EXPLORING POTENTIAL LINK BETWEEN NEONATAL MEDICINAL EXPOSURE & LONG TERM NEUROBEHAVIORAL OUTCOME: A PRECLINICAL STUDY ON FLUOXETINE

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Thesis submitted is partial fulfilment of the requirement for the Award of Doctor of Philosophy Degree in Pharmacology

DEPARTMENT OF PHARMACOLOGY
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2021
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MY FAMILY
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ACKNOWLEDGMENTS

Firstly, I would like to thank my Allah Almighty and his Prophet Hazrat Muhammad Mustafa S.A.W., who enabled me to accomplish this work. I would like express my gratitude to following people for their support and advice given during this study: My Supervisor Dr. Ghulam Abbas for his tireless, genius and genuine guidance and support, throughout my study from the development of the Research Proposal to final completion of this dissertation. I find him very cooperative and outclass in the Research field and I would say it was blessing of Allah Almighty for me that My supervisor quit HEJ and Joined Ziauddin University. In fact that was turning point of my Ph.D. journey. His untiring efforts and generous cooperation made my Ph.D. dream to come close of being true. I find no words to pay my thanks to my highly estimable sir. Indeed the journey of PhD was so tough, complicated, time taking and thorny at many stages. When I got weak and fragile, it was his able guidance, motivation, dedication and love, which inculcated a real spirit in me to achieve this milestone. Once again I will say that I am utmost beholden of this thoroughly a great gentleman.

I will pay vote of thanks to respected madam Shumaila Usman as well because her critical review and valuable comments on my research work made my Ph.D. journey to this point of time.

I am also thankful to Dr. Rehan Ahmed Siddiqui for his assistance and advice on this dissertations

I am very indebted to Sir Mohammad Harris Shoaib who is the professor of Pharmaceutics department and Ex-Chairman of Pharmaceutics department at faculty of Pharmacy, Karachi University for sharing his valuable comments guidance with me.

I am extremely grateful to Prof. Rafeeq Alam Khan, Dean Faculty of Pharmacy for his support and cooperation during the course of study.

I am also thankful to the teachers (especially Dr. Uzair) and staff (especially, Umar) of department of pharmacology for their help during my study.

Bundles of thanks to my friends i.e. Wasim, Hammad, Najeeb and Dawood for their timely assistance during tough days.

Last but not the least My parents and Family, who had always prayed and encouraged me on every stage of my study and for my late Father Haji Ali Akber who was very struggling labor and made great sacrifices for my higher studies and cherished very bright future for me, besides my dearest Aamah Sonan for her rich prayers whose prayers really brought wonders for me in addition to this my elder brother Abdul Sattar for his immeasurable love and everlasting encouragement who always cherished for my doctorate degree. Similarly My wife Dr Kanwal Baloch she always supported and stood shoulder to shoulder with me during my course of study and always kept my motivation intact.

Nadeem
Declaration

I declare this thesis doesn’t contain any material previously submitted for a degree or diploma in any university and to the best of any knowledge it doesn’t contain any material that published previously or written by another person except where due reference have cited in the text.

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This is to certify that thesis entitled “Exploring Potential Link Between Neonatal Medicinal Exposure & Long Term Neurobehavioral Outcome: A Preclinical Study on Fluoxetine” submitted by Nadeem for the award of degree of Doctor of Philosophy in pharmacology at Faculty of Pharmacy, Ziauddin university, is a bonafide record of the research work carried out by him under my supervision and guidance. The content of thesis in full or parts have not been submitted to any other institute or university for the award of any other degree or diploma.

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Professor Dr. Rafeeq Alam Khan
Meritorious Professor & Dean
Faculty of Pharmacy
Ziauddin University
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LIST OF ABBREVIATIONS

5HIAA - 5hydroxyindoleacetic Acid
5HT - 5hydroxytryptamine
ACTH - Adrenocorticotropic
ADHD - Attention Deficit Hyperactive Disorder
ANOVA - Analysis of Variance
C - Control
CA1 - Cornu Ammonis 1
CA3 - Cornu Ammonis 3
CBG - Corticosteroid Binding Globulin
cDNA - copy Deoxyribonucleic acid
CNS - Central Nervous System
CRF - Corticotrophin Releasing Factor
CRFR1 - Corticotropin Releasing Factor Receptor 1
CRFR2 - Corticotropin Releasing Factor Receptor 2
CRHR1 - Corticotropin Releasing Hormone Receptor 1
CS - Caesarean Section
CT - Cycle Threshold
DG - Dentate Gyrus
DLG4 - Discs Large Homolog 4 Dene
dNTP - Deoxyribonucleotide Triphosphate
DRN - Dorsal Raphe Nucleus
ECG - Electrocardiogram
EDTA - Ethylenediaminetetraacetic acid
ELISA - Enzyme Linked Immunosorbent Assay
EPM - Elevated Plus Maze
F - Forward
F10 – Fluoxetine 10 mg/kg
F100 – Fluoxetine 100 mg/kg
F50 – Fluoxetine 50 mg/kg
FDA - Food & Drug Administration
FKBP5 - FK Binding Protein 5
FST - Forced Swim Test
GAPDH - Glyceraldehyde 3-phosphate Dehydrogenase
GOI - Gene of Interest
GR - Glucocorticoid Receptor
H&E - Hematoxylin & Eosin
HCl - Hydrochloric Acid
HKG - Housekeeping Gene
HPA axis – Hypothalamic Pituitary Adrenal axis
HRP - Horseradish Peroxidase
ICD - International Classification of Diseases
IPA - Isopropyl Alcohol
KMNO4 - Potassium permanganate
mGluR1 - metabotropic Glutamate Receptor Subtype 1
mGluR5 - metabotropic Glutamate Receptor Subtype 5
MMP9 - Matrix metallopeptidase 9
mRNA - messenger Ribonucleic Acid
NCD - Non-communicable Disease
NMDAR - N-methyl-D-aspartate receptor
NR3C1 - Nuclear receptor subfamily 3, group C, member 1
OD - Optical Density
PBS - Phosphate Buffer Saline
PCR - Polymerase Chain Reaction
PSD 95 - Post-synaptic Density Protein 95
PVN - Paraventricular Nucleus
qPCR - quantitative Polymerase Chain Reaction
R - Reverse
rDNA - ribosomal Deoxyribonucleic Acid
RNA - Ribonucleic Acid
SEM - Standard Error Mean
SERPINA6 - Serpin A6 (Serpin Peptidase Inhibitor Clade A, Member 6)
SERT - Serotonin Transporter
SLC6A4 - Solute Carrier Family 6 (Neurotransmitter Transporter, Serotonin), Member 4
SNRIs – Serotonin Norepinephrine Reuptake Inhibitors
SPSS - Statistical Package for Social Sciences
SSRIs - Selective Serotonin Reuptake Inhibitors
TCAs - Tricyclic Antidepressants
TE - Tris Ethylenediaminetetraacetic acid
Tm - Melting temperature
TPH - Tryptophan Hydroxylase
TPH2 - Tryptophan Hydroxylase 2
UN - Urocortin
WHO - World Health Organization
ZF - Zebrafish
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SUMMARY

Trauma (physical, chemical or environmental) during early in life integrates into innervation patterns in the brain and therefore can chronically impact the mental health of victim. Long term neurobehavioral aspect of developmental toxicity is overlooked in the panel of testing requirements during drug development process. The perinatal depression renders the use of antidepressant inevitable and exposes the fetus and newborn to fluoxetine during vulnerable window of development through mother. Keeping this into account, the present study was designed to study the potential effect of pre-weaning fluoxetine exposure on mental health of offspring upon attaining adulthood. Briefly, the mother rats were administered with fluoxetine (10, 50 and 100 mg/kg) from post-natal day 1 to 21 (pre-weaning period). The litters were then shifted to regular food and water (drug free) and allowed to grow for three months (adulthood) followed by behavioral, biochemical, expression and brain morphometric assessments. Our behavioral data showed decreased depression (forced swim test) and anxiety (elevated plus maze test), while enhanced social behavior (social deficit test) in rats as compared to control. The effect was more pronounced in female rats. The biochemical data (ELISA) revealed increased tryptophan and decreased corticosterone levels. However, the levels of serotonin and its metabolite remained significantly unaltered as compared to control rats. The gene expression (qPCR) data revealed up regulation of NR3C1 (glucocorticoid receptor) and down regulation of PSD-95 (post-synaptic density protein) in the whole brain of fluoxetine expose rats as compared to control. However, the expression of SERT (serotonin receptor), 5-HT1A receptor, TPH (tryptophan hydroxylase), FKBP5 (FK binding protein 5), CRHR1 (corticotropin releasing hormone receptor 1) and CBG (calmodulin binding globulin) remained statistically non-significant. Assessment of hippocampal morphometry (hematoxylin-eosin and silver staining) revealed significant increase in the area, cell count and neuronal arborization in the dentate gyrus region of treated rats as compared to control. In conclusion, the present study demonstrate that pre-weaning fluoxetine exposure affect the depression, anxiety and social behaviors upon adulthood via perturbing tryptophan metabolism, corticosterone levels, NR3C1 and PSD-95 expression and hippocampal morphometry. Hence, early life fluoxetine exposure has the capacity to define the mental health attributes in the society, which stresses upon the need to incorporate long term neurobehavioral study as a component of developmental toxicity studies performed on active pharmaceuticals during drug development process.
Chapter 1
General Introduction
PREGNANCY & DEPRESSION

Depression is the central nervous system disorder primarily characterized by low mood / energy and changes in dietary / sleep patterns. In its worst form, it can lead to suicide. It will become the main cause of disability till 2030. Depression may victimize females during critical time of pregnancy, known as antenatal depression. An estimated 10 percent of the mothers suffer from depression during pregnancy or post-partum one year (Gaynes, Gavin et al. 2005). In Pakistan, the estimates are far higher with prevalence of antenatal depression was 37%, while that of postnatal depression was 30% (Atif, Halaki et al. 2021). Pregnancy itself was reported to induce relapse of depression in euthymic mothers. The situation becomes more severe, if the conceived mothers discontinue antidepressant therapy (Cohen, Altshuler et al. 2006, Silverman, Reichenberg et al. 2017). If left untreated, depression has its own implications such as low weight, preterm birth and inability of mother to take care of herself and baby in the womb (Li, Liu et al. 2009). The worst consequence of untreated depression is suicide, which account for approximately 20 % of post-partum deaths (Lindahl, Pearson et al. 2005). Furthermore, caesarean section (CS) is increasing around the globe with more numbers in the developing world (Niino 2011, Betrán, Ye et al. 2016). It is worth mentioning that the positive co-relation between CS, breast feeding discontinuation and post-natal depression was noted (Nam, Choi et al. 2017, Xu, Ding et al. 2017). The depression not only affects the mothers but also the progeny. The babies from depressed mother was shown to have altered temperament (Davis, Glynn et al. 2005), high sensitivity (Essex, Klein et al. 2002) and increase cortisol levels (Ashman, Dawson et al. 2002). Taken together, the antidepressant therapy could not be avoided during pregnancy and lactation. The gestational risk and consequences related with untreated maternal depression is tabularized (Table-1.1). This treatment with antidepressants exposes the baby towards chemicals during vulnerable window of development. This is the time when most of the physiological and behavioral parameters are fixed in the body. Any traumatic exposure (physical, chemical or environmental) could have a long lasting consequence. In this regard, the knowledge regarding teratogens among women of childbearing age was not found to be satisfactory (Alhamdan, Moukaddem et al. 2020).
### Table-1.1 Gestational risk factors and consequences related with untreated maternal depression

<table>
<thead>
<tr>
<th>MATERNAL DISORDER</th>
<th>GESTATIONAL RISKS &amp; CONSEQUENCES</th>
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<tbody>
<tr>
<td>DEPRESSION</td>
<td>Improper maternal weight gain (Bodnar, Wisner et al. 2009).</td>
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<tr>
<td></td>
<td>Compound abuse (Flynn, Chermack et al. 2008)</td>
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<td>Fetal distress (Jablensky, Morgan et al. 2005).</td>
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<td></td>
<td>High risk of cesarean deliveries; increased possibility of neonatal intensive care unit (NICU) submissions (Chung, McCollum et al. 2004)</td>
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**Antidepressants in Pregnancy**

The antidepressants consumption during pregnancy is increasing with time and an estimated 1 in 10 women use them (Cooper, Willy et al. 2007). Selective serotonin reuptake inhibitor, especially fluoxetine (Prozac) is commonly used to treat pregnancy associated depression. Search of literature revealed that the effect of antidepressants on physical being of fetus has been the focus of investigations. With an exception of paroxetine, other SSRIs were shown to have no effect on fetal malformations (Einarson and Einarson 2005, Gentile 2005, Sivojelezova, Shuhaiber et al. 2005, Rahimi, Nikfar et al. 2006). Although, the antidepressant use has been attributed to spontaneous abortion (Hemels, Einarson et al. 2005) and pulmonary hypertension in neonates (Chambers, Hernandez-Diaz et al. 2006). FDA has also given black box warning on the SSRIs because of their potential association with persistent pulmonary hypertension in neonates. However, their effect on the mental well-being is poorly studied and still the matter of debate. In this regard, there are very few mix and inconclusive reports on the effect of SSRIs in psychological outcome in both animals (Maudhuit, Hamon et al. 1995, Vogel, Hagler et al. 1996, Ansorge, Zhou et al. 2004, Maciag, Simpson et al. 2006) and humans (Nulman, Rovet et al. 1997, Nulman, Rovet et al. 2002, Misri, Reebye et al. 2006). There are some reports on the withdrawal / poor adaptation effects of antidepressants on neonates (Webster 1973, Moses-Kolko, Bogen et al. 2005). Moreover, the
antidepressants treatment during pregnancy was attributed to autism spectrum disorder (Croen, Grether et al. 2011) infant convulsion (Hayes, Wu et al. 2012) and altered ECG (Fukushima, Nanao et al. 2016). It is of note here that the FDA has issued black box warning to antidepressants, especially SSRIs for induction of suicidal tendencies in children. This is suggestive of the susceptibility of progeny towards the adverse outcomes attributed to antidepressants. Unfortunately, there is clear lack of insight on the effect of antidepressants on psycho-developmental outcome of progeny. It is worth noting that early fluoxetine exposure to zebrafish (ZF) caused the decrease in cortisol levels upon getting adults. It is of note that this effect remained evidence till three generations, who were not exposed to fluoxetine (Vera-Chang, Moon et al. 2019). In similar line, the offspring of fluoxetine exposed (perinatal) Sprague Dawley dams showed anxiety and depression type behaviors at the age of adolescence. This was also accompanied by region dependent expression changes of NMDA receptor, PSD-95, mGluR1 and mGluR5 (Millard, Lum et al. 2019).

