Identification and Characterization of Hormonal Response in Depression Induced Mice Models

Author

Noor-Ul-Ain Ilyas

Regn Number

328421

Supervisor

Dr. Aneeqa Noor

DEPARTMENT OF BIOMEDICAL ENGINEERING AND SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY

ISLAMABAD, PAKISTAN

October, 2022

Identification and Characterization of Hormonal Response in Depression Induced Mice Models

Author

Noor-Ul-Ain Ilyas

Regn Number

328421

A thesis submitted in partial fulfillment of the requirements for the degree of

MS Biomedical Sciences

Thesis Supervisor:

Dr. Aneeqa Noor

Thesis Supervisor's Signature:

_____

DEPARTMENT OF BIOMEDICAL ENGINEERING AND SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY

ISLAMABAD, PAKISTAN

October, 2022

DECLARATION

I certify that this research work titled ***"Identification and Characterization of Hormonal Response in Depression-Induced Mice Models"*** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged/referred.

Signature of Student _____

Noor-Ul-Ain Ilyas

Regn No: 328421

MS Biomedical Sciences

PLAIGIRISM CERTIFICATE (TURNITIN REPORT)

This thesis has been checked for Plagiarism. Turnitin report endorsed by Supervisor is attached.

Signature of Student
Noor-Ul-Ain Ilyas

Regn No: 328421

Signature of Supervisor

COPYRIGHT STATEMENT

- Copyright in text of this thesis rests with the student author. Copies (by any process) either in full, or of extracts, may be made only in accordance with instructions given by the author and lodged in the Library of NUST School of Mechanical & Manufacturing Engineering (SMME). Details may be obtained by the Librarian. This page must form part of any such copies made. Further copies (by any process) may not be made without the permission (in writing) of the author.
- The ownership of any intellectual property rights which may be described in this thesis is vested in NUST School of Mechanical & Manufacturing Engineering, subject to any prior agreement to the contrary, and may not be made available for use by third parties without the written permission of the SMME, which will prescribe the terms and conditions of any such agreement.
- Further information on the conditions under which disclosures and exploitation may take place is available from the Library of NUST School of Mechanical & Manufacturing Engineering, Islamabad.

DEDICATION

Dedicated to my beloved Parents

ACKNOWLEDGMENTS

I would like to acknowledge and give my warmest thanks to my supervisor (Dr. Aneeqa Noor) who made this work possible. Her guidance and advice carried me through all stages of writing my project. I would also like to thanks to committee members to make my defense enjoyable moment and for your brilliant comments and suggestion, thanks to you.

I would also like to give my special thanks to my parents, my husband and my family as a whole for continuous support and understanding when undertaking my research and writing project. Your prayers for me was what sustained me so me so far.

Finally I would like thank Allah for letting me through all the difficulties. I have experienced your guidance day by day. You are the one who let me finish my degree. I will keep on trusting you for my future.

ABSTRACT

Background: Early life stress is correlated with escalated peril for anxiety, mood, substance, impulse control and depressive disorders. Such disorders might spring from chemical imbalance in brain. A well-grounded mouse prototype of childhood adversity contributing to the everlasting behavioral changes in an individual would help understand the mechanism underlying these adverse effects. Maternal deprivation is frequently used paradigm of early neglect. Natriuretic peptides especially Atrial Natriuretic Peptide (ANP) released from Atrial myocytes have significant anxiolytic role corroborated by certain animal and human trails. The concentration of ANP has been found to be elevated by Levothyroxine (LT4) which treats the adverse symptoms of anxiety and depression.

Method and Results: In an attempt to contrive the paradigm of childhood adversity in mouse models with everlasting impacts on behavior of balb/c mice, maternal separation model followed by early weaning model were developed and behavioral tests were performed 60 days following maternal separation followed by early weaning (MSEW) paradigm for the validation of model. The experimental and control groups were further divided into 3 groups: MSEW group with drug administration, MSEW group with no drug administration and control group with no MSEW and no drug administration. Following a 7 days administration of LT4 at the concentration of 15 micrograms per mice to MSEW group and one of the control groups, decline in anxiety level in mice subjected to MSEW was observed in comparison to the MSEW group that was not administered LT4. These findings were validated by performing anxiety related paradigms after drug injection and the difference in the behavior was observed accordingly which suggested decrease in behavioral despair and anxiety related symptoms in mice.

Conclusion: Our findings elucidate that maternal separation model followed by early weaning contributes as a substantial paradigm to scrutinize the intricate behavioral anomalies in organisms with early life adversity history and by increasing the concentration of ANP by injection of LT4 cures the anxiety-related symptoms and provides a n apprehension for a futuristic therapeutic plan of actions.

TABLE OF CONTENT

CHAPTER 1 INTRODUCTION 1

CHAPTER 2 LITERATURE REVIEW 4

2.1 Depression and Anxiety 4

2.2 Maternal Separation Model of Depression 5

2.2.1 Depression caused by maternal separation 5

2.3 Natriuretic Hormones 7

2.3.1 Physiological functions of NP 7

2.3.2 Natriuretic peptides in brain 7

2.3.3 Role of Natriuretic peptides in behavior 8

2.4 Atrial Natriuretic Peptide and its Anxiolytic Role 8

2.5 Thyroid Hormone Increases ANP Concentration 9

2.5.1 Thyroid hormone for treatment of mental disorders 10

2.5.2 Thyroid hormone increases ANP concentration 10

3.1 Ethics Statement 12

3.2 Animals 12

3.3 Study Design 12

3.3.1 Dose determination 13

3.4 Behavioral Testing 14

3.4.1 Elevated Plus Maze 14

3.4.2 Forced swim test 15

3.4.3 Open field test 15

3.4.4 Light dark test 15

3.5 Statistical Analysis 16

3.6 *In silico* Approach to Determine Protein (ANP) and Ligand(LT4) Interaction 16

3.6.1 Software 16

3.6.2 Software and online resources 16
3.6.2.1 RCSB PBD 16
3.6.2.2 PubChem 17
3.6.2.3 Discovery Studio 17
3.6.2.4 PyRx 17
3.6.3 Selection of target protein 17
3.6.4 Selection of ligand or Drug target molecule 18
Figure 3: Structure of Levothyroxine downloaded from PyRx. Three-dimensional structure of Levothyroxine visualized from PyRx, chemical formula: C15H11I4NO4, weight: 776.87002, and number of atoms=35. 18
3.6.4 Preparation of Ligand and protein 18
3.6.6 Molecular docking through PyRx 20
3.6.6.1 PyRx 20
3.6.6.2 Docking 20
3.6.6.3 Minimization of energy 20
3.6.6.4 Docking 21
3.6.6.5 Ligand- receptor interaction 21
Chapter 4 Results 22
4.1 Mouse Model of Anxiety 22
4.2 Behavioral Analysis 22
4.2.1 Increased aversion of mice towards open and elevated areas 22
4.2.2 Reduced movements: 23
4.2.3 Diminished exploratory behavior and exposure to light 24
4.2.4 Reduced mobility as a consequence of MSEW 25
4.3 Effect of administration of LT4 to increase ANP level on behavior of mice 25
4.3.1 Behavioral elucidation after treatment with LT4 26
4.3.1.1 Decreased aversion to elevated areas 26
4.3.1.2 Increased movement: 28
4.3.1.3 Increased mobility 28
4.3.1.3 Increased exploratory behavior 29

4.4 In-Silico Results 30
4.4.1 PyRx in Vina interpretation.....30
4.4.2 Binding energy evaluation 30
4.4.3 Ligand and protein interaction evaluation.....31
4.4.4 Discovery studio visualization studies.....32
Chapter 5 Discussion 34
5.1 Elevated plus maze test.....35
5.2 Open field test depicts depressive response in mice.....35
5.3 Light/dark chamber test 35
5.4 Forced swim test.....36
CHAPTER 6 CONCLUSION.....38
Chapter 7 BIBLIOGRAPHY.....39

LIST OF FIGURES

Figure 1: Study timeline depicting 74 days long procedure for developing MSEW model……12
Figure 2: 3D structure of Atrial Natriuretic Peptide Receptor C complexed with Atrial……....17
Figure 3: Structure of LT4 downloaded from PyRx……18
Figure 4: 1YKO structure with A and B chain……18
Figure 5: Prepared receptor molecule (1YKO chain a) using BIOVA Discovery Studio…….....18
Figure 6: Chain A of 1YK0 molecule loaded in PyRx……19
Figure 7: Protein (1YK0) and ligand docked……20
Figure 8: MSEW effects on anxiety like behavior on EPM. (A) Number of entries in open arm, (B) Time spent in open arm……21
Figure 9: MSEW effects on anxiety like behavior on EPM. (A) Time spent in close arm,(B) Number of entries in close arm…….....22
Figure 10: MSEW effects on anxiety related response on LD test. (A) Total distance travelled, (B) Time spent in center zone……23
Figure 11: MSEW effects on the anxiety-related response on LD test. (A) Time spent in a bright chamber (B) Time spent in a dark chamber……..23
Figure 12: Impact of MSEW on depression In FST. (A) Immobility time, (B) Mobility time……..24
Figure 13: Effect of LT4 administration on depressive symptoms determined by EPM test. (A) Time spent in close arm, (B) Number of entries in close arms……26
Figure 14: Impact of LT4 administration on depression determined by EPM test. (A) Time spent in open arm, (B) Number of entries in open arms……26
Figure 15: LT4 impact on depressive symptoms determined by open field test. (A) Total distance travelled, (b) Time spent in the center of Open Field apparatus……...27
Figure 16:LT4 effect of depressive symptoms evaluated by FST (A) Immobility time, (B) Mobility time……27
Figure 17: Effect of LT4 administration on depression demonstrated by LD test. (A) Time spent in bright chamber, (B) Transition number between the compartments……28

Figure 18: Model 1 interaction with 1YKO…….31
Figure 19: Model 2 interaction with 1YKO…….31
Figure 20: Model 3 interaction with 1YKO…….32

LIST OF TABLES

Table 1: Software used for *in silico* analysis. 16

Table 2: Chemical properties of 1YK0 20

Table 3: Grouping of mice in accordance with LT4 administration and MSEW induction. 26

Table 4: Ligand and protein binding affinity 30

Table 5: 2D Analysis of 1YK0 interaction with LT4 31

CHAPTER 1 INTRODUCTION

Major depressive disorder accounts for one of the most crucial obstacles encountered by healthcare workers all around the globe influencing 18 million mortals in US and 340 million inhabitants all around the world (Greden, 2001). Given the gigantic impact of depression of individuals and healthcare system, the requisite is for efficacious therapy is translucent. Mental disorders such as anxiety and depression are the fundamental cause of disability and loss of productivity all around the world. Organization, W. H. (2017). It is often said that depression is the result of chemical imbalance but it does not elucidate how intricate the disease is. A great body of literature suggests that depression does not merely spring from having too little or much brain chemicals but there are various other causes of depression including faultiness of brain mood regulation, genetic susceptibility and stressful social environment. It is a school of thought that various these factors interconnect to cause depression (George et al., 1989; Nestler et al., 2002; Barnhofer et al., 2010).

The societal changes have reshaped the mother-child relationship. The separation of mother from newborn immediately after birth has been accustomed in hospitals (Odhong' et al., 2019). A growing mass of literature corroborates that early life adversity is positively associated with development of psychopathologies like depression and anxiety (Robinson et al., 2003; Coplan et al., 2014).The utilization of rodent model of childhood adversity would serve as a beneficial model for understanding the mechanism of behavioral and neurolobiological alterations associated with early life neglect. A vast majority of experimental protocols are available that induce behavioral alterations but these studies are conducted on rats (Trent et al., 2007; Kaufman et al., 2007).For example, 3 hours mother-infant separation in rats cause manipulation in functioning of hypothalamic pituitary adrenal (HPA) axis and certain behavioral alterations including anxiety, fearfulness and attention. But the same protocol caused the increase in mother care towards infants, diminishing the effect of maternal deprivation itself. So maternal separation combined with early weaning was selected in the current study as it

diminishes maternal care towards infants which was the reason for unconvincing results of 3 hours maternal separation (George et al., 2010).

