Characterization of atrial natriuretic peptide (ANP) response in depression-induced mice models

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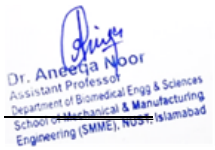
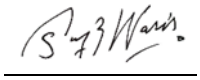
Supervisor Name: Signature: 

Name of Dean/HOD: Signature: 

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DEDICATION

Dedicated to every kindhearted individual I met along the way, as well as to my parents and beloved siblings, who always provided me with support and encouragement when things were tough.

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LIST OF SYMBOLS, ABBREVIATIONS and acronyms

ANP	Atrial natriuretic peptide
ANF	Atrial natriuretic factor
NPPA	Natriuretic peptide A
BNP	Brain natriuretic peptide
CNP	C-type natriuretic peptide
LT4	Levothyroxine 4
PCR	Polymerase chain reaction
RT PCR	Real time polymerase chain reaction
qPCR	Quantitative real time polymerase chain reaction
ECF	Extracellular fluid
HPA	Human pituitary axis
ACTH	Adrenocorticotropic hormone
CRH	Corticotropin releasing hormone
NRXN1	Neurexin 1
NUCB1	Nucleobindin 1
NFL	Neurofilament light chain
NMDA	N-methyl-D-aspartate
IRB	Institutional review board
MSEW	Maternal separation with early weaning
TST	Tail suspension test
LDL	Low density lipoprotein
HDL	High density lipoprotein
CRP	C-reactive protein
H & E	Haematoxylin and Eosin
PBS	Phosphate-buffered saline
DTT	Dithiothreitol
NCBI	National Center for Biotechnology Information
CDNA	Complementary DNA
PDB	Protein data bank
SD	Standard deviation
PND	Postnatal day

ABSTRACT

Depression is a complex psychological disorder that is also often link to the hormonal imbalances. Our research delves into the intricate relationship between depression and atrial Natriuretic Peptide (ANP), considering the multifaceted influences of neurological, genetic, and environmental factors. With a focus on hormonal imbalances as key contributors to depression, our investigation explores the potential therapeutic effects of Levothyroxine (LT4) in the context of ANP. Utilizing an early weaning mouse model involving maternal separation, we conducted a detailed examination of the anxiolytic effects of LT4, aiming to evaluate its efficacy in alleviating symptoms associated with anxiety and depression. Behavioral assessments and histological analyses were employed to comprehensively evaluate the impact of LT4 on ANP. This study also extends to molecular investigations using RT-PCR to analyze the distribution and expression of ANP within the mouse central nervous system, highlighting the cortex region. Our findings reveal significant differences in brain expression levels of ANP between treated mice and those exhibiting depressive symptoms. This insight suggests potential therapeutic applications of ANP for mitigating depression, presenting intriguing avenues for further research, particularly in the context of depression-induced mouse models through parental separation. This research contributes to an enriched understanding of the complex factors influencing depression and proposes interventions that extend beyond conventional approaches. The integration of behavioral assessments, histological analyses, and molecular investigations offers a holistic perspective, laying the groundwork for future exploration in the critical realm of mental health research.

Key words: Depression, ANP, Hypothyroidism, maternal separation, levothyroxine, thyroid hormone

CHAPTER: 1 INRODUCTION

1.1 Atrial natriuretic peptide (ANP)

The human NPPA gene encodes the natriuretic peptide hormone primarily secreted by the cardiac atria, also referred to as atrial natriuretic peptide (ANP) or atrial natriuretic factor (ANF). Members of the structurally linked family of hormones known as natriuretic peptides comprise ANP, brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP; Macchia, 1987). ANP predominantly reduces the volume of elevated extracellular fluid (ECF) by promoting renal sodium excretion. Cardiac muscle cells in the walls of the atria of the heart's chambers synthesize and secrete ANP and these cells' volume receptors respond to an upsurge in atrial blood volume, triggering the atrial wall to stretch significantly (Potter et al., 2009). A disulfide bond between the cysteine residues at positions 7 and 23 forms the 17-amino acid ring that is a characteristic of the 28-amino acid ANP peptide. Given that BNP and CNP share this structural feature, it is clear that they are closely related. Of the nine natriuretic hormones that are structurally related, seven come from the atria (Vesely DL, 2013).

