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学位論文（要約）

**Postnatal stress exposure infers depressive-like behavior  
and morphological changes in rats**

（ラット幼若期ストレスはうつ様行動と形態学的変化  
を引き起こす）

2015年3月

北海道大学

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## **Table of Contents**

<b>List of publications and presentations .....</b>	<b>1</b>
<b>General Introduction.....</b>	<b>2</b>
<b>Chapter 1</b>	
<b>Postnatal stress exposure induces depressive-like behavior.....</b>	<b>9</b>
<b>Chapter 2</b>	
<b>Effect of postnatal stress exposure on raphe nuclei tryptophan hydroxylase 2 (TPH2) and Glutamate Decarboxylase 67 (GAD67).....</b>	<b>24</b>
<b>General Discussion.....</b>	<b>37</b>

## **List of publications and presentations**

### **List of Presentations:**

1. Lyttle, K., Ohmura, Y., Izumi, T., Yoshida, T., Yoshioka, M. Adult Rats Subjected to Postnatal Stress Exposure are more likely to Exhibit Behavioral Despair. 26<sup>th</sup> Meeting of the Hokkaido Pharmacological Drug Action. July 28, 2012, Sapporo, Japan.
2. Lyttle, K., Ohmura, Y., Izumi, T., Yoshida, T., Yoshioka, M. Adult Rats Subjected to Postnatal Stress Exposure are More Likely to Exhibit Behavioral Dysfunction. 19<sup>th</sup> Meeting of Young Researchers in Neurobehavioral Pharmacology. March 20, 2013, Fukuoka, Japan.

## General Introduction

Depressive disorders are characterized as a complex, heterogeneous disorder characterized by protracted depressed mood, anhedonia and changes in psychomotor, sleeping and eating patterns, inability to concentrate, lack of energy, feelings of worthlessness or guilt, and thoughts of suicide<sup>1</sup>. Depressive disorders are highly prevalent and present a significant economic burden<sup>2,3</sup> as well as one of the leading causes of morbidity worldwide<sup>4</sup>. While significant steps have been made in identifying the neural mechanisms involved in depression, the low therapeutic response rate in depressed patients is an indicator of the need for further clarification of the pathogenesis of depression.

The early life environment has an important and distinctive influence on adult psychiatry with adverse early environments resulting in enhanced susceptibility to a range of psychiatric conditions including depressive and impulsive disorders, schizophrenia, and autism spectrum disorders<sup>5</sup>. The modulatory effects of the early environment on adult psychiatry are thought to be mediated through the disruption of neurotransmitters systems at time-specific critical points in development. The monoamine 5-hydroxytryptamine (5HT, serotonin) is of particular interest due to its wide range of influence on brain functions including neurogenesis, dendritic elaboration and cortical organization and modulatory effect on other neurotransmitter systems. The neurotransmitter is involved in the regulation of stress responsivity with altered serotonergic signalling a common biomarker of stress – related disorders including anxiety disorders and depressive mood disorders<sup>6</sup>.

When modelling psychiatric diseases in animals one is faced with two problems. The first concerns the mechanistics behind the induction of the symptoms of a psychiatric disease in a rodent and the second concerns detection of these symptoms. Although, several useful and informative models of early-life stress have been previously developed many of these models employ a period of separation from the mother, termed maternal separation. These models exploit the critical role of maternal influence on the activation of the molecular stress-mediators in the immature rat. However maternal separation models fail to reproduce the early-life psychological stress caused by abnormal interactions with the environment, such as is observed in cases of abuse. Here I employed the use of a model of postnatal stress exposure in juvenile rats to investigate the presentation of depressive-like behavior at the adult stage in animals exposed to

an early adverse stressor and to identify the neural mechanisms involved. Juvenile stress exposure was carried out at postnatal week three and will thus be referred to simultaneously as juvenile stress or three week footshock stress.

## **Rodent Correlates of Aspects of Depression**

### *Depressive-like behavior*

In rats, the forced swim test (FST) is a measure of behavioural despair/depressive-like behavior based on Porsolt's (1978)<sup>7</sup> observation of immobility following an initial period of escape-directed behaviour in rodents when placed in an inescapable container of water. The rat FST involves 2 exposures: a 15 min swim exposure on the first day which is termed an 'induction phase' in which the animal learns that escape is futile, the second day represents the test day characterized by increased immobility, decreased swimming and less attempts to escape seen as the measure of behavioural despair. The FST is a widely used test because of the ease of use as well as the reliability in identifying antidepressant activity of agents. False positives or negatives are easily excluded by the use of additional tests which assess changes in locomotor activity.

### *Anhedonia*

Anhedonia, the decreased capacity to experience pleasure, is a central feature of unipolar major depressive disorder<sup>1</sup>. The use of the term "anhedonia" in clinical psychiatry may be traced to over a century ago when Ribot, 1897<sup>8</sup>, first defined anhedonia as the "insensibility relating to pleasure alone" to distinguish it from analgesia, the inability to experience pain, and highlighted the role of anhedonia in the diagnosis of melancholia. As anhedonia is not specific to depression and is often observed across a spectrum of psychiatric illnesses, clinical reviews have focused on the relevance of anhedonia to depression. Two types of depression have been proposed: reactive (i.e., 'neurotic,' 'exogenous,' or 'atypical' depression) and endogenomorphic (i.e., 'classic,' 'endogenous,' or 'melancholic') depression. Anhedonia has been identified as a characteristic of the more severe endogenomorphic depression<sup>9</sup>. In rodent models, anhedonia may be assessed by 3 protocols: the sucrose preference test<sup>10</sup>, the cookie test<sup>11</sup> and the intracranial self-stimulation test<sup>12</sup>. Of the three paradigms, the sucrose preference test is long-standing, flexible and easy to implement. Intriguingly, rodents display hedonic reactions immediately after consuming sucrose that closely resemble responses made by humans and primates<sup>13, 14</sup>.

## **Sucrose preference test**

The sucrose preference test is a measure of anhedonia based on the observation that rats subjected to unpredictable chronic stress fail to show preference for a saccharin or sucrose solution<sup>15</sup>. This anhedonic effect is reversed by antidepressant treatment. The procedures used here are based on Willner's<sup>10</sup> method and involves a habituation period of 24 hrs in which animals are provided with 2 bottles of 1% sucrose solution (habituation). Animals are next provided with 1 bottle containing 200 ml of tap water & 1 bottle containing 200 ml of 1% sucrose solution and baseline measurements taken for 3 consecutive 24 hr periods with replenishment of tap water and sucrose solutions between measurements.

## **Treatment of depressive disorders**

Several therapeutic approaches for the treatment of depressive disorders exist. These include cognitive psychotherapies, deep brain stimulation (DBS) and pharmacotherapeutic approaches. Of the 3, pharmacotherapeutic approach has the advantage of being less invasive with a faster acting effect than that of cognitive psychotherapies. It involves the use of drugs to target the anatomical/morphological changes observed in depression. However, unlike DBS the effect is less specific with whole body responses to the effect of drugs.

Pharmacological treatment of depression is based on the antidepressant agents which increase monoamine levels. Common antidepressant drugs fall in the class of serotonin reuptake inhibitors (SSRIs), atypical antidepressants, tricyclic antidepressants (TCAs) or monoamine oxidase inhibitors (MAOIs). TCAs and MAOIs represent the oldest classes of antidepressants with a broader mechanism of action which involves inhibition of reuptake of serotonin, norepinephrine and dopamine. As a consequence of the broad mechanisms of action an extensive side effect profile and severe adverse effects are observed in the prescription of these agents.

The SSRI class of drugs are popular due to reduced side effects but are considered to be of similar efficacy to TCAs. The aim of pharmacological treatment of depression is the reduction of symptoms and improvement in the patients' quality of life with minimal risk. The SSRIs present an alternative to the life-threatening adverse reactions observed with TCAs and MAOIs. However increased suicide rates have been observed following SSRI prescription particularly in the treatment of young adults and adolescents. Fluvoxamine is an SSRI with potent action at the

sigma-1 receptor which is thought to mediate its pro-cognitive effects<sup>16</sup>. Particularly in cases of young adult depression it is important to prevent cognitive deterioration and fluvoxamine may provide an alternative.

### **Determination of baseline FST parameters in the presence of a prodepressant factor**

To investigate the increased depressive-like behavior in rats exposed to juvenile stress in the form of exposure to foot shock stress beginning at postnatal week three (3wFS) rats, the prodepressant agent [5 $\alpha$ ,7 $\alpha$ ,8 $\beta$ ]-*N*-methyl- -[7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec8-yl]-benzenacetamide (U-69593) was used to determine FST behavioral parameters. While the monoaminergic systems are predominantly implicated in depressive mood disorders, the endogenous opioid systems have been identified to regulate emotional and perceptual experience<sup>17</sup>. The endogenous opioid system is made up of the dynorphin and kappa opioid receptor systems. The prodepressant effect of  $\kappa$ -opioid receptors is believed to be due to inhibition of neurotransmitter release from mesolimbic DA neurons<sup>18,19,20</sup>. U-69593 demonstrates high selectivity for the  $\kappa$ -opioid receptor. Using the  $\kappa$ -opioid agonist U-69593, baseline prodepressant FST parameters were established.

In the present study, I employed a model of juvenile stress exposure, heretofore referred to as postnatal stress exposure or three week foot shock exposure to investigate the presence of behavioral impairments, more specifically, depressive-like behaviour at the adult stage in rats previously exposed to an early life stressor. Following behavioral assessments in which depressive-like behaviour was observed in rats exposed to early adverse stress I investigated the mechanisms underlying the observed behavioural impairments by focusing on the serotonergic raphe nuclei and the prefrontal cortex, two areas which have been identified to play significant roles in behavioral modulation. Specifically, I semi-quantitatively assessed raphe nuclei protein levels of tryptophan hydroxylase 2, the enzyme responsible for serotonin biosynthesis. I next investigated prefrontal cortex morphology by analysing infralimbic and prelimbic spine densities where I observed reduced infralimbic spine densities in rats exposed to postnatal stress exposure. 14 day administration of the selective serotonin reuptake inhibitor fluvoxamine recovered the depressive-like behavior and reduced spine densities observed in rats subjected to juvenile stress.