Natriuretic peptides have substantial role in modulation of hypothalamic pituitary axis and master anxiety like behaviors in various ways. The liberation of corticotrophin (ACTH) and corticotrophin releasing hormone (CRH) is inhibited by ANP (Fink et al., 1991). Inhibition of cortisol is also brought about by ANP whereas CNP increases cortisol concentration (Charles et al., 1995). The anxiety associated behaviors in rats have been found to be dwindled by central or peripheral administration of ANP (Ströhle et al., 1997). Patients with anxiety and panic disorders have declined concentration of ANP (Kellner et al.,1998). Higher ANP concentration has been reported in patients recovering from heart failure and had reduced anxiety level (Herrmann-Lingen et al., 2003). These discernments suggest the therapeutic role of ANP in anxiety and depressive disorders. The effect of ANP on treatment of anxiety has been mechanically associated with inhibitory role of ANP on HPA axis (Kellner et al., 2003). Gardner attempted to demonstrate the relation between thyroid hormone and ANP. Both watered and dehydrated thyroidectomized rats increased ANP concentration to about two folds and the cardiac ANP RNA was elevated to about three folds in dehydrated animals. This study indicated the elevation of secretion and genomic expression of ANP by thyroid hormone (Gardner et al., 1987).

Another study was conducted with the rationale to investigate the stimulating role of T4 in the synthesis and outpour of ANP by examining cell secretion and content into the substratum of immunoreactive ANP of rat and RNA concentration of ANP in the cultured atrial myocytes of neonatal rats. Cell secretion and content of IR ANP was increased by induction of T4 in a dose dependent manner (Mori et al.,1990). The possible interconnection between ANP and thyroid hormone was assayed by induction of hypo and hyperthyroidism in rat radioimmunoassay was performed to illustrate the concentration of ANP in atria, plasma and of hypothalamus of brain. The concentration of ANP in plasma was found to be substantially higher in hyperthyroid rats and lower in hypothyroid rat in comparison to the control group while atrial ANP concentration was declined in hyperthyroid rats when compared to control group and no substantial change in hypothyroid rats ANP concentration in atria was found. The level of ANP in

hypothalamus of hypothyroid rats was fundamentally declined in contrast to control group where as the concentration of ANP in hypothalamus of hyperthyroid rats remained unaltered (Muramatsu et al., 1990).

The following dissertation is based on experiments that scrutinize the effect of ANP on depressive behaviors. The study is based on creation of mice models of depression by daily 3 hours maternal separation of pups from dam from postnatal day (PND) 2 to PND 17 followed by early weaning at PND 17. In order to corroborate the creation of models of depression, mice would be subjected to behavioral tests which include elevated plus maze test (EPM), open field test (OFT), dark/light chamber test (LD) and forced swim test (FST).

After the creation of mice model of early neglect by separation followed by early weaning from mother and validation of model by behavior testing and the statistical outcomes, the control group and maternal separation followed by early weaning group were further segregated into 3 groups, one MSEW group was treated with LT4, (synthetic form of thyroxine, a hormone produced by thyroid gland) consecutively for seven days through intraperitoneal route of administration. The amount of LT4 injected was 15 µg per mice. The other two groups 1 control and 1 MSEW group were treated with vehicle alone with same amount of dose. After the successful administration of LT4, the mice were subjected again to behavior testing paradigms. These tests would indicate the impact of drug on the behavior of mice subjected to early neglect and controls and the difference was analyzed by using statistical parameters. Furthermore, *in silico* methodology was used in order to check the interaction of ANP with LT4.

CHAPTER 2 LITERATURE REVIEW

2.1 Depression and Anxiety

Depression is a well known brain disease with about 322 million individuals being identified with the illness per annum worldwide. Depression being the supreme reason of disability leads to the expenditure of massively greater health care cost being spent on depressed individuals as compared to other disorders (Cai et al., 2015; Unützer et al., 2009). Recently, the first line treatment options available for depression include selective serotonin reuptake inhibitors (SSRIs) and noradrenaline reuptake inhibitors (SNRIs) both of which need four to six weeks to be efficacious (Liu et al., 2015). Sleep disturbances and resistance to other diversities of antidepressants are the side effects of SSRIs and SNRIs (Chen et al., 2013). In order to determine the effectiveness of certain anti-depressants, depression had been induce in mice and rat models through various paradigms including learned helplessness, chronic unpredictable stress and many others. Early life adversity is also interconnected to stress resembling psychopathologies and studies have verified that rodents subjected to maternal separation developed depression like phenotype in adulthood (Vetulani, 2013; Alboni et al., 2017).

Depression typically develops in the growing age and halts and impairs person function and life quality. The two fundamental distinguishing symptoms of depression include anhedonia and depressed mood (Nestler et al., 2002). Above and over that depressed patients have increased susceptibility to physical sickness, diminished social working and elevated death rates (Nemeroff, 1998). Depression is further worsened by cormorbidities such as anxiety which influences the treatment, halts recovery and elevates the chances of recurrence (Hirschfeld, 2001).

It is often said that depression is the result of chemical imbalance but this fact does not elucidate how intricate the disease is. A great body of literature suggests that depression does not merely spring from having too little or much brain chemicals but there are

various other causes of depression including faultiness of brain mood regulation, genetic susceptibility and stressful social environment. It is a school of thought that various these factors interconnect to cause depression (George et al., 1989; Nestler et al., 2002; Ströhle et al., 1997; Barnhofer et al., 2010).

2.2 Maternal Separation Model of Depression

An authentic model of animal with depression can be implied as a valuable agent for interpreting the underlying mechanism of disease along with its testing and treatment. The maternal separation altercates hypothalamic pituitary adrenal axis regulation in which the depressive symptoms inculcated by 3 hours mother infant segregation cause hyperactivity of HPA axis (Lajud et al., 2012). It has been documented that rats induced with maternal separation of 3h per day suffer from behavioral despair, anomalous HPA axis working in response to stressors and long lasting changes in concentration of BNDF protein (Holmes et al., 2005). In addition, these alterations in rats are homogenous with the impacts of human maternal neglect and deprivation and it has been agreed that maternal neglect makes the children prone to depressive like symptoms in the later stage of life (Pesonen et al., 2007;Tyrka, 2008; Vetulani, 2013).

The depression induced in rodents by depriving the infants from mother has a striking mechanistic and pathogenic validity as it induces the alterations during the early developmental period, the perturbances, inducing depression in a similar manner as is induced in the initial stages of human lives reported by (Belzung et al., 2011).

Various antidepressants overturning the alterations inculcated by maternal deprivation corroborate the predictive validity of depression induced by mother-infant separation (Cotella et al., 2013; El Khoury et al., 2006).

2.2.1 Depression caused by maternal separation

The infant adversity in the shape of derelict is ubiquitous throughout the world. It is estimated that 24.4 out of 1000 children are being neglected during infancy (Ogawa et al., 2004) .

The clear-cut neurobiological mechanism of early life stress in inculcating depression in living being is intricate. In the perinatal stage of life, the organism's interconnection to the surroundings has a crucial impact on structural and functional development towards the best adaptations with surpassing magnitude of reproduction and succession. Any

change from optimal conditions during the first year of growth can have wrecking impact on morphon's structure and function (Colombo, 2019; Silva et al., 2014). Substantially, the aggravations during the perinatal period have been found to cause imperishable, rather than fugacious effects on the creature.

The alteration in neurobiology, physiology and sentimental etiquette by disruption in mother-pup interconnection during infancy is the striking example of childhood adversity documented to interfere with the maturation process of neuroendocrine system which has persistent effects on organism lasing throughout the life (Ellenbroek et al., 1998; Lyons et al., 1998; Enthoven et al., 2008). There is massive literature to inspect the everlasting percussions of early life adversity in rodent by enforcing maternal separation paradigms that flustered parent-offspring interaction leading to deprivation and neglect in child growing time span (Holmes & Mathews, 2005) . Maternal separation protocols implicate mother deprival but usually fluctuate in extent and quantity of segregations following two weeks after birth. Thus, depending upon theses fluctuations, some maternal separation procedures were found to inculcate everlasting anxiety and depressive symptoms and altercations in HPA feedback to exasperating environments (Deschesnes et al.,2008; Richardson et al., 2004). The ground-breaking research by Levine has revealed that the maternal care during infancy models the sentimental decorum and reaction to stress in the later life (Levine, 1957; Ackerman et al., 1978;Meany et al 1991;Plotsky & Meany, 1993; Lippman et al., 2007). Since then, the massive amount of literature have been catalogued the consequences of dam pup separation during the early stages of life in rats and mice. The most familiar maternal separation protocol involve 3 hours daily segregation of mother from pups from postnatal day 2 to 14 (Barna et al., 2003). However 3 to 8 hours and 24 hours separation has also be reported in other studies resulting in different degree of perceived stress depending upon the duration of separation and other tantrums of the protocol used (George et al., 2010).

Maternal separation followed by early weaning comprise of the segregation periods that occur over a vast degree of postnatal ages followed by subsidiary element of early weaning. This separation model implies far longer segregation periods compared to typical models of MS. Furthermore, offspring are weaned from mother at early age that further restrains the parental contact. Maternal separation followed by early weaning is a

pioneering protocol that enables the comprehensive investigation of behavioral and neurobiological impacts of parental deprivation in mice models.

The success of maternal deprivation models is dependent on precise environmental factors and genetic susceptibility, says (Schmidt, 2011). No depression model of early life stress is considered vigorous as the environment in the adult stage of life is of critical importance and the early adversity may have adaptive results in the dreadful adulthood environment by could be dysfunctional in the non-aversive environment.

2.3 Natriuretic Hormones

The very initial affirmation of endocrine connection between the heart and kidney came from ground breaking work of De Bold who discovered ANP in rats by injecting them with atrial homogenate that caused substantial diuresis and natriuresis (Sudoh, 1990). Later, the other members of family BNP (Brain Natriuretic Peptide) and CNP (C-type Natriuretic Peptide) were purified. ANP, BNP and CNP share the identical structure and are usually expressed in the form of pre-pro-hormones and then processed proteolytically to form mature proteins. The stretching of the walls of atria induce the ANP release while the ventricles are the major source for the release of BNP (De Bold et al 1981; Edwards et al., 1988).

2.3.1 Physiological functions of Natriuretic Peptides

ANP plays a pivotal role in blood pressure regulation. ANP brings about dieresis and natriuresis in the kidney by enhancing the glomerular filtration rate. ANP induces vasorelaxation by stimulating the relaxation of smooth muscle cell in blood vessels (Currie et al., 1983). It was demonstrated that ANP deprived mice developed hypertension sensitized to salt (John et al., 1995). The functional significance of BNP is not yet well understood although it activates same receptors as ANP does (Holtwick et al., 2003). BNP acts as a crucial biomarker in myocardial infarction and congestive heart failure as higher concentration of BNP have been reported in these conditions (Nagaya et al., 2000). CNP acts in autocrine fashion and has a potent role in inducing vasorelaxation (Drewett et al., 1995).

2.3.2 Natriuretic peptides in brain

Natriuretic peptides along with receptors are usually expressed in brain with CNP being most abundant in brain implying its role as neurotransmitters rather than a cardiac

hormone (Komatsu et al.,1991). ANP and BNP with neuromodulatory functions have been found in brain regions. ANP has been demonstrated to be present in hypothalamus, neurons and glia in cerebral cortex and in cerebellum Hypothalamus and cerebral cortex contains BNP (Tanaka et al., 1984; Standaert et al., 1986; McKenzie et al., 1994; McKenzie et al., 2001).

2.3.3 Role of Natriuretic peptides in behavior

Natriuretic peptides have substantial role in modulation of hypothalamic pituitary axis and master anxiety like behaviors in various ways. The liberation of corticotrophin (ACTH) and corticotrophin releasing hormone (CRH) is inhibited by ANP 75. Inhibition of cortisol is also brought about by ANP whereas CNP increases cortisol concentration. The anxiety associated behaviors in rats have been found to be dwindled by central or peripheral administration of ANP (Drewett et al., 1995). . Patients with anxiety and panic disorders have declined concentration of ANP. Higher ANP concentration has been reported in patients recovering from heart failure and had reduced anxiety level. These discernments suggest the therapeutic role of ANP in anxiety and depressive disorders (Fink et al., 1991). The effect of ANP on treatment of anxiety has been mechanically associated with inhibitory role of ANP on HPA axis. BNP has also been found to have anxiolytic effects by inhibiting cortisol concentration which is a major stress hormone but CNP has anxiogenic affects as it enhances cortisol secretion. Various studies verify the role of NP in brain functions which has opened thrilling novel venues for research and development of drugs.(Bhattacharya et al., 1996).