1.2 Role of ANP in Different Body Functions

ANP, the first member of the natriuretic peptide family, and the first indication of an endocrine connection between the heart and kidneys was discovered by De Bold. De Bold and associates also discovered that administering an atrial homogenate to rats resulted in notable diuresis and natriuresis (De Bold et al., 1981). The experiments prove that a key role in the process of blood pressure homeostasis is played by ANP. By raising the glomerular filtration rate and preventing the kidney from reabsorbing salt and water, ANP causes natriuresis and diuresis (Marin-Grez et al., 1986).

ANP acts as a functional antagonist of the renin-angiotensin-aldosterone system by blocking the kidney's release of renin and the adrenal glands' production of aldosterone. (Richards et al., 1988). Vasorelaxation results from its stimulation of smooth muscle cell relaxation in blood vessels (Currie et al., 1983). It also increases endothelium permeability,

which controls intravascular volume (Baron et al., 1989). It was discovered that ANP knockout mice exhibited salt-sensitive hypertension in line with these consequences (John et al., 1995). By preventing cardiac hypertrophy, ANP also has a direct impact on the heart.

1.3 Role of ANP in the central nervous system

Natriuretic peptides are generally expressed in the brain, with cerebral tissues exhibiting a significant prevalence of CNP. This high concentration implies a potential function for CNP as a neurotransmitter in addition to its primary function as a cardiac hormone. ANP expression was initially determined to be present in the hypothalamus, which is the primary source of natriuretic peptides in the brain. ANP has been identified in neurons and glial cells in the cerebral cortex and cerebellum. Moreover, ANP has been detected in neurons and fibers in several different places, such as the limbic areas, olfactory bulb, striatum, and thalamus.

Previous studies have shown that natriuretic peptides do regulate the neuroendocrine secretions and cardiovascular processes. By using radioimmunoassay and immunohistochemistry, ANP, which was first discovered in the heart and peripheral tissues, has been found in both human and rodent brains (Ito et al., 2011). A growing body of research suggests that cerebral ANP plays a significant role in controlling blood pressure, cerebral blood flow, brain water and electrolyte balance, and neuroendocrine functions. The homeostasis of the central nervous system may therefore be impacted by variations in cerebral ANP levels. Since peripheral clearance controls the amount of ANP in circulating blood, it is believed that both production and clearance also influence the cerebral level of ANP (Chiu et al, 1991).

1.4 Role of ANP in psychological disorders

In the setting of depression, complicated neuroendocrine interactions exist between ANP and the hypothalamic-pituitary-adrenal (HPA) axis. While ANP is most widely known for its effects on cardiovascular health, it also seems to have an impact on the HPA axis, which is essential to the body's stress response. The HPA axis is regulated by

natriuretic peptides, which additionally impact depression and behaviors that are addictive. Studies indicate that ANP may modulate the release of adrenocorticotropic hormone (ACTH) from the pituitary gland and subsequently downregulates corticotropin-releasing hormone (CRH) from the hypothalamus to have inhibitory effects on the HPA axis (Holsboer, 2000). One prevalent characteristic of depressed patients is dysregulation of the HPA axis, which results in a high concentration of cortisol, the final product of this pathway. (Fink et al., 1991; Vesely et al, 2001). Natriuretic peptides control the HPA axis at multiple levels. Moreover, ANP directly reduces cortisol release, which is a major stress hormone in the body, although CNP has the opposite effect.