## List of Abbreviations

GABA	$\gamma$ -amino butyric acid
3wFS	three week foot shock
5HIAA	5-hydroxyindoleacetic acid
5HT	5-hydroxytryptamine
5HT <sub>1A</sub>	5-hydroxytryptamine 1A
5-HTP	5-hydroxytryptophan
8-OH-DPAT	8-Hydroxy-2-(di-n-propylamino)tetralin
APA	American Psychiatric Association
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
CMS	chronic mild stress
CREB	cAMP response element-binding protein
DA	dopamine
DBS	deep brain stimulation
DRN	dorsal raphe nucleus
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders IV
DTT	dithreithiol
eGFP	Enhanced Green Fluorescent Protein
ERK ½	extracellular signal-regulated kinases 1 and 2
FLV	fluvoxamine
FS	foot shock

FST	forced swim test
FST1	forced swim test day 1
FST2	forced swim test day 2
GAD	glutamic acid decarboxylase
GAD65	glutamic acid decarboxylase 65
GAD67	glutamic acid decarboxylase 67
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GR	glucocorticoid receptor
IL	infralimbic
mPFC	medial prefrontal cortex
mRNA	messenger RNA
MAOI	monoamine oxidase inhibitor
MRN	median raphe nucleus
noFS	no foot shock
OFT	open field test
PFC	prefrontal cortex
PL	prelimbic
PND	postnatal day
PSB	protein solving buffer
PSB-TCEP	protein solving buffer- tris(2-carboxyethyl)phosphine
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulphate

SSRI	selective serotonin reuptake inhibitor
TBST	tris buffered saline
TCA	tricyclic antidepressant
U-69593	[5 $\alpha$ ,7 $\alpha$ ,8 $\beta$ ]-N-methyl- -[7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec-8-yl]-benzenacetamide

## **Chapter 1**

### **Postnatal stress exposure induces depressive-like behavior**

## Introduction

Major depressive disorder is a leading cause of disability worldwide, with a lifetime population prevalence as high as 20%<sup>21</sup>. Depression is a complex, heterogeneous disorder characterized by protracted depressed mood, anhedonia and changes in psychomotor, sleeping and eating patterns<sup>1</sup> and has been conventionally associated with disruption in monoamine neurotransmitter systems serotonin, norepinephrine, and dopamine. Research has proven this theory to be insufficient and has identified the role of non-monoaminergic neurotransmitter systems including GABA, glutamate, and peptide systems, BDNF, tropomyosin-related kinase B receptor, extracellular signal-regulated kinase, and Akt signalling pathways as well as the role of the hypothalamic-pituitary-adrenal (HPA) axis in depression, current pharmacotherapies are predominantly of the monoaminergic class. It has been observed that the monoaminergic agents represent the first step in the neural mechanisms involved in depressive disorders. Furthermore, the identification of other processes such as decreased neural plasticity (hippocampal neurogenesis, hippocampal and cortical synaptogenesis), altered glial function (eg, astrocyte deficit), and inflammation (excess of proinflammatory cytokines) in the pathogenesis of depression indicate an intricate and multi-layered process<sup>22</sup>.

The association between adverse life events and onset of major depression is well established in the literature<sup>23</sup>. In particular, the early environment has been identified as an important factor in the presentation of depressive disorder<sup>24</sup>, and epidemiological studies have identified strong associations between an early adverse exposure and an increased risk of depression<sup>25</sup>. Supporting evidence is provided by clinical studies which have identified a strong dose-response relationship between the number of experienced childhood adversities and the presentation of psychiatric conditions in adulthood<sup>26</sup>. It is purported that an adverse early environment may infer neural changes which confer a susceptibility to depressive disorders.

Animal models present a viable instrument in the identification of the pathogenetic mechanisms involved in psychiatric conditions. In the present study, I investigated the effect of an early adverse stress exposure on the presentation of depressive-like behavior. Rats were exposed to juvenile stress between postnatal days 21 – 25. Behavioral assessment of depressive-like behavior was performed between postnatal days 63 – 84. Anhedonia, one of the core symptoms of depression, was assessed using the sucrose preference test. The Porsolt FST was

used to assess depressive-like behavior. Baseline forced swim test measures were determined through the use of the prodepressant drug U-69593 following which depressive-like behavior assessment was performed in juvenile stressed rats.

## **Methods**

### **Animals**

Adult rats used in baseline experiments were Wistar/ST rats purchased from Nippon SLC. For juvenile stress experiments, rat pups were the offspring of timed-pregnant Wistar/ST rats bought from Nippon SLC. Animals were housed in a temperature controlled ( $21 \pm 2$  °C) animal room with lights on from 19:00 to 07:00 h. All testing was carried out in the dark period. Gender was determined on PND 10 and only male rats were used. Pups were weaned at postnatal day (PND) 21 and group-housed (4 – 5 per cage). Food and water were provided *ad libitum*. All procedures involving animals were carried out in accordance with the guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of the Hokkaido University.

### **Juvenile Stress**

Rat pups were divided into two groups, no foot shock exposure (noFS) and three week foot shock exposure (3wFS). Each pup was acclimated in the foot shock (FS) box for 5 mins and subjected to five shock stimuli (shock intensity, 0.5 mA; intershock interval, 30 sec; shock duration 2 sec). Each rat pup was held in the FS box 5 min after the last FS stimulation before being returned to the home cage. No-FS controls were exposed to the FS box for 12.5 min without FS stimuli. Pups were subjected to FS exposure for 5 continuous days. Behavioral experiments were carried out at the postadolescent period (10 - 14 weeks old). Animals were handled only during cage cleaning and drug administration.

### **Open Field Test**

#### *Effect of juvenile stress exposure on locomotor activity*

A 15 min open field test was carried out in a square chamber (90 cm long  $\times$  90 cm wide  $\times$  40 cm high) with illumination of 200 lux. Behavioral assessment began with a rat placed in the center of the chamber. Locomotor activity was assessed by measuring total distance travelled and the total number of crossings (crossings of the lines made by the division of the larger

chamber into 9 cm × 9 cm squares) dividing the open field chamber during a 15 min interval. LimeLight 2 software (Actimetrics USA) was used to score behavioral parameters. Analysis was divided in three time intervals to assess the time spent in 3 quadrants, centre, middle, periphery during the first, second and third 5 min intervals.

### **Prodepressant characterization of the FST - U69593 1 mg/kg i.p. administration**

U-69593 was dissolved in PBS-acetic acid solution and administered at a dosage of 1.0 mg/kg; 5 ml/kg dosage. U-69593 was intraperitoneally (i.p.) administered 1 hr before the second exposure to the FST. Control animals received the appropriate volume of PBS-acetic acid (i.p) vehicle 1 hr before the second exposure to the FST. 24 hr after FST1 rats were retested for 5 min (300 s) under identical swim conditions.

### **Forced swim test**

A cylindrical acrylic container (600 mm tall x 200 mm inner diameter) was filled to 450 mm with water ( $25 \pm 1$  °C). A videocamera placed above was used to capture data for subsequent offline analysis. Rats were subjected to a 15 min pre-test swim and re-exposed 24 hr later for a 5 min test<sup>7</sup>. After swimming, animals were dried with a towel and returned to the home cage. Forced swim test analysis was done by assessment of the occurring behavioral parameter at the end of each 5 sec interval. Behavioral parameters assessed included: (1) immobility, no additional activity other than required to keep the rat's head above water; (2) swimming, movement throughout the swim cylinder, including crossing quadrants of the cylinder; (3) climbing, upward directed movement of the forepaws, usually directed along the side of the swim cylinder<sup>27</sup>. Increased counts of immobile behavior is accepted as a depressive-like phenotype<sup>27</sup>.

### **Sucrose preference test**

Rats were singly housed for a period of 7 days prior to the start of sucrose preference testing. Rats were not deprived of food or water before or during the test. All sucrose preference testing was carried out over 24 hr intervals. Sucrose preference testing started with a habituation interval of 24 hrs. During the habituation period, rats were provided with 2 bottles of a 1% sucrose solution. Following habituation period 3 baseline measures of sucrose preference were taken. A 3 day baseline period was selected based on preliminary experiments. For assessment of baseline

sucrose preference, rats were provided with 200 ml of tap water and 200 ml of a 1% sucrose solution. Bottle positions were counterbalanced each successive day to prevent place preference. Following acquisition of stable baseline measurements (rats with sucrose preference less than 60% were removed from further experiments), sucrose preference was assessed following stress exposure. The consumption of water and sucrose solution was measured by weighing the bottles. Sucrose preference was calculated as a percentage of sucrose solution consumption divided by total consumption. Reduced sucrose solution consumption indicates an anhedonia-like state.

### **Statistical Analysis**

A Student's t test was used for analysis of most parameters. In the case of repeated measures analysis, a one way repeated measures ANOVA with the subsequent appropriate post hoc test was used. Probability values less than 5% was considered significant.

## Results

### Effect of U69593 on forced swim test behavior and locomotor activity

As shown in Figure 1, no significant differences were observed in locomotor activity parameters of total distance moved (Student's *t* test,  $P > 0.05$ ) nor number of crossings of the open field arena (Student's *t* test,  $P > 0.05$ ) between rats administered PBS-acetic acid vehicle and rats administered 1 mg/kg U69593. In the FST, 1 mg/kg U69593 resulted in significantly increased immobility, decreased swimming (both  $P < 0.01$ ) and climbing ( $P < 0.05$ ) in rats administered 1 mg/kg U69593, Figure 2.

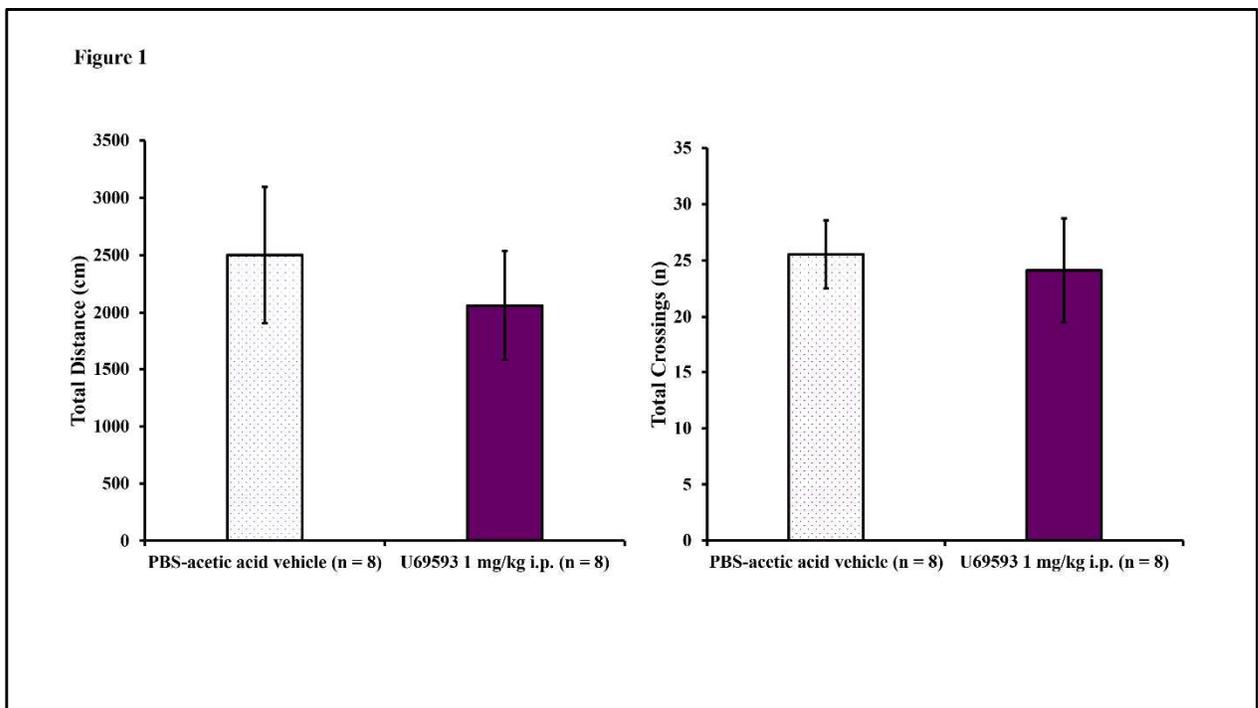


Figure 1. Mean counts of open field test behavioral parameters of total distance and total number of crossings. Data expressed as the mean  $\pm$  s.e.m. No significant differences in either total distance or total number of crossings were observed between the PBS-acetic acid (n = 8) and 1 mg/kg U69593 (n = 8) administered group as assessed by a Student's *t* test.

