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Psychotropic effects of *Lactobacillus plantarum* PS128 in early life-stressed and naïve adult mice

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ABSTRACT

Ingestion of specific probiotics, namely "psychobiotics", produces psychotropic effects on behavior and affects the hypothalamic–pituitary–adrenal axis and neurochemicals in the brain. We examined the psychotropic effects of a potential psychobiotic bacterium, *Lactobacillus plantarum* strain PS128 (PS128), on mice subjected to early life stress (ELS) and on naïve adult mice. Behavioral tests revealed that chronic ingestion of PS128 increased the locomotor activities in both ELS and naïve adult mice in the open field test. In the elevated plus maze, PS128 significantly reduced the anxiety-like behaviors in naïve adult mice but not in the ELS mice; whereas the depression-like behaviors were reduced in ELS mice but not in naïve mice in forced swimming test and sucrose preference test. PS128 administration also reduced ELS-induced elevation of serum corticosterone under both basal and stressed states but had no effect on naïve mice. In addition, PS128 reduced inflammatory cytokine levels and increased anti-inflammatory cytokine level in the serum of ELS mice. Furthermore, the dopamine level in the prefrontal cortex (PFC) was significantly increased in PS128 treated ELS and naïve adult mice whereas serotonin (5-HT) level was increased only in the naïve adult mice. These results suggest that chronic ingestion of PS128 could ameliorate anxiety- and depression-like behaviors and modulate neurochemicals related to affective disorders. Thus PS128 shows psychotropic properties and has great potential for improving stress-related symptoms.

Abbreviations: CFU, colony-forming unit; CORT, corticosterone; ELS, early life stress; EPM, elevated plus maze; FST, forced swimming test; HPA axis, hypothalamus–pituitary–adrenal axis; HPLC-ECD, high-performance liquid chromatography-electrochemical detection; IL, interleukin; LAB, lactic acid bacteria; MRS, de Man Rogosa and Sharpe; MS, maternal separation; NELS, non-ELS; OFT, open filed test; PD, postnatal day; SPT, sucrose preference test; TNF, tumor necrosis factor

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1. Introduction

Probiotics are living microorganisms known to have beneficial effects on the host when ingested in adequate amounts (FAO/WHO, 2001). Numerous studies have demonstrated the diverse benefits of probiotics (Vyas and Ranganathan, 2012), including anti-inflammatory effects (Liu et al., 2011), gut protection (Zareie et al., 2006), and the attenuation of metabolic dysfunctions (Barrett et al., 2012; Huang et al., 2013). In recent years, the influences of probiotics on the central nervous system (CNS) and behaviors via the microbiome–gut–brain-axis have been uncovered (Collins et al., 2012). In particular, there is an association between the gut microbiota and stress response, and the effects of probiotics on CNS could be mediated by humoral, immune, neural, and metabolic pathways (Moloney et al., 2014; Sudo et al., 2004). Based on the roles of probiotics in regulating stress responses and maintaining CNS homeostasis, we think there is great potential for utilizing beneficial bacteria to improve CNS-related abnormalities.

Several probiotics have been shown to have effects on normalizing stress-induced abnormal behaviors and regulating the hypothalamus–pituitary–adrenal axis (HPA axis) and the inflammatory responses in animal models (Ait-Belgnaoui et al., 2012; Arseneault-Breard et al., 2012; Bercik et al., 2011; Desbonnet et al., 2010). For example, pretreatment with probiotics in immune-deficient mice has been shown to normalize immune-mediated deficits in intestinal physiology and to affect CNS functions (Smith et al., 2014). Administration of *Lactobacillus helveticus*–containing probiotics reduces anxiety-like behaviors in rodents (Messaoudi et al., 2011; Ohland et al., 2013). Both *Bifidobacterium longum* 1714 and *Bifidobacterium breve* 1205 reduce anxiety-like behavior in an anxious mouse strain (Savignac et al., 2014). It has also been reported that the administration of probiotics restores monoamine levels in key brain regions of rats in a maternal separation (MS) model of depression (Bercik et al., 2011; Desbonnet et al., 2010). Also, probiotics have been shown to alter behavior and CNS function in naïve adult animals (Moloney et al., 2014). These findings not only reveal the complexity of gut–brain interactions upon stress challenge but also suggest that probiotics could be used to ameliorate stress-induced disorders.

Early life stress (ELS) is known to convey negative effects on brain development and cause behavioral changes in adulthood (Lupien et al., 2009). In rodents, MS is the most commonly used method to create ELS for the study of the physiological and psychological impacts of ELS (O'Mahony et al., 2011; Sanchez et al., 2001). The affected animals show long-lasting behavioral and physiological phenotypes, including enhanced stress responses, anxiety-like and depression-like behaviors, HPA axis hyperactivity, and abnormal neurochemical changes (Cryan and Holmes, 2005). In addition, MS also increases the immune response, as indicated by enhanced IL-6 release following concanavalin A (ConA) stimulation (Desbonnet et al., 2010). In humans, ELS is also associated with immune dysregulation (Fagundes et al., 2013). Furthermore, ELS is a well-known risk factor for psychopathology, including major depressive disorder and anxiety disorders (Green et al., 2010; McCrory et al., 2010; Tyrka et al., 2013). Moreover, in animal studies MS has been shown to affect the development of serotonergic and dopaminergic systems and the HPA axis in the brain (Bravo et al., 2014; Rentesi et al., 2010). Thus, an animal model of MS is ideal for investigating the psychopathology of stress-related disorders and for evaluating the psychotropic potentials of probiotics (O'Mahony et al., 2009).

Although several probiotics showing psychotropic effects in animal models have been classified as psychobiotics, which are defined as probiotics that produce health benefits in patients suffering from psychiatric illnesses, the field is still in its infancy (Dinan et al., 2013). In an attempt to screen for potential psychobiotics in our lactic acid bacteria (LAB) bank, we administered several LAB strains by oral gavage to mice that underwent ELS and evaluated their psychotropic potentials according to their ability to reverse depression-like behaviors. Among the strains tested, we found that *Lactobacillus plantarum* strain PS128 (PS128), isolated from spontaneously fermented mustard greens in Taiwan, shows the highest potential. To our knowledge, the ability of this strain to normalize stress-induced depression-like behaviors has not been reported previously. Therefore, in the current study we further characterize its psychotropic effects on the HPA axis, immune responses, and neurochemical changes in the brain. In addition, we also administered PS128 to naïve adult mice to evaluate its effects on unstressed normal mice.

