for GTP. Essential for the SPATA13-mediated regulation of cell migration and adhesion assembly and disassembly. The MEMO1-RHOA-DIAPH1 signaling pathway plays an important role in ERBB2-dependent stabilization of microtubules at the cell cortex. It controls the localization of APC and CLASP2 to the cell membrane, via the regulation of GSK3B activity. In turn, membrane-bound APC allows the localization of the MACF1 to the cell membrane, which

				is required for microtubule capture and stabilization
Entrez gene ID	Gene name	Full name	Fold change	Function
84046	Sox11	SRY (sex determining region Y)-box 11	-2.60	May play a role in regulation of oligodendrocyte development
29332	Stmn1	stathmin 1	-1.60	May promote microtubule depolymerization; may act as a signaling relay phosphoprotein for second messenger signaling pathways involved in cell proliferation and differentiation
24803	Vamp2	vesicle-associated membrane protein 2	-1.67	Plays a role in membrane fusion in neuronal exocytosis
308523	Tshz3	teashirt zinc finger homeobox 3	-1.80	Transcriptional regulator involved in developmental processes. Function in association with APBB1, SET and HDAC factors as a transcriptional repressor, that inhibits the expression of CASP4. TSHZ3-mediated transcription repression involves the recruitment of histone deacetylases HDAC1 and HDAC2. Associates with chromatin in a region surrounding the CASP4 transcriptional start site(s). Regulates the development of neurons involved in both respiratory rythm and airflow control. Promotes maintenance of nucleus ambiguus (nA) motoneurons, which govern upper airway function, and establishes a respiratory rhythm generator (RRG) activity compatible with survival at birth. Involved in

				the differentiation of the proximal uretic smooth muscle cells during developmental processes. Involved in the up-regulation of myocardin, that directs the expression of smooth muscle cells in the proximal ureter
Entrez gene ID	Gene name	Full name	Fold change	Function
309208	Lrrc10b	leucine rich repeat containing 10B	1.65	Uncharacterized
296284	Kif3b	kinesin family member 3B	1.54	Uncharacterized
494125	Rcn3	reticulocalbin 3, EF-hand calcium binding domain	2.37	Uncharacterized
290303	Spryd7	SPRY domain containing 7	2.03	Uncharacterized
296197	Dstnl1	destrin-like 1	1.60	Uncharacterized
361859	Cdc40	cell division cycle 40 homolog	2.81	Uncharacterized
314423	Bcl11b	B-cell CLL/lymphoma 11B (zinc finger protein)	3.99	Uncharacterized
312720	Akap3	A kinase (PRKA) anchor protein 3	1.98	Uncharacterized
300802	Aph1b	anterior pharynx defective 1 homolog B	1.54	Uncharacterized
502916	Rerg	RAS-like, estrogen-regulated, growth inhibitor	2.18	Uncharacterized
315162	Mei1	meiosis inhibitor 1	2.07	Uncharacterized
288150	Mtx2	metaxin 2	2.80	Uncharacterized
287925	Pkb2	plakophilin 2	5.13	Uncharacterized
363869	Ubl3	ubiquitin-like 3	3.26	Uncharacterized
362894	R3hdm2	R3H domain containing 2	-1.83	Uncharacterized
313783	Olfm2	olfactomedin 2	-2.65	Uncharacterized
305096	Klhdc8a	kelch domain containing 8A	-1.55	Uncharacterized
288455	Pomp	proteasome maturation protein	-1.69	Uncharacterized
501145	Satb2	SATB homeobox 2	-1.53	Uncharacterized
306508	Mfhas1	malignant fibrous histiocytoma amplified	-1.64	Uncharacterized

		sequence 1		
308578	Ap2a1	adaptor-related protein complex 2, alpha 1 subunit	-1.99	Uncharacterized
362931	Bai1	brain-specific angiogenesis inhibitor 1	-3.39	Uncharacterized
361251	Elmo1	engulfment and cell motility 1	-1.95	Uncharacterized
498095	Tctex1d2	Tctex 1 domain containing 2	1.59	Uncharacterized
Entrez gene ID	Gene name	Full name	Fold change	Function
362220	RGD1306991	LAMP family protein C20orf103 homolog	-2.74	Uncharacterized
500297	RGD 1560607(predicted)	env precursor (similiar)	2.35	Uncharacterized
292539	RGD1562905 (predicted)	60S ribosomal protein L17 (L23) (similar)	9.87	Uncharacterized
364253	RGD 1566348 (predicted)	RIKEN cDNA 1110003E01 (similar)	1.67	Uncharacterized
309570	RGD1306962 (predicted)	dJ55C23.6 gene product (similar)	1.73	Uncharacterized
362626	RGD1359529 (predicted)	chromosome 1 open reading frame 63 (similar)	3.07	Uncharacterized
499018	RGD1560015 (predicted)	glycoprotein, synaptic 2 (similar)	1.93	Uncharacterized
364242	RGD1561956 (predicted)	RIKEN cDNA 1110003E01 (similar)	1.51	Uncharacterized
361417	RGD1565387 (predicted)	Rgd1565387 (similiar)	3.09	Uncharacterized
303677	RGD1309310 (predicted)	mKIAA0195 (similiar)	-2.10	Uncharacterized
500039	RGD1562178 (predicted)	Adenylate kinase 2 (similiar)	-2.53	Uncharacterized
499513	RGD1565489 (predicted)	RGD1565489 (similiar)	-1.50	Uncharacterized