DEVELOPMENTAL TOXICITY

In early 1960s, the thalidomide incidence led to global realization that medicines have the potential to harm fetus. This has led to development of the field of developmental biology related to the pharmaceuticals, which deals with harmful actions of medicines on the growth of the organism (Klaassen and Watkins 2010). The interest in this area has led to the incorporation of guidelines for preclinical developmental toxicity testing for pharmaceutical agents. These toxicity studies primarily study the impact of medicines on body growth (organogenesis, morphogenesis) without much emphasis on changes in function of developed systems (Saghir and Dorato 2016). A lot of work has been done in the past in this regard leading to identification of numerous toxins (embryotoxins and teratogens) (Rao and Schwetz 1982). Even Food & Drug Administration (FDA) has assigned various categories (A, B, C, D, X) to all drugs to describe the intensity of risk for the fetus (Osborne, Leistikow et al. 2020), which are as follows:

Category A: No potential risk

Category B: No potential risk except reduction in fertility

Category C: Preclinical data showed adverse effects but not proven in clinical settings
Category D: Potential fetal risk

Category X: Contraindicated during pregnancy

**Neurobehavioral Aspect**

The long-term neurobehavioral aspect is an area, which unfortunately could not catch the attraction of the policy makers in past for devising testing rules for development of pharmaceutical agents. This is the reason why no such guidelines are available on this particular aspect for testing for pharmaceutical agents, a potential risk yet to be completely deciphered. However, this deficiency in testing has been acknowledged in the literature (Ulbrich and Palmer 1996). Search of literature revealed some reports which highlighted the importance of this promising area of developmental toxicity. One such study conclude that maternal influenza infection during second trimester may enhance possibility of getting schizophrenia or depression upon getting adult (Watson, Mednick et al. 1999). The children has higher probability of being affected with attention deficit hyperactive disorder (Liew, Ritz et al. 2014) psychomotor developmental abnormalities with internalize or externalize behavior (Brandlistuen, Ystrom et al. 2013) born from mothers who have used acetaminophen at the time of pregnancy. Hence, these reports are suggestive of the long term neurobehavioral toxicity of the medicines.

**DEPRESSION**

Major depression is a common disease that severely limits psychosomatic functioning and impairs quality of life. It was in 19th century when melancholia was seen as an independent disease and considered to be associated with sadness, suicide and preoccupation with past. After Second World War, the ICD (World Health Organization) as well as Diagnostic and Statistical Manual (American Psychiatric Association) clearly characterized the illness. As per described criteria, person is depressed if five of the given symptoms manifest for at least 2 weeks.

1. Depressed or irritable mood
2. Decrease interest in pleasurable stimuli
3. Lack of sleep or too much sleep
4. Increase or decrease in weight
5. Psychomotor agitation or retardation

6. Fatigue or low energy

7. Feelings of worthlessness or excessive guilt

8. Decreased ability to think or concentrate

9. Repeated thoughts of death or suicide

According to the recent global health metrics, an estimated 264 (3.4%) million are victimized by depression (James, Abate et al. 2018). In Pakistan, the prevalence of depression vary between 22% to 60% (Ahmed, Enam et al. 2016). The genetic and environmental factors also play instrumental role in the pathogenesis of depression. The ability to inherit is high i.e. 50 % (Kendall, Van Assche et al. 2021) and almost equivalent to diseases such as type II diabetes, hypertension and asthma (Nestler, Barrot et al. 2002). In practice, its identification, evaluation and treatment have been a challenge for most practitioners due to its numerous presentations, unpredictable courses, and variable treatment response.

**Pathogenesis**

Depression being a complex disorder, its etiology is not fully understood. Number of the factors like biological and genetic can play a role. People of any race, age and socio-economic status may be affected by depression (Norman and Ryrie 2013). Unlike other diseases (cardiovascular and gastrointestinal) depression is more complex and multiple hypotheses have been proposed underlying its pathogenesis. Among all the monoamine hypothesis received significant attention as reflected by the fact that most of the antidepressants acts via enhancing the levels of monoamine neurotransmitters in the brain.

**Biochemical Basis**

The journey of the monoamine hypothesis started with serendipity of iproniazid (Delay J, Laine B et al. 1952) as well as imipramine (Kuhn 1958). Iproniazid, an anti-tubercular drug was found to be a mood elevator. Later, it was investigated to inhibit the monoamine oxidase enzyme (Zeller, Barsky et al. 1952, Loomer, Saunders et al. 1957). Imipramine, a prescribed anti-psychotic was observed to be an antidepressant. The
aforementioned drugs were found to affect both catabolism and re-uptake of catecholamines, specifically of nor-adrenaline (Axelrod, Whitby et al. 1961) and the same were attributed towards their clinical effectiveness. Furthermore, the reserpine (antihypertensive) induced depression (Freis 1954, Muller, Pryor et al. 1955) was reversed by imipramine (Sulser, Bickel et al. 1964). Altogether, this laid the foundation of catecholamine hypothesis of depression (Sulser, Bickel et al. 1964) i.e. decrease catecholamines are responsible for depression, while their elevation back to normal the required by antidepressants to produce their action. Later on, the efficacy of imipramine was linked to its action on serotonin (5-HT, 5-hydroxytryptamine) levels (Carlsson, Fuxe et al. 1968) followed by loss of antidepressants efficacy in the presence of 5-hydroxytryptamine synthesis inhibitor (Shopsin, Gershon et al. 1975). These initial results transformed catechol to monoamine hypothesis of depression and 5-hydroxytryptamine (serotonin) became the most popular target for antidepressants drug discovery. This development subsequently led to the emergence of a block buster drug fluoxetine, the details of which are described at the end of this chapter.

**Genetic Basis**

The expression level of various components of serotonergic system has been attributed to the pathogenesis of depression as follows:

*Serotonin Transporter (SERT)*:

SERT is the sodium dependent serotonin transporter protein which cause re-uptake of serotonin from synapse to pre-synaptic neuron. It is encoded by the gene i.e. solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (SLC6A4), which and one of prime candidate for pharmacogenetics research in the field of depression (Schiele, Zwanzger et al. 2021). Epigenetic modulation i.e. enhanced hypomethylation of SLC6A4 promoter (leads to enhanced activity of reporter gene) has been attributed to the pathology of major depression (Philibert, Sandhu et al. 2008, Iga, Watanabe et al. 2016, Shi, Sun et al. 2017).

*5-HT$_{1A}$ receptor*:

It is a subtype of serotonin receptor which is coupled with Gi protein coupled. Alteration in the levels of this receptor are reported in depressed subjects (Albert and Lemonde 2004). These alterations are reported to be gender specific i.e. its protein levels was found
to be significantly reduced in prefrontal cortex of female subjects while remained unaltered in males (Szewczyk, Albert et al. 2009). Down regulation or loss of function of this receptor (somatodendritic and postsynaptic) was reported suicide subjects, while administration of SSRIs (selective serotonin reuptake inhibitors) normalize these deficits (Savitz, Lucki et al. 2009). The 5-HT$_{1A}$ receptor knockouts were found to be more anxious and less depressed in animal studies (Overstreet, Commissaris et al. 2003). However, a distinct role of 5-HT$_{1A}$ somatodendritic autoreceptor and postsynaptic heteroreceptor in anxiety and depression phenotypes was reported (Albert, Vahid-Ansari et al. 2014).

_Tryptophan hydroxylase (TPH):_

It is one of important enzyme, which regulate the synthesis of serotonin via transforming L-tryptophan into L-5-hydroxytryptophan, the precursor of serotonin. Among the various isoform, the TPH2 is produced more in the brain and regulates serotonin synthesis (Zhang, Beaulieu et al. 2004). The mutation in TPH2 leading to loss of function was reported in depressed subjects (Zhang, Gainetdinov et al. 2005). The increased expression and translation of TPH was noted in depressed subjects committed suicide, which is suggestive of homeostatic compensation for serotonin deficiency (Bach-Mizrachi, Underwood et al. 2006) and was more pronounced in DRN (dorsal raphe nucleus) (Bach-Mizrachi, Underwood et al. 2008). Similar compensatory action was reporter by others too (Boldrini, Underwood et al. 2005). In similar lines, the roles of strong homeostatic responses in the brain was reporter earlier by us (Abbas, Naqvi et al. 2011, Abbas, Naqvi et al. 2012)

**Animal Model of Depression**

The animal model used for assessment of depression-like phenotype is as follows:

**Forced Swim Test (FST)**

It was established by (Porsolt, Le Pichon et al. 1977), due to of simplicity, high precision and consistency, forced swim test (FST) is the most broadly used animal model of depression. In this test, the animal is subjected to an unpreventable environment i.e. water filled jar. Following preliminary escape oriented effort, the animals become immobile. This immobility is a mark of behavioral despair perceived in depressed subjected and stopped by antidepressants. The common FST does not perceive SSRI whereas; its
altered form with greater water level can in the form of improved swimming behavior. Moreover, the psychomotor tonics provoke false positive outcome in the test and should be checked out for locomotor activity in order to endorse the antidepressant potential. The test is appropriate for rats (Cryan, Markou et al. 2002). Recently, the calculate of latency to immobility was stated to develop the sensitivity of the test for TCAs and SNRIs but not for SSRIs, associated with insensitivity to stimulants (Castagné, Porsolt et al. 2009).

**Tail Suspension Test (TST)**

TST is another widely used animal model to assess antidepressant-like activity (Steru, Chermat et al. 1985). Its principle of action is similar to that of FST with a difference that only mouse can be used in this test. Additionally, the non-escapable scenario in TST is the hanging of animal by tail. In dissimilarity with FST, it can detect SSRIs as well. In similarity with FST, the motor stimulants produce false positive result in the test therefore this possibility must be ruled out in order to confirm the antidepressant like action.

**Chronic Unpredictable Mild Stress (CUMS)**

In CUMS, the experimental animals are repeatedly exposed to numerous stressors like restraining, decreasing environmental temperature, mild electric foot shocks, putting in water, lack of bedding or wet bedding and reversal of dark and light cycles (Katz, Roth et al. 1981, Willner 1997). However, it shall be ensured that the sequence of stressors should be random in order to avoid prediction and habituation in animals. The animals after CUMS for several weeks exhibit decline in the normal preference towards sucrose, the effect prevented or reversed by the use of antidepressants. The merits of CUMS includes good predictive (behavioral alterations are reversed by many antidepressants), face (most of the demonstrable symptoms of depression are produced), and construct (produce a general decline in responsiveness to rewards which is comparable with anhedonia) validities.

**Learned Helplessness (LH)**

LH is the behavior demonstrated by the animals after exposure to unavoidable repetitive aversive stimuli such as electric shock via electric grid or tail electrode. During this
behavior, the animal do not try to escape the aversive situation or show delayed escape response, which is considered as the symptom of depression (Seligman 1972, Wang, Timberlake II et al. 2017).

ANXIETY DISORDERS

According to American Psychiatric Association, anxiety is a standard response against stress and useful for coping up with the situation. However, sometimes this response involves excessive feelings of nervousness or anxiousness beyond the proportion of stimuli and hinders the routine functioning of subject. It is termed as pathological anxiety. The difference between anxiety and fear is that the former happen in anticipation of future event while later is the response against immediate threat. Additionally, anxiety is primarily caused muscle tension and avoidance coping, while fear involves fight or flight responses. Examples of anxiety disorders includes generalized anxiety disorder, panic disorder, specific phobias, agoraphobia, social anxiety disorder and separation anxiety disorder. According to the recent global health metrics, an estimated 284 (3.7%) million suffered from anxiety disorders (James, Abate et al. 2018). In Pakistan, the prevalence anxiety disorders vary between 22% to 60% (Ahmed, Enam et al. 2016).