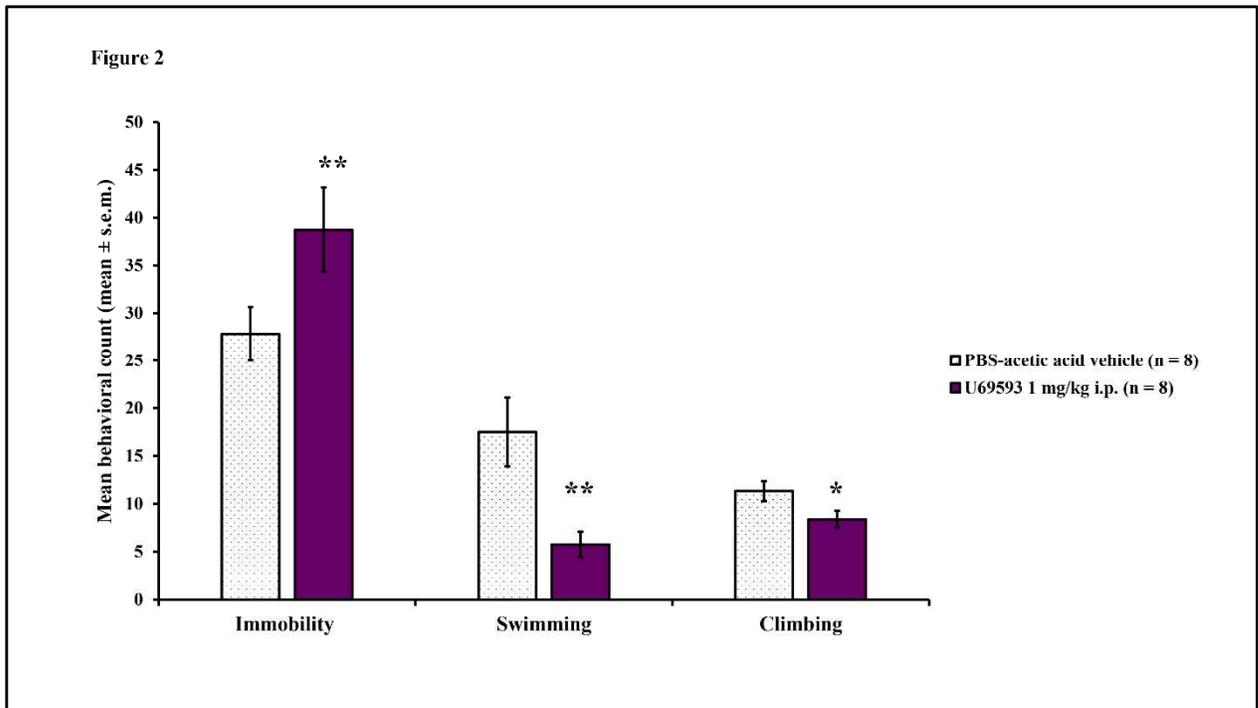


Figure 2. Mean counts of FST. behavioral measures of immobility, swimming, climbing and others assessed every 5 sec. of the 5 min FST. Data expressed as the mean ± s.e.m. \*:  $p < 0.05$  vs . PBS-acetic vehicle group; \*\*:  $p < 0.01$  vs. PBS-acetic vehicle group. On day 2 of the 5 min FST. Rats administered 1 mg/kg U-69593 displayed a significant increase in immobile behavior, reduced swimming and climbing behavior when compared to vehicle administered animals.

### Effect of postnatal stress on open field behavior

Figure 3 depicts OFT behavior in non-stressed and juvenile stressed rats exposed to foot shock stress at postnatal week three. A repeated measures ANOVA with time interval as the within subject factor and group as the between subjects factor identified a significant main effect of time on distance travelled,  $F_{(2, 32)} = 8.08$ ,  $P < 0.01$ . A significant difference was observed between time intervals 0 – 300 and 300 – 600; 0 – 300 and 600 – 900 seconds,  $P < 0.05$  for both, with the distance travelled successively reduced with each consecutive time interval. A significant interaction between stress group and time interval in the distance travelled  $F_{(2, 32)} = 6.34$ ,  $P < 0.01$ . Stress exposure did not have any significant main effect on distance per interval: 0-300 secs,  $F_{(1, 16)} = 2.34$ ,  $P > 0.05$ ; 300 – 600 secs,  $F_{(1, 16)} = 0.25$ ,  $P > 0.05$ ; 600 – 900 secs,  $F_{(1,$

$_{16}) = 0.70, P > 0.05$ . Bonferroni post hoc test did not identify any significant differences between the groups in any time interval.

A repeated measures ANOVA with time interval as the within subject factor and group as the between subjects factor identified a significant main effect of time on the number of crossings  $F_{(1.63, 32)} = 7.03, P < 0.01$  (Huyn-Feldt correction). There was a gradual reduction in the number of crossings with each successive time interval, 0 – 300 vs 300 – 600,  $P < 0.05$ ; 0 – 300 vs 600 – 900,  $P < 0.01$  indicative of acclimatization to the environment.

A significant interaction was observed between time interval and group  $F_{(1.63, 32)} = 4.37, P < 0.05$ . No significant differences were observed between noFS and 3wFS groups in the number of crossings for any time interval, 0 – 300,  $F_{(1, 16)} = 0.98$ ; 300 – 600,  $F_{(1, 16)} = 0.09$ , 600 – 900,  $F_{(1, 16)} = 1.28, P > 0.05$  for all time intervals.

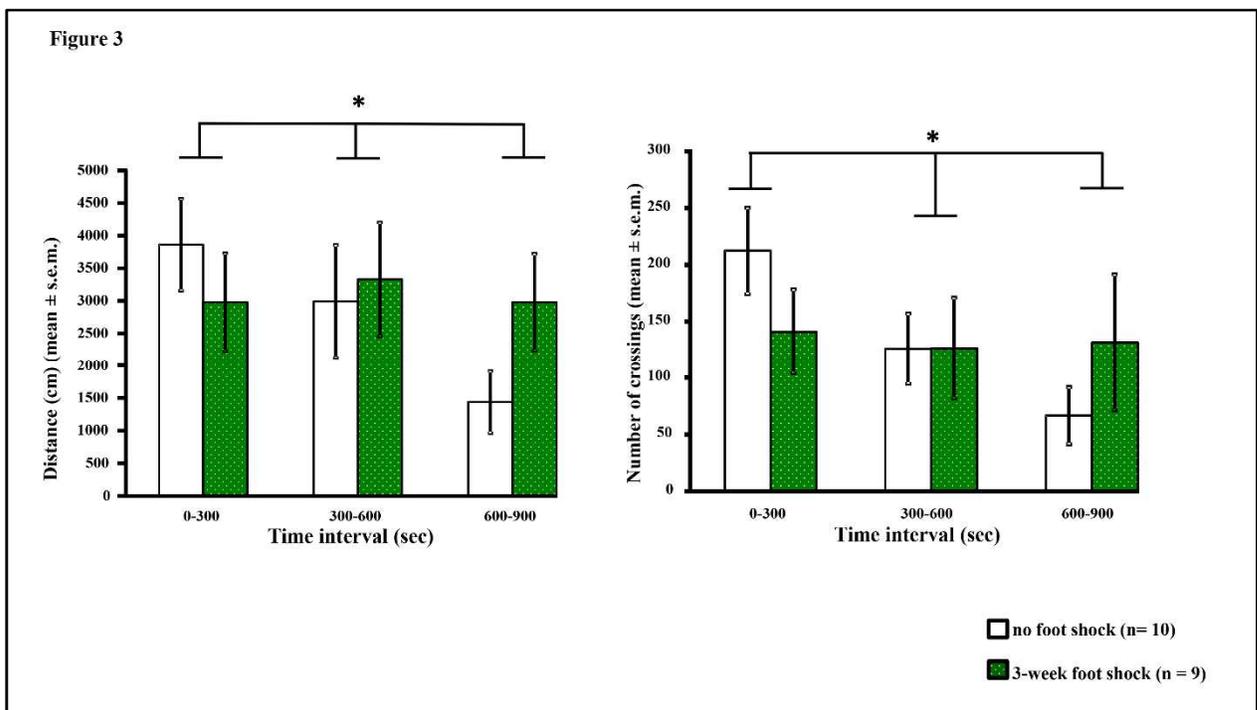


Figure 3. A repeated measures ANOVA with time interval as the within subject factor and group as the between subjects factor identified a significant main effect of time on distance travelled ( $F_{(2, 32)} = 8.08, P < 0.01$ ). A repeated measures ANOVA with time interval as the within subject factor and group as the between subjects factor identified a significant main effect of time on the number of crossings ( $F_{(1.63, 32)} = 7.03, P < 0.01$ ) (Huyn-Feldt correction).

There was a gradual reduction in the number of crossings with each successive time interval, 0 – 300 vs 300 – 600,  $P < 0.05$ ; 0 – 300 vs 600 – 900,  $P < 0.01$  indicative of acclimatization to the environment.

No significant differences in neither total distance nor total number of crossings was observed between noFS and 3wFS rats. (Figure 4).