361163	RGD1561271	LRRGT00078 (smiliar)	-1.62	Uncharacterized


NC vs. NE
WGE vs. NE

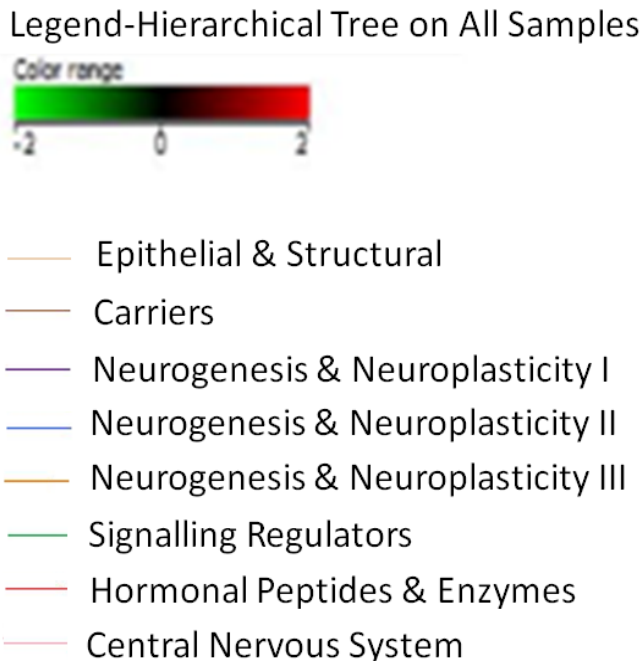


Figure 15 shows the heatmap of the 94 genes that have been altered in expression level upon treatment of WGE in the hippocampus analyzed by hierarchical clustering analysis. The genes were classified in 8 main groups based on gene ontology: epithelial & structural, golden-orange; carriers, brown; neuroplasticity & neurogenesis I, purple; neuroplasticity & neurogenesis II, blue; neuroplasticity & neurogenesis III, yellow; signaling regulations, green; hormonal peptides & enzymes, red; central nervous system, pink. As the legend shows, red means up-regulation, and green means down-regulation. The image is created by *Gene Spring*™.

Table 6 shows the possible KEGG pathways of genes regulated by WGE in the hippocampus, arranged in the order of increasing *p*-value.

The table is generated by *Pathway Studio*™.

Rank	Pathway name	Overlapping entities	*P*-value
1	Peripheral nervous system development	Sncg Pmp22 Bai1	4.76E-05
2	Positive regulation of neuron development	Rhoa Neurod1 Map1b	5.25E-05
3	Regulation of dopamine secretion	Sncg Chrna4	2.93E-04
4	Male meiosis I	Siah1 Mei1	2.93E-04
5	Positive regulation of cell motion	Stmn1 Bcl6	3.51E-04
6	Nervous system development	Neurod1 Stmn1 Sox11 Siah1 Map1b Neurod6	4.63E-04
7	Response to glucose stimulus	Rhoa Neurod1 Vamp2	4.83E-04
8	Transmembrane receptor protein	Bmbr2 Akap3	7.18E-04

9	Axon development	Rhoa Sncg Map1 Grm2	8.25E-04
10	Negative regulation of apoptosis	Gclc Neurod1 Bcl11b Bcl6	8.52E-04

(A)