Pathogenesis

Anxiety is the complex and heterogenous disorder and various biochemicals have been attributed to its developments. Among all, the cortisol appears to be the primary biochemical indiacatos of anxiety and its role in development of anxiety is as follows:

Biochemical basis

The hypothalamic-pituitary-adrenal axis (HPA-axis) plays the significant role in the generation of anxiety-like behavior (aan het Rot, Mathew et al. 2009) and helps to overcome stress under normal conditions (Figure-1.1).
Figure-1.1 The hypothalamic-pituitary-adrenal axis and its regulation
However, an enhanced HPA axis activity was reported to underlie the pathogenesis of anxiety disorders. The hyper-cortisolemia induce excito-toxic damage, which contributes to the reduction in hippocampal volume (Juruena, Eror et al. 2020). Moreover, the normalization of HPA axis was also reported to be involve in the anti-anxiety action of drugs (Tafet and Nemeroff 2020). Primarily preclinical research observed that the hippocampus is highly vulnerable to stress experiences. Chronic stress ultimately causes atrophy of apical dendrites in the CA1 and CA3 sub regions. In association, the chronic stress suppresses the neurogenesis (formation of new neurons from stem cells) in the dentate gyrus region of adult (Monroe and Harkness 2005, Samuels and Hen 2011, Dranovsky and Leonardo 2012, Boldrini, Santiago et al. 2013). The higher levels of stress hormone cortisol during prenatal life increased the likelihood of developing enhanced HPA axis activity and anxiety (Cottrell and Seckl 2009, Moisiadis and Matthews 2014). In case of depressed mothers, the increased basal salivary cortisol levels were noted (Ashman, Dawson et al. 2002). It is of note that the mother’s prenatal biochemistry is the important determinant of behavior and biochemistry of newborn (Field, Diego et al. 2004, Field, Diego et al. 2008), which in case of cognitive deficit in newborn was reported to be normalized by treatment with SSRIs (Ishiwata, Shiga et al. 2005).

**Genetic basis**

The expression of various components of HPA axis has also been attributed to the pathogenesis of anxiety as discussed below:

**Corticotrophin releasing factor (CRF):**

The CRFergic systems is composed of CRF, urocortin (UN), CRF receptor 1 (CRFR1) and 2 (CRFR2). Among all, the role of CRFR1 in development of stress response is extensively studied (Vasconcelos, Stein et al. 2020). The activation of this receptor leads to adrenocorticotropic hormone (ACTH) release, and subsequently the production of secretion from adrenal gland (Smith and Vale 2006). The CRHR1 is expressed in various brain regions and not limited to pituitary (Van Pett, Vial et al. 2000). The CRFR1-knockouts exhibit reduced anxiety and lower HPA-axis activation (Contarino, Heinrichs et al. 1999).
Nuclear receptor subfamily 3, group C, member 1 (NR3C1):

NR3C1 is the glucocorticoid receptor (GR), which plays an important role in stress relevant ailments e.g. its disruption in the CNS reduced anxiety (Tronche, Kellendonk et al. 1999). However, its deficiency during fetal life was attributed to anxiety-like behavior in the adulthood in a sex dependent manner (Schmidt, Lax et al. 2019). Similar association was also reported in animal model of chronic restraint stress (Chiba, Numakawa et al. 2012)

FK Binding Protein 5 (FKBP5):

It acts as a co-chaperone and regulate GR function in stress response (Zannas, Wiechmann et al. 2016). The alleles leading to upregulation of FKBP5 caused blunted stress response, which may contribute to stress associated mental disorders (Binder 2009).

Corticosteroid Binding Globulin (CBG):

CBG, also known as transcortin or serpin A6 (serpin peptidase inhibitor clade A, member 6) is a glycoprotein, which binds and transport glucocorticoids preferably as compared to albumin. It plays a role in stress related mood and behavior by buffering the availability of free corticosterone under stress (Breuner and Orchinik 2002, Meyer, Nenke et al. 2016). The CBG deficiency leads to reduced free corticosterone levels during rest and stressful situation (Richard, Helbling et al. 2010). Its plasma levels was found to be more in high reactive mice breed with enhanced HPA axis activity under stress (Mattos, Heinzmann et al. 2013). Furthermore, the CBG knockout animals display inadequate adaptive behavior via regulating availability of free glucocorticoid in the brain (Richard, Helbling et al. 2010, Minni, Dorey et al. 2012, Moisan 2013). Its expression was found to be downregulated upon exposure to stress (Neufeld, Breen et al. 1994, Spencer, Miller et al. 1996).

Gut-brain axis

The role of intestine in the health and diseases has been reported for long now. In this context, Hippocrates once said that “bad digestion is the root to all evil”. However, the function of non-self gut residents (microbiota) in health and disorders is recently shaping
the possible area of research in the drugs and therapeutics. This microbiota (normal flora) is available everywhere inside the human body, where inside meet outside. It is projected that the quantity of microbial inhabitants is 10 times larger ($10^{27}$ cells / gram tissue) than the total counts of human body cells. Within the human body, the deeply colonized part is gut, particularly colon (Sekirov, Russell et al. 2010). The developing body of literature also proposes network connection between flora and central nervous system through the process or system remained indefinable. The antibiotics, probiotics, symbiotic and dietary habits have been exposed to disturb the diversity of flora (Margolis, Cryan et al. 2021). As per different studies and evidences from the available literature have displayed the fluctuations in behavior in germ free animals, antibiotic-induced gut dysbiosis projects, probiotics used therapy and pathogen infected animals (Cryan, O’mahony et al. 2011). The part of gut microbiota in emerging anxiety like disorders is not understandable yet. According to Some analysis have shown the drop in anxiety-like behavior in germ free mice (Heijtz, Wang et al. 2011, Neufeld, Kang et al. 2011, Clarke, Grenham et al. 2013, Arentsen, Raith et al. 2015). However, some observations report no alteration in anxiety-like behavior (Bercik, Denou et al. 2011, Gareau, Wine et al. 2011). Hence, further research work is required to explain the effects of gut brain axis. Initial life in mammals shows a period of bacterial colonization and remain there whole life (Foster and Neufeld 2013). The stress can affect the composition of the gut microbiota (Cryan and Dinan 2012). Stress in early life can have the long-term results on the arrangement of the gut microbiota. It has been stated that the adults rats that had undergone maternal isolation for 3 hours per day from the postpartum days 2-12 have changed gut microbiota configuration which was confirmed by the 16s rDNA examination of the fecal samples when linked to the non-separated control rats (O’Mahony, Marchesi et al. 2009). A conceivable study provided the indication that the gut microbiota have the role in the development of the HPA axis, which performs an instrumental job in stress(Sudo, Chida et al. 2004). An amplified level of adrenocorticotrophic hormone and corticosterone, and decrease in basal levels of anxiety-like behavior was detected in the germ free mice, when crosschecked with control mice. These consequences were astonishingly challenging as corticosterone is a stress response hormone and normally its levels positively correlate with rise in anxiety levels (Neufeld, Kang, Bienenstock, & Foster, 2011).
Animal Model of Anxiety

The animal model should comply the similarity criteria in appropriate etiology, symptomatology and treatment of the particular disorders. (McKinney and Bunney 1969). It is very difficult to simulate the sign and symptoms of mental disorders. Despite of this fact various animal models are developed to assess the behavior of animals such as:

*Elevated Plus Maze (EPM)*

EPM is one of the most commonly used rodent model to assess the anxiety like behavior in rodents due to clarity, high selectivity and reliability (Pellow, Chopin et al. 1985). The apparatus consists of an arms in the shape of plus sign, which are elevated at a certain height from the ground. One arm of this sign has boundary walls while other is open. The model provides a conflict between two natural behaviors i.e. spontaneous exploration and fear from heightened open areas. The more time spent in the open arm is considered to represent less anxiety (Walf and Frye 2007).

**SOCIAL BEHAVIOR**

In all living beings, the communication with each other enhances their ability to have a social life, which is indispensable for their survival. This socialization primarily depends upon the drive to recognize and interact with other fellow beings. The brain structures responsible includes prefrontal cortex, amygdala, nucleus accumbens, anterior insula, anterior cingulate cortex, hippocampus, and temporal sulcus (Gao and Mack 2021). The social deficit, including both hypersocial and hyposocial behaviors, is one the hallmark of neurodevelopmental ailments e.g. Autism (Keifer, Mikami et al. 2020), Williams Syndrome (Jabbi, Kippenhan et al. 2012) and Schizophrenia (Murphy, Haigh et al. 2020). The etiology of this social issues is yet to be completely deciphered. Nonetheless, two mechanisms have been reported to modulate the social behavior i.e. the amygdala coordinated differentiation between fear and friendly social signals in order to generate appropriate behavioral response. Second one is dopamine coordinated reward and aversion phenomenon which ultimately decide to either approach or avoid the particular social contact (Toth 2019).
Pathogenesis

Search of literature revealed that the pathogenesis of social behavior alterations is not properly studied so far. In this context, no specific biochemical has been firmly attributed to development of social deficits. However, the genetic basis does exhibit some basis, which is as follows:

Genetic Basis

Post-synaptic density 95 (PSD-95) is one of the most widely studied protein in social behavior studies and considered as risk for the development of hypersociability, an action attributed to dysfunction of prefrontal cortex (Barak, Zhang et al. 2019). In glutamate synapse in the brain, it is the predominant scaffolding protein (Purcell, Moran et al. 2014, Rodzli, Lockhart-Cairns et al. 2020). Search of literature revealed that it cause maturation of synapses during neurodevelopment via trafficking glutamate receptors at post-synaptic membrane (Purcell, Moran et al. 2014). It is of note that PSD95 knock out cause robust hypersocial behavior in both genders of rat (Winkler, Daher et al. 2018). It is also known as SAP-90 (synapse associated protein 90), and is expressed by gene i.e. DLG4 (discs large homolog 4). It regulated as synaptic density and its stabilization in long term potentiation (Meyer, Bonhoeffer et al. 2014). Moreover, the social isolation was produces anxiety, which was attributed to upregulation of PSD-95 in forebrain (Zhang, Zu et al. 2012). In a study, the robust hypersocial behavior was observed in both genders of PSD95 knock outs. Furthermore, the male animals were more aggressive and territory conscious, while female makes more sound in the presence of sedated mouse (Winkler, Daher et al. 2018). A PSD-95 knockouts produced age dependent effect i.e. lower socialization during adolescence and vice versa in adults. This effect was ascribed to PSD-95 and NMDAR regulated developmental adjustment. (Coley and Gao 2019, Gao and Mack 2021). Furthermore, the loss of function alterations in PSD-95 was also reported to underlie compulsive grooming (obsessive-compulsive disorder) and enhanced anxiety (Welch, Lu et al. 2007), and behavioral and synaptic changes linked with psychostimulant drug addiction (Yao, Gainetdinov et al. 2004).

Animal model of Social behavior

The animal model used for assessment of various social behaviors is as follows:
**Social Deficit Test**

The social communication (verbal or non-verbal) is essential feature of living beings, which is dependent on the drive for interaction with other members of same specie as well as the ability to differentiate among different subjects (Lawande, Ujjainwala et al. 2020). In humans, altered social behavior can be observed in depression (Saunders and Roy 1999) and anxiety (White, Oswald et al. 2009). The animal studies provides an opportunity to obtain in-depth underlying mechanisms responsible for social deficits. In this test, the animals are placed in an enclosure and allowed to interact. The social (interactions with each other plus allo-grooming), non-social (self-grooming and rearing) and investigative (Sniffing and following) behaviors are noted and compared with control subjects to explore and deficit in social behavior (Cox and Rissman 2011).

**FLUOXETINE**

Fluoxetine is a block buster antidepressant, which enhance the concentration of serotonin in synapse by inhibiting its reuptake through serotonin transporter (SERT) (Rossi, Barraco et al. 2004, Cipriani, Brambilla et al. 2005). It is also used for the management of anxiety disorders (Chouinard, Saxena et al. 1999, Zou, Ding et al. 2013). Important adverse effects include insomnia, sexual dysfunction, pregnancy issues, suicide in below 25 years and arrhythmias (Sohel, Shutter et al. 2019). It is of note that the medicinal effects of fluoxetine has also been attributed to the phenomenon of neurogenesis (David, Samuels et al. 2009, Ohira, Takeuchi et al. 2013) and ageing was reported to abolish this effect (Couillard-Despres, Wuertinger et al. 2009). Search of literature revealed that the synaptic remodeling underlie the antidepressant action of fluoxetine, which includes the expression of several postsynaptic protein including PSD-95 (postsynaptic density protein 95) (Reinés, Cereseto et al. 2008). It is of note that PSD-95 is reported as the potential pharmacological target for the management of clinical depression (Doucet, Harkin et al. 2012, Doucet, Levine et al. 2013).

Search of literature revealed that early postnatal fluoxetine exposure caused reduction in effort-related motivation via improving dopaminergic activation (Menezes, Shah et al. 2021). In similar lines, it was reported that chronic fluoxetine administration caused anhedonia and hamper reward learning in mice. This affect was attributed to impairment in amygdalar plasticity, which was dependent on MMP-9 rendering the morphology of
dendritic spines in immature state, which most likely reflects the animal inability to adapt (Puścian, Winiarski et al. 2021).