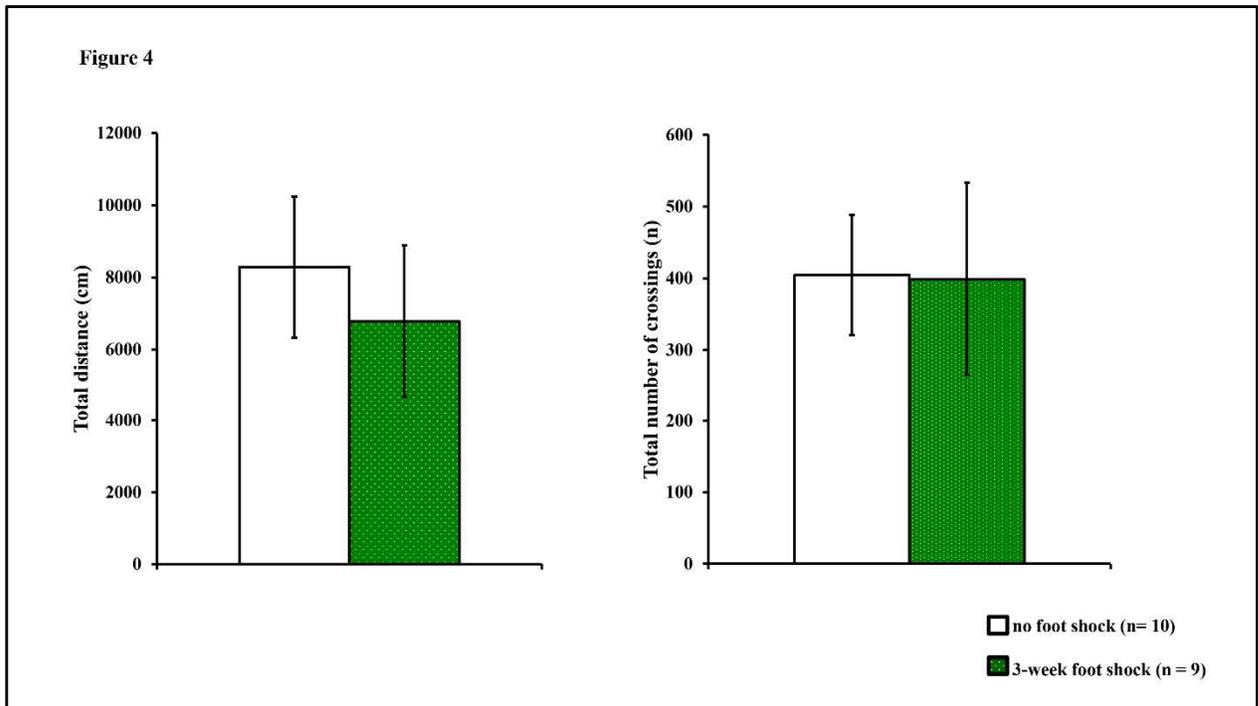


Figure 4. Open field parameters of total distance and total number of crossings in no foot shock and three week footshock rats. Data represented as the mean  $\pm$  s.e.m. A Student's t test identified no significant differences between the groups in neither total distance nor total number of crossings ( $P > 0.05$ ).

### Effect of postnatal stress exposure on depressive-like behavior

As shown in Figure 5, rats exposed to juvenile stress displayed increased depressive-like behavior as demonstrated by increased immobility counts (no foot shock  $26.14 \pm 1.87$ ; three week foot shock  $37.28 \pm 1.20$ ) and reduced swimming behaviors (no foot shock  $13.71 \pm 2.00$ ; three week foot shock  $6.14 \pm 1.53$ ). A Student's t test identified significant differences between no foot shock and three week foot shock rats in immobility and swimming behaviors ( $P < 0.01$ ).

for both). No significant differences were observed between the groups in forced swim test parameter climbing (no footshock  $15.57 \pm 1.57$ ; three week footshock  $13.28 \pm 1.77$ )  $P > 0.05$ .

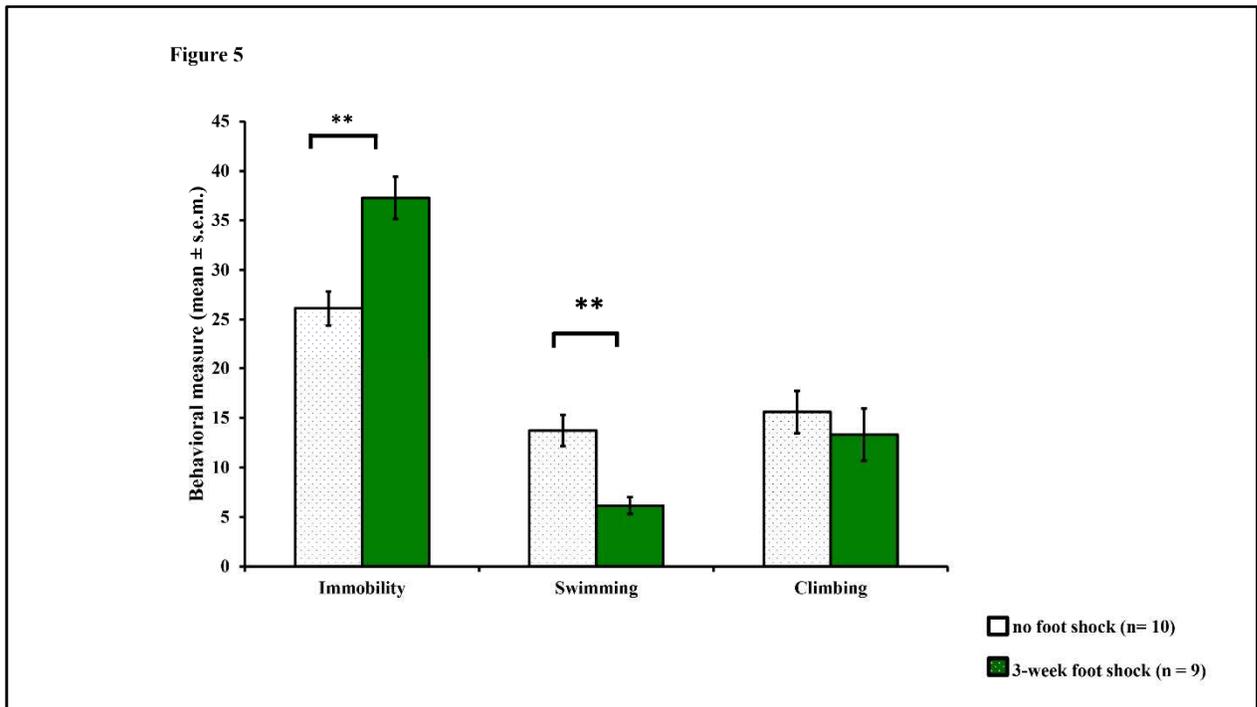


Figure 5. Mean counts of FST behavioral measures of immobility, swimming, climbing and others assessed every 5 sec. of the 5 min FST. Data expressed as the mean  $\pm$  s.e.m., \*\*:  $p < 0.01$  vs. non-foot shock animals. On day 2 of the 5 min FST, animals exposed to foot shock at three weeks of age displayed a significant increase in immobile behaviour and reduced climbing behaviour when compared to controls.

### Effect of postnatal stress exposure on sucrose preference

A two way repeated measures ANOVA identified successive significant increases in sucrose preference following FST1 and FST 2 exposures ( $F_{(2, 36)} = 10.16$ ,  $P < 0.01$ ) (Figure 6). noFS rats demonstrated a successive increase in sucrose preference, a similar trend was observed for 3wFS rats. I next investigated whether there was an effect of stress exposure on baseline sucrose preference. While 3wFS rats demonstrated reduced baseline sucrose preference this was not significantly different from that of noFS animals, ( $P > 0.05$ ; Student's t test) (Figure 7). No significant interaction was observed between stress exposure and sucrose preference ( $F_{(2, 36)} = 1.93$ ,  $P > 0.05$ ).

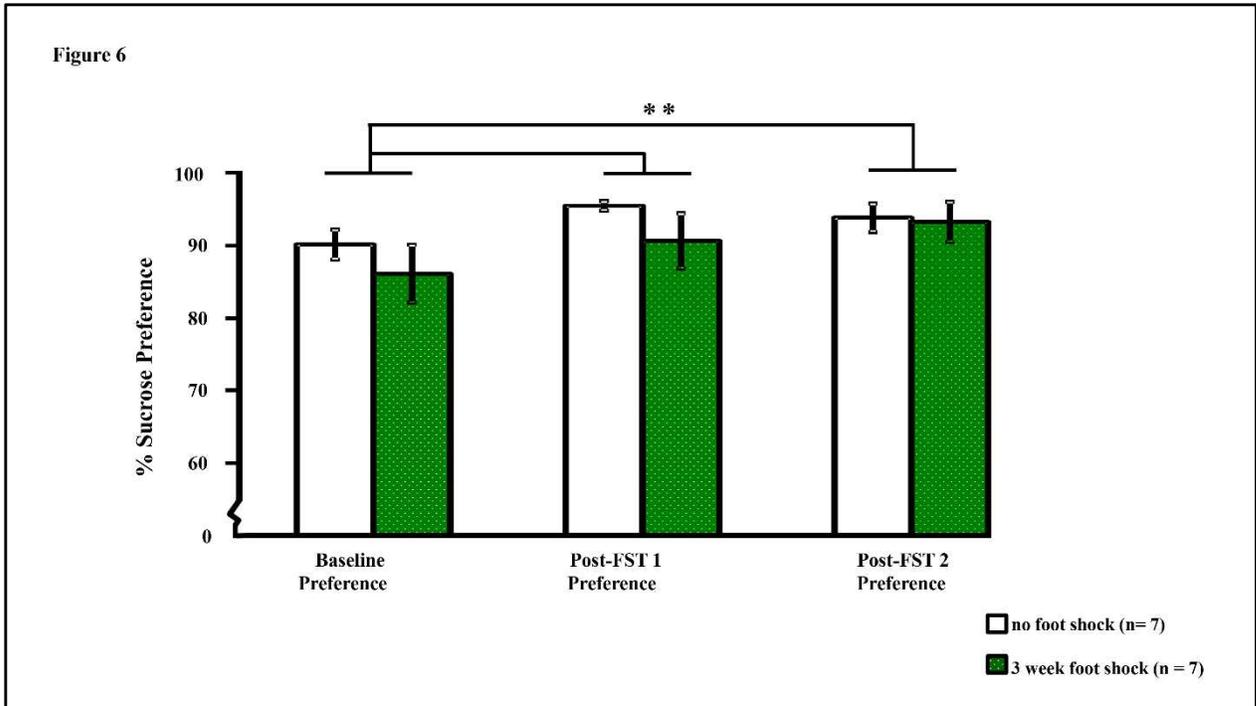


Figure 6. Sucrose preference of rats subjected to no foot shock versus three week foot shock exposure. Significant increases in sucrose preferences were observed in both groups following exposure to the two day forced swim test (FST1 and FST2) (two - way repeated measures ANOVA). Data expressed as the mean  $\pm$  s.e.m. \*\*:  $p < 0.001$ .