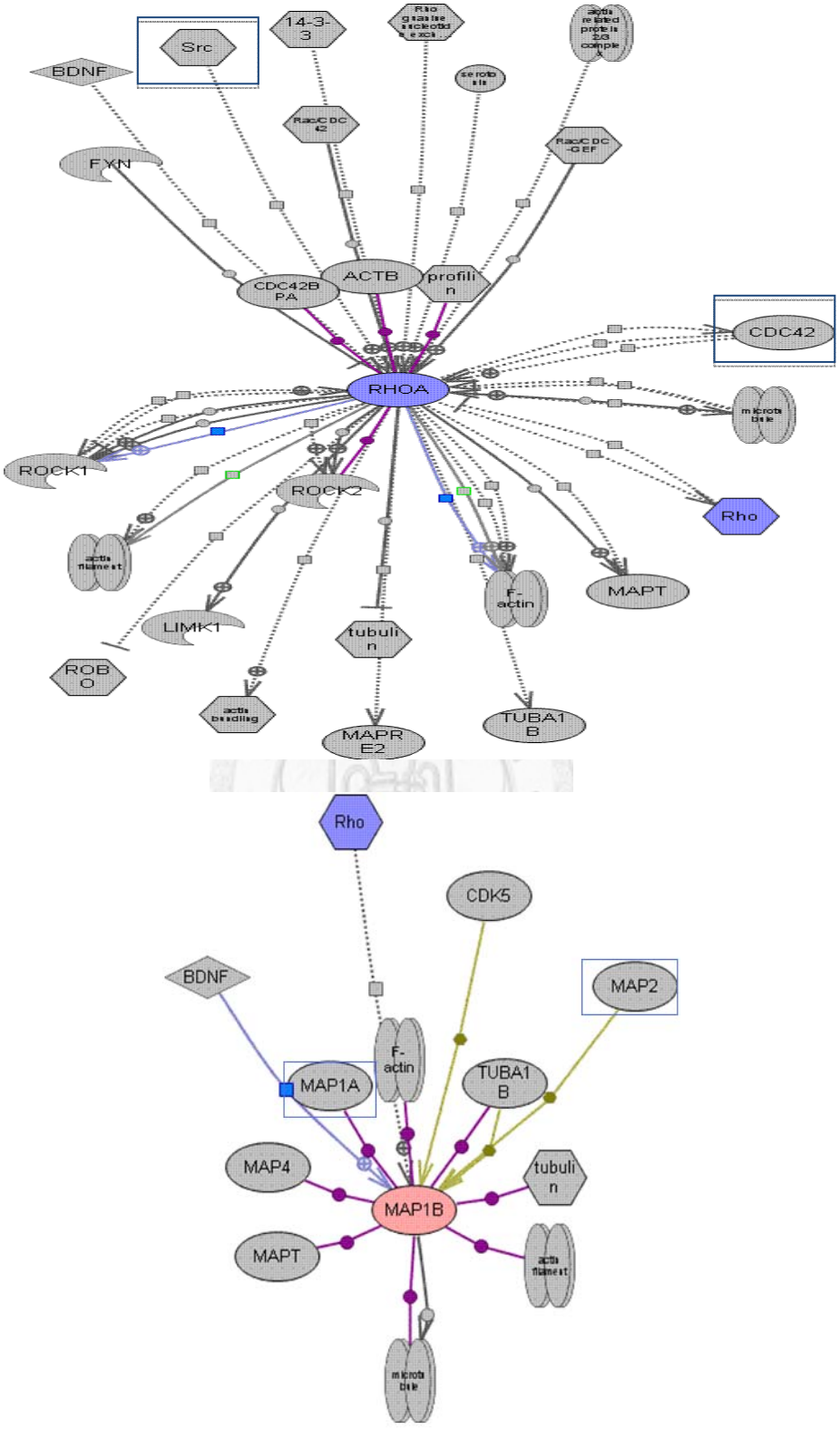


Figure 16 shows the gene network created by *Pathway Studio*™ showing the related genes altered by WGE in the hippocampus (A) The network of *Map1b* and related genes (B) The network of *RhoA* and related genes. After filtering out the unrelated genes, a gene network analysis was constructed, with *Map1b* and *RhoA* located

in the centre of the network. The two genes were selected because they are important regulators of neurogenesis. Boxes highlight other regulators of neurogenesis that were screened out by network analysis, which were one gene neighboring (either upstream or downstream) to the central gene in the network.

(A)

(B)

Figure 17 shows the Venn diagram that displays the numbers of genes altered by WGE in the striatum in both up and down regulations. The pink region represents the genes acquired from chip 1, and the teal region represents the genes acquired from chip 2. The overlapped region represents the genes altered in both chips (fold change > 1.5). (A) 42 genes are up-regulated. (B) 18 genes are down-regulated. The image is created by *Gene Spring*™.

Table 7 shows the list showing the genes that have been altered in expression level upon treatment of WGE in the striatum (fold change > 1.5, WGE vs. negative control). The table is generated by *Gene Spring*™.

Entrez gene ID	Gene name	Full name	Fold change	Functions
117029	Ccr5	chemokine (C-C motif) receptor 5	1.98	G protein-coupled receptor; involved in regulation of leukocyte activation and migration
116456	Dnajc2	DnaJ (Hsp40) homolog, subfamily C, member 2	1.78	Tumor rejection antigen for 9L gliosarcoma cells; may play a role in suppression of tumor growth
282838	Gm2a	GM2 ganglioside activator	1.83	Mouse homolog is involved in sphingolipid metabolism
306439	Gpm6a	glycoprotein m6a	1.63	A NGF-gated Ca^{2+} channel involved in neuronal differentiation
25135	Ncl	nucleolin	2.05	Nucleolar-specific protein; involved in cell growth
60373	Nop58	nucleolar protein NOP58	1.51	Component of the box C/D class of small nucleolar ribonucleoprotein particles (snoRNPs); may catalyze the modification of ribosomal RNAs
29527	Ptgs2	prostaglandin-endoperoxide synthase 2	1.69	Catalyzes the conversion of arachidonic acid products to prostaglandin; may play a role in response to endotoxic shock; may a role in long term potentiation
295619	Ermn	ermin, ERM-like protein	1.76	Plays a role in cytoskeletal rearrangements during the late wrapping and/or compaction phases of myelinogenesis as well as in maintenance and stability of myelin sheath in the adult. May play an important role in late-stage oligodendroglia maturation, myelin/Ranvier node formation during CNS development, and in the maintenance and plasticity of related structures in the mature
64640	Rpl30	ribosomal protein L30	10.10	Compoment of ribosome
64347	Sncg	synuclein, gamma (breast cancer-specific protein 1)	3.24	May play a role in the neurofilament network
114520	Strn3	striatin, calmodulin binding protein 3	2.04	Human homolog is an S, G2 phase nuclear autoantigen detected in a patient with lung and bladder cancer
117556	Sv2b	synaptic vesicle glycoprotein 2b	1.71	Proton cotransporter; mediates the uptake of neurotransmitters into vesicles

Entrez gene ID	Gene name	Full name	Fold change	Functions
289307	Tfb2m	transcription factor B2, mitochondrial	1.54	S-adenosyl-L-methionine-dependent methyltransferase which specifically dimethylates mitochondrial 12S rRNA at the conserved stem loop. Also required for basal transcription of mitochondrial DNA, probably via its interaction with POLRMT and TFAM. Stimulates transcription independently of the methyltransferase activity
29248	Tnni3	troponin I type 3 (cardiac)	1.75	Regulation of muscle contraction
286758	aqp11	aquaporin 11	-1.60	Member of the major intrinsic protein family
29131	cartpt	CART prepropeptide	-1.68	Neuronal protein that may play a role in brain development and may be regulated by testosterone
313648	Ddost	dolichyl-diphosphooligosaccharide-protein glycosyltransferase	-1.52	Human homolog catalyzes the transfer of a high-mannose oligosaccharide (GlcNac2Man9Glc3) onto the asparagine acceptor site within an Asn-X-Ser/Thr consensus motif
25431	Dlx5	distal-less homeobox 5	-1.63	Homeobox protein that is required for BMP-2-mediated oseoblast differentiation
116698	Trim28	tripartite motif-containing 28	-1.52	Mouse homolog is a transcriptional repressor; interacts with the Kruppel-associated box A (KRAB-A) domain of C2H2 zinc finger proteins
314313	rfp113a2	ring finger protein 113A2	-1.82	Contains RING and CCCH-type zinc finger domains
293291	RGD1563867 (predicted)	ribosomal protein L36 (similar)	1.60	Component of the large subunit of the mitochondrial ribosome
307766	Smarca5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	1.70	Uncharacterized
308968	Rbbp6	retinoblastoma binding protein 6	1.59	Uncharacterized
297942	Pnisr	PNN-interacting serine/arginine-rich protein	1.62	Uncharacterized
362484	Plekhf2	pleckstrin homology domain containing, family F (with FYVE domain) member 2	1.63	Uncharacterized
307016	Arhgap12	Rho GTPase activating protein 12	1.67	Uncharacterized