With reference to gut-brain axis, the effect of fluoxetine on gut microbiota is reported. In this regard, the stress-induced alterations in gut microbiota was reported to underlie pathogenesis of depression. It is of note that this dysbiosis reduced the efficacy of fluoxetine efficacy via changes in serotonergic pathway of tryptophan metabolism (Siopi, Chevalier et al. 2020). Additionally, the fluoxetine treatment at pregnancy as well as lactation was reported to modulate the gut microbiome similar to that of depression (Ramsteijn, Jašarević et al. 2020). It also caused selective depletion Lactobacilli in the gut; the microbe reported to be involved in the control of weight (Lyte, Daniels et al. 2019).

Fluoxetine treatment was reported to downregulation of 5-HT1 receptors (especially presynaptic 5HT1A) in the brain, but region dependent (Beasley, Masica et al. 1992, Shishkina, Kalinina et al. 2012). However, the expression of postsynaptic 5-HT1A receptor in forebrain remained unaltered (Johnson, Ingram et al. 2009)

The fluoxetine treatment (2 weeks) significantly decreased the expression of Serotonin transporter (SERT) in rat brain (Dygalo, Shishkina et al. 2006). Another study revealed the similar outcomes in all regions of the brain (Johnson, Ingram et al. 2009)

The fluoxetine treatment (2 weeks) downregulated tryptophan hydroxylase-2 (TPH2) in rat brain stem (Dygalo, Shishkina et al. 2006) and midbrain (Shishkina, Kalinina et al. 2007). However, age dependency was reported to influence its expression such as fluoxetine treatment was shown to enhance tryptophan hydroxylase immunoreactivity in adolescent animals and reduced immunoreactivity in adult animals (Klomp, Václavů et al. 2014). Fluoxetine was reported to increased TPH2 gene expression (Heydendael and Jacobson 2010).

The fluoxetine administration was reported to decrease the level of cortisol in responder patients (Piwowarska, Chimiak et al. 2012). However, it was shown to enhance the function of glucocorticoid receptor (GR) (Pariante, Kim et al. 2003). In another study, the fluoxetine treatment did not affect the corticosterone and glucocorticoid receptor expression (Mitic, Simic et al. 2013). Fluoxetine upregulated GR in the hippocampus following 4 weeks (Yau, Noble et al. 2004). Another study revealed decreased
glucocorticoid receptor expression (Heydendael and Jacobson 2010). The increase in GR expression along with decrease in corticosterone levels was also reported (Brady, Gold et al. 1992). These are differences are probably due to regional differences in the brain and duration of study.

Fluoxetine treatment was reported to increase the secretion of corticotrophin releasing factor (CRF) in hypophysial portal plasma (Gibbs and Vale 1983). Another study revealed no increase in CRF expression in paraventricular nuclei of rats (Marar and Amico 1998). On the contrary, the increase in CRF expression was also reported in PVN of rats (Brady, Gold et al. 1992). A study also reported that fluoxetine the expression of FK506 binding protein 5 (Park, Heah et al. 2012).

VULNERABILITIES IN BRAIN DEVELOPMENT

The brain development is a miraculous process which involves migration and synaptic connectivity of cells in order to prepare for encoding information during entire life. In mammals, the brain architecture (synapses and receptors) is overproduced and eradicated up to 50%, firstly at the time of birth and secondly after birth till adulthood. This phenomenon reflects vulnerable times in the brain development for the incoming stressor (environmental or chemical) to produce long lasting psychopathology, an area of research still in its infancy. Hence, the time of contact of risk factor is extremely crucial in determining the emergence of pathology depending upon the brain structure and function under development. Although, it is not knows in detail the transformation of from childhood to adolescence but it is well established that the pre-pubertal brain is extremely plastic and thus more vulnerable. Hence, the extreme plasticity of developing brain should be considered while exposing to any chemical intervention, the potential challenge for the clinicians to take decision regarding administration of drugs. Whether, such exposure of immature brain to drugs may produce long lasting enduring impact on the development of brain is the question yet to be answered. However, emerging concepts believe that immature brain takes incoming information different from that of mature brain which is again suggestive of time spans of vulnerability (Andersen 2003). Early life trauma integrates into the neural connectivity whereas the later life events only produce functional changes, which are adaptive (Figure-1.2). Therefore, the treatment strategy shall ideally be aimed to restore the disorderly trajectory on normal path without not much emphasis on symptomatic management. Another sign for a vulnerable time span
for the effect of early stress on hippocampal volume revealed the age till 5 years (Humphreys, King et al. 2019). In this regard, the maltreatment of childhood was reported to influence the activity HPA axis on long term basis via epigenetic modification of NR3C1 gene (Perroud, Paoloni-Giacobino et al. 2011).

Figure-1.2 Development of brain & window of vulnerability
AIMS & OBJECTIVES

Depression is a psychiatric illness, which may victimize females during the critical course of pregnancy. It may lead to abortion, pre-term birth, post-partum suicide and inability of mother to take care of herself and baby. These consequences compelled to opt for the treatment with antidepressants, which exposes the fetus to chemicals during vulnerable window of development. It is of note that most of the bodily parameters, physiological and psychological, defined during fetal and early post-natal life. Any intervention (chemical or environmental) during this vulnerable life span can have a long-term consequence on mental health of progeny. SSRIs, especially fluoxetine has been used for managing depression in pregnant mothers. Search of literature revealed few reports on the effect of this drug on physique and behavior of offspring. However, no single report found on the long-term consequence of this exposure on the neuro-behavioral wellbeing of offspring upon attaining adulthood. Keeping this in view, the present study was designed to assess the impact of early antidepressant exposure on behavioral manifestations on progeny. In conclusion, the present study may explain the underlying cause(s) of social attributes that prevail in a society. It shall also help in devising the health policy for the treatment of pregnancy associate depression.
Chapter 2

Materials & Methods
CHEMICALS

The following chemicals were used in the study:

<table>
<thead>
<tr>
<th>Reagents / Chemicals</th>
<th>Company Name</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Sigma, Germany</td>
<td>A-9539</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Scharlau, Spain</td>
<td>CL 0205</td>
</tr>
<tr>
<td>DNA Ladder (100bp)</td>
<td>Thermo Fisher Scientific, USA</td>
<td>USA RO621</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Thermo Fisher Scientific, USA</td>
<td>USA 64-17-5</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>MP Biomedical, USA</td>
<td>USA 04802511</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>Lilly Pharmaceuticals, Pakistan</td>
<td>3105</td>
</tr>
<tr>
<td>Taq Green Master Mix</td>
<td>Promega, USA</td>
<td>USA M7122</td>
</tr>
<tr>
<td>TRIzol reagent</td>
<td>Invitrogen, USA</td>
<td>15596026</td>
</tr>
</tbody>
</table>

ANIMALS

Female Sprague Dawley (Pregnant) Rats were obtained from Animal Resource Facility of International Center for Chemical and Biological (ICCBS), University of Karachi. They were accommodated separately with adlibitum access to food and water. The temperature was kept at 25°C while 12 hour dark and light cycle was maintained throughout the course of study. The F1 generation obtained, after weaning begins, were separated from the dams and housed gender-wise in a group of 5 rats per cage. All experiments were performed according the ethical guidelines provided the Animals Ethics Committee of the University (Approval No. 2019-004).

EXPERIMENTAL DESIGN

At post-natal day 1, the dams (F0 generation) were given fluoxetine at 10, 50 or 100 mg/kg daily for 3 weeks (average pre-weaning time) by dissolving the required amount of drug in the minimum drinking water (approximately 5 ml). The control group received drinking water alone. After 3 weeks, the pups were fed with regular diet and allowed to reach adulthood (3 months). The 5 males and 5 females rats from F1 generation was subjected to behavioral, biochemical, gene expression and morphometric assessments (Table-2.1). The schematic diagram (Figure-2.1) of the experimental design is as follows:

---

**Figure-2.1 Schematic diagram of the experimental design**
Table 2.1 Grouping of experimental animals

<table>
<thead>
<tr>
<th>Treatment Groups (F0 Pregnant Mothers)</th>
<th>Offspring (F1 Pups)</th>
<th>Adulthood (F1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Control (n=5)</td>
<td>5♂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5♀</td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td></td>
<td>Behavioral,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biochemical,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expression &amp;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Morphometric</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Studies</td>
</tr>
<tr>
<td>10 mg/Kg (n=5)</td>
<td>5♂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5♀</td>
<td></td>
</tr>
<tr>
<td>50 mg/Kg (n=5)</td>
<td>5♂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5♀</td>
<td></td>
</tr>
<tr>
<td>100 mg/Kg (n=5)</td>
<td>5♂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5♀</td>
<td></td>
</tr>
</tbody>
</table>

**BEHAVIORAL STUDY**

All behavioral studies were conducted between 9am to 1pm and recorded (using Handycam) for later estimations. The following tests were performed:

**Forced Swim Test**

The forced swim test (FST) was used to assess the behavioral despair as described earlier (Porsolt, Le Pichon et al. 1977). Briefly, the rats were individually placed in the Plexiglas cylinder (25 x 10 cm), filled with water (17 cm depth) set at 25 ± 1°C, for 6 minutes. The recording of last 5 minutes was used for the estimation of immobility time, providing initial 1 minute for acclimatization.

**Elevated Plus Maze**

The elevated plus maze (EPM) was performed to evaluate the anxiety-like phenotype as described earlier (Pellow, Chopin et al. 1985). The maze consisted of four arms (length and width of 50 cm and 10 cm respectively) arranged in plus shape structure and elevated at the height of 80 cm. Two arms are closed with walls around them while the other two are open. The rats were individually placed at the middle of EPM for 10 minutes. The recordings were used to estimate the time spent and entrances in open arms.
Social Deficit Test

The social behavior of the rats were assessed as described earlier (Cox and Rissman 2011). Briefly, the rat from each treatment group were placed together in the cage (50 x 50 x 40 cm, pre-cleaned with ethanol 70%) and allowed to interact with each other for 10 minutes. Recording were used to assess various behaviors such as:

1. Social behavior such as interaction and allo-grooming.
2. Non social behavior such as self-grooming and rearing with support of the walls
3. Investigative behavior such as sniffing (nose, body, ano-genital) and following and Follow (walking behind and following the other mouse around the cage)

After behavioral estimations, the blood and brain was harvested for biochemical, gene expression and morphometric assessments as follows:

BIOCHEMICAL STUDY

The day after the last behavioral assessment, the rats were decapitated (9 am) to collect trunk blood and brain for biochemical studies, which are as follows:

Serotonin, 5-HIAA & Tryptophan Levels

One half of the brains’ homogenates were used for measuring the serotonin, 5-hydroxyindoleacetic acid (5-HIAA) and tryptophan concentrations using ELISA kits (Bioassay Technology Laboratory, England) as described in the manufacturer protocol. For homogenization, the hemisphere was dipped in chilled phosphate buffer saline (PBS, 1ml) and homogenized using small volume homogenizer (Hand Homogenizer Shanghai, China) followed by centrifugation at 7500 rpm to obtain the supernatant, which was stored at -80°C till analysis. Briefly, all reagents were prepared as described in the protocol and kept at room temperature. The required number of strips were inserted into the frames. The standard (50μl) and sample (40μl) were added into respective wells followed by addition of anti-ST antibody and streptavidin-HRP (50μl). After mixing, the plate was shielded with sealer and incubated (1 hour). Afterwards, the wells were washed 5x with wash buffer and blotted. After addition of substrate solution A (50μl) and substrate solution B (50μl), the plate was sealed and incubated again for 10 minutes in the dark. After addition of stop solution (50μl), the optical density (OD) value was determined immediately at 450 nm using microplate reader (Multiskan Sky Spectrophotometer, Thermo Fisher Scientific, USA).
**Corticosterone Levels**

Heparinized tubes were used for the collection of blood, which was centrifuged (3000 rpm for 15 minutes) to obtain plasma. The corticosterone was measured using ELISA kit as described in the manufacturer protocol (Bioassay Technology Laboratory, England)

**GENE EXPRESSION STUDY**

The expression levels of the genes of interest especially related to serotonergic system (SERT, 5-HT1A receptor and Tryptophan hydroxylase) and corticosterone (FKBP5, NR3C1, CRHR1 and CBG/SERPINA6), synaptic plasticity (PSD-95) and housekeeping gene (GAPDH) were estimated using qPCR as follows:

**RNA Isolation**

Prior to RNA isolation, pipettes, glassware and bench top was cleaned with RNAase spray. One hemisphere of brain was obtained and immediately washed with chilled and sterile PBS. After removal of PBS, the TRIzol reagent was added and homogenized for 15 minutes. After suspending Trizol reagent (1 ml), Chloroform (200 µ) was added (per 1ml of TRI zol-1/5 volume) to the mixture, vortexed (15 seconds) and incubated (20 minutes). The phases were separated by centrifuging (11,000 g for 30 minutes at 4 °C). The aqueous phase was carefully transferred into eppendorf tube. In this mixture, absolute isopropyl alcohol (0.5-1 ml) was added followed by incubation (30 minutes) and centrifugation (11,000 g for 30 minutes at 4 °C). After removal of supernatant, 70% ethanol (1ml) was added followed by brief vortexing. After centrifuged (11,000 g for 30 minutes at 4 °C), the supernatant was removed and air dried the RNA pallets inside the safety cabinet. The dried pellets were re-suspended in sterile nuclease free water (40 μl) and preserved (-80˚C) till further analysis.