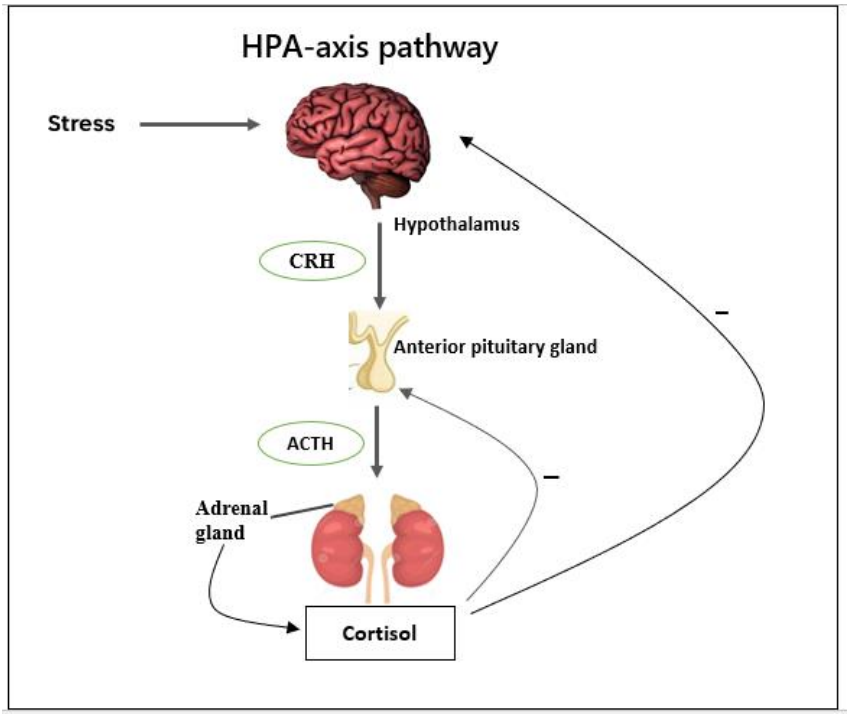

Figure 1.1 HPA-axis pathway The HPA axis operates in a stepwise manner: the pituitary gland releases ACTH in reaction to stress after the brain releases CRH. The adrenal glands then create and release cortisol into the bloodstream in response to ACTH. By imposing a negative feedback loop on the pituitary and hypothalamus, cortisol controls the stress response and upholds homeostasis.

In an experiment, the rats' anxiety-related behavior was reduced when ANP was administered centrally or peripherally (Ströhle et al., 1997). Human patients with anxiety-related disorders, heart failures, and depression have been shown to have reduced levels of anxiety when their ANP levels are high (Levinson et al., 2003; Herrmann-Lingen et al.,

2003). Increases in plasma ANP levels were seen after experimentally generated panic attacks; these increases were more rapid and noticeable in patients with panic disorder (Kellner et al., 1998). These findings point to the possibility of using ANP agonists therapeutically to address diseases associated with stress and depression (Kellner et al., 2003). Moreover, pretreatment with intravenous ANP dramatically decreased the number of experimentally produced panic attacks in both healthy individuals and patients with panic disorder (Ströhle et al., 2003). These discernments suggest the therapeutic role of ANP in anxiety and depressive disorders. The effect of ANP on the treatment of anxiety has been mechanically associated with the inhibitory role of ANP on the HPA axis (Kellner et al., 2003).

1.5 The connection between thyroid hormone and ANP

There are intricate interactions within the endocrine system between thyroid hormones and ANP. Evidence suggests that ANP may potentially affect thyroid function, even though its primary function is the regulation of the cardiovascular system. Gardner employed rat experiments to illustrate the connection between ANP and thyroid hormone. Rats with thyroidectomies that were either watered or dehydrated showed a two-fold rise in ANP concentration, and dehydrated rodents showed a three-fold increase in cardiac ANP RNA. Following this investigation, thyroid hormone stimulates an upsurge in ANP production and genomic expression (Gardner et al., 1987). An additional investigation evaluated cell secretion and content into the rat substratum of immunoreactive ANP as well as the RNA levels of ANP in the incubated atrial myocytes of newborn animals to explore the significance that T4 serves in enhancing the synthesis of ANP. T4 administration led to a dose-dependent rise in cell production and ANP concentration (Mori et al., 1990).

The potential relationship between thyroid hormone and ANP has been studied through the induction of hypo- and hyperthyroidism in rodents. The quantity of ANP in the brain hypothalamus, plasma, and atria was demonstrated using the radioimmunoassay technique. Unlike control group, the quantity of ANP in plasma was found to be significantly higher in hyperthyroid rats and significantly lower in hypothyroid rats. The quantity of ANP in the atria of hyperthyroid rats reduced, while the level of ANP in the

atria of hypothyroid rats did not significantly change. In comparison to the control group, the concentration of ANP in the hypothalamus of hyperthyroid rats did not change, whereas the level of ANP in the hypothalamus of hypothyroid rats decreased significantly (Muramatsu et al., 1990).