362819	Sarnp	SAP domain containing ribonucleoprotein	2.26	Uncharacterized
Entrez gene ID	Gene name	Full name	Fold change	Functions
303010	Cpeb4	cytoplasmic polyadenylation element binding protein 4	1.57	Uncharacterized
363869	Ubl3	ubiquitin-like 3	1.80	Uncharacterized
259228	SYT14	synaptotagmin XIV	1.53	Uncharacterized
298074	Xpa	xeroderma pigmentosum, complementation group A	1.72	Uncharacterized
361920	Ythdf3	YTH domain family, member 3	1.74	Uncharacterized
304337	zfp68	zinc finger protein 68	1.54	Uncharacterized
367160	Cpne4	copine IV	-1.80	Uncharacterized
500720	LOC500720	LOC500720	1.59	Uncharacterized
500867	RGD1561573	LRRG00116	1.98	Uncharacterized
501087	RGD1562999	RGD1562999	1.90	Uncharacterized
360435	LOC360435 (predicted)	LOC360435 (similiar)	1.57	Uncharacterized
361942	LOC361942 (predicted)	ORF4 (similiar)	1.77	Uncharacterized
361912	RGD1561614 (predicted)	LRRG00116 (similiar)	1.96	Uncharacterized
362315	RGD1566109 (predicted)	Retrovirus-related POL polyprotein (similiar)	1.81	Uncharacterized
362543	RGD1566046 (predicted)	LRRG00116 (similiar)	1.69	Uncharacterized
363492	RGD1559815 (predicted)	Ac1262 (similiar)	1.74	Uncharacterized

Entrez gene ID	Gene name	Full name	Fold change	Functions
498048	RGD1563925 (predicted)	ORF4 (similiar)	1.81	Uncharacterized
499554	RGD1565765 (predicted)	ORF2 consensus sequence encoding endonuclease and reverse transcriptase minus RNaseH (similar)	1.54	Uncharacterized
500297	RGD1560607 (predicted)	env precursor (similar)	1.70	Uncharacterized
500717	RGD1566401 (predicted)	Uncharacterized	1.74	Uncharacterized

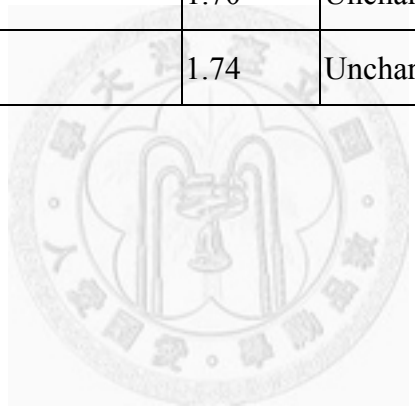


Table 8 shows the possible KEGG pathways of genes regulated by WGE in the striatum, arranged in the order of increasing *p*-value.

The table is generated by *Pathway Studio*™.

Rank	Pathway name	Overlapping Entities	*p*-value
1	rRNA methyltransferase activity	Tfb2m Nol5	0.00001
2	rRNA modification	Tfb2m Nol5	0.00001
3	positive regulation of epinephrine secretion	Cartpt	0.00018
4	endosomal lumen acidification	Aqp11	0.00035
5	protein amino acid terminal N-glycosylation	Ddost	0.00035
6	nucleic acid binding	Cpeb4 Ncl Zfp68 Smarca5 Eg625193 Rbbp6	0.00047
7	regulation of systemic arterial blood pressure by ischemic conditions	Tnni3	0.00075
8	cyclooxygenase pathway	Ptgs2	0.00075

9	beta-N-acetylgalactosaminidase activity	Gm2a	0.00093
10	dimethyladenosine transferase activity	Tfb2m	0.00093