**RNA Quantification**

The strength and purity of isolated RNA was checked by Multi Scan Sky spectrophotometer, Thermo Fisher Scientific, USA.

**cDNA Synthesis**

The cDNA was synthesized using Revert Aid First Strand cDNA synthesis Kit according to the manufacturer’s protocol (Thermo Fisher, USA). RNA (1 μg) was used for the
synthesis of cDNA in pre-chilled nuclease free micro-centrifuge tubes (200 μl) micro centrifuge tubes. The cDNA mixture was prepared as per following protocols (Table-2.2):

Table-2.2 Reaction mixtures for the cDNA synthesis

<table>
<thead>
<tr>
<th>Volume/concentration</th>
<th>Reagents/Mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μg</td>
<td>RNA</td>
</tr>
<tr>
<td>1 uL</td>
<td>Random Hexonucleotide Primer (50 μM)</td>
</tr>
<tr>
<td>10 μL</td>
<td>Nuclease Free Water</td>
</tr>
<tr>
<td>12 μL</td>
<td>Total volume</td>
</tr>
</tbody>
</table>

After short spin at 4°C, first incubation for 5 min at 70°C. Then positioned back on ice immediately.

| 4 μL                 | 5X Reaction buffer                                  |
| 1 μL                 | RiboLock RNase Inhibitor (20 U/ μL)                 |
| 2 μL                 | dNTPs Mix (10 m M)                                  |
| 19 μL                | Total Volume                                        |

Afterwards, the Revert Aid enzyme (1uL) was added in the mixture, the brief centrifugation was done followed by incubation (42°C for 1h). The reaction was stopped by heating (70°C) for 5 minutes. The synthesized cDNA was stored (-20°C) till further analysis (Macedo and Ferreira 2014).

**Primer Designing**

Primers were designed using the primer3 design program at http://frodo.wi.mit.edu/primer3/ (Koressaar and Remm 2007, Untergasser, Cutcutache et al. 2012) (Table-2.3). Each primer was reconstituted in 10 mM Tris-HCl/EDTA (TE) buffer (pH 8). Initially, the primer stocks (100 μM) was prepared from aforementioned 10mM vials followed by dilution (10 μM) in TE buffer (pH 8.0). The formula used for calculation of melting temperature (Tm) is as follows:

\[ Tm = 4(G + C) + 2(A + T) \]
### Table-2.3 Primer Sequences of the genes of interest

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing Temperature</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAPDH</td>
<td>F</td>
<td>GTGGACCTCATGGCCTACAT</td>
<td>57°C</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>GGATGGGAATTGTGAGGGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>TH</td>
<td>F</td>
<td>ACTGGCCACGTGCTATTCTTCT</td>
<td>56°C</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>TTGGAAGGTTGGTGATAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NR3C1</td>
<td>F</td>
<td>TACCACAGCTCACCTACC</td>
<td>56°C</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>AGCAGGGTCATTTGGTCATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CRHR1</td>
<td>F</td>
<td>GTTTATGGGCCCTGTGAGA</td>
<td>58°C</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>GTGACCCCTGCTTTCGCTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CBG/SERPINA6</td>
<td>F</td>
<td>GAATGAGACAAGCACCAGTG</td>
<td>58°C</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>TGTGTCCCGACTAAGTGCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>SERT</td>
<td>F</td>
<td>AGCGATGTGAAGGAGATGCT</td>
<td>56°C</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>ATGCAGTAGCCCAAGACGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5-HT1A</td>
<td>F</td>
<td>GGCTTTTCTCACCTCCATCC</td>
<td>55°C</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CCTTTTTCCACCTCCTCCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>FKBP50</td>
<td>F</td>
<td>CCTCCTTGTACGCAGTGTA</td>
<td>57°C</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>GAGCGAGGTATCTGCTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>PSD95</td>
<td>F</td>
<td>TGGGATGAGGTTAGGATGAG</td>
<td>57°C</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>AGAAACAGAGCAGGGAGATG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### qPCR

Expression level of genes of interest were analyzed by quantitative PCR. Experiment was carried out in triplicates for each gene of interest. The cDNA (0.4 μl) was added in 10 μl of 1X SYBR green Master in a PCR tube. In this mixture, the 9.6 μL of a particular primer in diluted form (1:100) was incorporated to make up total volume equals to 20 μl. The 40 cycles of denaturation, annealing and extension was run to obtain the CT values (Schmittgen and Livak 2008). Relative gene expression and relative fold change was calculated for each gene in all groups. GAPDH was used to normalize the expression. The fold change value was obtained with the help of following standard formula:

\[
\text{Ct gene of interest (GOI)} - \text{Housekeeping gene (HKG)}
\]

\[
\Delta \text{ACT} = \text{GOI-HKG}
\]

\[
\Delta \Delta \text{CT} = (\Delta \text{CT treated}-\Delta \text{CT untreated})
\]

\[
\text{Fold change} = 2^{\Delta \Delta \text{CT}}
\]
MORPHOMETRIC ANALYSIS

The brain of some rats were also used for histopathological and morphometric analysis as follows:

Collection & Preservation of Brains

After decapitation, one hemispheres of brain was dissected, washed and quickly placed in the fixative i.e. neutral buffered formalin (20 times greater than tissue volume). The samples were kept in fixative overnight and processed on next day.

Tissue Processing & Embedding

The tissue samples were dehydrated in graded alcohol using the following steps:

- 70% isopropyl alcohol for one hour; 3x
- 90% isopropyl alcohol for one hour; 1x
- 100% isopropyl alcohol for one hour; 3x
- Xylene for one hour; 3x
- Xylene + paraffin wax (1:1) for 20-30 min at 68°C in hot air oven
- Paraffin wax (100%) for overnight at 68°C in hot air oven

On the next day, the sample was embedded in the paraffin wax and tissue blocks were made using tissue molds and tissue embedding cassettes. The prepared tissue blocks was stable at room temperature and was subsequently used for sectioning.

Tissue Sectioning

In order to visualize the samples, 5 µm thin tissue sections were cut with the help of a microtome (Thermo Shannon Microtome, China). Sections were floated on the surface of the water having temperature 42°C for 5 minutes to remove the wrinkles. The sections were then fished out on gelatin coated glass slides and kept on slide warmer at 42°C for overnight to allow the proper attachment of the sections on the slides. After 24 hours, slides were places in slide box till further analysis.

Staining

Hematoxylin & Eosin (H&E) and silver staining were performed as follows:
**H&E Staining**

The following steps were performed for this staining:

1. Xylene I (10 to 15 minutes)
2. Xylene II (5 to 10 minutes)
3. 100% isopropyl alcohol (2 to 3 minutes)
4. 90% isopropyl alcohol (2 to 3 minutes)
5. 70% isopropyl alcohol (2 to 3 minutes)
6. Distilled water (15 to 20 minutes)
7. Hematoxylin (3 minutes)
8. Washed with distilled water
9. Eosin (30 seconds)
10. Washed with distilled water
11. 70% isopropyl alcohol (3 to 4 dips)
12. 90% isopropyl alcohol (3 to 4 dips)
13. 100% isopropyl alcohol (1 minute)
14. Xylene I (3 to 4 dips)
15. Xylene II (1 to 2 minutes)

At the end, the slides were mounted with DPX mounting media and kept on a clean tissue paper for 30 minutes for drying.

**Silver Staining**

The following steps were performed for this staining:

1. Xylene I (10 to 15 minutes)
2. Xylene II (5 to 10 minutes)
3. 100% isopropyl alcohol (2 to 3 minutes)
4. 90% isopropyl alcohol (2 to 3 minutes)
5. 70% isopropyl alcohol (2 to 3 minutes)
6. Distilled water (15 to 20 minutes)
7. Potassium permanganate (KMNO₄ for 5 minutes)
8. Potassium nitrate sulphate (2 minutes)
9. Iron Alum (10 minutes)
10. 10% silver nitrate (1 minute)
11. Formalin (1 minute)
12. Mounting with DPX
Finally, the slides were mounted using DPX and stored at room temperature for further analysis.

**Microscopy and Morphometry**

The aforementioned stained slides were examined under bright field microscope (Nikon Eclipse Inverted Microscope, Japan). Images were captured by using NIS-Elements D software and processed using Adobe Photoshop software. The images were used for the measurements of the following:

**Area & Cell Count**

These were used to calculate the total hippocampal area, total dentate gyrus (DG) area and granular cells count in the DG region.

**Sholl’s Analysis**

These were used for morphological changes in the neuronal dendritic tree using Sholl’s analysis (und Halbach 2013) as shown in figure 2.2.

![Figure-2.2 A representative diagram of Sholl’s analysis](image)

After the neuronal image was taken, it was placed on the trace paper pre-printed with the circles. The counting of ring intersections was noted to prepare Sholl profile, which gives an idea about dendritic alterations among various treatment groups.
STATISTICAL ANALYSIS

The data is presented as mean ± SEM of n = 5, 5, 3 and 3 per group for behavioral, biochemical, gene expression and histological assessments respectively. Differences among various means were computed using one-way ANOVA followed by post-hoc analysis (Least significant difference) using software (SPSS Version 20, SPSS Inc, Chicago, IL, USA).
Chapter 3

Results
BEHAVIORAL STUDY

The results of the behavioral study is as follows:

**Forced Swim Test**

The fluoxetine treatment (10, 50 or 100 mg/kg) caused dose dependent decrease in the immobility time of rats in both genders. However, the effect appeared to be more pronounced in female rats. (Figure-3.1)

![Figure-3.1 Effect of pre-weaning fluoxetine exposure on the immobility time of rats in FST upon adulthood](image)

The figure shows mean ± SEM (n=5 per group) of immobility time (IT) of adult rats in FST, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The IT shows dose dependent reduction in both genders as compared to control (C) group. * p<0.05 and *** (p<0.005) as compared to control.
Elevated Plus Maze

The fluoxetine treatment (10, 50 or 100 mg/kg) caused significant dose dependent increase in the time spent (Figure-3.2) and number of entries (Figure-3.3) in the open arm of elevated plus maze as compared to control, the effect more pronounced in female rats.

Figure-3.2 Effect of pre-weaning fluoxetine exposure on the time spent in open arm of elevated plus maze by rats upon adulthood

The figure shows mean ± SEM (n=5 per group) of time spent in open arm by adult rats in EPM, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows dose dependent increase in both genders as compared to control (C) group. * p<0.05 and *** (p<0.005) as compared to control.
Figure 3.3 Effect of pre-weaning fluoxetine exposure on the number of entries in open arm of elevated plus maze by rats upon adulthood

The figure shows mean ± SEM (n=5 per group) of number of entries in open arm by adult rats in EPM, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows dose dependent increase in both genders as compared to control (C) group. *** (p<0.005) as compared to control.
Social Deficit Test

The fluoxetine treatment (10, 50 or 100 mg/kg) caused significant dose dependent increase in the social behaviors of rats in both genders (Figure-3.4). The non-social behaviors showed significant increase in female rats alone (Figure-3.5), while the investigative behaviors remained statistically non-significant (Figure-3.6).

![Social Behavior (Male)](image)

**Figure-3.4 Effect of pre-weaning fluoxetine exposure on the social behavior of rats upon adulthood**

The figure shows mean ± SEM (n=5 per group) of duration of social behaviors (social interaction and allo-grooming) by adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows dose dependent increase of social behaviors in both genders as compared to control (C) group. * (p<0.05) and ** (p<0.01) as compared to control.
Figure 3.5 Effect of pre-weaning fluoxetine exposure on the non-social behavior of rats upon adulthood

The figure shows mean ± SEM (n=5 per group) of duration of non-social behaviors (self-grooming and rearing) by adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows dose dependent increase of non-social behavior in female rats only as compared to control (C) group. * (p<0.05) as compared to control.
Figure 3.6 Effect of pre-weaning fluoxetine exposure on the investigative behavior of rats upon adulthood

The figure shows mean ± SEM (n=5 per group) of frequency of investigative behaviors (sniffing and following) by adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows non-significant alterations as compared to control (C) group.
BIOCHEMICAL STUDY

The results of the biochemical study is as follows:

**Serotonergic Levels**

The fluoxetine treatment (10, 50 or 100 mg/kg) caused declining trend in brain serotonin levels, which achieve statistical significance in male rats alone at highest tested dose (Figure-3.7).

![Serotonin Levels Graph](image)

Figure-3.7 Effect of pre-weaning fluoxetine exposure on the brain serotonin levels of rats upon adulthood

The figure shows mean ± SEM (n=5 per group) brain serotonin levels in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows declining trend in both genders, which became statistically significant at highest tested dose as compared to respective control (C). * (p<0.05) as compared to control.
The 5-hydroxyindoleacetic acid (5-HIAA, metabolite of serotonin) also exhibit declining trend in the brain (Figure-3.8).

Figure-3.8 Effect of pre-weaning fluoxetine exposure on the brain 5-HIAA levels of rats upon adulthood

The figure shows mean ± SEM (n=5 per group) brain serotonin levels in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows declining trend (statistically non-significant) in both genders as compared to respective control (C).
The precursor of serotonin i.e. tryptophan showed significant dose dependent increase in both genders in both brain (Figure-3.9) and plasma (Figure-3.10).