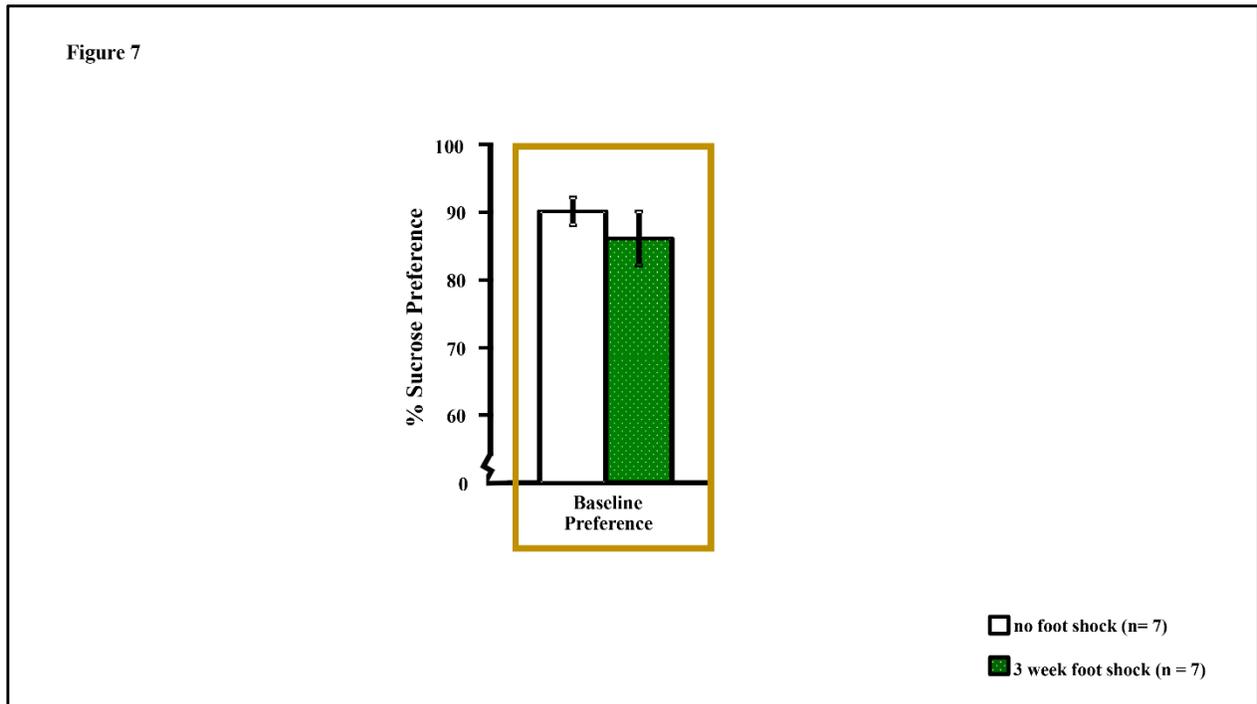


Figure 7. Baseline sucrose preference of animals subjected to no foot shock versus three week foot shock exposure. No significant difference in baseline sucrose preference was noted between groups; Student's t test ( $p = 0.13$ ). Data expressed as the mean  $\pm$  s.e.m.

## Discussion

The OFT has been extensively validated, ethologically and pharmacologically and is a well-established method for the assessment of general locomotor activity in rodents<sup>28</sup>. Using the open field test no significant differences between noFS and 3wFS animals in locomotor activity parameters of total distance travelled and the number of crossings were observed. As observed in noFS animals, upon introduction to the arena, animals tend to explore the novel environment actively<sup>28</sup>, characterized by a high number of crossings and large distance covered in the initial phases of the test followed by a gradual reduction in activity as the animals habituate to the open field arena. While noFS animals showed a gradual reduction in locomotor activity this effect was not observed in 3wFS animals. These results show a disruption in the ability to adapt to a novel environment or a dysfunction in the processes regulating habituation. This observation of delayed habituation demonstrates behavioral impairment in rats exposed to stress at the juvenile stage. While we cannot directly state the reason and effect of the non-habituation effect on the open field test previous reports have demonstrated that rats raised in social isolation demonstrate behavioral deficits such as reduced cognition and reduced habituation to the open field test<sup>28</sup>. Similarly, delayed habituation to the open field test may be a consequence of juvenile stress exposure. However, importantly, no significant differences in total locomotor activity were observed between noFS and 3wFS animals.

In the prodepressant paradigm, 1 mg/kg U69593 administration significantly increased FST depressive-like behaviors with no effect on locomotor activity. U-69593 is a selective agonist for the  $\kappa$ -opioid receptor. The  $\kappa$ -opioid receptor along with the dynorphins represent the receptor subtype and ligand for the endogenous opioid receptor system. Recent studies have identified a modulatory role of the opioid systems in the regulation of emotional and perceptual experience<sup>17</sup>. The  $\kappa$ -opioid receptors and dynorphin are enriched in the ventral tegmental area (VTA), nucleus accumbens and prefrontal cortex (PFC); brain regions that regulate mood and motivation. Agonistic activity at the  $\kappa$ -opioid receptor is thought to mediate depressive-like behavior through  $\kappa$ -opioid receptor – mediated inhibition of mesolimbic neuronal dopamine (DA) release<sup>18,19,20</sup>.

DA is also implicated in early adverse stress-induced susceptibility to behavioral depression as observed in the increased ventral striatal DA concentrations following a psychosocial stressor in patients who report poor early-life maternal care<sup>29</sup>. Additionally,  $\kappa$ -opioid receptor activation produces inhibition of VTA neuron dopamine release which is thought to contribute to the presentation of depression-related behavior including anhedonia.  $\kappa$ -opioid receptor activation is thought to mediate increased CREB levels within the nucleus accumbens, which is associated with increased immobility in the rodent FST<sup>30</sup> and anhedonia-like behavior<sup>31</sup>. Additionally,  $\kappa$ -opioid receptor activation induces ERK 1/2 phosphorylation in the nucleus accumbens<sup>32</sup> which mediates depressive – like behavior in the FST. Stress exposure has been observed to activate dynorphin release and receptor binding in dorsal raphe nucleus, basolateral amygdala, hippocampus, ventral pallidum, ventral tegmental area, nucleus accumbens, and bed nucleus of the stria terminalis<sup>33</sup>. Dynorphins are released in stressful aversive situations<sup>34,35</sup> and mediate the stress response<sup>36,37</sup>.

Postnatal stress exposure increased the presentation of a depressive-like state (i.e., immobile posture) and reduced active forms of coping (i.e., swimming and climbing). The expression of increased depressive-like behavior in 3wFS rats is congruent with other studies which have demonstrated that adverse early-life stress results in behavioral deficits characterized by the predominance of passive behavioral response to aversive stimulation at later life stages<sup>24,38,39</sup>. Moreover, clinical studies have identified a strong dose–response relationship between the number of experienced childhood adversities and the presentation of psychiatric conditions in adulthood with up to a third of cases involving the presentation of psychiatric disorders across the life course directly attributable to adverse childhood experiences<sup>40-43</sup>. The early life period is characterized by the development and maturation of neurotransmitter systems and neural circuitries which are essential to the modulation of behavior. During the early life period the brain is highly plastic and reacts strongly to environmental signals; in particular, the early brain is characterized by increased cortical glucocorticoid receptor expression<sup>44</sup> and a more prolonged and exacerbated corticosterone response following acute stress<sup>45</sup>.

The serotonergic (5HTergic) neurotransmitter system is one of the most widely distributed in the mammalian brain, with the axons of serotonergic neurons of the midbrain raphe nuclei reaching almost every brain structure and a profuse innervation of the cerebral cortex<sup>46,47</sup>, as well

as areas crucial for the regulation of emotion and stress-coping behavior, such as the amygdala and the paraventricular nucleus of the hypothalamus<sup>48,49</sup>. Consequently, the 5HTergic system is involved in a large number of functions including direct modulation of the stress response<sup>50</sup>. There is mounting evidence of altered 5HT transmission in depressive disorders and animal experiments have implicated altered 5HT release as well as increased DRN 5HTergic neuronal activity in forced swim test behavioral depression model. The conventional theory behind depression postulates a monoamine – serotonin deficiency as the cause of depression. Current pharmacotherapeutic agents enhance monoamine levels and thus reinforce the theory of a monoamine deficiency. However there have been discrepancies in this theory with the observation of a correlation between inhibition of 5-HT release and active coping in the forced swim test<sup>51</sup>, activation of 5HT<sub>1A</sub> receptors by infusion of the selective serotonin<sub>1A</sub> (5-HT<sub>1A</sub>) receptor agonist, 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) was associated with reduced forced swim test immobility<sup>52</sup>, whereas other findings point to depressive-like behavior associated with 5HT deficiency<sup>53</sup>. However, many factors are involved in 5HTergic neurotransmission and reduced 5HT levels may be a marker or end-point of upstream dysregulated mechanisms (such as altered receptor densities and sensitivity) and the mechanism of antidepressant agents, in particular, SSRI's is dependent on the downregulation 5HT<sub>1A</sub> autoreceptor activity with a resultant increase in extracellular 5HT levels.

Raphe nuclei ascending 5HTergic projections are evident from embryonic day 12 (E12) and 5HT system ontogeny can be divided into 3 phases: (1) early 5HT neurogenesis and primitive pathway development, (2) late neurogenesis and selective pathway development (E16 -19), (3) terminal field development (E19 – 21)<sup>54</sup>. 5HT terminal field development/or neuronal arborization is particularly important as the role of 5HT as a neuromodulator requires tightly regulated and precise end terminal development to maintain fine control. Previous studies have noted disrupted hippocampal laminar and columnar development with increased 5HT activity following prenatal 5-methoxytryptamine administration<sup>55</sup>. Conversely, reduced developmental 5HT neurotransmission is associated with severe anatomical abnormalities as demonstrated by Migliarini et al.<sup>56</sup> who observed severely reduced serotonergic innervation to the suprachiasmatic and thalamic paraventricular nuclei and marked serotonergic hyperinnervation in the nucleus accumbens and hippocampus of Tph2::eGFP mutants. It is conceivable that stress-induced

exaggerated increases in 5HT levels during postnatal day 21 – 25 may negatively affect 5HT terminal development particularly cortical arborization with subsequent effects on cortical – raphe nuclei circuitry.

### **Assessment of anhedonia-like behavior using the sucrose preference test**

Using the traditional 1% sucrose solution, both noFS and 3wFS rats displayed significant increases in sucrose preference following FST1 and FST2 exposures. The sucrose preference test is consistently used as a model of anhedonia, a marker of depression and exploits the endogenous liking for a sweet solution. While other neurotransmitter systems may be involved, anhedonia is directly dependent on the dysregulation of dopaminergic systems<sup>67,68</sup> and anhedonia can be considered to be a specific mood disorder explained by hypoactivation of the mesolimbic dopamine reward pathway<sup>69</sup>. Decreased consumption of sucrose following stress is mediated in part by the nucleus accumbens<sup>70</sup>. In the present results I did not observe a reduction in sucrose preference in rats exposed to stress. This may be a consequence of experimental design as reduced sucrose preference is commonly observed after experimental paradigms of chronic stress exposure such as chronic mild stress (CMS) exposure or stronger adverse stimuli such as repeated social defeat<sup>10</sup>. Although anhedonia, the decreased capacity to experience pleasure, is a central feature in the diagnosis of depression, a diagnosis of depression is possible with only the presence of depressed mood. Furthermore due to the heterogeneity of depression subtypes, not all depressed patients present with anhedonia<sup>71</sup> and anhedonia is not specific to depression but also observed in schizophrenia<sup>72</sup> and Parkinson's Disease<sup>73,74</sup>. This data suggests conservation of dopaminergic function in rats exposed to juvenile stress.

### **Conclusion**

In summary, 3wFS exposure resulted in behavioral impairment as expressed by an increased depressive-like behavior in the FST and altered environmental habituation in the OFT. As the 5HTergic system is undergoing final arborization during the period of footshock exposure I next investigated the effect of juvenile stress exposure on two potential modulators of raphe nuclei 5HT neurotransmission, TPH2 and GAD67 protein levels.