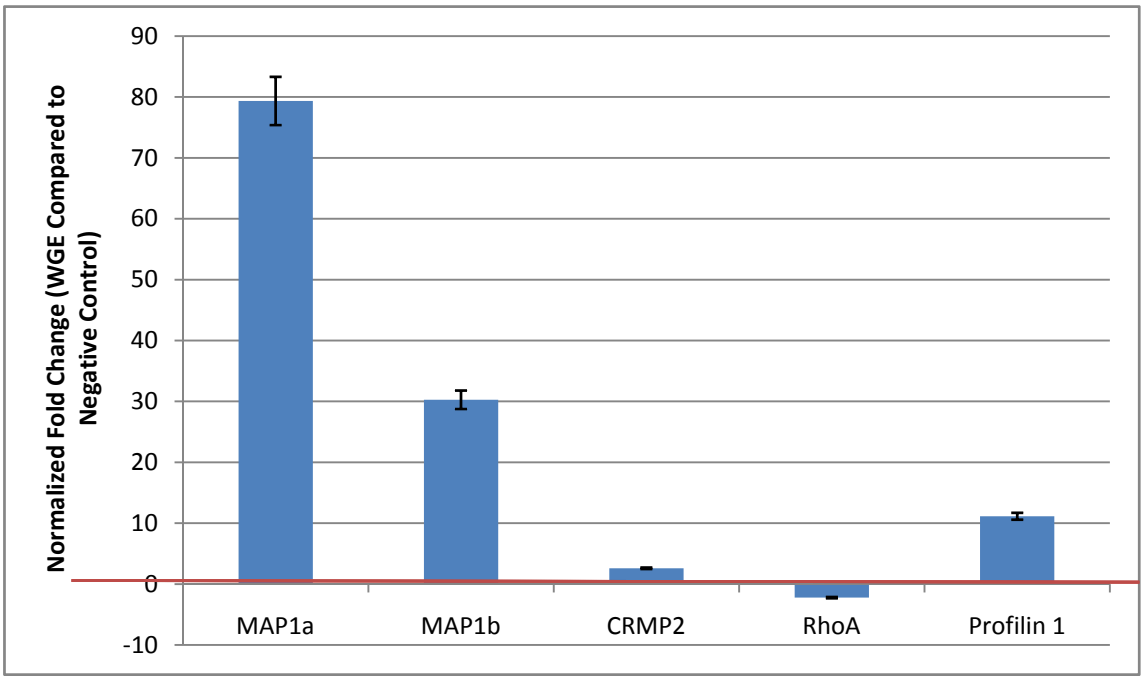


Figure 18 shows the expression level of genes related to neuroplasticity in the cortex in the QPCR experiment. Blue bars represent the treatment group. Genes labeled with asteroids are significantly up or down-regulated compared to the control. Data are represented as mean +/- SD (n=3) and analyzed statistically using Student t-test.

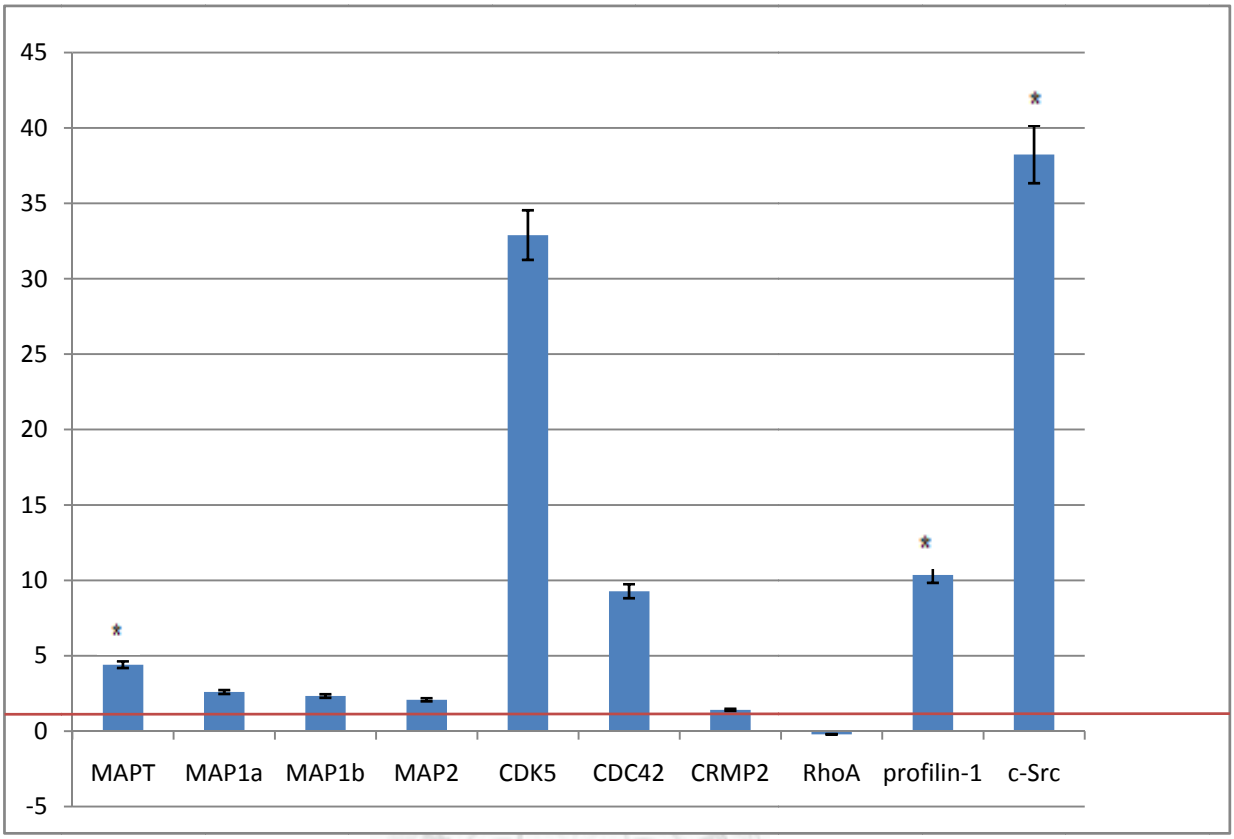


Figure 19 shows the expression level of genes related to neuroplasticity in the hippocampus in the QPCR experiment. Blue bars represent the treatment group, and the control is normalized to the baseline level. Genes labelled with asteroids are significantly up or down-regulated compared to the control. Data are represented as mean +/- SD (n=3) and analyzed statistically using Student t-test.

7 Discussion

7.1 Effect of *Gastrodia elata* Bl. on weight change and food intake

As mentioned previously in the earlier sections, antidepressants can cause several side-effects – one of them is the effect of significant weight loss and loss of appetite by SSRIs, as pointed out in the study of Khawam and his colleagues (Khawam et al., 2006). *Gastrodia elata* Bl., however, does not demonstrate such effect. As the results show, there is no significant difference in the weight change over the 3 weeks during the experiment among the treatment group (WGE), the positive control group (PC), the negative control group (NE), and the normal control group (NC) (Figure 6). In addition, there is no significant effect of food intake between the WGE group and the NE group each week over the 3 weeks period (Figure 2). There is, however, a significant decrease in food intake between the PC group and the NE group in weeks two and three (Figure 7). According to Mascand and Gupta, fluoxetine can cause anorexia (lack of appetite) (Mascand and Gupta, 2000), but usually only in the early treatment, because it activates 5-HT2C receptors, which are serotonin receptors associated with appetite control (Stahl, 2000; Stahl, 1998). On the other hand, there has not been much evidence of side-effects caused by GEB (Zhao, 2005; Mears, 2005; Wang and Yan, 2010).