2. Results

2.1. PS128 causes distinct alterations in behavior in ELS and naïve adult mice

We administered PS128 to ELS and naïve adult mice for 4 weeks and then started the behavioral tests to evaluate the behavioral effects of chronic PS128 ingestion. The behavioral tests were conducted starting from the least stressful to the most stressful test in the following order: SPT, OFT, EPM, and FST. In the SPT, the ELS mice showed a clear reduction in sucrose preference compared to NELS littermates; chronic ingestion of PS128 could revert this reduction (Fig. 1A). However, chronic ingestion of PS128 had no effect on sucrose preference in the naïve mice (Fig. 1B). In the OFT there was no difference in the total distance traveled by the ELS and NELS mice. However, the ELS mice showed a reduction in the time spent in the center (Fig. 2A and B). Chronic PS128 treatment in ELS mice significantly increased the total distance traveled by the ELS mice but did not reverse the time spent in the center by the mice (Fig. 2A and B); however, this treatment significantly increased the total distance traveled and the time spent in the center by the naïve mice (Fig. 2C and D).

From the EPM test we observed that ELS mice spent significantly more time in the closed arms than the NELS group, indicating more anxiety-like behaviors in the ELS mice (Fig. 3A). Although we did not observe any change in the PS128-treated ELS mice, this treatment significantly reduced the closed arm time and increased open arm time of the naïve mice (Fig. 3A and B). In contrast, in the FST, PS128

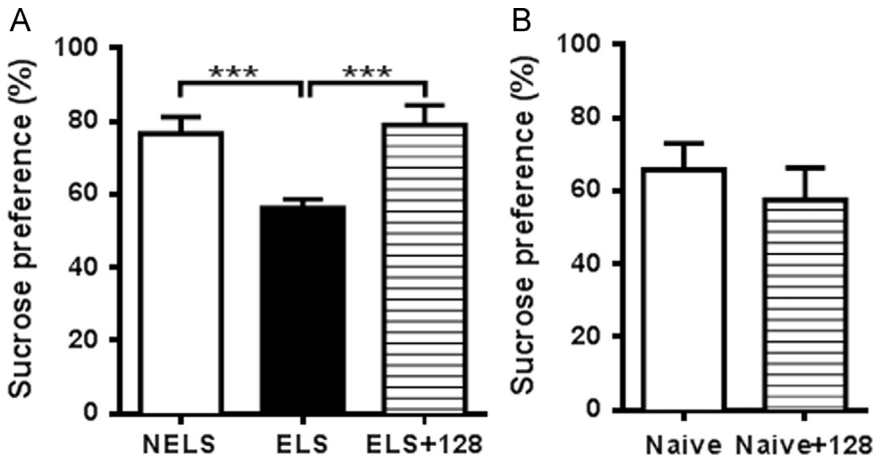

Fig. 1 – PS128 administration reversed the reduced sucrose preference in the ELS mice but has no effect on naïve mice. (A) ELS mice show a clear reduction in sucrose preference compared to NELS mice, and chronic PS128 ingestion reverts this reduction to a level similar to that of NELS mice. (B) Chronic PS128 ingestion has no effect on sucrose preference in naïve mice. ELS, early life stress; ELS+128, ELS mice administered PS128; NELS, non-ELS; Naïve+128, naïve mice administered PS128. Data in the ELS mice experiments were analyzed by one-way ANOVA; data in the naïve mice experiments were analyzed by *t*-test. $^{***}p<0.001$.

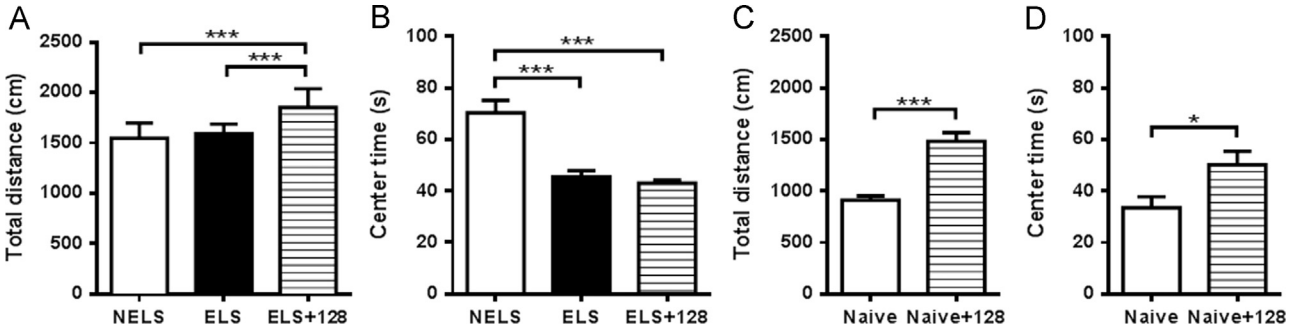

Fig. 2 – Open field test indicates that chronic PS128 ingestion increases locomotor activities in both ELS and naïve mice and reduces anxiety-like behaviors in naïve mice. (A) In the open field test the total travel distance is increased in ELS mice after PS128 administration. (B) The time spent in the center area is significantly reduced in ELS mice, and PS128 administration had no effect on the center time of ELS mice. (C) The total travel distance in naïve mice is also increased following PS128 administration. (D) Time spent in the center area is significantly increased in naïve mice after chronic PS128 administration. ELS, early life stress; ELS+128, ELS mice administered PS128; NELS, non-ELS; Naïve+128, naïve mice administered PS128. Data in the ELS mice experiments were analyzed by one-way ANOVA; data in the naïve mice experiments were analyzed by *t*-test. $^{*}p<0.05$; $^{***}p<0.001$.

treatment significantly reduced the immobile time in the ELS mice, reverting the immobility to a level similar to that of the NELS group, whereas such treatment had no effect on the naïve mice (Fig. 4A and B). Taken together, chronic PS128 treatment increased locomotor activity in both ELS mice and naïve rats (Fig. 2). For anxiety-like behavior evaluated by the center time of OFT and open arm time of the EPM, PS128 had a significant effect on naïve mice but not ELS mice (Figs. 2 and 3). Conversely, PS128 significantly reduced depression-like behaviors in ELS mice but had no effect on naïve mice (Figs. 1 and 4).