There has been an established connection between thyroid hormone and depression for several decades now. Depression-related symptoms are more prevalent among individuals with thyroid conditions. According to earlier research, individuals with hypothyroidism have four times greater chance of major depressive disorder than the general population (Chueire et al., 2007). This suggests that low thyroid concentration exacerbates depression. Thyroid hormones were closely linked to suicide attempts made by individuals with significant depressive illnesses, according to (Zhou et al., 2021). Patients with refractory or uncomplicated depression typically have subclinical thyroid dysfunction (Fountoulakis et al., 2006). The researchers have examined the potential link between mental health issues and thyroid hormones for more than 100 years. Thyroid disorders have been linked to higher occurrences of nerve affectations. Thyroid function has a significant impact on psychological wellness, including memory and emotions, as has long been widely understood. According to estimates, 2 to 40% of those individuals suffering from mental illnesses experience clinical depression, while 1 to 4% of individuals with mental disorders experience hypothyroidism (Wolkowitz & Rothschild, 2008).

A strong positive correlation between the amount of ANP and thyroid hormone concentration has been established. It has been demonstrated that people with hyperthyroidism have significantly greater ANP concentrations, whereas individuals with hypothyroidism have lower ANP levels which becomes normal after adequate therapy of ANP (Koukoulis et al., 2002). LT4 administration had beneficial results in depressive patients contrary to fluoxetine (Barak et al., 1996) and improved depressive symptoms in female depressed patients whose response to serotonergic antidepressants was inadequate (Łojko & Rybakowski, 2007).

1.6 Hormonal response in psychiatric disorders

Depression is a multifactorial psychological disorder with environmental, genetic, and neurological components. Hormones serve as vital for mood regulation and mental health, and depression is frequently associated with changes in hormonal balance. It's crucial to remember, though, that there are many facets to the association between depression and hormones, and this area of research remains under development. Reduced concentrations of neurotransmitter serotonin are seen in those with depression (Belmaker & Agam, 2008). Chronic stress can cause dysregulation of the stress hormone cortisol, which can impact the HPA axis and exacerbate symptoms of depressive disorders (Anacker et al., 2011). Numerous antidepressants target the abnormal functioning of reward-related neurotransmitters, dopamine, and norepinephrine, which are connected to depression (Nestler et al., 2002). The significance of thyroid hormones in controlling metabolism is well-established; hypothyroidism has been scientifically correlated with manifestations of depression (Bauer et al., 2002). Recognizing the diversity of depression and the complex interactions between hormones, environment, and heredity is essential. For an in-depth comprehension and enhanced therapeutic approaches for depressive disorders, ongoing study is crucial.

1.7 Depression

Depression is a prevalent and debilitating mental illness that continues to be a major global public health concern. People of any age, gender, or ethnicity can suffer from depression. According to Kessler et al. (2003), it has been defined by persistent emotions of sadness and hopelessness together with an overall lack of enthusiasm or enjoyment in mundane activities. Depression is a complex illness that is challenging to fully comprehend and cure due to its complex interplay of genetic, environmental, and neurological components. Numerous studies have looked into the intricate reasons for depression, including information about alterations in neurotransmitter systems, neuroplasticity, and inflammatory pathways associated with the condition (Krishnan & Nestler, 2008). Numerous studies have also been conducted on the impact of stress, trauma, and life events on the onset and severity of depressive symptoms. Major depressive disorder is a

significant problem for medical professionals globally, with 18 million instances in the US and 340 million cases worldwide (Greden, 2001).