7.2 Effect of *Gastrodia elata* Bl. on Rodents' Performance in FST

Forced swimming test is a reliable animal model that can accurately imitate the human depression symptom of behavioral despair. It is often applied to evaluate the efficacies of antidepressants (Lucki, 1997; Borsini and Meli, 1988). Therefore, FST was used to induce depression and to evaluate the efficacy of WGE in this experiment.

There are three behaviors that can be seen in this model: immobility, struggling or climbing, and swimming. During the immobility period, the rodent remains inactive and floats on the water surface, without any conspicuous movement of struggling, climbing, or swimming. The immobile behavior is similar to the human state of despair. The longer the immobility time, the more serious the level of depression is (Porsolt, 1977a; Porsolt, 1977b; Porsolt, 1978a; Porsolt, 1978b). In the struggling/climbing state, the rat fiercely waives its fore and back limbs in an attempt to escape the water tank. Because the definition of "struggling intensively" is subjective and hard to be defined, the definition of immobile in this research was defined to be completely immobile – so that the definition will be easier and less subjective.

The administration of most antidepressants can lower the immobility time and thus improve the rodent's inactive performance in the FST. In Figure 8, the immobility times

of the WGE and the fluoxetine groups are significantly shorter than the negative control group. Based on this result, the rodents were successfully induced of depression-like behavioiurs, and the WGE treatment had preventive effects of depression.

While the FST has been questioned that whether the immobility behavior could be a result of fatigue rather than of despair, studies have shown that the FST is effective in screening the effects of various classes of antidepressants. In the study of Cryan et al., (2005), patterns of animal behaviors in the FST resulted by administration of different classes of medication have been classified, indicating that antidepressants can regulate the level of neurotransmitters, and that specific patterns of animal behaviors are indeed results of different neurotransmitters (Cryan et al., 2005). For example, a number of studies have shown that SSRIs administrations demonstrate significantly shorter immobility times, longer swimming periods, and constant struggling/climbing periods (Detke and Lucki, 1996; Page et al., 1999; Lopez-Rubalcava and Lucki, 2000; Cryan et al., 2005; Jama et al., 2008). Likewise, several studies have shown that NRRIs administrations lead to significantly shorter immobility times, longer struggling/climbing periods, and constant swimming periods (Detke and Lucki, 1996; Cryan et al., 2005; Jama et al., 2008). SSRIs and NRRIs prevent the reuptake of serotonins and norepinephrines, respectively, and therefore increase the serum serotonin

and norepinephrine levels. From these specific behavioral patterns in response to different concentrations of neurotransmitters, it is fair to conclude that different behavioral patterns, specifically immobility, are not random actions but responses to neurotransmitters change.

7.3 Advantages and Disadvantages of Microarray Analysis

In this study, microarray technology was used to investigate the anti-depressant effects of WGE at the transcriptional level, so that the potential mechanisms of GEB can be further understood. Microarray is a powerful technology that can measure the transcription levels of multiple genes simultaneously; in other words, it can genotype multiple regions of a genome. As opposed to traditional molecular biology tools, such as Northern blotting, the conventional method can only measure the transcriptional level qualitatively of one gene at a time.

However, microarray has its shortcomings. Microarray technology, similar to Northern blotting, contains either DNA fragments, or synthesized oligonucleotides, attached to the substrate, and then probed to a known gene or fragment. Therefore, the probes may cross-hybridize to target mRNAs in the sample different from what they are supposed to hybridize to, generating false-positive results. However, since we are still

unclear about the mechanism of WGE at the genomic level, we needed to use microarray to screen for a most possible mechanism out of the hits given by the KEGG database. The shortcomings of microarray were solved by validating the results using the QPCR technique.

7.4 Microarray Analysis on the Potential Mechanism in the Cortex

As oligonucleotide microarray data analysis shows (Table 2), WGE mainly regulates 3 neuroplasticity-related and 1 neurotransmitter-related pathways in the cortex; specifically, it negatively regulates microtubule depolymerization, and MAPK activity, and it positively regulates axogenesis. As for the neurotransmitter aspect, it regulates dopamine concentration. However, only the aspect of neurogenesis will be solely discussed in this thesis.

According to the KEGG pathway analysis (Table 2), the most probable pathway in the cortex is axongenesis, followed by negative regulation of microtubule depolymerization. Microtubule and actin polymerization are part of the axon and dendrite formation (Dotti et al., 1988). Specific signaling pathways are activated to wire the nervous system, consequently reorganizing the neuronal cytoskeleton, specifically, the axon morphology (Zhou et al., 2004). The specific process of neurogenesis will be

discussed later in this section. KEGG database was chosen because it accommodates with more signaling pathways than other databases. The specific process of neurogenesis will be discussed later in this section.

7.5 Microarray Analysis on the Potential Mechanism in the Hippocampus

Based on the oligonucleotide microarray data (Table 4), WGE positively regulates axongenesis and dopamine secretion, and it negatively regulates apoptosis in the hippocampus. However, only the aspect of neurogenesis will be solely discussed in this thesis.

Hippocampus is one of the main brain regions where neurogenesis in adult brain has been documented across several vertebrate species, including rats, monkeys and humans (Stevens, 2008; Gould et al., 2001; Eriksson et al., 1998). In fact, adult hippocampal neurogenesis also contributes functionally to cognitive capacity. Shors et al., (2001) reported that inhibition of neurogenesis in adult rat hippocampus, in the absence of the destruction of existing neurons, caused impaired memory function. The specific process of neurogenesis will be discussed later in this section.