## **Chapter 2**

### **Effect of postnatal stress exposure on raphe nuclei tryptophan hydroxylase 2 (TPH2) and Glutamate Decarboxylase 67 (GAD67)**

## Introduction

The neurotransmitter 5HT has been implicated in the pathophysiology and treatment of major depression. The midbrain raphe nuclei is the major source of central 5HT and consists of nine subnuclei designated B1 – B9<sup>75</sup> which receive synaptic input from specific forebrain and brainstem structures and reciprocally give rise to efferents to specific forebrain and brainstem structures. In particular, the dorsal raphe is the largest serotonergic nucleus, containing more than half of the estimated 20,000 serotonin-producing neurons in the rat<sup>76</sup>. In addition to 5HTergic cells, the raphe nuclei consists of a heterogeneity of cell types. Predominantly studies have been done in the DRN and have demonstrated the presence of a variety of other neurotransmitters and neuromodulators including dopamine, norepinephrine, glutamate, GABA, enkephalin, substance P, neuropeptide Y, thyrotropin-releasing hormone, vasoactive intestinal polypeptide, cholecystokinin, gastrin and neurotensin<sup>57</sup>. In actuality, 5HTergic cells constitute at most two thirds the cell population of the DRN<sup>57,76</sup>. These non-5HTergic systems are important in the modulation of 5HTergic neuronal activity, however, main control of 5HTergic neuron activity is controlled by 5HT<sub>1A</sub> autoreceptors in the raphe nuclei.

The synthesis of 5HT is initiated by the hydroxylation of the essential amino acid tryptophan to 5-hydroxytryptophan (5-HTP) which is further decarboxylated to 5HT. Tryptophan hydroxylase (TPH) catalyzes the first, rate limiting step of 5-HT synthesis. TPH belongs to a superfamily of aromatic amino acid hydroxylases, together with phenylalanine and tyrosine hydroxylase<sup>77,78</sup>. There are 2 isoforms, TPH1 and TPH2<sup>79</sup>. TPH1 is predominantly expressed in the periphery and the pineal gland, whereas TPH2 is the predominant isoform found in the brain and is localized to the raphe nuclei and in some cases its projection areas<sup>79-81</sup>.

TPH2 plays an important role in the modulation of 5HT neurotransmission due to its role as the rate-limiting enzyme for central 5HT synthesis and thus may provide the most direct target of the brain 5HT system. As the modulator of the rate-limiting step in 5HT synthesis, TPH2 tightly controls 5HT availability in peripheral tissues and central neurotransmission. A functional, well-balanced system for 5HT production is essential for mental health and disruption or dysfunctions in TPH2 mechanics is strongly correlated with affective disorders<sup>81,82</sup>. Disruption in TPH2 functions, either due to a mutation in the TPH2 gene<sup>81-83</sup> or, due to an abnormal mRNA or protein

level of TPH2<sup>84-87</sup> may result in altered 5HT release in target areas, 5HT turnover and feedback regulation, as well as 5HT autoreceptor and target cell receptor densities.

$\gamma$ -amino butyric acid (GABA) is the main inhibitory neurotransmitter in the CNS and a high density of GABAergic elements is observed in the raphe nuclei<sup>58</sup>, and GABA neurons have been observed to suppress the activity of 5HT neurons<sup>61</sup>. GABA is synthesized by glutamic acid decarboxylase (GAD) which is present as 2 isoforms; GAD65 and GAD67. The 2 isoforms are thought to be functionally distinct, GAD67 responsible for production of a basal pool of GABA, whereas GAD65 is activated to produce extra GABA when required<sup>88</sup>. GABAergic signalling is present and functional very early in the brain and persistent presence of endogenously secreted GABA may provide tonic excitatory drive for trophic functions before synapse formation<sup>89</sup>. Furthermore, GAD67 is regulated in developmental, activity-dependent<sup>90</sup> and experience-dependent<sup>91,92</sup> manner by changes in GAD67 gene transcription. Activity-dependent GAD67 expression could result in the adjustment of the cellular and vesicular GABA pool for release and signalling. Interestingly, developing neural tissues are enriched in GABA<sup>93</sup>, which could be supplied by circulation through an immature blood – brain barrier<sup>94</sup> and an inefficient clearance of secreted GABA by immature uptake mechanisms<sup>95</sup>.

In particular, GABA neurons are thought to play an important role due to their high presence in the raphe nuclei. GABAergic terminals are present<sup>58</sup> and form synaptic connections with 5HTergic neurons in the DRN<sup>59</sup> that also express GABA-A receptors<sup>60</sup>. Furthermore the DRN receives GABAergic inputs from neurons located in a large number of distant regions from the forebrain to the medulla in addition to the local ventral pontine periaqueductal gray, including the DRN itself. Local application of GABA receptor agonists in the DRN inhibits 5HTergic neuronal activity<sup>61</sup> and 5HT release and metabolism in the forebrain<sup>62</sup>. These effects are blocked by specific GABA receptor antagonists<sup>61</sup>. Local GABAergic neurons in the DRN are thought to provide an inhibitory relay for converging inputs that exert top-down control over 5HT function<sup>96-99</sup>. Dysfunctions of the GABAergic system has also been identified in psychiatric disorders and stress related disorders<sup>63,64</sup>. Studies suggest that major depressive disorder is associated with reductions in GABAergic transmission and findings of reduced plasma and cerebrospinal fluid GABA levels have been observed in depressed patients. Additionally, detailed studies have

identified reductions in occipital cortical GABA levels in depressed patients<sup>65</sup> and post-mortem studies have identified reduced numbers of GABA neurons in layer II of the orbitofrontal cortex<sup>66</sup>.

While various anomalies in serotonergic biomarkers have been observed, previous research has demonstrated inconsistent data on TPH2 mRNA expression in depression<sup>85,86</sup>, and it remains unclear the effect of early adverse experiences on the expression of TPH2 protein levels. The enzyme is the rate-limiting factor in the biosynthesis of serotonin and consequently the elucidation of the effect of early adverse stressors on TPH2 expression may clarify the pathophysiology of neuropsychiatric disorders, and may also provide new strategies for the treatment of stress-associated diseases. GAD67 catalyzes basal GABA biosynthesis and altered GABA levels observed in depressive disorders may be indicative of upstream anomalies in GAD67 levels. Physiologically, GABAergic afferents exert spontaneous inhibitory tone on serotonergic neurons<sup>100</sup> and, it is conceivable that modulation of GABAergic afferents may influence 5HT release. This view is strengthened by the observation that GABAergic neurons have a strong tonic inhibition on serotonergic neurons in the DRN<sup>62</sup>. Neural activity significantly regulates GAD67 transcription and protein levels which may have consequences for GABA production and release. DRN GABAergic neurons are involved in the modulation of 5HTergic neuronal firing. Consequently, I assessed raphe nuclei TPH2 and GAD67 protein levels as a marker of altered neurotransmission of their respective neurotransmitters which may clarify the neural basis of juvenile stress associated depressive-like phenotype. As the DRN and MRN display distinct neurocircuitries, which may have consequences on stress sensitivity, independent protein analysis of both regions was performed<sup>46,49,57</sup>.

## **Method**

All procedures involving animals were carried out in accordance with the guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of the Hokkaido University.

### **Juvenile Stress**

Juvenile stress was performed as previously described in Chapter 1. In brief, rat pups were subjected to five shock stimuli (shock intensity, 0.5 mA; intershock interval, 30 sec; shock duration 2 sec) between PND 21 - 25. Each rat pup was held in the FS box 5 min after the last FS stimulation before being returned to the home cage. No-FS controls were exposed to the FS box for 12.5 min without FS stimuli. Pups were subjected to FS exposure for 5 continuous days. Behavioral experiments were carried out at the postadolescent period (10 - 14 weeks old).

### **Behavioral Assays**

#### **Open field test**

The open field test was carried out according to previously outlined procedures (Chapter 1).

#### **Forced swim test**

The two-day rat forced swim test was carried out following the same procedure as previously outlined (Chapter 2).

### **Tissue Preparation**

Rats were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). Referencing the Paxinos and Watson rat brain atlas, 2  $\mu$ m sections of the raphe nuclei were quickly dissected under dry ice. An incision separating the dorsal and median raphe was made and samples stored separately at -80 °C until processing for Western blotting.

### **Tissue processing**

Tissue processing was carried out using the NucleoSpin<sup>®</sup> Triprep kit. To detail the procedure, tissue samples were mechanically homogenized in a RP1 - Dithreithiol (DTT) buffer

using the Qsonica Q55 sonicator. DTT was prepared as a 500 mM preparation. The lysate was centrifuged for 1 min, 11,000 x g. 350  $\mu$ L ethanol (70 %) was added to the homogenized lysate and mix by pipetting up and down (approx. 5 times). The lysate was centrifuged for 30 s at 11,000 x g, filtrated and the flow-through collected for further protein isolation. 350  $\mu$ l of protein precipitator was added to the protein solution and the solution mixed vigorously. The mixture was incubated at room temperature for 10 minutes. Centrifugation of the solution (11,000 x g.) was carried out. The protein isolate was then washed by addition of 500  $\mu$ L of 50 % ethanol to the protein pellet and centrifuged at 1 min at 11,000 x g. The supernatant was completely removed and the protein pellet allowed to completely dry at room temperature by keeping container lid open. The protein sample was prepared by addition of 100  $\mu$ L of a PSB-TCEP solution. The mixture was incubated for 3 min at 95 °C to enable complete denaturation and dissolution of protein. The mixture was allowed to cool to room temperature, centrifuged for 1 min at 11,000 x g and the supernatant recovered and stored at 20 °C until protein quantification.

### **Protein Quantification and Sample Preparation**

Total protein quantification was carried out to enable comparison of juvenile stress exposure on TPH2 and GAD67 levels. Protein quantification was carried out using Macherey-Nagel® protein quantification system. Prior to quantification samples were heated to 30 °C and then vortexed vigorously to remove any possible SDS precipitate. A bovine serum albumin (BSA) reference solution was made by dissolving 1 mg of BSA in 1 ml of protein solving buffer (PSB) solution to produce a 1 mg/ml BSA stock solution. Protein quantification was carried out using the Macherey-Nagel microplate assay procedure. In short, a reference protein dilution series of 7 protein concentrations from 0, 0.031, 0.063, 0.125, 0.250, 0.500, 1.00  $\mu$ g/ $\mu$ l was prepared by dissolving appropriate quantities of BSA stock solution to 50  $\mu$ l of protein solving buffer (PSB). 20  $\mu$ l of each dilution series solution was pipetted into Eppendorf tube. 20  $\mu$ l of each protein sample was pipetted into a conical flask and 40  $\mu$ l PSB was then added to both dilution series and protein sample preparations. 40  $\mu$ l of quantification reagent was added to both dilution series and protein sample preparations, the mixture gently disturbed/mixed to ensure a complete and equal throughout color change from blue to yellow. The eppendorf tube containing mixtures were incubated at room temperature for 30 minutes. Light extinction was measured at 595 nm using the GE® GeneQuant 100 spectrophotometer. Following protein quantification, all

samples were prepared at 1 µg/µl using the Macherey-Nagel™ protein solvating buffer (PSB) as loading buffer. Protein samples were stored at -20 °C until electrophoresis and blotting.