The devastating impact that depression has on patients and healthcare systems shows how urgently effective therapies are needed. Two of the primary psychological issues that cause disability and reduced efficiency worldwide are anxiety and depression (World Health Organization, 2017). Depression affects not just the subjective symptoms that sufferers describe, but also how well they function in the interpersonal, professional, physiological, and cognitive domains. According to research, depression is more likely to result in a decline in health than major chronic physical conditions such as angina, diabetes, asthma, and arthritis. An individual with depression experiences severe reductions in their ability to work due to emotional, motivational, and cognitive effects. This results in lower income for the individual and their family, as well as a lessened ability to contribute to the community through professional and tax-related means. The broader societal repercussions include a decline in self-worth and confidence as well as an increase in reliance on assistance and benefits. Social impairments, such as reduced communication skills and trouble maintaining relationships, have a cascading effect even after an episode ends (Moussavi et al., 2007).

Furthermore, individuals with persistent or recurring depressive disorders may encounter long-term impairment in their ability to function socially, underscoring the long-term consequences of depression on the community. Pain, misery and disability associated with medical conditions can be rendered harsher by depression, which can negatively impact outcomes overall. Long-term physical wellness issues combined with depression exacerbate health decline by more than a single physical sickness or even a combination of illnesses (Moussavi et al., 2007). Moreover, an increased probability of death is associated with concurrent depression for multiple physical health problems (Cassano & Fava, 2002). According to Nielsen et al. (2006), there is a correlation between depressive illnesses and an 80% increased risk of coronary heart disease and death from it in later life. This correlation is partially explained by shared contributing factors. Sometimes aggressive acts towards others, including homicide, are the result of depressive periods.

According to Ramachandani and Stein (2003), parental depression can lead to child neglect and severe disruptions in children, which can negatively affect family dynamics. Suicide makes up nearly 1% of all fatalities, and over two-thirds of suicide deaths include depressed individuals (Sartorius, 2001). When taken into consideration, the risk of suicide for those suffering from depression is greater than four times higher than for the general population. That risk increases to nearly a twentieth for the sickest patients (Bostwick & Pankratz, 2000). Despite the prevalent belief that depression stems from a chemical imbalance, the condition is more complex than it first appears. A plethora of evidence indicates that depression is not solely caused by an imbalance in brain chemistry. Instead, several intricate factors are involved, including genetic predisposition, social setting stress, and cognitive irregularities of mood. These variables may collaborate and contribute to the development of depression, according to research (Nestler et al., 2002; George et al., 2010).

1.7.1 Presentation and symptoms of depression

Depression's cognitive and physical symptoms are often associated with crying, rage, withdrawal from social interactions, exacerbation of pre-existing problems, and pain from tightening of the muscles. Reduced energy, fatigue, and reduced productivity are further symptoms. Anxiety is considerably elevated, and restlessness is frequently prevalent (Lewinsohn et al., 2000). In general, there's reduced appetite and sleep, which might sometimes lead to a discernible decrease in weight. However, it is reported that some people claim to be hungry more frequently and sleeping longer. Feelings of guilt, unworthiness, and rightful punishment coexist with a broad lack of interest in and enjoyment from daily living. It is not rare to experience suicidal thoughts, diminished self-worth, confidence problems, powerlessness, or attempts at suicide or self-harm. Modifications in behavior may encompass a loss of concentration, a shorter attention span, and depressive ideas regarding themselves, the past, and future aspirations; they can also include cognitive laziness and fantasizing (Cassano & Fava, 2002).