2.2. *PS128 normalizes ELS-induced exaggerated corticosterone release*

Maternal neglect is known to increase stress reactivity in offspring (Meaney, 2001). Thus, we measured the serum corticosterone levels to access the HPA axis reactivity. During the basal unstressed condition, the corticosterone level of the ELS mice was significantly higher than that of the NELS mice (Fig. 5A). Chronic PS128 treatment significantly reduced the baseline corticosterone level in the ELS mice to a degree similar to that of NELS mice (Fig. 5A). Under stressed conditions, in this case 30 min after forced swimming, ELS mice also showed significantly higher corticosterone levels than did the NELS mice (Fig. 5A). Again, chronic PS128 treatment could significantly reduce the elevated corticosterone level in stressed ELS mice (Fig. 5A). In the naïve mice, chronic ingestion of PS128 had no effect on the serum corticosterone level either at baseline or under stressed conditions (Fig. 5B). These results indicated that the HPA axis in the ELS mice was dysregulated and administration of PS128 could normalize the elevated corticosterone level (Fig. 5A).

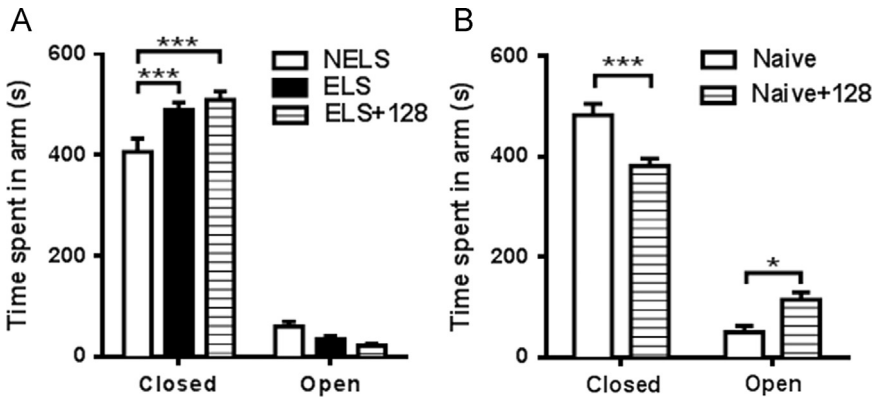

Fig. 3 – Elevated plus maze test shows PS128 administration decreases anxiety-like behaviors in naïve mice but not ELS mice. (A) Time spent in closed arm is significantly increased in ELS mice and PS128 administration has no effect on ELS mice. (B) In naïve mice, PS128 administration significantly reduced the time spent in the closed arm and increased the time spent in the open arm. ELS, early life stress; ELS+128, ELS mice administered PS128; NELS, non-ELS; Naïve+128, naïve mice administered PS128. Data of closed or open arm in the ELS mice were analyzed separately by one-way ANOVA; data of closed or open arm in the naïve mice experiments were analyzed separately by *t*-test. $^{*}p<0.05$; $^{***}p<0.001$.

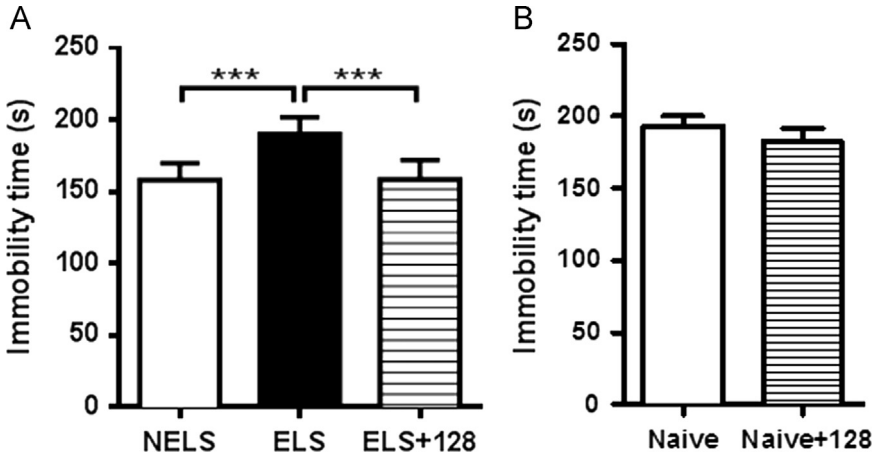

Fig. 4 – Forced swim test indicates PS128 administration reduces depression-like behaviors in ELS mice but not in naïve mice. (A) The immobile time is significantly increased in ELS mice, and PS128 administration reduces the immobile time to the level similar to that of NELS mice. (B) PS128 administration has no effect on the immobile time of naïve mice. ELS, early life stress; ELS+128, ELS mice administered PS128; NELS, non-ELS; Naïve+128, naïve mice administered PS128. Data in the ELS mice experiments were analyzed by one-way ANOVA; data in the naïve mice experiments were analyzed by *t*-test. $^{***}p<0.001$.

2.3. *PS128 decreases ELS-induced inflammation*

To evaluate the effects of PS128 on the immune system of ELS mice, we measured the levels of the pro-inflammatory cytokines TNF-α and IL-6 and the anti-inflammatory cytokine IL-10 in serum and in mitogen-stimulated splenocytes. The ELS mice showed a significant reduction in serum TNF-α, whereas the IL-6 level was increased compared with that of NELS mice (Fig. 6A and B). The IL-10 concentration of ELS mice seemed lower than that of the NELS mice; however, this did not reach statistical significance (Fig. 6C). After chronic administration of PS128, the ELS mice showed no significant change in TNF-α level (Fig. 6A). However, this treatment significantly reduced the IL-6 level and increased the IL-10 level in the ELS mice (Fig. 6B and C). In the cultured splenocytes, which were obtained from NELS, ELS, and PS128-treated ELS mice, we collected culture medium and measured the aforementioned cytokines. We found that in the unstimulated culture there were no differences in TNF-α, IL-6, and IL-10 concentrations among different groups (Fig. 6D, E and F). When we stimulated the culture with ConA, we found that splenocytes from ELS mice produced significantly more TNF-α than splenocytes from NELS mice (Fig. 6D). In splenocytes from PS128-treated ELS mice, this enhancement was attenuated (Fig. 6D). The level of IL-6 in LPS-stimulated splenocytes from ELS mice was not different from that of NELS mice; however, the IL-6 response of splenocytes from PS128-treated ELS mice was significantly higher than that of the other two groups (Fig. 6E). The levels of IL-10 in LPS-stimulated splenocytes from ELS mice showed a trend of reduction; however, the difference was not statistically significant. The IL-10 levels of splenocytes from the PS128-treated ELS group were significantly higher than those of the ELS mice (Fig. 6F). These results showed that PS128 modulated immune responses in ELS mice. However, PS128 treatment showed no clear effects on cytokine levels in