2.4 ANP and its Anxiolytic Role

Natriuretic pepties are being progressively recognized in psychiatric studies because of their role in central nervous system (Bandelow et al., 2017). ANP is a protein comprising of 28 amino acids produced by cardiomyocytes and regulates blood pressure. Decline in the level of ANP has been interconnected with heart diseases, increase in blood pressure and resistance to insulin. Hypothalamus, brain stem, cerebral cortex and cerebellum express ANP in CNS (Meyer et al., 2017).

ANP has been found to be a major contributor to the regulation of chemical feedback in sympathetic nervous system and hypothalamic pituitary adrenal axis, the two crucial stress systems (Wisen et al., 2011).

Intraventricular administration of ANP in rodents demonstrated the anti-anxiety response CNS (Bhattacharya et al., 1996; Meyer et al., 2017). Reduced level of basal ANP was reported in patients with panic attacks in comparison with healthy controls but after administering CRH, no difference was visualized (Kaczmarczyk et al., 2019). Experimentally induced panic attack by administering Cholecystokinin Tetrapeptide (CCK-4) led to a sturdy escalation of ANP level in patients suffering from panic disorder in contrast in healthy individuals, says Wiedemann. Administration of ANP before the inculcation of CCK-4 decreased the intensity of panic attacks in patients. This augmentation in ANP level is interconnected to the decline in HPA axis (Kellner et al., 2001). Kellner reported the decline of basal ANP concentration in patients with post-traumatic stress in his study in 2006. No information is available on the contribution of ANP in major depressive disorder to date. Wisen in his study in 2011 demonstrated the diminished ANP levels have been reported in depressed patients (Wisen et al.,2011). Enervated NT-proANP feedback was document in depressed patients by Krogh in 2011 (Krogh et al., 2011). Acute childhood adversity has also connection with changes in ANP release (Otte et al., 2016) .

2.5 Thyroid Hormone Increases ANP Concentration

The release of ANP from cardiomyoctes is affected by thyroid hormone. Certain experimentations on rat models stipulate that ANP secretion is enhanced by thyroid hormones (Hodes & Lichtstein, 2014). Substantially higher concentration of ANP has been documented in hyperthyroid patients and ANP level is dwindled in hypothyroid patients. George Koukoulis found a robust positive concurrence between thyroid hormone concentration and ANP level. Increased concentration of ANP was reported in patients with hyperthyroidism and decreased ANP level was documented in hypothyroid patients which were returned to normal after adequate therapy (Koukoulis et al., 2002). . In order to demonstrate the participation of thyroid hormone on the secretion of ANP, immunoreactive concentration in atria and plasma of hyper and hypothyroid rats was measured by Kohno. The level of ANP in plasma was elevated in hyperthyroid rats and mitigated in hypothyroid rats compared to euthyroid rats (M Kohno et al., 1986). The study conducted by Wong had similar results indicating increased ANP concentration in hyperthyroidism and viceversa (Wong et al., 1989).

2.5.1 Thyroid hormone for treatment of mental disorders

The interrelation between depressive disorders and the role of thyroid hormone has been recognized for decades. Patients with thyroid diseases are more liable to develop depressive symptoms. Previous studies show that low thyroid concentration aggravates depression. Major depressive disorder has been found to be four times more prevalent in hypothyroid patients than healthy population (Chueire et al., 2007) . Suicide attempts by patients suffering from Major depressive disorders had close interconnection with thyroid hormones, says Zhou et al (Zhou et al., 2021). Subclinical hypothyroidism is quite frequently present in patients having refractory or simple depression (Fountoulakis et al., 2006). Traditionally the relation between thyroid hormone and mental disorders has been under consideration far more than 200 years. Increased incidences of nervous affectations have been documented in thyroid diseases. At present, it is a well-established fact that psychiatric health including sentiments and memory is substantially affected by thyroid function. It is estimated that 1 to 4 percent of the patients with mental disorders suffer from hypothyroidism whereas 2 to 40 percent of these patients suffer from clinical depression (Wolkowitz & Rothschild, 2008).

Administration of L-Thyroxine to depressed females in whom the results of sertotonergic antidepressants were unsatisfactory had amelioration in depressive symptoms (Łojko & Rybakowski, 2007). Thyroxine administration had beneficial results in depressive patient contraey to flouxitene (Barak et al., 1996).

2.5.2 Thyroid hormone increases ANP concentration

The release of ANP from cardiomyocytes is affected by thyroid hormone. Certain experimentations on rat models stipulate that ANP secretion is enhanced by thyroid hormones (Argentin et al., 1987; Hodes, 2014). Substantially higher concentration of ANP has been documented in hyperthyroid patients and ANP level is dwindled in .hypothyroid patients (Diekman, Harms, Endert, Wieling, & Wiersinga, 2001; Masakazu Kohno et al., 1987; Koukoulis et al., 2002; Rolandi et al., 1992; Widecka, Gozdzik, Dutkiewicz, Majewska, & Czekalski, 1990). George Koukoulis found a robust positive concurrence between thyroid hormone concentration and ANP level. Increased concentration of ANP was reported in patients with hyperthyroidism and decreased ANP level was documented in hypothyroid patients which were returned to normal after

adequate therapy (Koukoulis et al., 2002). In order to demonstrate the participation of thyroid hormone on the secretion of ANP, immunoreactive concentration in atria and plasma of hyper and hypothyroid rats was measured by Kohno. The level of ANP in plasma was elevated in hyperthyroid rats and mitigated in hypothyroid rats compared to euthyroid rats (M Kohno et al., 1986). The study conducted by Wong had similar results indicating increased ANP concentration in hyperthyroidism and vice versa (Wong et al., 1989).

The current study aimed to determine the interaction between Levothyroxine and Atrial Natriuretic Peptide by *in silico* analysis for confirming the drug for treatment. Creation of mice model of depression by maternal separation coupled by early weaning followed by behavior testing and then treatment with Levothyroxine was also the present study aim.

CHAPTER 3 METHODOLOGY

3.1 Ethics Statement

All the experiments were performed in accordance with the rulings of Institute of laboratory Animal Research. All protocols employed were approved by Internal Review Board (IBR), School of Mechanical and Manufacturing Engineering (SMME) and Atta-ur-Rehman School of Applied Biosciences.

3.2 Animals

All male and female mice of strain Balb/c were purchased from animal house of Atta ur Rehman School of Applied Biosciences in National University of Science and Technology Pakistan and were habituated in plastic cages of mice. The temperature was kept incessant (23^oC) and the room was ventilated. In addition the mice were retained in 12 hours dark/light cycle with water and food *ad libitum.*

3.3 Study Design

The maternal separation model coupled with early weaning was in accordance with the protocol concocted by George et al in his paper in his paper in 2010 (George et al., 2010). A total of 48 pups were divided in two groups; experimental and control group. The experimental group consisted of 1 group with 16 pups and a dam in each group. The control group also consisted of 2 groups with 16 pups and a dam in each. The experimental group was subjected to maternal separation from postnatal day 2 to 17 followed by weaning at day 17. The mice were then subjected to anxiety related paradigms to ascertain the validation of MSEW model. The anxiety related paradigms included Elevated Plus Maze test, Open Field Test, Forced Swim Test and Light/Dark Chamber test (Joffe, 2022).

The synthetic form of thyroxine called as LT4 was administered intraperitonealy for 7 days at the dosage of 15 µg per mice for treatment of MSEW induced anxiety (Gardener et al., 1987). For this purpose the two groups; experimental and control group were further divided into 3 groups as follows:

1. Control group without maternal separation and drug administration

2. Experimental group with MSEW and drug administration
3. Experimental group with MSEW but without drug administration

The mice were again tested by anxiety associated paradigms to illustrate the effectiveness of LT4 in increasing ANP level and subsequently analyzing the impact on behavioral despair and anxiety like response in mice models subjected to maternal deprivation coupled with early weaning.

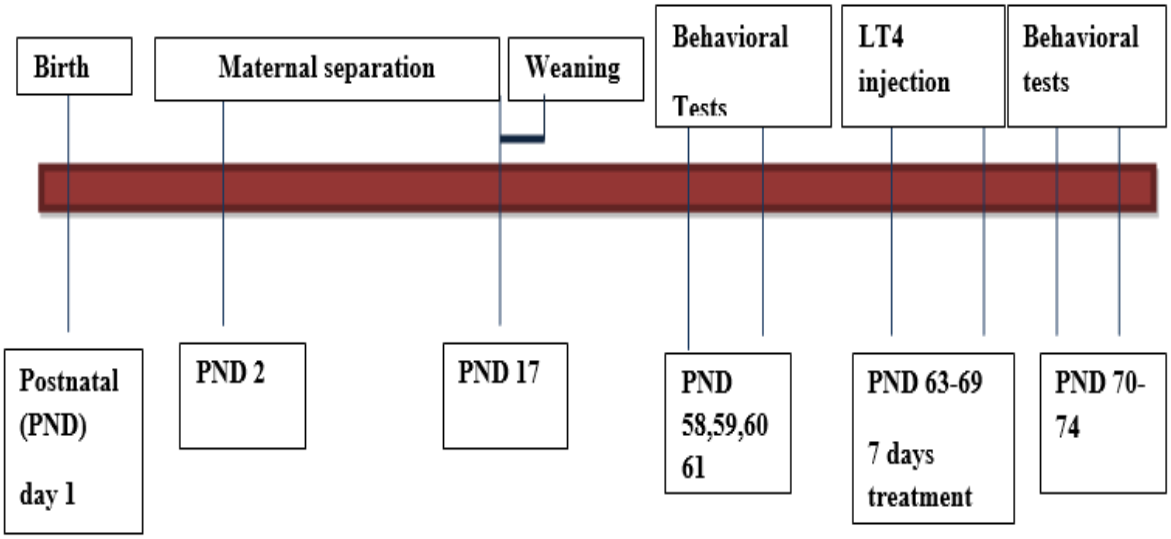


Figure 1: Timeline of the proposed study. Study timeline depicting 74 days long procedure for developing MSEW model, assessment of behavior through anxiety related tests, treatment with LT4 and again behavioral testing to examine anxiety changes.

3.3.1 Dose determination

Sodium hydroxide (0.1 Molar) was prepared by dissolving 4 g of sodium hydroxide in 100 ml distilled water. 15 micrograms of Levothyroxine was dissolved in 0.1 Molar NaOH solution. The solution was freshly prepared every morning and was injected in mice at 11 a.m. The mice received LT4 injection via intraperitoneal route of administration for 7 days consecutively from Monday to Sunday.

3.4 Behavioral Testing

Behavioral tests were conducted consecutively for five days from postnatal day 58 to postnatal day 62 with the interval test of 24 hours always from 9 a.m. ahead. The mice were acclimatized to the experimental room and were habituated to the experimenter 48 hours prior to conducting the tests. Iso propyl alcohol was used to clean all the apparatuses used after testing each animal. Video recordings were made using the digital video camera to analyze the results of the tests and determine the experimental conditions and observations that the observer was blind of during the experiment. Only those animals were included who had completed their tasks.

3.4.1 Elevated Plus Maze (EPM)

Elevatd Plus Maze employs the pattern of inborn aversions to open spaces and exploratory capability of mice to evaluate anxiety related responses. The mice positive with anxiety and fear disorders would avoid the open arms. Since closed arms characterize the protection areas, it becomes plausible to see the anxiety like behavior after exposure to EPM.