7.6 Microarray Analysis on the Potential Mechanism in the Striatum

As the microarray analysis shows (Table 7), there was no depression-related pathway matched with the KEGG database. Neurogenesis is generally accepted to occur in the hippocampus (Taupin and Gage, 2002). However, recent studies have reported that neurogenesis may occur in the other parts of the adult brain under normal conditions as well, such as the neocortex (Gould et al., 1999) and the amygdale (Bernier et al., 2002). In addition, brain insults have been shown to induce formation of new neurons in the striatum (Parent et al., 2002; Fallon et al., 2000) and in the cortex (Magavi et al., 2000; Jiang et al., 2001). Yet, several studies have not been able to replicate the results (Koketsu et al., 2003; Kornack and Rakic, 2001).

7.7 Neurogenesis, Neuroplasticty, Neurotransmitters, Antidepressants, and Depression

In the present study, the microarray data explicitly indicated one of the most probable antidepressant mechanisms was the neurogenesis/neuroplasticity mechanism in the cortex and the hippocampus. Also, neurogenesis-related genes were regulated in the direction favoring the process in the WGE treated group. Specifically, QPCR results validated that there was significant up-regulation in the expression of MAP1b

and MAPK in the cortex and hippocampus, respectively (Figure 19). MAPs are important regulator proteins as they stabilize microtubules by promoting microtubule binding and microtubule and actin interactions (Dehmelt, 2004). They are one of the most abundant axonal microtubule axonal proteins, and they are important for axonal elongation (Stunden et al., 2001). In fact, studies have shown that disruption of MAP1b in C57B1/6J mice revealed dysgenesis in axon tracts (Stunden et al., 2001). In addition to MAPs, neurotrophins also contribute to neuron development. Neurotrophins activate both the MAPK and PI3K by binding to their specific receptor: tyrosine kinase (TRK) (Zhou et al., 2004).

Neurogenesis can be characterized as three successive stages: proliferation of endogenous stem cells and precursors, differentiation into neurons, and neuron maturation with formation of viable synaptic connections (plasticity). By taking into account all stages of neurogenesis, then the hippocampal volume loss in depression could potentially be caused by 1) inhibition of the endogenous hippocampal stem cell proliferation in the dentate gyrus, 2) inhibition of differentiation and dendrite development and 3) by loss of neurons (apoptosis) and their dendritic structure. Though apoptosis is observed in depression, hippocampal apoptosis as measured by DNA fragmentation from depressed patients appears to play only a minor role in the volume

loss (Lucassen et al., 2001). The term neuroplasticity describes either functional or structural changes of neurons and glial cells that occur in developing and adult brains, in order to adjust to external and internal stimuli (Mesulam, 1999; Nestler et al., 2002). Structural changes are achieved by changes in the strength of connections among brain cells – either by adding or removing connections, or by adding new cells (Von Bohlen and Halbach, 2009). As neuroplasticity is related to neurogenesis, the two processes were discussed together.

Whether neurogenesis can reverse atrophy brain tissues remains uncertain today (Duman and Monteggia, 2006). Many studies observed the existence of a natural repair pathway by means of neurogenesis in mature mammalian brains, upon inducing neurogenesis by degenerative conditions (Fisar and Hroudova, 2010). For example, seizures, which are a memory degenerative condition and are induced by electroconvulsive shock, increased neurogenesis in dentate gyrus of hippocampus in adult brains (Scott et al, 2000; Madsen et al, 2000). These observations highlight the likelihood that a cellular mechanism for neurogenesis within adult human CNS, especially in hippocampus, does exist both as a normal physiological process and as a self-repairing pathway. However, this fact remains controversial as Duman and Monteggia remained reserved about reverse atrophy. They pointed out that although

numerous studies have analyzed the neurotrophic effects of antidepressants on hippocampal and other neural tissue, there is still not any longitudinal study that can prove the ability of antidepressants to reverse atrophy of brain structures (Duman and Monteggia, 2006). Since we have concluded that WGE takes the neurogenesis mechanism, it is fair to conclude that WGE not only prevents depression, but it might also be able to alleviate the depression symptoms by means of repairing the neurological system through neurogenesis.

Neurogenesis can not only achieve anti-depressant effects, but it can also prevent and improve other cognitive problems, such as memory consolidation. A decline in neurogenesis due to ageing is observed in adults (Kuhn et al., 1996). The phenomenon can be best observed with the transplantation of human stem cells into aged rats, initiating improved water maze learning and retention (Qu et al., 2001). This is probably why WGE can also achieve memory consolidation by means of neurogenesis, as Malberg and Czeh pointed out that antidepressants can improve diseases presenting cognitive-deficits. Interestingly, Lee and Van Praag further pointed out that neurogenesis can also be promoted by diet restriction (Lee et al., 2000), exercise (van Praag et al., 1999), or growth factor addition (Lichtenwalner et al., 2001) improves learning and memory in adult or aged rats.

One hypothesis that may account for the slow-onset of the antidepressants' therapeutic activity is that they work by promoting hippocampal neurogenesis. It is expected that neurogenesis would require a number of weeks for stem cells to divide, differentiate, migrate and establish connections with post-synaptic neurons. The neurogenesis theory of depression then postulates that antidepressant effect is brought about by structural changes in the hippocampal circuitry contributed by newly generated neurons stimulated by antidepressants (Malberg et al., 2000; Czeh et al, 2001; Santarelli et al, 2003).