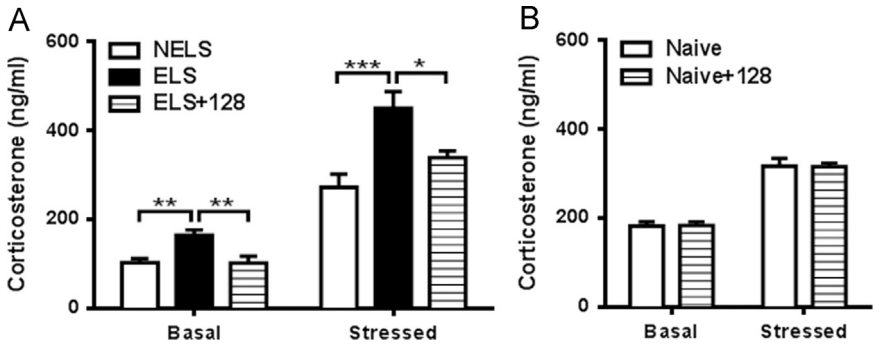

Fig. 5 – Chronic PS128 administration reduces corticosterone levels in ELS mice but not naïve mice. (A) ELS treatment significantly elevates blood corticosterone levels under both basal and stressed states. PS128 administration effectively reduces the corticosterone levels in the ELS mice to a level similar to that of NELS mice under both states. (B) PS128 administration has no effect on the corticosterone levels in naïve mice in both basal and stressed states. ELS, early life stress; ELS+128, ELS mice administered PS128; NELS, non-ELS; Naïve+128, naïve mice administered PS128. Data of basal or stressed state in the ELS mice experiments were analyzed separately by one-way ANOVA; data of basal or stressed state in the naïve mice experiments were analyzed separately by *t*-test. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

serum or stimulated splenocytes from naïve mice (data not shown).

2.4. *PS128 altered the serotonin and dopamine systems in both ELS and naïve adult mice*

Because ELS has been reported to increase serotonergic and dopaminergic activities in the PFC, and to increase dopaminergic activity in the striatum (Rentesi et al., 2013), we also investigated whether PS128 could alter the ELS-induced changes in the serotonin and dopamine systems. We analyzed levels of 5-HT, DA, and their metabolites in the PFC and striatum by HPLC-ECD. In the PFC, ELS mice showed significantly reduced 5-HT levels and had no change in their 5-HIAA concentration. As a result, the ratio of 5-HIAA to 5-HT was significantly increased in ELS mice compared to that of NELS mice, indicating an increase in serotonergic activity (Table 1). Chronic PS128 ingestion did not increase the reduced 5-HT in ELS mice, but instead reduced the 5-HIAA concentration and consequently reduced the ratio of 5-HIAA to 5-HT to the level similar to that of NELS mice (Table 1). However, in the naïve adult mice PS128 increased 5-HT concentration and decreased both the 5-HIAA concentration and the ratio of 5-HIAA to 5-HT (Table 2). The serotonin system in the striatum was not significantly different in ELS, NELS, PS128-treated ELS mice, or naïve mice (Table 1).

In the dopaminergic system, the DA concentration of PFC tended to decrease in ELS mice compared with NELS mice; however, this difference was not statistically significant (Table 1). The concentration of DA metabolite DOPAC was not changed in ELS mice compared to NELS mice (Table 1). However, the ratio of DOPAC to DA was significantly higher than that of the NELS mice (Table 1). Although the level of HVA was significantly reduced in ELS mice, the HVA-to-DA ratio was not significantly different from that of the NELS mice (Table 1). Following chronic PS128 ingestion, the treated ELS mice had a significantly elevated DA level compared to untreated ELS mice (Table 1). The DOPAC and HVA concentrations of PS128-treated ELS mice were also significantly higher than those of the untreated ELS mice. As a result, the ratio of DOPAC to DA and HVA to DA were both significantly lower than that of the untreated ELS mice (Table 1). In particular, the ratio of HVA to DA in PS128-treated mice was even lower than that of the NELS mice (Table 1).

In naïve adult mice, PS128 treatment significantly increased the concentration of DA, DOPAC, and HVA (Table 2). However, the DA turnover rate was not different from that of the saline-treated mice (Table 2). In addition, there was no change in the dopaminergic activity in the striatum in ELS, PS128-treated ELS, and naïve mice groups (Tables 1 and 2).

3. Discussion

In the present study we demonstrated the psychotropic effects of PS128 on ELS and naïve adult mice. We used MS as the adverse event to create ELS. MS is a well-established paradigm used in rat models of ELS and has been shown to result in prolonged and consistent dysfunctions in the gut–brain-axis (O'Mahony et al., 2011). Hence, it proved to be a useful platform for screening psychobiotics (Dinan et al., 2013). For that reason, we had adapted the rat MS procedure with some modifications because our mice had ELS. It has been reviewed that MS in mice is difficult to work with because it produces unreliable phenotypes that may be caused by either different stressor protocols or mice strains (Millstein and Holmes, 2007; Savignac et al., 2011). However, we have consistently found that mice that underwent our modified MS procedure developed reliable abnormal behaviors (Fig. 1). In addition to behavioral changes, we also found dysfunctions in the HPA axis, immune response, and neurotransmitters in the ELS mice. These findings suggest that the modified MS procedure could be used in mice for understanding the adverse effects of ELS and for screening psychobiotis.

Our ELS mice showed both anxiety-like and depression-like phenotypes because they had reduced center time in OFT, increased closed arm time in EPM, increased immobile time in the FST, and reduced preference for sucrose (Figs. 1–4). Most