Elevated plus maze testing was conducted in a mode homologous to that narrated by (Simen et al., 2006). The test was based on placing the mouse in central portion of the apparatus also designated as neutral zone with the head of mouse pointed towards one of the open arms. Ten minutes were allotted to mice to explore the elevated plus maze contrived of white Plexiglass in accordance with the dimensions of mouse mazes which are commercially available the most for instance, the Panlab Harvard apparatus. The plus maze, at the height of 31.5 cm from the ground possessed two open arms and two closed arms hinged by a square of 6 cm length. The high black walls of 28 cm surrounded closed arms. An opaque testing box, positioned in the center of the room that was dimly lit and did not have any sort of apparent perceptible cues was used as a substrate for placing the maze inside it. The digital video was made by a video camera that was placed 3 feet above the elevated plus maze and the video was later viewed by the observer. The behavior of the mice was accessed in 5 minutes interval for the total time spent in closed arms and the total time spent in open arm as well as number of transitions between closed and open arms were being analyzed.

3.4.2 Forced swim Test (FST)

Forced swim test was conducted by minor alterations in the procedure documented by Porsolt in his papers published in 1977 (Porsolt et al., 1977). The mice were kept in a 16 cm in diameter glass cylinder (4 liter) and were filled with water up to the depth of 10 cm. the temperature of water was about 25 ^{0}C. After testing each mouse for the interval of 15 minutes, the water in the cylinder was wasted and cylinder was refilled with clean water. After the completion of protocol, mice were removed from water, shortly dried and before returning them to their home cage, the mice were placed in a holding cage and warmed for 30 minutes on a heating lamp. For the observer who was blind to the experimental situation, video was recorded in 5 minute duration. Behavior was divided into immobile (defined as the movement absence except for what was needed to keep the head of mice above water) and active swim/mobile (defined as all four limbs motion).

3.4.3 Open field test (OFT)

Open field test is acknowledged as a broadly utilized test to analyze the scouting activity and anxiety related response in mice models. Open field test comprised was putting down the mice in the pivot of quadrangle translucent box (33×33cm), placing the mice in it to free explore for apparatus in a period on 20 minutes. Longer duration was used in open field test to finely analyze the alterations in behavior and diminish the impact of anxiety and stress by subjection to novel environment. The parameters such as anxiety related response and exploratory behavior were demonstrated by the total distance covered during the test and the total time spent in the central area of the square (Walsh & Cummins, 1976).

3.4.4 Light dark (LD) test

Light dark chamber test is customarily employed test for determination of stress and anxiety related response and is dependent on the dissension between the inborn inclination of an mice to explore novel environment compared to the intrinsic repugnance to vivid environment. The LD test comprised of an instrument which is a rectangular body segregated into two chambers of equivalent size with a tiny unlatched doorway which permits the mice survey the both chambers. The mice were kept in light chamber

and were permitted to survey the both compartments for the duration of 10 minutes. In order to habituate, all mice were placed in the dark room, one hour prior to conducting the test. The light intensity was about 390 lux in light chamber and 4 lux in dark chamber. The time spent in dark chamber, the time spent in bright chamber and the total number of transitions between light and dark chamber was being assayed.

3.5 Statistical Analysis

The data were expressed as mean and standard deviation. Independent student T-test and one-way-ANOVA was conducted for the analysis of comparative groups. All results were analyzed considering the p-value below 0.05 to be significant. Graph Pad Prism version 8.0.1 was used for analysis and visualization.

3.6 *In silico* Approach to Determine Protein (ANP) and Ligand(LT4) Interaction

3.6.1 Software

The *in silico* resources utilized for the current study have been summarized in Table 1.

Table 1: Software used for *in silico* analysis.

Software	Application	Developer
PyRx	Molecular docking	Source Forge
Discovery Studio	Visualization and 2D analysis	Biova

3.6.2 Software and online resources

3.6.2.1 RCSB PBD

RCSB PBD site is a data bank of proteins which contains three- dimensional shapes of nucleic acids and proteins. It is usually a sole dossier containing all the structural details of biological molecules and consists of structures demonstrated from diverse techniques including X-ray crystallography, NMR and cryoelectron microscopy (Feng & Weissig).

3.6.2.2 PubChem

This DrugBank is usually a bioinformatics database consisting of complete detail of drug's structures and their protein targets. The database comprises of more than 5000 entries and 1000 entries are approved by FDA .

3.6.2.3 Discovery Studio

Biova Discovery Studio allows researchers to perform computation of substance and material's characteristics. It helps emulate and dissect natural and artificial frameworks and delivers the end products to various researchers (Haque et al., 2022).

3.6.2.4 PyRx

Autodock Vina in PyRx is a virtual screening tool that docks ligands or drugs to protein. The tool comprises of docking Wizard and convienent interface which plays a crucial role in Computer Aided Drug Design (CADD).

3.6.3 Selection of target protein

The target identification process has a crucial role in discovery of drug that can be performed by biochemical methods, genetic interactions, and computational techniques. The following study utilized computational interpretations to explore a target protein based on the sequence length and stability of protein. Natriuretic peptide Receptor C complexed with Atrial Natriuretic Peptide (1YKO) was selected as a target protein with target resolution of 2.40 Angstrom, R- Value Free: 0.284, R-Value Work: 0.240 and R-Value Observed: 0.24 determined by X-ray diffraction technique. The file was downloaded in PBD format from RCSB protein data bank for utilization in molecular docking described later (Dev et al., 2020).

Figure 1: 3D structure of Atrial Natriuretic Peptide Receptor C complexed with Atrial Natriuretic Peptide (1YK0). The protein was download in PBD form from RCSB protein data bank and was visualized using Discovery Studio.

3.6.4 Selection of ligand or Drug target molecule

The ligand was selected by reviewing the literature and unearthing the advancing inquisitive drugs that increase ANP concentration and treat depression from PubChem and DrugBank. DrugBank is a highly elucidative asset that integrate ample drug statistics with extensive drug target and drug action information. Drug Bank has been widely utilized to favour the prediction of drug interaction, drug docking and insilico drug discovery. The 3D structural file of Levothyroxine was downloaded in SDF format.

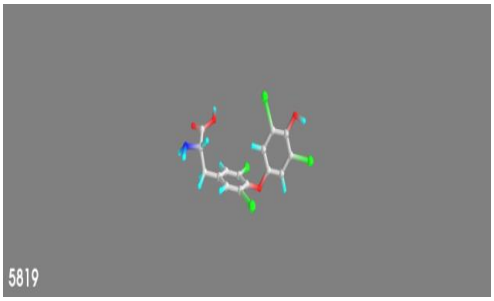


Figure 2: Structure of LT4 downloaded from PyRx. Three-dimensional structure of Levothyroxine visualized from PyRx, chemical formula: C15H11I4NO4, weight: 776.87002, and number of atoms=35.

3.6.4 Preparation of Ligand and protein

Three-dimensional structure of 1YK0 in PDB format was taken from RCSB protein data bank and 3D- structure of Levothyroxine was downloaded in SDF format from PubChem.

The structure of 1YK0 was additionally purified for the purpose of docking by deleting the water molecules and hetatoms using BIOVA Discovery Studio to keep the active site open and was saved in PDB format for additional interpretation.

Figure 4: 1YKO structure with A and B chain. After removing water, hetatoms and remaining chains, A and B chains were left in order to empty the binding sites.

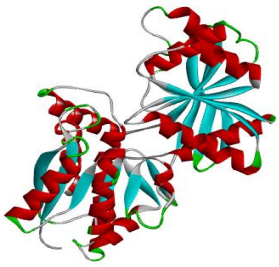


Figure 5: Prepared receptor molecule (1YKO chain a) using BIOVA Discovery Studio. The only chain selected for docking with LT4 was A chain which was downloaded in PBD format from discovery studio and loaded in PyRx.

Table 2: Chemical properties of 1YK0

Protein	**Atrial Natriuretic Peptide C complexed with Atrial Natriuretic Peptide**
PBD ID	1YK0
Chain	A
Sequence length	480
Organism	*Homo sapien*

3.6.6 Molecular docking through PyRx

3.6.6.1 PyRx

PyRx is computer aided drug discovery software employed for the purpose of docking ligands or drugs with protein.

3.6.6.2 Docking

Docking using PyRx software was performed by following the protocol documented by (Dallakyan & Olson, 2015). The prepared protein 1YK0 with a chain that was saved in pdb format was loaded in Pyrx and autodocked into macromolecule. As a result the receptor molecule (protein) was converted into PDBQT format.

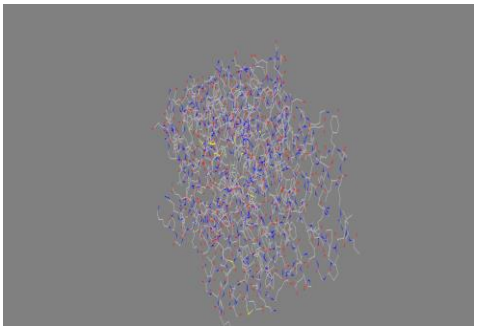


Figure 6 Chain A of 1YK0 molecule loaded in PyRx. The molecule containg chain A was download fron Discovery Studio and then loaded in PyRx and converted into macromolecule by autodocking.

3.6.6.3 Minimization of energy

The ligand molecule was loaded in PyRx. The energy of ligand was minimized in Open Babel software package that is implanted in PyRx and then the ligand was converted to PDBQT format

3.6.6.4 Docking

The Vina Wizard tool of PyRx was utilized for docking. The protein and ligand was selected and docked. The protein was covered with the gridbox, the dimensions of which were extended to cover the entire protein molecule and vina wizard was being run.

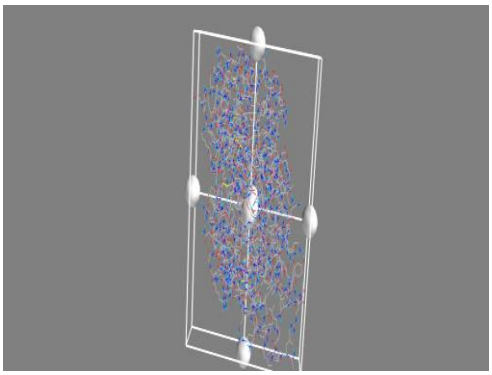


Figure 7: Protein (1YK0) and ligand docked. The gridbox elongation and further processing in In Vina Wizard was performed for determining binding energy.

3.6.6.5 Ligand- receptor interaction

For the demonstration of interaction between ligand and receptor, docked molecule was inserted in BIOVA discovery Studio for visualization by modeling and miniature of 2D interactive picture.

CHAPTER 4 RESULTS

4.1 Mouse Model of Anxiety

The protocol to establish mice model of anxiety was optimized using previously described methodology. The mice 2 days old were subjected to maternal separation and weaned at 17th PND in order to induce behavioral despair and anxiety like response. The methodology documented by George et al., 2010 was selected for the generation of mice model of anxiety as it was more significant in inducing behavioral alterations in contrast to maternal separation paradigm used alone.

4.2 Behavioral Analysis

4.2.1 Increased aversion of mice towards open and elevated areas

Elevated plus maze paradigm was used to access the effect of maternal separation followed by early weaning on mice behavior. The analysis on EPM indicated substantial decline in the number of entries in open arm in mice subjected to maternal separation coupled with early weaning in comparison to control group. The total time spent in closed arms was also greater in mice subjected to MSEW and less time was spent by mice subjected to MSEW in open arms. Statistical analysis data of EPM provided the evidence of decreased number of entries and time spent in open arm

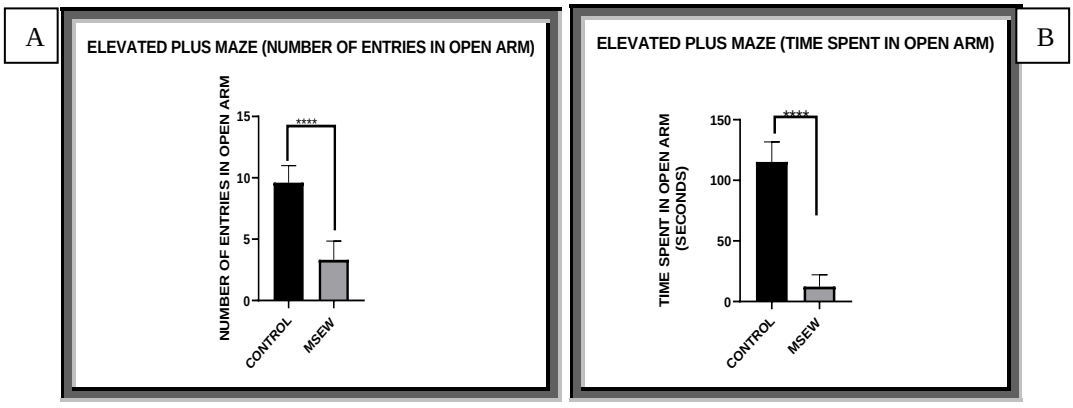


Figure 8: MSEW effects on anxiety like behavior (a) Number of entries in open arm, (b) Time spent in open arm in EPM test. Independent t-test was applied. Mice exposed

to MSEW made fewer entries in open arms and spent less time in open arms during every entry. p< 0.05 for MSEW× control group differences in t-tests. Number of animals per group: control, n=16; MSEW= 32. Results are expressed as mean ± SD.