**Figure-3.9 Effect of pre-weaning fluoxetine exposure on the brain tryptophan levels of rats upon adulthood**

The figure shows mean ± SEM (n=5 per group) brain tryptophan levels in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows dose dependent increase in both genders as compared to respective control (C). *(p<0.05), ** (p<0.01) and *** (p<0.005) as compared to control.
Figure 3.10 Effect of pre-weaning fluoxetine exposure on the plasma tryptophan levels of rats upon adulthood

The figure shows mean ± SEM (n=5 per group) plasma tryptophan levels in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows dose dependent increase in both genders as compared to respective control (C). *(p<0.05) and *** (p<0.005) as compared to control.
**Corticosterone Levels**

The fluoxetine treatment (10, 50 or 100 mg/kg) caused dose dependent decline in the basal plasma concentration of corticosterone levels in both genders as compared to control (Figure-3.11).

**Figure-3.11 Effect of pre-weaning fluoxetine exposure on the basal plasma corticosterone levels of rats upon adulthood**

The figure shows mean ± SEM (n=5 per group) basal (early morning) plasma corticosterone levels in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows dose dependent decrease in both genders as compared to respective control (C). *(p<0.05), ** (p<0.01) and *** (p<0.005) as compared to control.
GENE EXPRESSION STUDY

The results of gene expression study is as follows:

Serotonergic Genes

The fluoxetine treatment (10, 50 or 100 mg/kg) did not significantly alter the expression of serotonin transporter (SERT, Figure-3.12), 5HT\textsubscript{1A} receptor (Figure-3.13) and tryptophan hydroxylase (TPH, Figure-3.14) in the brain of both genders.

**Figure-3.12 Effect of pre-weaning fluoxetine exposure on brain SERT expression levels of rats upon adulthood**

The figure shows mean ± SEM (n=3 per group) of fold change of SERT expression in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows non-significant alterations as compared to control (C) in both genders.
Figure-3.13 Effect of pre-weaning fluoxetine exposure on brain 5HT1a expression levels of rats upon adulthood

The figure shows mean ± SEM (n=3 per group) of fold change of 5HT1a expression in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows non-significant alterations as compared to control (C) in both genders.
Figure-3.14 Effect of pre-weaning fluoxetine exposure on brain tryptophan hydroxylase expression levels of rats upon adulthood

The figure shows mean ± SEM (n=3 per group) of fold change of tryptophan hydroxylase expression in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows non-significant alterations as compared to control (C) in both genders.
**HPA-axis Genes**

The fluoxetine treatment (10, 50 or 100 mg/kg) did not significantly changed the expression of FKBP5 (Figure-3.15), CHRH1 (Figure-3.16) and SERPINA6 (Figure-3.17) in the brain of both genders. However, the NR3C1 expression was found to be significantly elevated in dose dependent manner as compared to control (Figure-3.18).

![FKBP5 (Male) Graph](image1)

![FKBP5 (Female) Graph](image2)

**Figure-3.15 Effect of pre-weaning fluoxetine exposure on FKBP5 expression levels of rats upon adulthood.**

The figure shows mean ± SEM (n=3 per group) of fold change of FKBP5 expression in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows non-significant alterations as compared to control (C) in both genders.
Figure 3.16 Effect of pre-weaning fluoxetine exposure on CRHR1 expression levels of rats upon adulthood.

The figure shows mean ± SEM (n=3 per group) of fold change of CRHR1 expression in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows non-significant alterations as compared to control (C) in both genders.
Figure 3.17 Effect of pre-weaning fluoxetine exposure on SERPINA6 expression levels of rats upon adulthood.

The figure shows mean ± SEM (n=3 per group) of fold change of SERPINA expression in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows non-significant alterations as compared to control (C) in both genders.
Figure 3.18 Effect of pre-weaning fluoxetine exposure on NR3C1 expression levels of rats upon adulthood.

The figure shows mean ± SEM (n=3 per group) of fold change of NR3C1 expression in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows significant dose dependent increase in the expression as compared to control (C) in both genders. *(p<0.05) and ** (p<0.01) as compared to control.
Post-synaptic Density 95 Gene

The fluoxetine treatment (10, 50 or 100 mg/kg) caused decline trend in the expression of PSD-95, which was found to be statistically significant in female rats at the doses of 10 and 50 mg/kg (Figure-3.19).

**Figure-3.19 Effect of pre-weaning fluoxetine exposure on PSD-95 expression levels of rats upon adulthood**

The figure shows mean ± SEM (n=3 per group) of fold change of PSD-95 expression in adult rats, whom mothers were treated with fluoxetine at doses of 10 (F10), 50 (F50) and 100 (F100) mg/kg during pre-weaning period (post-natal day 1 to 21). The data shows the declining trend, which was found to be significant in female rats alone at the doses of 10 and 50 mg/kg as compared to control (C). *(p<0.05) as compared to control.
MORPHOMETRIC STUDY

The fluoxetine treatment (10, 50 or 100 mg/kg) produced significant dose dependent rise in the morphometry (area and cell count) of dentate gyrus (DG) region of the brain in both genders (Table-3.1, Figure-3.20).

Table-3.1 Effect of pre-weaning fluoxetine exposure on brain morphometry of adult rats

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<td>Area (µm)</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>F50</td>
<td>244 ± 3***</td>
<td>668226 ± 17638***</td>
</tr>
<tr>
<td>F100</td>
<td>265 ± 3***</td>
<td>704599 ± 8819***</td>
</tr>
<tr>
<td>FEMALE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>161 ± 5</td>
<td>411913 ± 26458</td>
</tr>
<tr>
<td>F10</td>
<td>182 ± 2***</td>
<td>674599 ± 14530***</td>
</tr>
<tr>
<td>F50</td>
<td>245 ± 4***</td>
<td>684798 ± 18559***</td>
</tr>
<tr>
<td>F100</td>
<td>290 ± 2***</td>
<td>886681 ± 37118***</td>
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Figure-3.20a Effect of pre-weaning fluoxetine exposure on brain morphometry of female rats using H&E staining

The figure shows significantly decreased number of cells in dentate gyrus (DG) cells in control group as compare to fluoxetine treated (10, 50mg/kg and 100mg/kg) female rats.
Figure-3.20b Effect of pre-weaning fluoxetine exposure on brain morphometry of male rats using H&E staining

The figure shows significant increase in the fluoxetine treated rats (10, 50 and 100mg/kg) as compare to control group in male gender.
Figure-3.20c Effect of pre-weaning fluoxetine exposure on neuronal arborization of female rats using silver staining

The figure shows significant increase in neuronal arborization of fluoxetine treated (10, 50 and 100mg/kg) female rats as compare to control group.
Figure-3.20d Effect of pre-weaning fluoxetine exposure on neuronal arborization of male rats using silver staining

The figure shows significant increase in neuronal arborization of fluoxetine treated (10, 50 and 100mg/kg) male rats as compare to control group.
Additionally, the significant enhancement in the branching and arborization of hippocampal neurons were noted in both genders using Sholl’s analysis (Figure-3.21).

Figure-3.21 A representative drawing of Sholl’s analysis in various treatment groups

The figure shows the representative sketching of Sholl’s analysis for each treatment group [C (control), F10, F50 and F100 means 10, 50 and 100 mg/kg treatment groups]. An increase branching and arborization of neurons can be observed in a dose dependent fashion in both genders.
Chapter 4

General Discussion
Early (pre and post-natal) life of developing being is the most vulnerable time of life. The exposure of trauma (physical, chemical or environmental) during this sensitive life span can have long lasting impact on the wellbeing (physical and mental) of an individual in the later life to come. Emerging literature suggests that ignoring this concept during toxicological evaluation of pharmaceuticals is jeopardizing the mental health of human race. In this context, the inevitable use of antidepressants by depressed mothers leads to unwanted exposure of fetus or newborn to the drugs capable of interfering with the chemical process in the brain. Keeping in view the aforesaid scenario, the study was designed to assess the impact of pre-weaning fluoxetine (the most commonly used antidepressants) exposure on certain mental health indicators (depression, anxiety and social behavior) in offspring upon attaining adulthood.

Forced swim test (FST) is the widely used tool for the assessment of behavioral despair, a phenotypic presentation of depressive behavior (Porsolt, Le Pichon et al. 1977). Our data showed that litters exposed to fluoxetine in our experimental design gave lower immobility times (behavioral despair) in dose dependent manner as compared to control (Figure-3.1). This suggests that antidepressant like action intended for depressed mothers was engraved in the brains of litters, and was noticeable even after three months of drug free period given for reaching adulthood before behavioral assessment (Figure-2.1). Furthermore, our data revealed that female rats showed pronounced behavioral despair as compare to the male rats, which is in agreement with our earlier work suggesting that female rats are more sensitive to stressors while male rats exhibit more resilience (Zaman, Ahmad et al. 2017). Depression is a complex disorder and a large number of biochemical have been attributed to its pathogenesis. Among all, the serotonin neurotransmitters has been the key pharmacological target of antidepressant (Gronemann, Petersen et al. 2021). Therefore, the serotonergic system was selected in present study for subsequent biochemical and gene expression studies. Our data showed that the levels of serotonin (5-HT, 5-hydroxytrptamine) as well as its metabolite 5-HIAA (5-hydroxyindoleacetic acid) in the brain did not exhibit significant alterations (Figure-3.7 and 3.8, respectively). In similar lines, the level of serotonin in the brain was reported to have no association with the behavioral manifestations in FST (Abbas, Naqvi et al. 2011). Fluoxetine was reported to affect the composition of gut microbiota (Ramsteijn, Jašarević et al. 2020), which plays an important role in the metabolism of tryptophan (a synthetic precursor of serotonin). Therefore, the tryptophan levels were also measured and a
significant dose dependent rise was in both brain (Figure-3.9) and plasma (Figure-3.10) as compared to control. This suggest that early life fluoxetine exposure perturbs the microbiota composition to the one, which makes more tryptophan availability in both center and periphery. Keeping in view the increasing importance of gut-brain axis in health and disease, this outcome needs to be comprehensively studied. It is of note that despite of higher availability of tryptophan in the brain, there was lack of increase in serotonin levels. Hence, the tryptophan utilization in other pathways like kynurenine and its implication on mental wellbeing warrants further studies (Więdłocha, Marcinowicz et al. 2021). In search of genetic basis, important proteins (SERT, 5HT1A and TH) of serotonergic system were selected via literature review based on their involvement in depressive disorders (For details, refer to Chapter-1). Our data showed that the expression of none of these proteins were found to be significantly altered as compared to control (Figure-3.12, 3.13 and 3.14, respectively). This suggests that the transcriptome and proteome is strongly regulated and compensated along time following trauma. Another possibility for such outcome is due to the assessment of protein expression in whole brain samples, which may have masked any significant difference in the regions predominantly involved in depression (Rosa-Neto, Diksic et al. 2004, Levone, Moloney et al. 2021).

Elevated plus maze is the widely used tool to assess the anxiety like phenotype in rodents (Pellow, Chopin et al. 1985). Our data showed that early life fluoxetine exposure caused significant increase in the time spent (Figure-3.2) and entries (Figure-3.3) in the open arm of EPM. This is suggestive of decrease anxiety-like behavior as reported earlier (Dulawa, Holick et al. 2004). In similarity with FST data, the effect appeared be engraved in the brain of offspring. Anxiety is a complex phenomenon with both somatic and central effect and a number of biochemical were reported to underlie its pathogenesis. Among all, the corticosterone (regulated by HPA-axis) over-secretion has been considered as biomarker of anxiety (Myers and Greenwood-Van Meerveld 2010, Humer, Pieh et al. 2020). Our data showed significantly dose dependent reduction in basal plasma corticosterone levels in both genders of rats (Figure-3.11). This explains the lower anxiety levels observed in the rats under study. In search of the genetic basis, certain protein related to HPA-axis were selected from literature based on their reported role in anxiety development (For details, refer to Chapter-1) and their expression was noted in whole brain. Our data showed three of selected genes (FKBP5, CRHR1, SERPINA6) remained significantly unaltered (Figure-3.15, 3.16 and 3.17 respectively) as compared to
control. However, the NR3C1 (glucocorticoid receptor) upregulation was noted in both genders (Figure-3.18). This appears to be a homeostasis response to increase the corticosterone signal against its lower levels as supported by existing literature (Brady, Gold et al. 1992, Yau, Noble et al. 2004).

Social behavior deficit is one of the hallmark of developmental abnormalities such as autism spectrum disorder. Our data showed significant hyper-sociability in treated group as compared to control rats (Figure-3.4) as reported earlier in social deficit gerbils (Hendrie, Pickles et al. 2003). It is of note that this enhanced socialization supports our earlier depression and anxiety results. A subject with reduced anxiety and depression is more likely to socialize than vice versa. Furthermore, the female rats in our study showed more social behavior as compared to male rats, which is in line with existing literature on the role of gender in socialization (Peshkovskaya, Myagkov et al. 2017, Borland, Aiani et al. 2019). Furthermore, in female rats alone, the non-social behavior (self-grooming and rearing) showed increasing trend and received mild statistical significant at highest dose (Figure-3.5). This is in line the existing literature which demonstrate more self-care behavior in female subjects (Cameron, Sethares et al. 2017). The investigative behavior remained unaltered (Figure-3.6).