### **Western Blotting**

Protein samples were defrosted on ice and, heated to 95 °C for 5 minutes, mixed briefly and spun in a microcentrifuge. 10 µl of each protein samples was loaded onto Bio-Rad Mini-Protean® TGX™ Precast Gel for electrophoresis. 5 µl of molecular weight marker protein marker containing a 1: 9 ratio preparation of Invitrogen MagicMark™ XP Western Protein Standard (20-220 kDa) (Cat #. LC 5603) and Precision Plus Protein™ All Blue Standards (Bio – Rad) (Cat # 161-0373) standard was loaded into end lanes of the precast gel. For protein samples, Macherey – Nagel PSB was used as protein sample loading buffer due to previous background signalling observed with a 1% preparation of Biovision 3X SDS – PAGE loading buffer (2108-10). Samples were loaded slowly to prevent sample leakage or the presence of air bubbles. Samples were electrophorised using a Powerpac 300 (Bio-rad) power generator (200 volts, 300 mA) for 50 min. Blotting buffer was prepared using 1.52 g Trizma – base (Sigma Cat #. T1503-500G) and 7.2 g Glycine (G7126-500G) to 500 ml millipore purified water. When the dye front had reached the bottom of the gel the power supply was disconnected and the gel was placed into the prepared blotting buffer solution. GE Amersham Hybond-P PVDF (RPN 2020P) membrane was used for protein transfer from gel to membrane (catalog RPN 2020F). The membrane was activated by short exposure to methanol which was removed by rinsing double distilled water (Millipore™). Biorad extra thick blot paper (1703695) and gel was prepared for protein transfer by immersion in blotting buffer for 15 min. a transfer sandwich was made and blotting performed for 30 min with the use of BIO-RAD Trans – Blot SD Semi – dry Transfer Cell (power settings 25 volts, 300 mA). After blotting the membrane was washed for 30 min in 1 X TBST preparation (10X TBST Cell Signaling #9997; 100 ml + 900 ml double distilled water (Millipore™)). Washing buffer was prepared, 100 ml of 10X TBST Cell Signaling #9997 with 900 ml of double distilled water (Millipore™). A blocking buffer was prepared using 5% skim milk in Tris-buffered saline containing Tween-20 (TBST) was prepared. Blocking buffer was prepared using Hokkaido MegMilk skim milk powder, 2.5 g dissolved in 50 ml of 1X TBST. The PVDF membrane containing the transferred proteins was immersed in blocking buffer and subjected to gentle agitation for 60 min at room temperature to prevent non-specific protein binding. The

membrane was then washed twice and incubated with primary antibodies overnight at 4 °C in a rotary shaker/ rotating shaker. For each sample, 3 proteins were assessed, GAD67, TPH2 and GAPDH. Primary antibodies used were Millipore Anti-Glyceraldehyde-3-Phosphate Dehydrogenase monoclonal antibody MAB374 (Cat. #AB2302) at 1: 1 000 000 dilution. Anti-rabbit anti-tryptophan hydroxylase 2 antibody (TPH2, Hasegawa et al., unpublished antibody) at 1: 100 000 dilution, Chemicon® International Laboratories/ Millipore anti-mouse anti-GAD67 antibody MAB5406 at 1:5000 dilution. On the following day, the membrane was exposed to the respective horse radish peroxidase conjugated secondary antibodies (anti-rabbit for TPH2) and anti-mouse for GAD67 and GAPDH for 1 hr at room temperature using the Amersham ECL Plus Western Blotting Reagent Pack (Cat. # RPN 2124). The Amersham ECL Plus Western Blotting Reagent Pack contains sheep derivative anti mouse IgG peroxidase linked whole antibody and anti - rabbit IgG peroxidase linked whole antibody from donkey. The membrane was then washed and developed using the Amersham™ ECL™ Prime (Cat #. RPN2232) kit by a 1 min exposure to a mixture containing equal volumes of Solution A (luminol solution, 50 ml) and Solution B (peroxide solution, 50 ml). The intensity of peroxidase reactions was visualised by exposure of membranes to Amersham Hyperfilm ECL High Performance Chemiluminescence Film (Cat #. 28-9068-36). Film exposure time for GAD67 and TPH2 were 5 minute and 1 minute respectively.

### **Image analysis & Quantification of protein expression**

All digital dark – field/ light – field images were captured by an Olympus BX50 light microscope operated by MCID Basic Version 7.0 Application Guide:Gel Analysis software using the direct band analysis mode.

### **Statistical analysis**

Experimental values are given as the mean  $\pm$  s.e.m. Optical density values are presented normalized to the endogenous protein GAPDH. A Student's t test was used for comparing DRN and MRN protein densities. Probability values less than 5% was considered significant.

## Results

### Effect of juvenile stress exposure on TPH2 and GAD67 levels in the DRN

Figure 1 depicts normalized protein levels of TPH2 (A) and GAD67 (B) in the DRN. As shown, TPH2 levels were significantly increased in 3wFS rats ( $1.18 \pm 0.24$ ) compared to noFS rats ( $0.61 \pm 0.07$ ); ( $P < 0.05$ ), however no significant differences in GAD67 protein levels were observed between noFS ( $0.64 \pm 0.07$ ) and 3wFS ( $0.59 \pm 0.04$ ) rats ( $P > 0.05$ ).

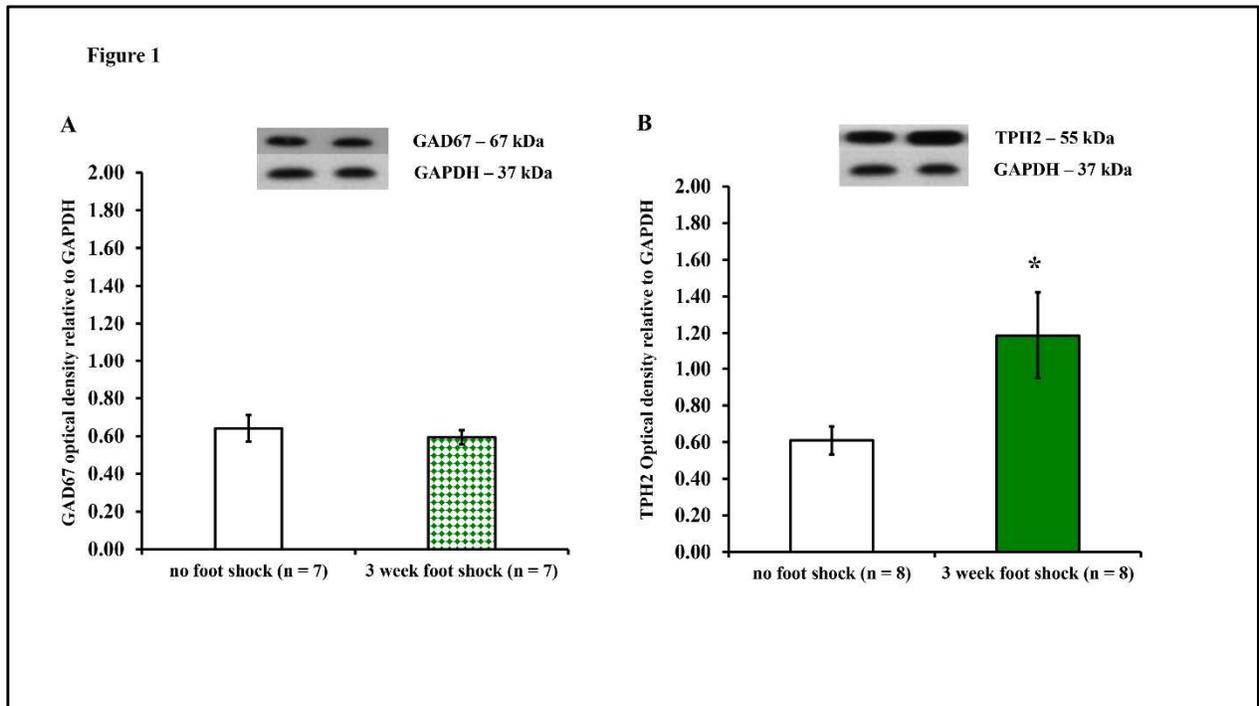


Figure 1. Dorsal raphe nucleus protein expression levels of GAD67 and TPH2 in no foot shock and three week foot shock animals. Data represented as the mean  $\pm$  s.e.m. A Student's t test identified no significant difference between no foot shock and three week foot shock animals in GAD67 expression ( $p > 0.05$ ) whereas three week foot shock animals displayed significantly elevated TPH2 protein levels ( $p < 0.05$ ).

### Effect of juvenile stress exposure on TPH2 and GAD67 levels in the MRN

As shown in Figure 2 juvenile stress exposure did not result in any significant differences in GAD67 and TPH2 levels in the MRN. A student's t test indicated there was no significant difference between noFS ( $0.53 \pm 0.02$ ) and 3wFS ( $0.51 \pm 0.02$ ) groups in MRN GAD67 protein

levels,  $P > 0.05$ . Similarly a student's t test identified no significant differences between noFS ( $0.75 \pm 0.21$ ) and 3wFS ( $0.63 \pm 0.20$ ) rats;  $P > 0.05$ .

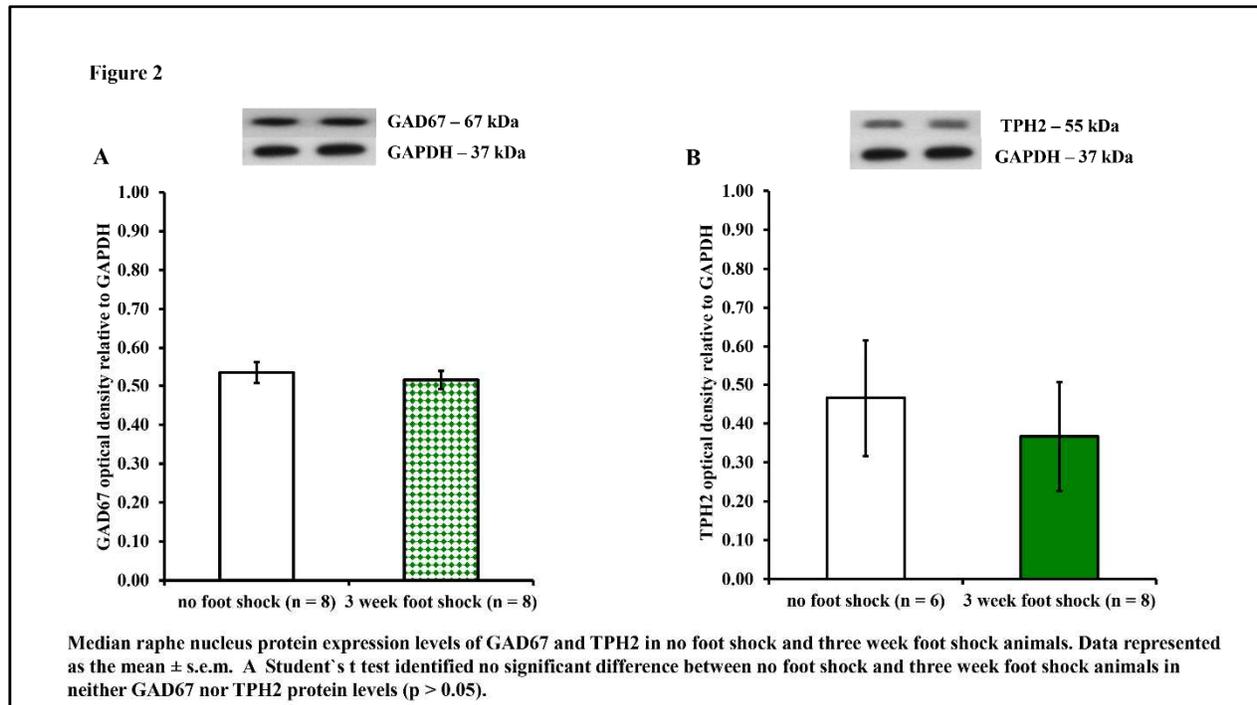


Figure 2. Median raphe nucleus protein expression levels of GAD67 and TPH2 in no foot shock and three week foot shock animals. Data represented as the mean  $\pm$  s.e.m. A Student's t test identified no significant difference between no foot shock and three week foot shock animals in neither GAD67 nor TPH2 protein levels ( $p > 0.05$ ).