Contrary to open arm entries, the findings of closed arm entries and time spent in closed arms elucidated greater number entries performed by mice subjected to MSEW in comparison to control group.

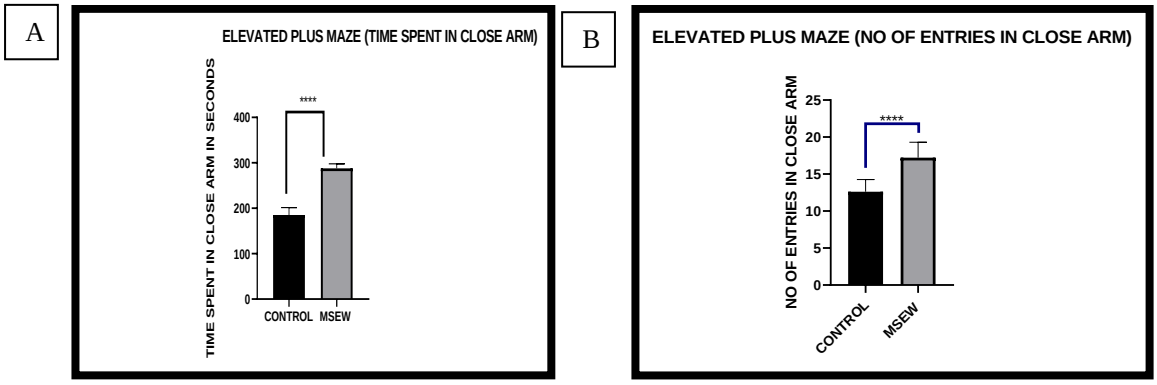


Figure 9: MSEW on anxiety like behavior on EPM. (A) Time spent in close arm,(B) Number of entries in close arm. Independent t test was used. p< 0.05 for MSEW× control group. Sample size: control, n=16; MSEW, n= 32. Results are expressed as mean± SD. The graph depicts greater number of entries in closed arms in mice subjected to MSEW as compared to control group indicating hyperactivity in MSEW mice.

4.2.2 Reduced movements:

Open field analysis reveals substantial effects of maternal separation on the speed. The mice subjected to maternal separation followed by early weaning moved faster and covered more area than controls and the time spent in central zone of the apparatus was declined as compared to controls.

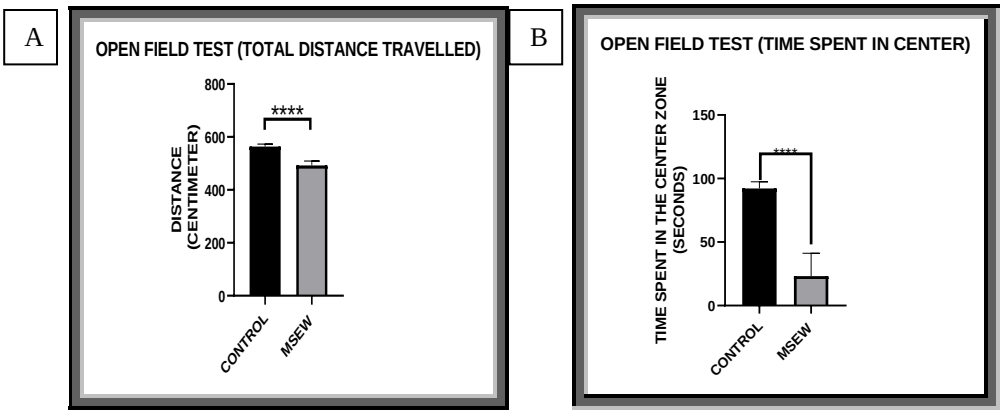


Figure 10: MSEW effects on anxiety related response. (A) Total distance travelled, (B)Time spent in center zone in OFT. p value<0.05 for MSEW and control group. Number of animals per group: control, n= 16; MSEW, n=32. Results are expressed as mean± SD. The mice subjected to MSEW spent less time in the center zone and were highly hyperactive moving with greater speed as compared to control group.

4.2.3 Diminished exploratory behavior and exposure to light

In light dark chamber test, MSEW group spent less time in exploring the bright compartment and declined quantity of rearing behaviors in the bright chamber. The time spent in bright chamber was greater in MSEW group. In addition, number of transitions was reported to be lesser in MSEW group as compared to control group.

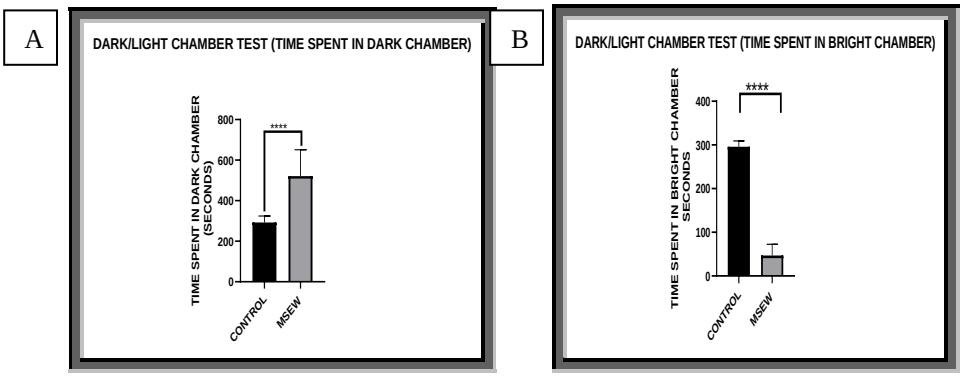


Figure 11: MSEW effects on the anxiety-related response on LD test. (A) Time spent in a dark chamber (B) Time spent in a bright chamber.

The value of p<0.05 for MSEW and the control group. Number of animals per group: control, n= 16; MSEW, n=32. Results are expressed as mean± SD. The mice subjected to

MSEW spent more time in the dark chamber and less time in the bright chamber and show fewer transitions between both compartments as compared to the control group.

4.2.4 Reduced mobility as a consequence of MSEW

The findings of forced swim test elucidated that the period of time spent immobile was increased in MSEW group and the duration of time spent in motility or active swim was decreased in comparison to control group.

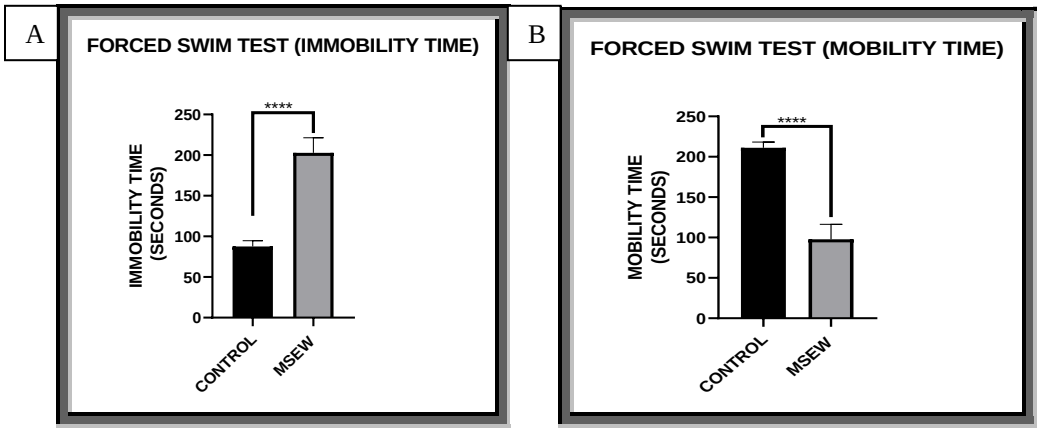


Figure 12: Impact of MSEW on depression. (A) Immobility time, (B) Mobility time in FST. Independent T test was employed. $p < 0.05$ for control and maternally separated group. MSEW group showed greater immobility and less time in active swim in comparison to control group. Sample size, n=48. Data is expressed as mean ± standard deviation.

4.3 Effect of administration of LT4 to increase ANP level on behavior of mice

After the administration of 15 µg LT4 per mice for 7 days consecutively, the impact of the drug on anxiety like response was determined using behavioral analysis tests.

Table 3: Grouping of mice in accordance with LT4 administration and MSEW induction.

Groups	Treatment
1. Control group with no MSEW and no LT4 administration.	15 µg vehicle
2. Experimental group with MSEW and LT4 administration.	15 µg LT4
3. Experimental group with MSEW but no LT4 administration	15 µg vehicle

4.3.1 Behavioral elucidation after treatment with LT4

4.3.1.1 Decreased aversion to elevated areas

After 7 days of treatment with LT4, EPM data showed substantial reduction in anxiety as the mice started to spent greater time in open arms as compared to the number of entries in open arm also increased in the MSEW group subjected to LT4 administration and the statistical analysis suggested the data to be comparable to control group. The MSEW group with no LT4 administration remained depressed with no significant change in behavior and time spent in open arms and number of entries in open arms remained lower than control group with no LT4 injection. In the similar manner the time spent in close arms was seen to be reduced in MSEW group after LT4 injection with values comparable to control group. In MSEW group with no LT4 injection and control group with LT4 injection, increased time spent in closed arms was found. In cases of the entries in close arm, MSEW group treated with LT4 showed increased number of entries in closed arms. The MSEW group with no LT4 treatment showed reduced entry number and in closed arm.

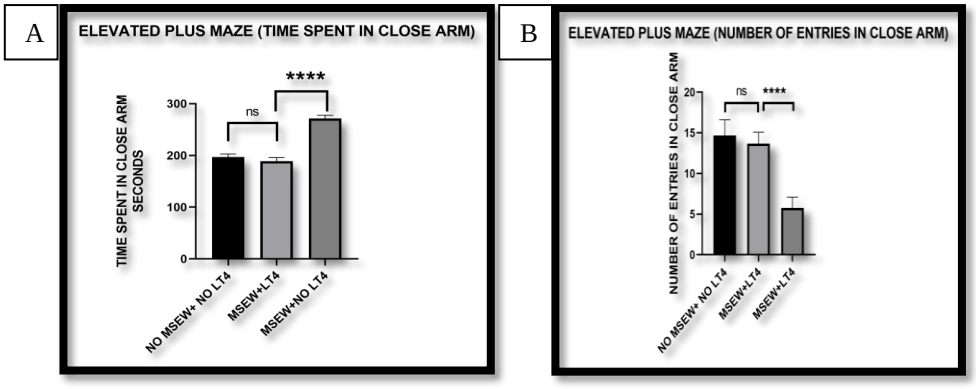


Figure 13: Effect of LT4 administration on depressive symptoms determined by Elevated Plus Maze test. (A) Time spent in close arm, (B) Number of entries in close arms. The p value was non-significant for control group (NO MSEW+ NO LT4) × LT4 treated depressed group (MSEW + LT4). The p value was less than 0.05 for (MSEW + LT4) × depressed group with no LT4 treatment (MSEW+ NO LT4). Data expression was in the form of mean and SD. Depressed mice subjected to Lt4 administration showed decreased time spent in elevated plus maze and the number of entries were increased.