In search of biochemical indicator, the search of literature revealed no specific biomarker. However, in case of genetic basis, the post-synaptic density protein (PSD-95) was identified as highly relevant gene linked with social abnormality, specifically hyper-sociability (Barak, Zhang et al. 2019). Hence, it was selected for the study and was found to be down regulated in the brain of both genders with predominant effect in female rats (Figure-3.19). In conformity, the PSD-95 knockouts led to hyper-social behavior in both genders in rats (Winkler, Daher et al. 2018). Further to it, another study also conclude that the early life fluoxetine exposure down regulated hippocampal PSD-95 (Millard, Lum et al. 2019).

Fluoxetine is among the few medicine, which were reported to enhance neuron density (count, branching and arborization) specifically in dentate gyrus (DG) region hippocampus of the brain; an area known to possess the stem cells and therefore extensively considered in neurogenesis studies (Abbott and Nigussie 2020, Licht, Kreisel et al. 2020). Therefore, the hippocampal morphometry of rats were also performed. As expected, a significant increase in DG area and count along with greater dendritic
arborization was observed in treated rats as compared control ones (Table-3.1 and Figure-3.20-21). Furthermore, the enhanced neurogenesis have been linked with anti-depression action of fluoxetine (de Oliveira, Bolzan et al. 2020, Zavvari, Nahavandi et al. 2020). In the absence of serotonergic elevation, this enhanced neurogenesis may have contributed to the reduced immobility time observed in the FST.

The primary limitations of the present study includes the following:

- Use of single behavioral paradigm for particular mental condition
- Use of whole brain rather than specific regions for expression studies
- Taking data at one age end point i.e. adulthood

The future studies includes:

- Compensation for aforementioned limitations
- Along with expression, the translation shall also be measured
- Evaluating the effect of pre-natal fluoxetine exposure also
- Evaluation of microbiota composition in the gut
- Evaluation of anxiety and depression subjects born with early life fluoxetine exposure.

CONCLUSION

The present study deduced that the pre-weaning fluoxetine exposure affected the basal depression, anxiety and social behavior upon adulthood via perturbing tryptophan metabolism, corticosterone levels, NR3C1 and PSD-95 expression and hippocampal morphometry. It appears like this early life chemical stress permanently integrates with the basal functioning of brain and change the trajectory of development away from normal course. This deeply engraved impact was noticeable at all levels (behavior, biochemistry and gene expression) even at adulthood despite of long drug free time span in between fluoxetine exposure and experimental assessments. Hence, the present study highlights the potential of early life fluoxetine exposure in defining the mental health attributes in the society thereby stressing upon the need to incorporate long term neurobehavioral aspect as a component of developmental toxicity studies performed on active pharmaceuticals during drug development process.
REFERENCES


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APPENDICES
January 1st, 2020

To,
Mr. Nadeem,
Department: Pharmacology,
Faculty of Pharmacy,
Ziauddin University,
Clifton Campus,
Karachi.

Subject: Approval of research study by BASR

Dear Mr. Nadeem,

This is to inform you that the 272nd BASR, Ziauddin University has approved your PhD synopsis in pharmacology, titled, “Exploring Potential Link Between Neonatal Medicinal Exposure & Long Term Neurobehavioral Outcome: A Preclinical Study On Fluoxetine”. Your supervisor is Dr. Ghulam Abbas and co-supervisors are Dr. Shumaila Usman and Dr. Rehan Ahmed Siddiqui.

Prof. Dr. Saeeda Bajir,
(Secretary BASR).
October 07, 2019

Dr. Nadeem
Ph.D. Student,
Department of Pharmacology,
Faculty of Pharmacy,
Ziauddin University

Subject: Animal Study Protocol (ASP) Approval

Protocol No.: 2019-004
Project Title: EXPLORING POTENTIAL LINK BETWEEN NEONATAL MEDICINAL EXPOSURE & LONG TERM NEUROBEHAVIORAL OUTCOME: A PRECLINICAL STUDY ON FLUOXETINE

Approved Animal Number/Species: 40 Sprague Dawley Rats

The Ziauddin University Animal Ethics Committee (AEC) approves your ASP referenced above for 18 months period (w.e.f. BASR approval). You may use the animals as per approved ASP.

For the Committee,

[Signature]

Prof. Dr. Anwar Ejaz Beg
In-charge
Animal Ethics Committee (AEC)
Ziauddin University

ST, A-8, Block - 6, Scheme 5, CI Hort, Karachi.
Phone: 9221 35862937, Fax: 9221 35833672, Email: info@zu.edu.pk, Web: www.zu.edu.pk
Subject: Letter of Recommendation by RAC

Dear Dr. Nadem,

Thank you for submitting your study proposal to RAC Ziauddin University. RAC has reviewed the project and recommends its further processing.

Title of study: “Exploring Potential Link Between Neonatal Medicinal Exposure & Long Term Neurobehavioral Outcome: A Preclinical Study On Fluoxetine”.

Supervisor: Dr. Ghulam Abbas
Co-supervisor: Dr. Rehan Ahmad, Dr. Shumaila Usman

Prof. Talat Mirza
Chairperson RAC
Ziauddin University, Karachi.

Date: 8th/10
Certificate of Originality Checked by "Turnitin"

Name: Nadeem
Father Name: Haji Ali Akher
Department: Pharmacology
College: Faculty of Pharmacy
Program Name: PhD
Registration. No: 9-4/2015/001
Supervisor Name: Dr. Ghulam Abbas
Designation: Associate Professor
Title of the Research: Exploring Potential link between Neonatal Medicinal Exposure and long term Neurobehavioral outcome: A Preclinical Study on Fluoxetine.

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Faculty of Pharmacy
Date:
EXPLORING POTENTIAL LINK BETWEEN NEONATAL MEDICINAL EXPOSURE & LONG TERM NEUROBEHAVIORAL OUTCOME: A PRECLINICAL STUDY ON FLUOXETINE

by Mr. Nadeem
EXPLORING POTENTIAL LINK BETWEEN NEONATAL MEDICINAL EXPOSURE & LONG TERM NEUROBEHAVIORAL OUTCOME: A PRECLINICAL STUDY ON FLUOXETINE

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Pre-weaning fluoxetine exposure perturbs social behavior at adulthood via altering hippocampal morphometry and PSD-95 expression in rats

Nadeem1,2, Rehan Ahmed Siddiqi2, ShumailaUsman3, Uzair Nisar1 and Ghulam Abbas4*
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2Institute of Pharmacy, Shauheed Mohtarma Benazir Bhutto Medical University, Larzina, Pakistan
3Department of Research, Ziaoddin University, Karachi, Pakistan

Abstract: The depression during and after pregnancy cause significant exposure of fluoxetine to the child at early life through mother. This exposure to the child, during the vulnerable window of development, can have a long lasting impact on overall mental wellbeing. Long term neurobehavioral aspect of developmental toxicity is neglected as the part of testing requirements in the process of drug developmental. In this context, the present study was designed to study the possible effect of pre-weaning fluoxetine exposure on the social behavior of rats upon adulthood followed by assessing hippocampal morphometry (hematoxylin-eosin and silver staining) and post-synaptic density protein 95 (PSD-95) expression (using qPCR). Our data showed that the fluoxetine exposure (10, 50 and 100mg/kg) caused predominant increase in the social behavior of rats; the effect more pronounced in female rats. The morphometric analysis revealed significant increase in cell population and count of dentate gyrus (DG) region of hippocampus along with enhanced dendritic arborization. Furthermore, the PSD-95 expression was found to be down regulated in the fluoxetine treated group as compared to control. In conclusion, the present study demonstrate that the early post-natal exposure to fluoxetine cause hypersociability upon attaining adulthood, which may be attributed to enhanced neuronal proliferation and decrease PSD-95 expression in the hippocampus.

Keywords: Pregnancy, depression, fluoxetine, hypersociability, brain morphometry, PSD-95.

INTRODUCTION

The communication among living being enhances their capacity to establish and maintain a social life, which is essential for survival. This social communication heavily depends upon the motivation to recognize and interact fellow beings; the phenomenon coordinated by various brain structures including prefrontal cortex, amygdala, nucleus accumbens, anterior insula, anterior cingulate cortex, hippocampus, and temporal-mucus (Gao and Mack 2021). However, under certain neurodevelopmental disorders such as Autism spectrum disorders (Keifer et al., 2020), Williams Syndrome (Jabbi et al., 2012) and Schizophrenia (Murphy et al., 2020), the abnormal social behavior (both hypo and hypersociability) can be observed. The basis of such social deficit is not completely understood. However, two major underlying processes have been identified as regulator of social behavior. Firstly, the discrimination between fear and friendly social signal; the process coordinated by amygdala to produce a suitable behavioral response. Secondly, the reward and aversion mechanism, which is primarily under the regulation of dopamine. This system cause the subject to approach or avoid the social contact. Hypersociability can be explained as the developmental abnormality causing failure in discrimination between familiar and stranger or increased reward or reduced aversion signals (Toth, 2019).

Post-synaptic density protein 95 (PSD-95) is the most abundant scaffolding protein in the excitatory glutamatergic synapses in the central nervous system (Purcell et al., 2014, Rodzi et al., 2020). During neurodevelopment, it was reported to cause synaptic maturation via recruiting glutamatergic receptors at post-synaptic membrane (Purcell et al., 2014). Search of literature revealed the robust hypersocial behavior in PSD95 knock out rats, both males and females (Winkler et al., 2018). PSD-95 is considered as a risk gene for hypersociability due to its role in malfunction of prefrontal cortex (Barak et al., 2019).

Depression is the central nervous system disorder, which may victimize females during critical time of pregnancy with an estimated prevalence of 10% in a year (Gaynes et al., 2005, Vitte and Mutuz, 2021). In Pakistan, the estimates are far higher i.e. 37% for antenatal depression and 30% of postnatal depression (Atil et al., 2021). Presumably, the use of antidepressants during pregnancy is increasing with time and an estimated 1 in 10 women use them, especially fluoxetine (Cooper et al., 2007). It is a block buster antidepressant, which enhance the levels of serotonin in the synapse by inhibiting its uptake through serotonin transporter (SERT) (Cipriani et al., 2005, Rossi et al., 2004). The pregnancy associated depression ultimately exposes the new born to the drugs (chemicals) during vulnerable window of development (Wu Jong and Einarson, 2021). This is the time, when most of the physiological and psychological parameters are set in the

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body. Any exposure (physical chemical or environmental) could have a long lasting impact on the mental wellbeing of the progeny.

In early 1960s, the thalidomide incidence led to global realization that medicines have the potential to harm fetus. Presumably, the interest in this area has led to the incorporation of guidelines for preclinical developmental toxicity testing for pharmaceutical agents. Hence, the Food & Drug Administration (FDA) has assigned various categories (A, B, C, D, X) to all drugs based on the level of risk the drug poses to the fetus (Osborne et al., 2020).

The long-term neurobehavioral aspect of developmental toxicity is an area, which unfortunately could not catch the attention of the policy makers in past. This is the reason why no such guidelines are available for said testing for pharmaceutical agents, a potential risk yet to be completely deciphered. However, this deficiency in testing was acknowledged in the literature (Ulrich and Palmer, 1996). Search of literature revealed few reports which are suggestive of aforementioned behavioral deficits. One such study conclude that that a 2nd-trimester maternal influenza infection may increase risk for adult schizophrenia or major affective disorder via disrupting the development of the fetal brain (Watson et al., 1999).

An important study reported higher incidence of attention deficit hyperactive disorder (ADHD) in children from the mothers administer acetaminophen (over-the-counter analgesic) during pregnancy (Lie et al., 2014). Another study has linked adverse developmental outcomes such as psychomotor development, externalization behavior in children exposed to acetaminophen during pregnancy (Brandts et al., 2013). Hence, these reports are suggestive of the long term neurobehavioral toxicity of the medicines and associated ailments. In this context, the present study was designed to observe the long lasting impact of pre-weaning fluoxetine exposure on the social behavior of rats along with evaluation of potential underlying causes at expression and morphometric levels.

MATERIALS AND METHODS

Chemicals

The following chemicals were used in the study: Agarose was obtained from Sigma (Germany); Chloroform from Scharlau (Spain); DNA ladder (100bp) and ethanol from Thermo Fisher Scientific (USA); Ethidium bromide from MP Biomedical (USA); Taq green master mix from Promega (USA) and Trizol reagent from Invitrogen (TISA). Fluoxetine was obtained from Lilly pharmaceuticals (Pakistan).

Animals

Female Sprague Dawley (Pregnant) Rats were obtained from Animal Resource Facility of International Center for Chemical and Biological (ICCBS), University of Karachi. They were housed separately with free access to food and water. The temperature was kept at 25°C while humidity was 60%. The 12 hour light and dark cycle was also maintained throughout the course of study. The F1 generation obtained, after weaning begins, were separated from the dams and housed gender-wise in a group of 5 rats per cage. All experiments were performed according the ethical guidelines provided the Animal Ethics Committee of the University (Approval No. 2019-004).

Experimental design

At post-natal day 1, the dams (F0 generation) were given fluoxetine at 10 (F10), 50 (F50) or 100 (F100) mg/kg daily for 3 weeks (average pre-weaning time) by dissolving the required amount of drug in the minimum drinking water (approximately 5 ml). The control group (C) received drinking water alone. After 3 weeks, the pups were fed with regular diet and allowed to reach adulthood (3 months). The males and females rats from F1 generation was subjected to behavioral, biochemical, gene expression and morphometric assessments. The schematic diagram of the experimental design is shown in fig. 1.