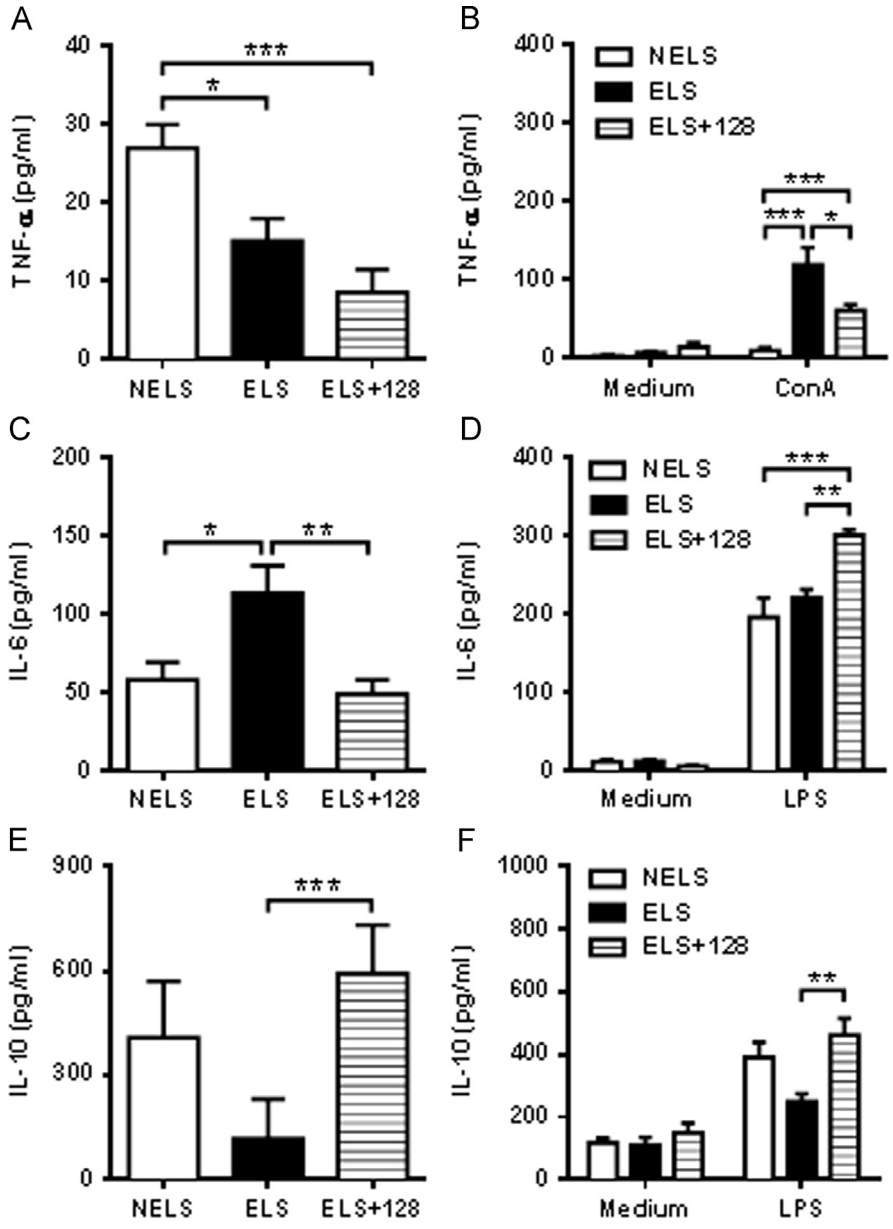

Fig. 6 – Effects of PS128 administration on serum cytokines and cytokines released from stimulated splenocytes from ELS and naïve mice. (A) ELS treatment reduces serum TNF-α concentration, and PS128 administration has no effect on the TNF-α level of ELS mice. (B) Serum IL-6 level is significantly elevated in ELS mice, and chronic PS128 administration reversed this elevation. (C) Chronic PS128 administration increases serum levels of anti-inflammatory cytokine IL-10 in ELS mice. (D) ConA stimulation significantly induced TNF-α release in cultured splenocytes from ELS mice. Splenocytes from PS128-treated ELS mice show a significant TNF-α release. (E) IL-6 release is significantly elevated in splenocytes from PS128-treated ELS mice following LPS stimulation. (F) Splenocytes from ELS mice show reduced IL-10 release following LPS stimulation, whereas the IL-10 release in splenocytes from PS128-treated ELS mice is similar to that of NELS mice. ConA, concanavalin A; LPS, lipopolysaccharide; ELS, early life stress; ELS+128, ELS mice administered PS128; NELS, non-ELS. Data of serum cytokine levels in the ELS mice experiments were analyzed by one-way ANOVA; data of medium or ConA/LPS stimulated splenocyte experiments were analyzed separately by one-way ANOVA. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

studies using MS show a single trait consisting of either anxiety-like or depression-like behavior (as reviewed in Moloney et al., 2014). In addition, the serum corticosterone levels in our ELS mice were significantly higher in both basal and stressed states (Fig. 5). However, most of the MS animals in previous studies showed increased corticosterone levels only during the stressed state (Rees et al., 2006). These results suggest that our ELS procedure produces a more robust behavioral phenotype with severely dysregulated stress response.

In our ELS mice we found a significant increase in serum IL-6 levels accompanied by decreased serum IL-10 levels (Fig. 6). These changes have not been previously described in MS rats (Desbonnet et al., 2010; O'Mahony et al., 2009).

Table 1 – Alteration of 5-HT and DA neurochemicals in brain regions of ELS mice.

	Prefrontal cortex			Hippocampus			Striatum		
	NELS	ELS	ELS+PS128	NELS	ELS	ELS+PS128	NELS	ELS	ELS+PS128
Monoamines and metabolites									
5-HT	88.5±45	42.3±14***	50.1±30*	69.7±4.7	52.7±	57.6±24	564±109	603±156	654±120
5-HIAA	21.3±7.2	25.8±9.0	12.5±4.7**,###	62.5±21	51.9±18	39.7±14	77.1±43	98.7±50	88.8±57
DA	168±114	66.1±33**	190±133##	36.0±14	29.8±24	33.0±12	4069±1176	3931±938	3999±939
DOPAC	50.4±11	59.9±29	71.2±28*	46.3±21	36.2±9.0	34.8±14	417±95	466±224	333±65
HVA	158±51	129±46	167±71	72.7±22	57.4±10	59.0±39	459±225	687±242	519±104
Turnover ratio									
5-HIAA:5-HT	0.301±0.38	0.615±0.29**	0.229±0.12###	0.930±0.29	1.08±0.55	0.734±0.42	0.0924±0.020	0.180±0.13	0.130±0.058
DOPAC:DA	0.468±0.28	0.830±0.36**	0.416±0.18###	1.06±0.51	1.04±0.36	0.930±0.29	0.0923±0.0069	0.123±0.064	0.0858±0.019
HVA:DA	1.60±1.1	2.31±1.2	0.977±0.72##	1.81±0.71	1.68±0.54	1.45±0.35	0.161±0.016	0.179±0.057	0.133±0.028

Concentrations of monoamines, metabolites and turnovers (ng/g wet tissue) are expressed as mean±SEM. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HT, serotonin or 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid. Data were analyzed by one-way ANOVA. Statistically significant values are highlighted in gray.
* $p<0.05$;
** $p<0.01$;
*** $p<0.001$ vs. NELS;
$p<0.01$;
$p<0.001$ vs. ELS.

Table 2 – Alterations of 5-HT and DA neurochemicals in brain regions of normal adult mice.