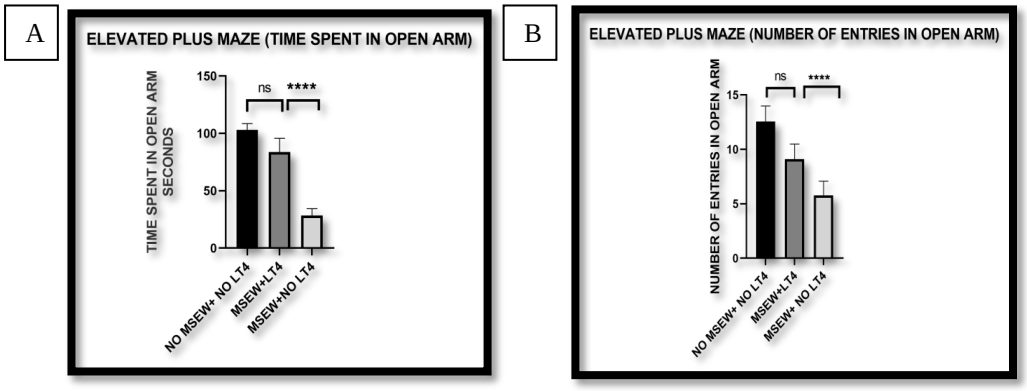


Figure 14: Impact of LT4 administration on depression as determined by EPM test. (A) Time spent in open arm, (B) Number of entries in open arms. The p value was non-significant for control group (NO MSEW+ NO LT4) × LT4 treated depressed group (MSEW + LT4). The p value was less than 0.05 for (MSEW + LT4) × depressed group with no LT4 treatment (MSEW+ NO LT4). Data expression was in the form of mean and SD. Depressed mice subjected to Lt4 administration showed increased time spent in open arms with greater number of entries in open arms.

4.3.1.2 Increased movement:

Open field analysis revealed increased movement in MSEW mice subjected to LT4 treatment as the distance travelled by them was significantly enhanced and the time spent in central portion was also elevated. In MSEW group with no LT4 injection movement remained the same as tested before and the time spent in central zone was also not increased.

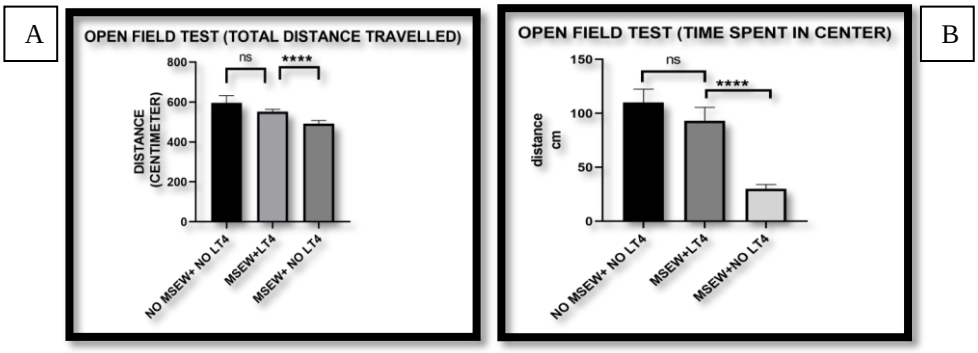


Figure 15: LT4 impact on depressive symptoms determined by OFT. (A) Total distance travelled, (b) Time spent in the center.

MSEW group injected with LT4 covered more distance and spent more time in center as compared to depressed mice with no Lt4 administration. $p< 0.05$ for MSEW+ NO LT4 group× MSEW+LT4 group. Number of animals per group: non treated controls, n=16, MSEW+LT4=16, MSEW+ no LT4= 16. Results are expressed as mean± SD.

4.3.1.3 Increased mobility

In forced swim test analysis MSEW+ LT4 group showed a substantial elevation in the time spent mobile or active swim and values were somehow comparable to control group with no LT4 administration. MSEW+ no LT4 injection group showed less time in active swim and greater time spent immobile than treated and control group.

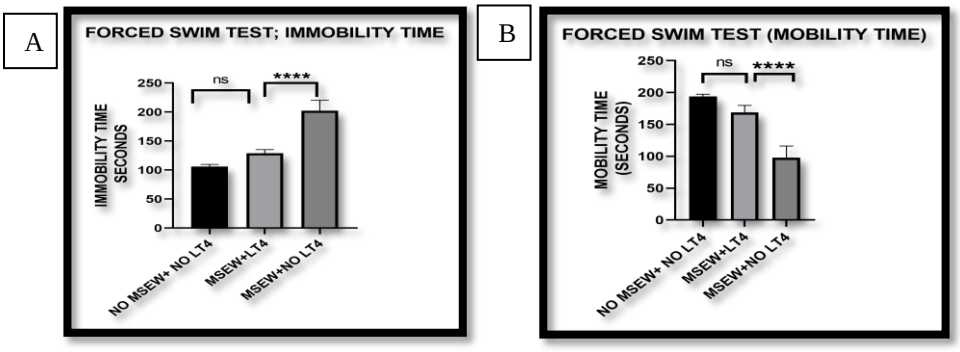


Figure 16: LT4 effect of depressive symptoms evaluated by FST. (A) Immobility time, (B) Mobility time. After LT4 injection depressed mice spent more time in active swim and reduced immobility time was recorded in comparison to non-treated depressed mice. . p< 0.05 for MSEW+ NO LT4 group× MSEW+LT4 group. Sample size: non treated controls, n=16, MSEW+LT4=16, MSEW+ no LT4= 16. Results are expressed as mean± SD.

4.3.1.3 Increased exploratory behavior

In light dark chamber analysis, the time spent in bright compartment of the apparatus was elevated and increase in number of transition between light and dark compartment was observed in MSEW group subjected to LT4 treatment in contrast to MSEW group with no treatment. This group showed decreased time spent in the bright compartment and increased time spent in the dark chamber of apparatus. The control group exposed to LTR injection showed reduction in time spent in bright chamber and number of transitions between dark and light chamber were also reduced in comparison to control group with LT4 treatment.

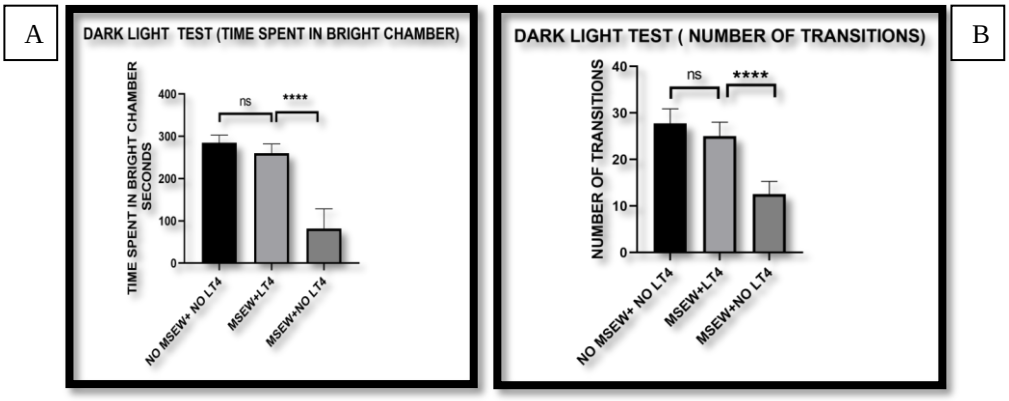


Figure 17: Effect of LT4 administration on depression demonstrated by LD test. (A) Time spent in bright chamber, (B) Transition number between the compartments. Independent t test was applied. Depressed mice subjected to LT4 administration (MSEW+ LT4) and non- treated controls (NO MSEW+ NO LT4) spent more time more time in bright chamber, less time in darks chamber and showed greater number of transitions as compared to mice not treated with LT4 (MSEW+ NO LT4). p< 0.05 for

MSEW+ NO LT4 group× MSEW+LT4 group. Number of animals per group: non treated controls, n=16, MSEW+LT4=16, MSEW+ no LT4= 16. Results are expressed as mean± SD.

4.4 *In Silico* Results

Atrial Natriuretic peptide receptor C complexed with ANP(1YK0) was selected as a receptore and Levothyroxine as ligand. PyRx was used for molecular docking and drug discovery studio was used for 2d and 3d visualization of protein ligand interaction.

4.4.1 PyRx in Vina interpretation

PyRx in vina tool was utilized for interpretation of 1YKO and for comparing the binding energy of LT4.

Table 4: Ligand and protein binding affinity

Ligand	**Binding Affinity (kcal/mol)**	**rmsd/ub**	**rmsd/lb**
Model 1	-7.1	0	0
Model 2	-7	2.284	1.13
Model 3	-6.9	2.228	0.938
Model 4	-6.6	5.91	4.926
Model 5	-6.6	5.819	4.836
Model 6	-6.5	6.115	4.836
Model 7	-6.5	2.914	0.986
Model 8	-6.4	18.68	16.722
Model 9	-6.4	18.668	16.614

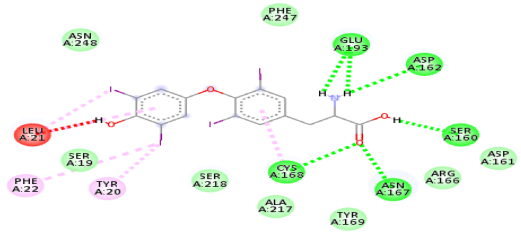
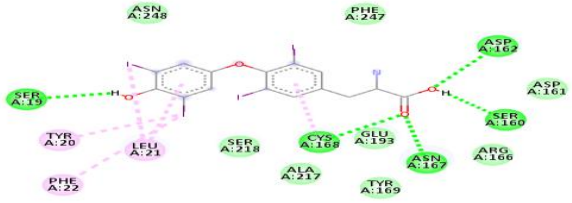
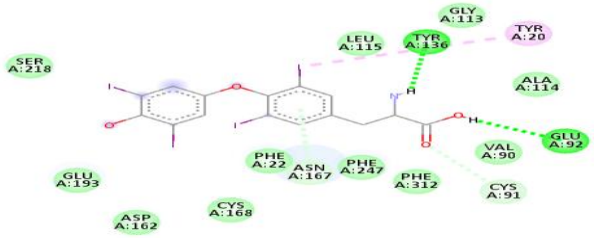
4.4.2 Binding energy evaluation

Listed in the figure is the comparative view of binding energy in kcal/mol obtained by docking Levothyroxine with 1YK0 protein. The model 1 has the highest binding energy of - 7.1 kcal/mol indicating the strongest interaction with of ligand (LT4) with protein.

4.4.3 Ligand and protein interaction evaluation

Active residues of 1YK0 that with levothyroxine using drug discovery software are Ser-160, Asp-162, Glu-193, Cys-168, Leu-21, Asn-167. Detailed analyses of interaction of protein with ligand are documented in the table below where contact of amino acid residue with ligand, bonding type and energy are elaborated.

Table 5: 2D Analysis of 1YK0 interaction with LT4

Ligand	2D diagram	Bonding
Model 1		**Hydrogen bonding, van dar waal interaction and weak donar- donar forces**
Model2		**Hydrogen bonding and van dar waal interaction**
Model 3		**Hydrogen bonding and van dar waal interaction**

4.4.4 Discovery studio visualization studies

Pyrx was utilized in docking 1YK0 with Levothyroxine (LT4). The PBDQT file generated by PyRx was opened for visualization in discovery studio. The model having least binding affinity was selected as it is the indicator of successful docking.

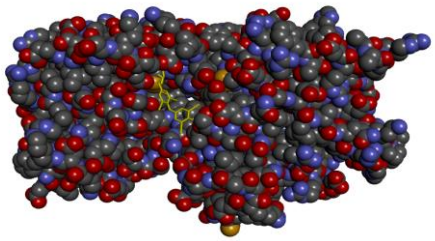


Figure 18: Model 1 interaction with 1YKO. The binding energy of – 7.1 kcal/mol obtained by using BIOVA Discovery Studio**.**

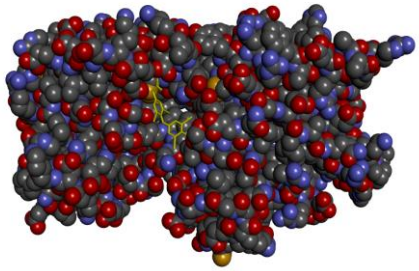


Figure 19: Model 2 interaction with 1YKO. The binding energy of – 7.0 kcal/mol obtained by using BIOVA Discovery Studio.