Social behavior study

The social behavior of the rats were assessed as described earlier (Cox and Rissman, 2011). Briefly, the rat from each treatment group were placed together in the cage (50 x 50 x 40 cm, pre-cleaned with ethanol 70%) and allowed to interact with each other for 10 minutes. The following behaviors were noted:

1. Social behavior such as social interaction and allo-grooming
2. Non-social behavior such as self-grooming and rearing with support of the walls
3. Investigative behavior such as Ano-genital sniff (sniffing the other rats' ano-genital region) Nose sniff (sniffing the other rats' nose), Body sniff (sniffing the other rats' body in any part other than nose or the ano-genital region) and Follow (walking behind and following the other animal in the cage).

![Fig. 1: Schematic diagram of the experimental design](image-url)
All behavioral studies were conducted between 9am to 1pm and recorded using HandyCam for later estimations. After behavioral estimations, the brain was harvested for morphometry gene expression studies.

**Morphometric analysis**
The brains of rats were used for morphometric analysis as follows:

**Collection, Preservation & Processing**
After decapitation, one hemisphere of brain was dissected, washed and quickly placed in the fixative i.e. neutral buffered formalin (20 times greater than tissue volume). The samples were kept in fixative overnight and processed on next day. The tissues were dehydrated in graded alcohol followed by embedding in paraffin and tissue blocks were made using tissue molds and tissue embedding cassettes. The prepared tissue blocks were stable at room temperature and was subsequently used for sectioning (5 mm) with the help of a microtome (Thermo Shannon Microtome, China). The sections were collected on the slides for staining as follows:

**H&E Staining**
After rehydration of sections, the sections were placed in hematoxylin (3 minutes) followed by eosin (30 sec) after washing with distill water. At the end, the slides were mounted with xylene soluble DPX mounting media and kept on a clean tissue paper for 30 minutes for drying. These were used to calculate total dentate gyrus (DG) area and granular cells count in the DG region present in the hippocampus of the brain.

**Silver Staining**
After rehydration of sections, the sections were placed in permanganate (KMnO4, for 5 minutes), potassium nitrate sulphate (2 minutes), iron alum (10 minutes), silver nitrate (10% for 1 minutes) and formalin (1 minute) followed by mounting with DPX and stored at room temperature for further analysis. These were used for morphological changes in the neuronal dendritic tree using Sholl’s analysis (und Halbach, 2013) as shown in fig. 2. After the neuronal image was taken, it was placed on the trace paper containing circles. The number of branches and counting of ring intersections were noted to prepare Sholl’s profile; which gives an idea about dendritic alterations among various treatment groups.

**Microscopy**
The aforementioned stained slides were examined under bright field microscope (Nikon Eclipse inverted Microscope, Japan). Images were captured by using NIS-Elements D software and processed using Adobe Photoshop software.

**Gene expression studies**
The expression levels of post-synaptic density protein (PSD-95) was estimated using qPCR. GAPDH was used as housekeeping gene. Briefly, the total RNA was isolated using TRIzol reagent followed by assessment of its concentration and purity using Multi Scan Sky spectrophotometer, Thermo Fisher Scientific, USA. Afterwards, the cDNA was synthesized using Revert Aid First Strand cDNA synthesis Kit according to the manufacturer’s protocol (Thermo Fisher, USA) (Macedo and Ferreira, 2014). The synthesized cDNA was stored at -20°C till further analysis.

![Fig. 2: A representative diagram of Sholl’s analysis](image)

**Primer designing**
Primers have been designed using the primer3 design program at [http://frodo.wi.mit.edu/primer3/](http://frodo.wi.mit.edu/primer3/) (Untergasser et al., 2012, Koressaar and Remm, 2007) and the sequences are given below. Each primer was reconstituted in 10mM Tris-HCl/EDTA (TE) buffer (pH 8). The primer stocks (100μM) was prepared from master primer vials and further diluted to 10μM in TE buffer (pH 8.0). Melting temperature (Tm) for each primer was calculated by using the following formula:

\[ Tm = 4(G+C) + 2(A+T) \]

**qPCR**
Expression level of genes of interest were analyzed by quantitative PCR. Experiment was carried out in triplicates for each genes of interest. The cDNA (0.4μl)
Pre-weaning fluoxetine exposure perturbs social behavior at adulthood via altering hippocampal morphometry

Fig. 3: Effect of pre-weaning fluoxetine exposure on social behavior of rats upon adulthood. The fig. depicts the effect of pre-weaning fluoxetine (10, 50 and 100mg/kg) on the social behaviors (social, non-social and investigative) of rats. Both social and non-social behavior either showed elevated trend or significant rise. Data is presented as mean ± SEM (n = 5). Asterisks showed the significant difference as compared to control, *p<0.05 and **p<0.005

was added in 10μl of 1X SYBR green Master in a PCR tube. In this mixture, the 9.6μL of a particular primer in dilute form (1:1000) was added to make up total volume of 20μl. The 40 cycles of denaturation, annealing and extension were run to obtain the CT values (Schmittgen and Livak, 2008). Relative gene expression and relative fold change was calculated for each gene in all groups. GAPDH was used to normalize the expression.

STATISTICAL ANALYSIS

The data is presented as mean ± SEM of n = 5, 3 and 3 per group for behavioral, morphometric and expression studies, respectively. Differences among various means were computed using one-way ANOVA followed by post-hoc analysis (Least significant difference) using SPSS software (Version 20, SPSS Inc, Chicago, IL, USA)

RESULTS

Social behavioral study

Our data showed that pre-weaning fluoxetine exposure significantly enhanced the social behavior in both genders in dose dependent manner (fig. 3). The effect appeared to be more pronounced in female rats. The non-social behavior also exhibited increasing trend in male rats while it became significantly elevated in case of female rats at highest tested dose. However, the investigative behavior remained unaltered.

Morphometric study

The fluoxetine treatment (10, 50 and 100 mg/kg) caused significant increase in the dentate gyrus morphometry (cell count and area) in dose dependent manner in both genders (table 1). The sholl’s analysis also revealed significant rise in neuronal branching and dendritic arborization in both genders (fig. 4).

Gene expression study

The pre-weaning fluoxetine exposure caused down regulation of PSD-95 gene in dose dependent manner in both genders as compared to control (fig. 5). The difference was found to be significant at the dose of 10 and 50 mg/kg in female rats only.

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DISCUSSION

Pre-natal and early post-natal life is the vulnerable window of development in the life of a developing being. Any trauma (physical, chemical or environmental) during this stage can have long lasting consequence on the overall wellbeing of an individual. In this context, the long term neurobehavioral impact of pharmaceuticals is ignored as the component of developmental toxicity studies. Emerging concepts revealed that this lack of testing is jeopardizing the mental health of humans by exposing them to harmful pharmaceuticals. Keeping this in view, the present study was designed to study the impact of pre-weaning fluoxetine exposure on the social behavior upon attaining adulthood.

Deficit in social behavior, either hypo and hyper, has been attributed to developmental abnormalities and is observed in certain indications such as autism spectrum disorder. Our data showed that the early post-natal fluoxetine exposed rats predominantly demonstrated hypersociability upon adulthood (fig. 5). In similar lines, the fluoxetine treatment was reported to enhance social behavior in pre-existing social deficit gerbils (Hendrie et al., 2003). On the contrary, the reduced social behavior was reported in prairie voles adults exposed early to fluoxetine (Lawrence et al., 2020). Keeping aforementioned in view, one possible explanation could be the decisive role of specie in defining the outcome of fluoxetine on social behavior. However, the experiment on prairie voles involved subcutaneous painful injections to the mother and higher anxiety levels were observed in the animals too, which may have contributed to reduce socialization as described earlier (Toth, 2019). On the contrary, our experimental design does not involve any painful procedure for the administration of fluoxetine, which may result in opposite outcome. Our data further showed that female rats are more sensitive towards hypersociability induced ability of fluoxetine, which is suggestive of the role of gender in defining the intensity of effect. In similar lines, the vulnerability of female gender towards behavioral alterations following chemical and environmental stressors has been reported earlier (Dagh, 2013, Zaman et al., 2017). It is of note that non-social behavior demonstrated increasing trend, which became mildly significant at the highest tested dose in case of female. One possibility for this outcome is enhanced locomotor activity, which may have contributed in enough physical activity to do self-grooming along with the grooming of other rats at higher doses.

Fluoxetine was reported to enhance neurogenesis and dendritic arborization; the effect attributed to its antidepressant action (Zavvari et al., 2020, de Oliveira et al., 2020). Dentate gyrus is an area in the hippocampus of the brain, which was reported to possess the stem cell and has been extensively studied in neurogenesis studies (Licht et al., 2020, Abbott and Nigussie, 2020). Keeping this in view, the hippocampal morphometry of rats were performed. In line with the existing literature, the developmentally fluoxetine exposed rats revealed significantly higher area and cells of DG along with superior dendritic arborization (fig. 4, table 1). The abnormally enhanced neurogenesis has been attributed to underlie pathogenesis of social disorder (such as autism) during developmental stage (Packer, 2016, Kanishk and Zarbalis, 2016). Although, the connection between neurogenesis and social disorder is yet to be established, our study provides the direct evidence reporting co-existence of enhanced hippocampal density (cellular count, area and neuronal arborization) and hypersociability in same animals, an outcome worthy of further investigations to delineate the underlying mechanisms of social disorders.

![PSD-95 (Male)](image)

**Fig. 5:** Effect of pre-weaning fluoxetine exposure on PSD-95 expression in rat brain. The fig. depicts the PSD-95 expression in the brains of rats subjected to fluoxetine (10, 50 and 100 mg/kg) during pre-weaning time period and compared to control. The general down regulation of the gene was observed, which was found to be statistically significant in female rats at 10 and 50 mg/kg. The data is presented as mean ± SEM of relative fold change as compared to control. Asterisks represent significant difference as compared to control (*p<0.05).

Post-synaptic density protein (PSD-95) has been considered as an important gene predisposing subject to social deficits, especially hypersociability (Barak et al., 2019). Therefore, its expression was also studied in the brain of rats. Our data showed its general down regulation in the brains of both gender, with slight significant changes in females (fig. 5). In similar lines, the PSD-95 knockouts were previously shown to demonstrate hypersocial behavior in both males and females rats.
Pre-weaning fluoxetine exposure perturbs social behavior at adulthood via altering hippocampal morphometry

Table 1: Effect of pre-weaning fluoxetine exposure on brain morphometry

<table>
<thead>
<tr>
<th>Group</th>
<th>Dentate Gyrus (DG) Morphometry</th>
<th>Sholl’s Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DG cells (count)</td>
<td>DG area (μm²)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MALE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>161±3</td>
<td>41858±213858</td>
</tr>
<tr>
<td>F10</td>
<td>187±2***</td>
<td>60793±215275</td>
</tr>
<tr>
<td>F50</td>
<td>244±3***</td>
<td>66822±17636</td>
</tr>
<tr>
<td>F100</td>
<td>263±3***</td>
<td>70459±8819***</td>
</tr>
<tr>
<td>FEMALE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>161±5</td>
<td>411913±26558</td>
</tr>
<tr>
<td>F10</td>
<td>182±2***</td>
<td>65459±14536</td>
</tr>
<tr>
<td>F50</td>
<td>245±4***</td>
<td>684798±18550</td>
</tr>
<tr>
<td>F100</td>
<td>290±2***</td>
<td>886681±371137</td>
</tr>
</tbody>
</table>

Fig. 4: A representative diagram of Sholl’s analysis. The fig. depicts the representative sholl’s analysis for morphometric assessment of neurons following silver staining. The “C” represents control, while F10, F50 and F100 represents the treatment groups who have received fluoxetine at the dose of 10, 50 and 100 mg/kg during pre-weaning time period. Enhanced neuronal branching and arborization was observed in test group in a dose dependent manner.

(Winkler et al., 2018). Furthermore, the early life fluoxetine exposure was also reported to reduce the expression of PSD-95 in the hippocampus (Millard et al., 2019). It is of note that our data revealed slightly increasing trend in PSD-95 expression at highest testes dose of 100 mg/kg. Search of literature also revealed that fluoxetine was reported to slightly increase the expression of PSD-95 (O’Leary et al., 2009) and was reported to underline its antidepressant action too in adult animals (Reinicke et al., 2008). The aforementioned literature is suggestive of age dependent effect of fluoxetine on the expression of PSD-95. This is in line with emerging concept that immature brain incorporates information into its structure and function differently than the mature brain (Andersen, 2003). Hence, age dependency can possibly be explained in a manner that extensive neuronal growth and proliferation at pre-synaptic level may be balanced by reduction in post-synaptic density at early age. However, the adult predominantly post mitotic brain does not need such homeostatic manipulation of the PSD-95 gene. In this regard, the decisive role of strong homeostasis in brain has been reported earlier (Abbasi et al., 2012, Abbas et al., 2011). However, further work is required to delineate this explanation.

CONCLUSION

the present study demonstrate that pre-weaning fluoxetine exposure predominantly caused hypersociability upon adulthood, which can be attributed to enhanced hippocampal morphometry and reduced expression of PSD-95. Hence, the present study highlights the potential underlying role of fluoxetine in setting the social behavior observed within the society.

REFERENCES