	Prefrontal cortex		Hippocampus		Striatum	
	Saline	PS128	Saline	PS128	Saline	PS128
Monoamines and metabolites						
5-HT	47.8±19	95.6±35**	78.4±18	99.6±48	829±146	868±217
5-HIAA	25.3±16	17.6±6.3	N/D	N/D	65.3±73	39.3±50
DA	142±66	301±157*	50.2±20	62.8±49	8080±1070	7364±1883
DOPAC	95.8±39	121±51	57.0±12	65.0±18	316±48	351±89
HVA	123±37	147±42	47.1±7.1	54.6±20	521±76	469±93
Turnover ratio						
5-HIAA:5-HT	0.638±0.64	0.160±0.037	N/D	N/D	0.0770±0.040	0.0780±0.044
DOPAC:DA	0.692±0.34	0.540±0.22	1.31±0.60	1.47±0.67	0.0396±0.0077	0.0457±0.017
HVA:DA	0.823±0.38	0.679±0.28	1.07±0.40	0.981±0.53	0.0647±0.0084	0.066±10.0051

Concentrations of monoamines, metabolites and turnovers (ng/g wet tissue) are expressed as mean±SEM. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine or serotonin. Data were analyzed by *t*-test. Statistically significant values are highlighted in gray. N/D, not detected.
* $p<0.05$;
** $p<0.01$ vs. Saline.

Elevation of serum IL-6 is associated with stress-related disorders in humans who had early adverse experiences during childhood (Coelho et al., 2014). It has been shown that adults with childhood maltreatment and patients with depressive disorders have increased serum IL-6 levels (Carpenter et al., 2010; Pace et al., 2006). In addition, a clinical study has demonstrated a higher ratio of serum IL-6 to IL-10 and a lower serum IL-10 level in patients with major depression compared to patients without major depression (Dhabhar et al., 2009). Therefore, our data suggest that the ELS in mice could induce an immune alternation that is similar to changes found in clinical studies. Furthermore, the HPA axis is also known to directly interact with the immune system (Moloney et al., 2014). Increased secretion of circulating glucocorticoids, including corticosterone, could inhibit both innate and adaptive immune responses (Sorrells and Sapolsky, 2007). Thus, there may be a complex cross-regulation between the HPA axis and the immune system, and this cross-regulation may lead to the modulation of animal behavior. Sustained elevation of corticosterone might also result in the reduced serum TNF-α observed in the ELS mice (Fig. 6A).

In addition to these findings, concentrations of neurotransmitters DA and 5-HT were significantly reduced in the PFC of ELS mice, but there was an increase in the turnover rate of both transmitters (Tables 1 and 2). The increased DA

and 5-HT turnover rate has also been reported in the maternal deprivation model in rats (Rentesi et al., 2013). The serotonergic system has long been linked to anxiety, and insufficient 5-HT concentration may lead to hyperexcitability in the amygdala, a condition that is associated with adulthood anxiety (Etkin and Wager, 2007). Thus, decreased 5-HT levels in the PFC may result in dysregulated excitability in the amygdala and consequently produce anxiety-like behaviors in ELS mice (Fig. 3). Taken together, our ELS procedure produced mice with multiple deficits, including abnormal behaviors, HPA dysregulation, immune alternation, and changes in neurochemicals in the PFC.

Following the daily gavage of PS128 for 4 weeks, we found that chronic administration of PS128 reduced the ELS-induced depression-like behaviors, normalized the HPA axis and immune systems, and modulated the changes in the DA and 5-HT system in the PFC. From previous studies, ELS is known to modulate gut microbiota and disrupt the integrity of the gut barrier, thus resulting in increased inflammation and abnormal behaviors (O'Mahony et al., 2011), both of which can be restored by the ingestion of beneficial bacteria. Reports have shown that consumption of a commercially available probiotic combination of *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 increases the amount of gut lactobacilli, reduces permeability in the colon, and reduces the elevated serum corticosterone induced by MS in rat pups (Gareau et al., 2007). A recent study also shows that administration of the commensal bacterium *Bacteroides fragilis* improves gut barrier integrity and normalizes anxiety-like behaviors in a mouse model known to display features of autism spectrum disorder (Hsiao et al., 2013). Also, administration of *Lactobacillus farciminis* has been shown to attenuate gut hyperpermeability and the abnormal activation of the HPA axis induced by psychological stress (Ait-Belgnaoui et al., 2012). These results suggest that beneficial bacteria in the gut contribute to the improvement of the stress response. Thus, it is possible that PS128 normalizes the MS-induced dysregulation of the HPA axis via modulating the gut microbiota and strengthening the gut barrier. We did not directly examine the gut integrity of PS128-treated mice; however, judging by the lack of effect on levels of corticosterone in PS128-treated naïve mice, which would have normal gut microbiota and gut barrier functions, it is likely that the effect of PS128 on the HPA axis of ELS mice involves the improvement of gut integrity.

Chronic treatment of PS128 also alleviated the increased serum IL-6 concentration in ELS mice. The serum concentration of IL-6 is positively correlated with depression severity in patients (Pace et al., 2006). Preclinical animal studies have shown that reduction of IL-6 is associated with reducing depression-like behaviors. For example, previous studies show that the administration of *Bifidobacterium infantis* 35624 to MS rats attenuated IL-6 release and reduced immobility in FST (Desbonnet et al., 2010). Similarly, we found that chronic PS128 administration also reduced serum IL-6 and improved depression-like behaviors in ELS mice (Figs. 4A and 6B). We also found that administration of PS128 significantly increases IL-6 production in LPS-stimulated splenocytes, suggesting PS128 treatment could increase the function of the immune system against foreign pathogens (Fig. 6E).

The effects of PS128 on ELS mice are clearly different from that of the naïve mice. We found PS128 could reduce anxiety-like behaviors in naïve mice but not ELS mice (Fig. 3). However, the depression-like behaviors were reduced in PS128-treated ELS mice but not naïve mice (Fig. 4). In addition, PS128 treatment reduced serum corticosterone levels under both basal and stressed conditions in ELS mice but had no effect on naïve mice (Fig. 5). Furthermore, PS128 treatment increased the 5-HT concentration in PFC only in naïve mice (Table 2). Because the corticosterone response of the HPA axis in naïve mice was not changed, the reduction of anxiety-like behaviors in naïve mice cannot be explained by regulating the HPA axis response. Instead, it might be related to the increased 5-HT level in the PFC for the reason mentioned previously, that is, that 5-HT could modulate the activity of amygdala, which is associated with anxiety-like behavior (Etkin and Wager, 2007). However, in ELS mice the reduced 5-HT concentration in PFC cannot be reversed by administration of PS128, and we did not see a reduction in anxiety-like behavior (Fig. 3 and Table 1).