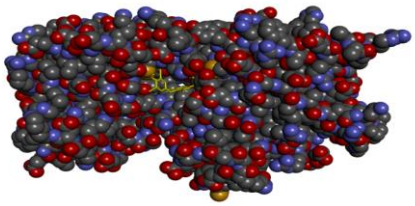


Figure 20: Model 3 interaction with 1YKO. The binding energy of – 6.9 kcal/mol obtained by using BIOVA Discovery Studio.

CHAPTER 5 DISCUSSION

The present study was outlined to describe and experimental fabrication that would contrive striking behavioral anomalies in mice of strain balb/c that would serve as an amenable paradigm of early childhood adversity and would subsequently be treated by raising the level of ANP by the administration of LT4, a synthetic form of thyroxine produced in body by thyroid hormone.

The foregoing studies have documented that maternal separation daily for the period of 3 hours increases the mother care towards infants that diminishes the effects of maternal separation (R. A. Millstein et al., 2007). Previous studies examining the impacts of maternal separation concluded that MS does not produce reliable behavioral alterations and long lasting despair (Vickers, 2017). We surmised that the effectiveness of the maternal separation paradigm would be enhanced by completely separating the mother from infants at PND 17 after the end of maternal segregation period. Ancient studies have corroborated that early weaning of brood from mother inculcates everlasting behavioral despair, hyper activity, elevated aggressive and anxiety related response by causing certain neuroendocrine manipulations (Carola et al., 2002; Kikusui et al., 2006; Kikusui et al., 2004). In the current study we developed the maternal separation paradigm coupled with early weaning that proved to be more effective paradigms of inducing behavioral despair, hyperactivity and anxiety with durable impacts in adulthood in mice.

It has also been validated in the current study that intraperitoneal administration of thyroid hormone (LT4) exerts anxiolytic effects on mice by increasing the concentration of ANP as determined by their effects of a variety of paradigms, exposed to in-depth crucial evaluation and verifies as clinical paradigms of anxiety (Bodnoff, et al., 1988). Previous studies have documented the decrease the anxiety like response and behavioral despair by intraceribroventricular injection of 200 to 500ng ANP to rats in diverse paradigms of anxiety including open field test, forced swim test and elevated plus maze test. The results of the present study are congruent with the previous documentations that validate the anxiolytic role of ANP. Another former study documented the anxiolytic role of ANP and the results are consistent with the present study (Biro et al., 1995).

5.1 Elevated plus maze test

Suggests increased anxiety in mice subjected to MSEW procedure and ANP administration masks these behavioral anomalies. We employed elevated plus maze test to evaluate behavioral despair and anxiety- related response in maternally separated mice and further injected the mice with Levothyroxine to increase ANP level and again tested mice in elevated plus maze apparatus to demonstrate the effect of ANP in suppression of anxiety and behavioral despair. In EPM test performed after inducing maternal separation followed by early weaning, substantial decrement in the time spent in open arms and number of entries in open arms was observed in comparison to the control group. The mice subjected to maternal separation spent greater part of time in closed arm (Orso et al., 2020). After one week of intraperitoneal administration of Levothyroxine, the effectiveness of the drug was again tested by elevated plus maze apparatus which corroborated a substantial decrease in behavioral despair and anxiety. The amount of time to be spent by mice subjected to MSEW significantly increased in comparison to the MSEW group that was not treated with Levothyroxine. There was found no signification difference in the before and after results of control group with no drug injection.

5.2 Open field test depicts depressive response in mice

The mice subjected to maternal separation followed by early weaning showed comparable difference from the control group in the parameter of total distance covered by mice in open field apparatus. So, this parameter remained significant indicating the retarded movements of mice exposed to MSEW. However, there was a decline observed in time spent in the central zone of open field apparatus in mice subjected to MSEW in contrast to control group (Orso et al., 2020). After administration of drug to MSEW group, depressive symptoms dropped as depicted by increase in movement and increase in the time spent in center portion of open field apparatus in contrast to the MSEW group that was not administered drug. However, the time spent in central area by control group treated with drug was reduced.

5.3 Light/dark chamber test

The maternal separation in mice brought a signification decline in the time spent in bright chamber of dark/light chamber test in comparison to the control group. More duration of time was spent in bright compartment of the apparatus and decline in transitions between

the two compartments was being observed. After treatment of MSEW group with LT4, time spent in bright chamber by mice substantially increased and the number of transitions also elevated suggesting increased exploratory activity in mice after drug administration which resulted in improvement in behavior of mice and decrease in despair and anxiety in contrast to the MSEW group not given the drug. The control group given the drug showed decline in activity with lesser number of transitions but time spent in bright and dark chambers remained equivalent (Kestering-Ferreira et al., 2021).

5.4 Forced swim test

MSEW group showed greater immobility in forced swim test in contrast to the control group in which substantial mobility time was being recorded. When MSEW group received the injection of LT4 substantial increase in mobility was observed in contrast to MSEW group that did not receive drug injection suggesting the anxiolytic effects of the drug. However, a minor reduction in mobility of control group given drug was observed (George et al., 2010). This finding verifies the striking anxiolytic function of ANP by anxiety related tests in mice models subjected to maternal separation coupled with early weaning.

ANP and its complementary receptors have been found in areas of central nervous system that regulate sentimental states such as amygdala (Langub J et al., 1995). Studies conducted on pregnant women supports the anxiolytic nature of ANP that circulates from periphery with 3 folds increment of ANP concentration in plasma with subsequent decline in panic disorders (Northcott & Stein, 1994). Further studies conducted in past few years demonstrated low basal ANP concentration in patients suffering from panic disorders. Intraceribroventricularly administering ANP and its congruent fragments have been demonstrated to reduce anxiety in elevated plus maze paradigms in animal models. (Kellner et al., 1995; Kellner et al., 1998).

Another important finding of our experiment was the positive correlation between thyroid and ANP concentration. The synthetic form of thyroxine, a hormone produced in body by thyroid gland was administered to mice exposed to MSEW which found to considerably increase ANP concentration in depressed mice. As is evident from historic studies that thyroid hormone in the form of LT4 when administered to thyroidectomized rats,

increased ANP concentration by 2 folds (Gardner et al., 1987). Another preliminary study indicated that LT4 at the concentration of (10(-8)-10(-6) M) increased ANP concentration in a dose dependent manner suggesting the stimulating impacts of LT4 on cellular contents of ANP (Mori et al., 1990).

As discussed above, there has been striking involvement of ANP in brain functions related to emotions and behavior. These considerations open the thrilling novel locuses for research and development of drugs. The mysterious connection between diverse cellular systems and ANP needs to be evaluated in depth. Although peripheral release of ANP is well understood but there is a lesser amount of literature on locally produced ANP other than cardiomyocytes, particularly brain. The underlying mechanisms involved in complex interaction of ANP with dopaminergic neurotransmitters, negative association of ANP with cortisol, involvement of ANP in HPA axis manipulation during stress response are yet to be evaluated in depth to provide the better understanding of ANP role in stress system and further paving the way for the development of ANP containing drug to normalize the imbalance of hormones and malformations in central nervous system caused by anxiety, stress, behavioral despair, depression and substance disorders.

CHAPTER 6 CONCLUSION

The maternal separation coupled with early weaning serves as a reliable model for incorporating everlasting behavioral anomalies and anxiety related responses in mice models as validated through diverse behavioral testing paradigms which suggest behavioral despair and anxiety induced in mice model by MSEW. These mice models when treated with ANP increasing drug LT4 for 7 days via intraperitoneal route of administration conforms certain behavioral changes and reduction in despair and anxiety when tested again through behavioral test paradigms validating the effectiveness of drug in curing anxiety. *In silico* analysis reveal the striking binding affinity of Levothyroxine with protein (1YK0) which validated the interaction of ligand (LT4) with protein (1YK0) and hence the influence of LT4 on ANP. In short, ANP concentration increased via LT4 administration induces antidepressant effect on mice subjected to maternal separation followed by early weaning.

CHAPTER 7 BIBLIOGRAPHY

Argentin, S., Drouin, J., Nemer, M. J. B., & communications, b. r. (1987). Thyroid hormone stimulates rat pro-natriodilatin mrna levels in primary cardiocyte cultures. *146*(3), 1336-1341.

Bandelow, B., Baldwin, D., Abelli, M., Bolea-Alamanac, B., Bourin, M., Chamberlain, S. R., . . . Fineberg, N. J. T. W. J. o. B. P. (2017). Biological markers for anxiety disorders, ocd and ptsd: A consensus statement. Part ii: Neurochemistry, neurophysiology and neurocognition. *18*(3), 162-214.

Barak, Y., Stein, D., Levine, J., Ring, A., Hadjez, J., Elizur, A., . . . Experimental. (1996). Thyroxine augmentation of fluoxetine treatment for resistant depression in the elderly: An open trial. *11*(6), 463-467.

Barna, I., Bálint, E., Baranyi, J., Bakos, N., Makara, G. B., & Haller, J. J. B. R. B. (2003). Gender-specific effect of maternal deprivation on anxiety and corticotropin-releasing hormone mrna expression in rats. *62*(2), 85-91.

Belzung, M. J. B. o. m. (2011). Criteria of validity for animal models of psychiatric disorders: Focus on anxiety disorders and depression. *1*(1), 1-14.

Bhattacharya, S. K., Chakrabarti, A., Sandler, M., & Glover, V. J. N. (1996). Anxiolytic activity of intraventricularly administered atrial natriuretic peptide in the rat. *15*(2), 199-206.

Cai, N., Bigdeli, T. B., Kretzschmar, W., Li, Y., Liang, J., Song, L., . . . Hu, Z. J. N. (2015). Sparse whole-genome sequencing identifies two loci for major depressive disorder. *523*(7562), 588-591.

Chen, J., Zhou, C., Wu, B., Wang, Y., Li, Q., Wei, Y., . . . Zou, D. J. P. r. (2013). Left versus right repetitive transcranial magnetic stimulation in treating major depression: A meta-analysis of randomised controlled trials. *210*(3), 1260-1264.

Chueire, V. B., Romaldini, J. H., Ward, L. S. J. A. o. g., & geriatrics. (2007). Subclinical hypothyroidism increases the risk for depression in the elderly. *44*(1), 21-28.

Colombo, J., Gustafson, K. M., Carlson, S. E. J. A. o. N., & Metabolism. (2019). Critical and sensitive periods in development and nutrition. *75*(1), 34-42.

Currie, M. G., Geller, D. M., Cole, B. R., Boylan, J. G., YuSheng, W., Holmberg, S. W., & Needleman, P. J. S. (1983). Bioactive cardiac substances: Potent vasorelaxant activity in mammalian atria. *221*(4605), 71-73.

Da Silva, M., De Souza, J., Dos Santos, L., Pinheiro, I., Borba, T., Da Silva, A., . . . Disease. (2014). Effects of maternal separation on the dietary preference and behavioral satiety sequence in rats. *5*(3), 219-228.

Dallakyan, S., & Olson, A. J. (2015). Small-molecule library screening by docking with pyrx. In *Chemical biology* (pp. 243-250): Springer.

Dev, S., Khan, B. J. J. o. m. P., & Sci, A. (2020). Molecular docking analysis of natriuretic peptide receptor-c towards the design of potential atrial fibrillation inhibitors. *974*, 2595-2600.

Diekman, M., Harms, M., Endert, E., Wieling, W., & Wiersinga, W. J. E. j. o. e. (2001). Endocrine factors related to changes in total peripheral vascular resistance after treatment of thyrotoxic and hypothyroid patients. *144*(4), 339-346.

Drewett, J. G., Fendly, B. M., Garbers, D. L., & Lowe, D. G. J. J. o. B. C. (1995). Natriuretic peptide receptor-b (guanylyl cyclase-b) mediates c-type natriuretic peptide relaxation of precontracted rat aorta. *270*(9), 4668-4674.