In the present study, the microarray data explicitly indicates the activation of the neurotransmitters related genes in the WGE treated group. Most antidepressants are thought to work by increasing the levels of monoamines available for post-synaptic receptors. Examples of classes of agents working apparently by the “monoaminergic hypothesis of depression” include the SSRIs like fluoxetine, the mixed noradrenaline/serotonin transporter blockers like tricyclic agent imipramine and noradrenaline uptake inhibitors like desipramine. The antidepressant-induced increase in intraneuronal biogenic amines occurs quite rapidly. However, the antidepressant-induced improvement in clinical behavior requires weeks of daily administration.

7.8 Overall Discussion

The neurotransmitters, apoptosis, and neurogenesis mechanisms can be seen established to explain the pathology of depression (Hayley et al., 2005). In this research, these mechanisms have also been spotted in the microarray results, as they matched the KEGG database with the lowest *p*-values. Based on the highest likelihoods among these mechanisms, neurogenesis was designated the lowest *p*-value. As the network analysis showed that more genes surrounded the central component genes were neuroplasticity-related than those that were neurotransmitter-related or apoptosis-related, the target genes chosen to be performed in QPCR to start with were some of the neuroplasticity-related genes; specifically, *MAP1*, *MAPK*, *RhoA*, *CDC42*, *c-Src*, and *profilin-1*. The QCPR results showed that these genes were regulated in the directions that favored the neurogenesis theory. Therefore, the microarray data were validated and neurogenesis was determined to be the anti-depressant mechanism that WGE took. In this thesis, a preliminary discovery of neuroplasticity has been achieved solely based on the microarray data, with a partial validation of QPCR, only on the neuroplasticity-related genes. A closer investigation involving tissue staining of neurons will be performed to validate neurogenesis being the mechanism that WGE takes.

8 Conclusion

This study was divided into two main parts: the animal study on the behavioral change upon the gavage of WGE, and the systematic biology study of the gene expression profile at the transcriptional level. The FST results showed that the WGE group demonstrated shorter immobility time, indicating that the FST had successfully induced depression in the experimental animals. Further investigation was done by systematic biology using cDNA microarray, and the results suggested that the possible mechanisms in which WGE altered were neurogenesis, nervous system development, and dopamine secretion, based on the highest probabilities from the match results within the KEGG data base. QPCR results supported the neurogenesis mechanism, as shown by the neuroplasticity-related genes, such as *RhoA*, *CRMP2*, *profilin-1*, *CDK5*, and *CDC42*, were significantly altered in gene expression ($p<0.05$) by WGE in the frontal cortex and the hippocampus of the rodents. The results provided an innovative fundament for the Oriental herb, *Gastrodia elata* Bl., in the aspect of its antidepressant mechanism at the transcriptional level. The neurogenesis/neuroplasticity mechanism, therefore, is an interesting scope in a further study, which can be done at the translational level and by gene-knock out. After the neuroplasticity mechanism has been confirmed at the protein level, *Gastrodia elata* Bl., a multi-functional herb that can be used to prevent depression without significant side-effects, can then be applied onto clinical trials.

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Transcriptomic Profile of Antidepressant Effects of Gastrodia elata Bl. in Rat Cortex, Hippocampus, and Striatum

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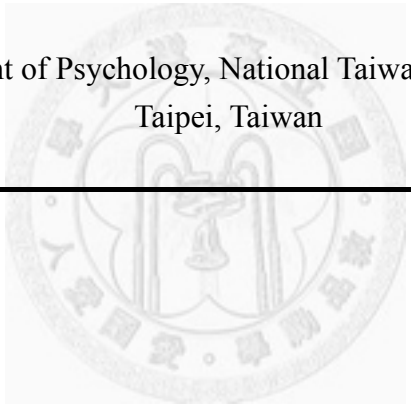

Abstract

Depression has been a serious issue, as the annual worldwide reported cases of depression have been constantly increasing, according to the statistics published by WHO. However, antidepressants generally demonstrate serious side-effects; therefore, it is important to find an effective way to prevent the occurrence of depression. In this research, *Gastrodia eleta* Bl., an Oriental herb that has been shown to demonstrate anti-depression effects, was investigated in the form of water extraction. The water extract of *Gastrodia eleta* Bl. (WGE) was orally administered to Sprague-Dawley rats at

the dose of 0.5 g/kg BW each day for 21 consecutive days. Forced swimming test (FST) was performed to induce depression in the rodent model, and results showed that the WGE group demonstrated shorter immobile time compared to the negative control group, indicating that FST has successfully induced depression-like behaviours in these rats. Total mRNA samples were then obtained from the frontal cortex, hippocampus, and striatum, after having sacrificed the rats after 21 days. In order to deduce a possible anti-depression pathway at the genomic level, cDNA microarray was performed to generate gene expression profiles of depression relevant brain regions: cortex, hippocampus, and striatum. To confirm the findings, real-time polymerase chain reaction (QRT-PCR) analysis of several neuroplasticity-related, differentially expressed genes, was performed. The microarray data showed that WGE altered axongenesis/neurogenesis, nervous system development, and dopamine secretion pathways in cortex and hippocampus, from the evidence that they yield the lowest *p*-values from all other pathway matches with the KEGG database. However, there were no depression-related pathways shown in the results of the microarray data for the striatum. QRT-PCR results validated that genes involved in neurogenesis, such as *Map1b*, *RhoA*, *profilin-1*, and *CRMP2* were significantly altered ($p < 0.05$) in the cortex and hippocampus. Therefore, neuroplasticity might be the mechanism that WGE takes. The neuroplasticity effects of WGE in the rodent model of antidepressant action

strengthen the case for further testing the results in the same context using other tools, and can serve as the basis of future antidepressant drugs, especially in the area of WGE demonstrating anti-depressant effects in the aspect of genomics.

Keywords: depression; forced swimming test; *Gastrodia elata* Bl.; microarray analysis; neuroplasticity