Administration of PS128 increased locomotor activity in both ELS mice and naïve mice. Previous studies have shown that the administration of DA or its analog into specific brain regions such as the striatum or the nucleus accumbens stimulates locomotion in animals (Mabrouk et al., 2014; Woodruff et al., 1976). Because we did not observe changes in the striatal dopaminergic activity (data not shown), this locomotor enhancement probably did not involve the striatum. However, a previous study has shown that the administration of *Bifidobacterium infantis* 35624 or citalopram to MS rats modulated the level of noradrenaline in the brain stem and normalized depression-like behavior (Desbonnet et al., 2010). Changes in the noradrenaline system could also potentially modulate the locomotor activity and should be further explored in future studies of PS128.

The most interesting finding in this study is that PS128 significantly reduced the hyperactive HPA axis response and the depression-like behaviors in ELS mice (Figs. 1, 4 and 5). We also found an increased DA concentration and DA turnover rate in the PFC of PS128-treated ELS mice (Table 1). PFC is known to be a highly evolved brain region that is responsible for regulating working memory, decision-making, and emotions, including stress response (Shansky and Lipps, 2013). Normal PFC function, especially regarding the reward/avoidance emotional responses, requires an adequate DA concentration (Arnsten, 2009). In addition, the mesocortical DA system may have a direct influence on the HPA axis (Feenstra et al., 1992). Thus, the hyperactive HPA axis and depression-like behaviors seen in ELS mice might be directly associated with their altered DA function in the PFC (Table 1). Interestingly, following PS128 administration, the DA system of ELS mice was restored to the control level, and so were the HPA axis responses and depression-like behaviors (Figs. 4, 5 and Table 1). Hence, PS128 might suppress the exaggerated release of corticosterone and normalized depression-like behavior through increasing dopaminergic activity in the PFC.

In summary, we assessed the psychotropic effects of PS128 in both ELS and naïve adult mice in an attempt to identify novel psychobiotic strains. We demonstrate that PS128 could normalize ELS-induced HPA axis hyperactivity

and depression-like behaviors, and that in naïve mice it reduced anxiety-like behaviors. Some of the ELS-induced serum cytokine and PFC neurochemical changes are also restored by chronic PS128 administration. The psychotropic effects of PS128 on ELS and naïve adult mice suggest great potential for PS128 to be used to improve affective behaviors under both normal and diseased conditions. In addition, to our knowledge, PS128 is the first psychobiotic that increases locomotor activity and modulates both serotonergic and dopaminergic systems, which further expands its possible application in the treatment of psychiatric and neurological disorders.

4. Experimental procedure

4.1. *Preparation of PS128*

PS128 was isolated from fermented mustard greens, which is a traditional Hakka ethnic food product. It was identified as a novel strain by phylogenetic classification of its 16S rDNA sequence. The isolated PS128 was inoculated in Man Rogosa Sharpe broth (MRS; BD Difco, Becton-Dickinson, Sparks, MD MD, USA), cultured at 37 °C for 18 h, and then harvested by centrifugation at $6000 \times g$ for 10 min. The pellet was re-suspended in MRS plus 12.5% glycerol to a final concentration of 5×10^9 colony-forming units per milliliter. The re-suspended solution was then aliquoted in freezer tubes and stored at −20 °C until use. When in use the aliquot was pre-warmed to 37 °C for 1 h and re-suspended in saline before being administered to mice.

4.2. *Animals and housing*

Timed-pregnant female and naïve adult male C57BL/6J mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). On arrival, the mice were accommodated in the specific pathogen-free room at the Laboratory Animal Center of National Yang-Ming University. The room was kept at 22±2 °C, 50–60% humidity, and under 12 h light/dark cycle. The mice were provided with water and chow *ad libitum* (LabDiet Autoclavable Rodent Diet 5010; PMI Nutrition International, Brentwood, MO, USA). After 1 week of acclimation the experiments were started. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, National Yang-Ming University (IACUC No. 1001102).

4.3. *Maternal separation stress*

We adapted MS procedures from a rat model of ELS described previously and made some modifications (Desbonnet et al., 2010). Briefly, between postnatal day (PD) 2 and PD 14, male and female neonates in the stress group were separated from their mothers and littermates and placed in a small glass bottle (5 cm in diameter) for 3 h per day (11:00–14:00) at room temperature without a heating pad (Supplementary Fig 1). The no MS group was left with their mother undisturbed. For the next 2 weeks, all the mice were left undisturbed except for the routine bedding change and were weaned at PD 28. On PD 29, only males pups were selected and were randomly assigned to the ELS experimental group (ELS+PS128, $n=10$) and ELS control group (ELS, $n=12$). Mice without MS were regarded as the no ELS control group (NELS; $n=10$).

4.4. *Experimental design and sample collection*

To evaluate the psychotropic effects of PS128, we administered PS128 to the ELS experimental group and to a separate naïve adult male group (naïve). The ELS and naïve experimental groups were given saline re-suspended PS128 daily (10^9 CFU/mouse/day) by gavage for 4 weeks from PD 29 and from 8 weeks old, respectively, whereas the ELS and naïve control groups were given saline by gavage during the same period. At the end of the PS128 treatment period, when ELS group mice were 8 weeks old and naïve group mice were 12 weeks old, the mice underwent a battery of behavioral tests. The tests were given in sequence from the least stressful to the most stressful in the following order: sucrose preference test (SPT); open field test (OFT); elevated plus maze (EPM); and forced swimming test (FST). After the 4-day SPT the mice were subjected to OFT on the next day. EPM test were conducted immediately after OFT on the same test day. On the next day the first session of FST began. All the tests were conducted during the light phase.

On the last day of FST, before the swim start, the mice were subjected to orbital sinus blood collection to obtain the basal state samples. Then, the mice underwent a 6-min forced swim and were returned to their home cage. After 30 min, blood samples were collected again to obtain the stressed state samples. All blood sampling was conducted within the same 3-h period in an effort to minimize the effect of circadian rhythm on corticosterone release. The collected whole blood was centrifuged at $3000 \times g$ for 10 min at 4 °C, and then the serum was stored at −80 °C until use. Following blood collection, the mice were sacrificed by cervical dislocation. The brains were quickly removed and placed on dry ice. The ice-cold brain was dissected on a filter paper placed on a glass dish on ice. The prefrontal cortex (PFC), hippocampus, and striatum samples were dissected out and immediately preserved in ice-cold 0.6% perchloric acid and stored at −80 °C until use. Spleen tissues were also collected, temporarily stored in ice-cold RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA), and then processed in splenocyte experiments (see mitogen-stimulated splenocyte section).