## Discussion

### Juvenile stress exposure produces elevated DRN TPH2 protein levels

Juvenile stress exposure resulted in increased TPH2 levels within the DRN whereas no significant changes in GAD67 levels were observed. Investigation of MRN TPH2 and GAD67 levels identified no significant differences between noFS and 3wFS rats in either enzyme protein levels.

### Relationship between TPH2 and 5HT

The monoamine hypothesis of depression postulates a central 5HT deficiency. This hypothesis originally derived from the observation that drugs that enhance 5HT

neurotransmission, by inhibiting the serotonin-reuptake transporter (SERT) or monoamine oxidase (MAO), displayed antidepressant activity<sup>102</sup>. However, the observation of behavioral improvements following antidepressant-induced enhancement of 5HT neurotransmission function does not necessarily mean that the associated disorder is a result of 5HT deficiency as several other factors are essential to 5HT neurotransmission including synthesis, storage/release, signalling, reuptake, and metabolism. Stress exposure, particularly uncontrollable stress produces intense activation of DRN 5HT neurons<sup>50</sup>, leading to large accumulations of extracellular 5HT within the DRN<sup>103</sup> and projection regions<sup>104</sup>. This activation sensitizes DRN 5HT neurons for 24–72 h. Subsequent stressful exposures results in an exaggerated release of 5HT in projection regions<sup>105</sup>.

Altered 5HTergic expression and functions have been observed in anxiety, depression and aggression-related disorders<sup>106</sup>. One factor mediating 5HT neurotransmission is the enzyme TPH2. TPH2 catalyses the rate-limiting reaction in the synthesis of 5HT, the hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP). The enzyme amino acid decarboxylase then converts 5-HTP to 5HT. Of note, TPH2 is susceptible to environmental stimuli and adverse exposures have been observed to result in either an increase or decrease in TPH2 mRNA and/or protein levels<sup>107,108</sup>. In line with the current results of increased TPH2 protein levels in the DRN, clinical studies of drug-free, depressed suicide victims identified elevated TPH2 protein and TPH2 mRNA expression in the dorso-caudal DRN subregions<sup>86,87</sup>. The previous studies' findings of increased TPH2 expression seems to contradict that of earlier studies of reduced 5HT and 5-hydroxyindole acetic acid (5HIAA) levels in the midbrain of suicide victims and in the cerebrospinal fluid of suicide attempters<sup>109,110</sup> and is an indication of the complexity involved in 5HTergic neurotransmission. Of note, however, elevated TPH2 protein levels may not necessarily translate to increased 5HT levels in target regions, increased TPH2 protein levels does not imply conserved enzymatic activity and, under basal conditions, TPH2 is only about 25–50% saturated with its substrate tryptophan<sup>111,112</sup>. Additionally, 5HT neurotransmission includes a multiplicity of pre- and postsynaptic receptor systems, the serotonin reuptake transporter and neuronal cell firing. These systems are tightly linked to compensate for homeostatic changes and may be directly or indirectly affected by stress exposure.

In fact, 5HT deficiency may be secondary to dysregulated mechanisms such as altered receptor sensitivity and previous research has demonstrated increased 5HT1A receptor mRNA expression in the amygdala and reduced expression in the DRN of rats subjected to maternal separation between postnatal days 2 -12<sup>113</sup>. The increased sensitivity of the juvenile brain to corticosterone levels may have compensatory effects on a number of factors regulating 5HT neurotransmission and it has been observed that prolonged corticosterone exposure reduced serotonin responsiveness in hippocampal target areas<sup>114</sup>.

The impact of the corticosteroid system is particularly important as it relates to the observation of increased TPH2 in the DRN. The DRN is known to receive a profuse CRF innervation. The raphe nuclei express a high density of corticoid receptors and the HPA system and the 5HT system are closely cross-regulated<sup>115,116</sup>. At physiological levels of corticosterone release mineralocorticoid receptors (MR) are bound whereas in stress states the glucocorticoid receptor (GR) is also bound<sup>117</sup>. MR receptors are found mainly in limbic regions such as the hippocampus<sup>118</sup>, whereas the GR has a more widespread distribution in the brain<sup>119</sup> particularly in the hippocampus, the paraventricular nucleus, the locus coeruleus and the DRN<sup>119,120</sup>. GR occupation increases serotonin responses. Increased tonic activity of the HPA system may result in an exaggerated increase in the firing of 5HTergic neurons with a resultant compensatory effect of increased TPH2 levels. The expression of GR mRNA and protein exclusively in raphe nuclei 5HTergic neurons is suggestive of regulation of gene expression and may have implications for altered TPH2 expression. Steroid modulation of TPH2 protein is specific to the glucocorticoid receptor as co-administration of the glucocorticoid antagonist mifepristone blocked the effects of dexamethasone. This is exemplified by studies which have demonstrated corticosterone-induced increased TPH activity<sup>121</sup> glucocorticoid-modulated raphe TPH2 mRNA expression<sup>122</sup> and corticosterone-induced elevations in TPH2 mRNA associated with presentation of depressive behaviors in rats<sup>123</sup>.

The lack of effect on MRN may be related to the differential sensitivity of DRN vs. MRN 5HTergic axons. The DRN and MRN are subserved by distinct neural circuits with differing projection areas; the DRN receives inputs from areas involved in stress and behavioral control such as the mPFC, central amygdala nucleus, lateral hypothalamus, while the MRN receives innervation from the anterior cingulate, prelimbic cortex, hippocampus<sup>50,113,124</sup>.

Protein level quantification failed to identify any effect of juvenile stress exposure on GAD67 protein levels in either DRN or MRN. GABA neurons within the raphe nuclei receive efferents from the cortico-raphe glutamatergic neurons or the habenulo-raphe pathway leading to inhibition of 5HTergic cell firing<sup>59,97</sup>. In particular, DRN GABAergic afferents are from the periaqueductal gray area and the amygdala<sup>58,125,126</sup>. The lack of any significant changes in GAD67 levels may translate to differential sensitivity and modulatory criteria of 5HTergic versus 5HT-GAD67 expressing neurons in the modulation of depressive-like behavior. This is exemplified by Warden et al., 2012<sup>127</sup> who identified increased immobile behaviors in the FST following activation of mPFC-DRN direct projecting neurons whereas activation of 5HT-GABAergic neurons did not produce any effects on FST behavior.

### **Conclusion**

Here I provide evidence of juvenile stress-induced change in the serotonergic biomarker, increased TPH2 levels within the dorsal raphe nuclei. As the dorsal raphe nuclei receive and send extensive innervation to other structures in the subcortical limbic system such as the amygdala and the hippocampus, this may have consequential effects on emotional stress response.

## General Discussion

The goal of these studies were to investigate the effect of an early adverse/juvenile stress exposure on the presentation of depressive-like behaviors in adult rats and identification of the underlying neural mechanisms. First and foremost, early adverse exposure produced behavioral impairments observed by increased FST immobility behaviors and altered locomotor activities. Behavioral impairment was accompanied by a lack of changes in GAD67 raphe nuclei protein levels but an increase in TPH2 dorsal raphe nucleus protein levels. Consistent with the behavioral changes, pyramidal neurons of the IL region of the mPFC displayed reduced spine densities. The IL region is found within the ventral part of the mPFC, and previous studies have indicated an important role in the mediation of depressive-like behavior. No significant changes in prelimbic spine densities were observed and this may relate to the relative refractoriness of the PL region to stress.

Dendritic pattern and distribution are important determinants of the functionality of cortical neurons. Future assessments of dendritic branching and dendritic branch length may provide insights into the cortical effects of juvenile stress induced dendritic anomalies. As dendritic branching and dendritic length are important parameters of network activity, clarification of the effect of juvenile stress on these parameters would provide plausible explanation for juvenile stress induced dysfunctions in cortical neuronal circuitry. Furthermore, electrophysiological investigations may clarify the effect of disrupted layer II/III neuronal morphologies and how this affects intra- and interlayer circuitry. In particular, layer V/VI pyramidal neuronal morphology and electrophysiological characteristics are of interest due to the predominant output role of this layer characterized by profuse afferent and efferent projections to subcortical structures which are highly implicated in depression. Electrophysiological investigations of PL layer II/III and V/VI may also clarify reasons for the apparent lack of juvenile stress exposure on prelimbic pyramidal neuron morphology.

Further studies may also investigate the morphology of other structures known to be highly involved in depression such as the hippocampus and amygdala. This is made all the more pressing as these structures demonstrate strong interconnectivity with the PFC. While unexpected, the findings of elevated TPH2 protein levels in the dorsal raphe have previously been indicated in adult animals which expressed increased TPH2 mRNA levels following stress exposure<sup>107,108</sup>. As,

elevated TPH2 protein levels does not implicitly translate to increased protein activity, other biomarkers of serotonin neurotransmission such as baseline and evoked extracellular 5-HT levels may provide more direct explanation of the serotonergic neural mechanisms involved in depression associated with juvenile stress. Additionally, raphe nuclei 5HT<sub>1A</sub> and prefrontal cortex 5HT<sub>2A</sub> receptor densities may provide more insight into the effect of juvenile stress exposure on serotonergic neurotransmission.

Repeated 14 day administration of FLV recovered the behavioral and morphological changes observed in juvenile stressed rats. In addition to being a selective serotonin reuptake inhibitor, fluvoxamine is an agonist at the sigma-1 receptor and activity at this receptor may be related to BDNF-related recovery of depressive-like behavior and IL spine densities. While I postulate neurotrophic factors responsible for FLV-mediated behavioral and morphological recovery, the exact mechanisms remain to be identified. Initial effects of antidepressant agents involve increased intrasynaptic monoamine levels, however the delayed mood-elevating effects are evidence of downstream processes necessary for antidepressant activity. As previous studies have identified sigma-1 receptor mediated elevations in BDNF levels, coadministration of a sigma-1 antagonist such as NE-100 (N,N-dipropyl-2-(4-methoxy-3-(2-phenylethoxy)phenyl)ethylamine hydrochloride) would clearly demonstrate that FLV-mediated stimulation of sigma-1 receptor is involved in the observed behavioral and morphological recovery. The present characterization of a behavioral phenotype of depressive-like behavior associated with a prior stress exposure at the juvenile stage provides an opportunity for detailed investigations into causative neurobiological mechanisms. These findings may provide useful pathways for the identification of more effective pharmacotherapeutic approaches in the treatment of depressive disorders.