Edwards, B. S., Zimmerman, R. S., Schwab, T. R., Heublein, D. M., & Burnett Jr, J. C. J. C. r. (1988). Atrial stretch, not pressure, is the principal determinant controlling the acute release of atrial natriuretic factor. *62*(2), 191-195.

Ellenbroek, B. A., van den Kroonenberg, P. T., & Cools, A. R. J. S. r. (1998). The effects of an early stressful life event on sensorimotor gating in adult rats. *30*(3), 251-260.

Feng, H. B. J. W. Z., & Weissig, G. G. T. B. H. J. N. A. R. In shindyalov pe bourne (2000). *28*, 235-242.

Fink, G., Dow, R., Casley, D., Johnston, C., Lim, A., Copolov, D., . . . Dick, H. J. J. o. e. (1991). Atrial natriuretic peptide is a physiological inhibitor of acth release: Evidence from immunoneutralization in vivo. *131*(3), R9-R12.

George, E. D., Bordner, K. A., Elwafi, H. M., & Simen, A. A. J. B. n. (2010). Maternal separation with early weaning: A novel mouse model of early life neglect. *11*(1), 1-14.
Greden, J. F. J. J. o. C. P. (2001). The burden of recurrent depression: Causes, consequences, and future prospects. *62*, 5-9.
Herrmann-Lingen, C., Binder, L., Klinge, M., Sander, J., Schenker, W., Beyermann, B., . . . Pieske, B. J. P. M. (2003). High plasma levels of n-terminal pro-atrial natriuretic peptide associated with low anxiety in severe heart failure. *65*(4), 517-522.
Hirschfeld, R. M. J. T. P. C. C. f. C. D. (2001). The comorbidity of major depression and anxiety disorders: Recognition and management in primary care. *3*(6), 24412.
Hodes, A., & Lichtstein, D. J. F. i. e. (2014). Natriuretic hormones in brain function. *5*, 201.
Holmes, E. A., & Mathews, A. J. E. (2005). Mental imagery and emotion: A special relationship? , *5*(4), 489.
Holtwick, R., van Eickels, M., Skryabin, B. V., Baba, H. A., Bubikat, A., Begrow, F., . . . Kuhn, M. J. T. J. o. c. i. (2003). Pressure-independent cardiac hypertrophy in mice with cardiomyocyte-restricted inactivation of the atrial natriuretic peptide receptor guanylyl cyclase-a. *111*(9), 1399-1407.
Joffe, R. T. J. D. i. c. n. (2022). Hormone treatment of depression.
John, S. W., Krege, J. H., Oliver, P. M., Hagaman, J. R., Hodgin, J. B., Pang, S. C., . . . Smithies, O. J. S. (1995). Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *267*(5198), 679-681.
Kaczmarczyk, M., Otte, C., Wiedemann, K., Kuehl, L., Schultebraucks, K., Spitzer, C., & Wingenfeld, K. J. P. (2019). Major depression and atrial natriuretic peptide: The role of adverse childhood experiences. *101*, 7-11.
Kohno, M., Murakawa, K.-I., Yasunari, K., Nishizawa, Y., Morii, H., & Takeda, T. J. T. A. j. o. m. (1987). Circulating atrial natriuretic peptides in hyperthyroidism and hypothyroidism. *83*(4), 648-652.
Kohno, M., Takaori, K., Matsuura, T., Murakawa, K., Kanayama, Y., Takeda, T. J. B., & Communications, B. R. (1986). Atrial natriuretic polypeptide in atria and plasma in experimental hyperthyroidism and hypothyroidism. *134*(1), 178-183.
Komatsu, Y. J. C.-t. n. p. i. r., & Endocrinology, h. (1991). Nakao k, suga s, ogawa y, mukoyama m, arai h, shirakami g, hosoda k, nakagawa o, hama n, kishimoto i, and imura h. *129*, 1104-1106.
Koukoulis, G., Polymeris, A., Tzavara, I., Pappas, D., & Thalassinos, N. J. H.-A.-. (2002). Normalization of thyroid hormone levels in patients with either hyper-or hypothyroidism results in a profound change of atrial natriuretic peptide (anp) levels. *1*, 104-112.
Krogh, J., Ströhle, A., Westrin, Å., Klausen, T., Jørgensen, M. B., & Nordentoft, M. J. P. (2011). N-terminal pro-atrial natriuretic peptide response to acute exercise in depressed patients and healthy controls. *36*(5), 656-663.
Liu, T., Zhong, S., Liao, X., Chen, J., He, T., Lai, S., & Jia, Y. J. P. o. (2015). A meta-analysis of oxidative stress markers in depression. *10*(10), e0138904.
Łojko, D., & Rybakowski, J. K. J. J. o. A. D. (2007). L-thyroxine augmentation of serotonergic antidepressants in female patients with refractory depression. *103*(1-3), 253-256.
Marin-Grez, M., Fleming, J., & Steinhausen, M. J. N. (1986). Atrial natriuretic peptide causes pre-glomerular vasodilatation and post-glomerular vasoconstriction in rat kidney. *324*(6096), 473-476.
Meaney, M. J., Diorio, J., Francis, D., Widdowson, J., LaPlante, P., Caldji, C., . . . Plotsky, P. M. J. D. n. (1996). Early environmental regulation of forebrain glucocorticoid receptor gene expression: Implications for adrenocortical responses to stress; pp. 61–72. *18*(1-2), 61-72.
Meaney, M. J., Mitchell, J. B., Aitken, D. H., Bhatnagar, S., Bodnoff, S. R., Iny, L. J., & Sarrieau, A. J. P. (1991). The effects of neonatal handling on the development of the adrenocortical response to stress: Implications for neuropathology and cognitive deficits in later life. *16*(1-3), 85-103.
Meyer, T., Herrmann-Lingen, C. J. V., & hormones. (2017). Natriuretic peptides in anxiety and panic disorder. *103*, 131-145.
Mikics, É., Barsy, B., Barsvári, B., Haller, J. J. H., & behavior. (2005). Behavioral specificity of non-genomic glucocorticoid effects in rats: Effects on risk assessment in the elevated plus-maze and the open-field. *48*(2), 152-162.
Millstein, R., Ralph, R. J., Yang, R. J., Holmes, A. J. G., Brain, & Behavior. (2006). Effects of repeated maternal separation on prepulse inhibition of startle across inbred mouse strains. *5*(4), 346-354.
Millstein, R. A., Holmes, A. J. N., & Reviews, B. (2007). Effects of repeated maternal separation on anxiety-and depression-related phenotypes in different mouse strains. *31*(1), 3-17.

MORI, Y., NISHIKAWA, M., MATSUBARA, H., TAKAGI, T., TOYODA, N., OIKAWA, S., & INADA, M. J. E. (1990). Stimulation of rat atrial natriuretic peptide (ranp) synthesis by triiodothyronine and thyroxine (t4): T4 as a prohormone in synthesizing ranp. *126*(1), 466-471.

Muramatsu, H., Suzuki, Y., Tsuchiya, T., Ohtake, R., Hashigami, Y., Kobori, H., . . . Shimoda, S. J. N. N. G. Z. (1990). Altered alpha atrial natriuretic peptide (anp) concentrations in plasma, atria and hypothalamus in experimentally induced hyper-and hypothyroid rats. *66*(3), 168-174.

Nagaya, N., Nishikimi, T., Uematsu, M., Satoh, T., Kyotani, S., Sakamaki, F., . . . Nakanishi, N. J. C. (2000). Plasma brain natriuretic peptide as a prognostic indicator in patients with primary pulmonary hypertension. *102*(8), 865-870.

Nakamura, K., Kikusui, T., Takeuchi, Y., & Mori, Y. J. J. o. v. m. s. (2003). The influence of early weaning on aggressive behavior in mice. *65*(12), 1347-1349.

Nemeroff, C. B. J. S. A. (1998). The neurobiology of depression. *278*(6), 42-49.

Nestler, E. J., Barrot, M., DiLeone, R. J., Eisch, A. J., Gold, S. J., & Monteggia, L. M. J. N. (2002). Neurobiology of depression. *34*(1), 13-25.

Newport, D. J., Stowe, Z. N., & Nemeroff, C. B. J. A. J. o. P. (2002). Parental depression: Animal models of an adverse life event. *159*(8), 1265-1283.

Northcott, C. J., & Stein, M. B. J. T. J. o. c. p. (1994). Panic disorder in pregnancy.

O'Boyle, N. M., Banck, M., James, C. A., Morley, C., Vandermeersch, T., & Hutchison, G. R. J. J. o. c. (2011). Open babel: An open chemical toolbox. *3*(1), 1-14.

Odhong', C., Wilkes, A., van Dijk, S., Vorlaufer, M., Ndonga, S., Sing'ora, B., & Kenyanito, L. J. F. i. S. F. S. (2019). Financing large-scale mitigation by smallholder farmers: What roles for public climate finance? , *3*, 3.

Ogawa, H., Qiu, Y., Ogata, C. M., & Misono, K. S. (2004). Crystal structure of hormone-bound atrial natriuretic peptide receptor extracellular domain: Rotation mechanism for transmembrane signal transduction. *J Biol Chem*, *279*(27), 28625-28631. doi:10.1074/jbc.M313222200

Organization, W. H. (2017). *Global diffusion of ehealth: Making universal health coverage achievable: Report of the third global survey on ehealth*: World Health Organization.

Otte, C., Gold, S. M., Penninx, B. W., Pariante, C. M., Etkin, A., Fava, M., . . . Schatzberg, A. F. J. N. r. D. p. (2016). Major depressive disorder. *2*(1), 1-20.

Rolandi, E., Santaniello, B., Bagnasco, M., Cataldi, A., Garibaldi, C., Franceschini, R., & Barreca, T. J. E. J. o. E. (1992). Thyroid hormones and atrial natriuretic hormone secretion: Study in hyper-and hypothyroid patients. *127*(1), 23-26.

Schmidt, M. V. J. P. (2011). Animal models for depression and the mismatch hypothesis of disease. *36*(3), 330-338.

Ströhle, A., Jahn, H., Montkowski, A., Liebsch, G., Boll, E., Landgraf, R., . . . Wiedemann, K. J. N. (1997). Central and peripheral administration of atriopeptin is anxiolytic in rats. *65*(3), 210-215.

Unützer, J., Schoenbaum, M., Katon, W. J., Fan, M. Y., Pincus, H. A., Hogan, D., & Taylor, J. J. J. o. t. A. G. S. (2009). Healthcare costs associated with depression in medically ill fee-for-service medicare participants. *57*(3), 506-510.

Widecka, K., Gozdzik, J., Dutkiewicz, T., Majewska, U., & Czekalski, S. J. A. o. c. b. (1990). Atrial natriuretic factor in untreated hyperthyroidism. *27*(4), 313-317.

Wisén, A. G., Ekberg, K., Wohlfart, B., Ekman, R., & Westrin, Å. J. J. o. a. d. (2011). Plasma anp and bnp during exercise in patients with major depressive disorder and in healthy controls. *129*(1-3), 371-375.

Wishart, D. S., Knox, C., Guo, A. C., Shrivastava, S., Hassanali, M., Stothard, P., . . . Woolsey, J. J. N. a. r. (2006). Drugbank: A comprehensive resource for in silico drug discovery and exploration. *34*(suppl_1), D668-D672.

Wolkowitz, O. M., & Rothschild, A. J. (2008). *Psychoneuroendocrinology: The scientific basis of clinical practice*: American Psychiatric Pub.

Wong, N., Huang, D., Guo, N., Wong, E., Hu, D. J. A. J. o. P.-E., & Metabolism. (1989). Effects of thyroid status on atrial natriuretic peptide release from isolated rat atria. *256*(1), E64-E67.

Zhou, Y., Ren, W., Sun, Q., Yu, K. M., Lang, X., Li, Z., & Zhang, X. Y. J. T. p. (2021). The association of clinical correlates, metabolic parameters, and thyroid hormones with suicide attempts in first-episode and drug-naïve patients with major depressive disorder comorbid with anxiety: A large-scale cross-sectional study. *11*(1), 1-7.