4.5. *Sucrose preference test*

SPT was modified from a previous study by Yu et al. (2012). To habituate the mice to drinking sucrose-containing solution, on the first day of the test the mice were given two bottles of 1% sucrose solution (g/mL) at 18:00 for 24 h. On the second day, one of the sucrose bottles was replaced with tap water and left for 24 h. On the third day, the mice were water-deprived for 18 h from 18:00 until 12:00 next day. On the fourth day, immediately after water deprivation, the mice were given one bottle of 1% sucrose and one bottle of tap water for 5 h. The position of the bottles was switched to avoid location preference. The sucrose and water consumed during the 5-h test were measured. The sucrose preference (%) was calculated as follows: (weight of sucrose consumed/total weight of sucrose and water consumed) × 100.

4.6. Open field test

Locomotor activity of the mice was examined by the OFT. In this test the mouse was placed in the open field activity chamber for 10 min (Tru Scan Activity System; Coulbourn Instruments, Whitehall, PA, USA). The square activity chamber ($25.4 \times 25.4 \times 38$ cm^3) is made of Plexiglas walls with two photobeam sensor bars on each side. The box was cleaned with 70% ethanol after each test. The activities were automatically recorded and quantified with the Tru Scan 2.2 software (Coulbourn Instruments). The total distance traveled, moving time, and center distance and time were measured by the Tru Scan Activity System. The center area was defined as a region in the center measuring 12.5×12.5 cm^2.

4.7. Elevated plus maze test

The elevated plus maze is composed of two closed arms and two open arms (height, 45 cm; full arm length, 66 cm; arm width, 10 cm; wall height of closed arm, 30 cm). This test was used to assess anxiety-like behavior of the mice. The mouse was placed in the center arm crossing area (10×10 cm^2) and allowed free exploration in the maze for 10 min. The maze was cleaned with 70% ethanol after each test. The mouse activity was recorded by a video camera mounted on the ceiling of the maze center. The recorded activity was later analyzed by EthoVision video tracking software (Noldus Information Technology, Wageningen, the Netherlands). The total travel distance and duration spent in the open and closed arms were quantified.

4.8. Forced swimming test

The FST was used to assess the depression-like behaviors in the mice. Briefly, mice were put in a transparent acrylic cylinder (height, 30 cm; internal diameter, 10 cm) containing 15 cm water (23–25 °C) to swim for 6 min on the first day. After the swim, the mice were dried with tissue paper and returned to their home cage. The next day, the mice were given a second swim session for 5 min. Only naïve adult mice were subjected to the first and second sessions of FST. Mice in the ELS experiments underwent only the first day session. The swim sessions were recorded by a video camera and the behavior was analyzed by EthoVision video tracking software. The immobile time were quantified from the first day session of ELS groups and from the second day session of naïve adult mice.

4.9. Serum corticosterone

The serum was diluted first, and then we used a commercial CORT EIA kit (Cayman Chemical, Michigan, MI, USA) to analyze corticosterone concentration. The corticosterone concentration was interpolated using the standards provided in the kit following the manufacturer's instructions.

4.10. Serum cytokine

We measured cytokine levels in the serum samples collected during the baseline period and in the culture medium of stimulated splenocytes. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 were measured using commercial ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

4.11. Mitogen-stimulated splenocytes

Spleen preserved in ice-cold RPMI 1640 medium was first squashed by a sterile piston and then filtered through a sieve mesh cell strainer (Becton, Dickinson and Company, East Rutherford, NJ, USA) to isolate single cells. The preparation was centrifuged at 1000 rpm for 5 min. The pellet was collected and treated with lysis buffer for 5 min to remove erythrocyte. Then, the preparation was centrifuged again to remove lysis buffer and washed in serum-free RPMI 1640 medium. After the final centrifugation to remove serum-free medium, the splenocyte pellet was re-suspended in RPMI 1640 medium plus 10% fetal bovine serum and seeded at 2×10^6 cells/200 μL/well in 96-well flat-bottom plates. The stimulated splenocytes were cultured with mitogen concanavalin A (ConA; 4 μg/mL) or lipopolysaccharide (LPS; 0.6 μg/mL) in the culture medium for 48 h at 37 °C (5% CO_2). The control splenocytes were cultured for the same period of time without mitogens.

4.12. Quantification of monoamines and metabolites

The frozen PFCs, hippocampus, and striata were thawed and homogenized with a micro-sonicator (Q125 sonicator; Qsonica, Newton, CT, USA). The homogenized samples were then centrifuged at $12{,}000 \times g$ for 10 min at 4 °C. The supernatants were filtered through a 0.22-mm polyvinylidene difluoride membrane (4-mm syringe filter; Millex-GV; Millipore, Billerica, MA, USA). After proper dilution we took 20-μl samples to measure the concentrations of monoamines and their metabolites by the high-performance liquid chromatography-electrochemical detection system (HPLC-ECD). The HPLC-ECD comprised a micropump (CMA-100; CMA, Stockholm, Sweden), an online injector (CMA-160), a Microtech LC-pump (Microtech Scientific, Sunnyvale, CA, USA), a BAS-4C electrochemical detector (Bioanalytical Systems, Inc., West Lafeyette, IN, USA), and a reversed-phase column (Kinetex C_{18}, 2.6 μm, 100×2.1 mm I.D.; Phenomenex, Torrance, CA, USA) as previously described (Cheng et al., 2000). The potential for the glassy carbon working electrode was set at +650 mV with respect to an Ag/AgCl reference electrode at room temperature (25 °C). The mobile phase containing 0.1 M NaH_2PO_4, 8% methanol, 0.74 mM SOS (1-octanesulfonic acid, sodium salt), 0.03 mM EDTA, and 2 mM KCl was adjusted to pH 3.74 with H_3PO_4. Diluted filtrates were then injected (20 μL) into the chromatographic system at a flow rate of 0.2 mL/min. Concentrations of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) in the samples were interpolated using standards (Sigma-Aldrich, St. Louis, MO, USA) ranging from 1 to 100 ng/mL.

4.13. Statistical analysis

All data were expressed as mean ± SEM. Differences between groups were analyzed by one-way or two-way analysis of variance (ANOVA) with Bonferroni's post-test or two-tailed *t*-test when appropriate.

Contributions

WSL, YCJ, and YCT designed the study. WSL, YCJ, YCW, and HPT performed the experiments. WSL, SW, and YCJ analyzed and interpreted the data. YWL, SW, WSL, CCW, YCJ, and YCT wrote the manuscript.

Disclosure

The authors have declared no conflict of interest related to this study.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2015.11.018.

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