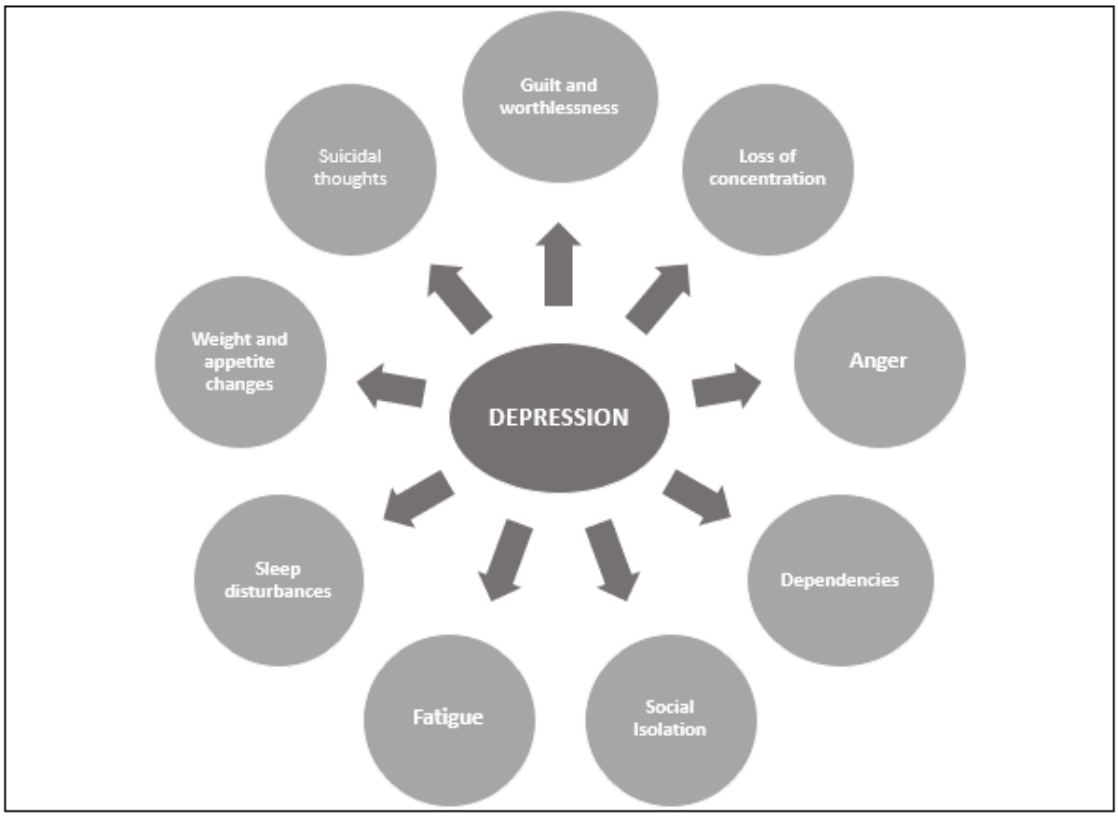

Figure 1.2 Symptoms of depression. This figure represents the symptoms possessed by individuals with depression.

Atypical presentations include impulsive behavior, increased appetite, weight gain, and chronic sleep deprivation in certain individuals. Additionally, according to Barnhofer and Chittka (2010), these individuals possess a personality factor that makes them vulnerable to refusal. Major depression is the diagnosis determined by these symptoms. Severe depression may also be a contributing factor in psychotic symptoms, such as hallucinations or delusions. The depressive thoughts and low mood that characterize severe depression are fundamentally linked to these symptoms. When a person exhibits psychotic symptoms that are irrelevant to their state of emotions, it can be challenging to differentiate severe depression from psychotic diseases such as schizophrenia (Andrews & Jenkins, 1999).

1.7.2 Prevalence and incidence

The World Health Organization reports that 121 million people globally struggle with depression, ranking it among the top ten indicators of morbidity and mortality (Rosenzweig-Lipson et al., 2007). The proportion of the global population that is predicted to experience depression at some time in their lives varies substantially among studies and contexts. However, the most accurate projections put the estimated prevalence for mild recurring depressive symptoms at 2.5 to 5% and severe depression at 4 to 10% (Waraich et al., 2004). Inconsistencies in this data may arise from differences in methods of evaluation and real disparities between nations. In the UK, 2.6% of individuals between the ages of 16 and 74 experienced an approximate point rate of an episode of depression in 2000 (2.8% for females, 2.3% for males). However, these percentages dramatically increased to 11.4% (13.6% for females, 9.1% for males) when the more extensive classification of mixed anxiety and depression was considered (Singleton et al., 2001).

It has been repeatedly demonstrated that women had 2.5 times a higher incidence of clinical depression than men. Moreover, there has been no variation in these rates across the years between the ages of 18 and 64 (Waraich et al., 2004). Taking ethnic status into consideration has not resulted in any appreciable variation in the prevalence rates of mood disorders or mixed anxiety and depression. Still, it is significant that, from a statistical perspective, there were a higher number of South Asians among individuals with either depression or anxiety than among those without it (Singleton et al., 2001).

1.7.3 Etiology of depression

The appearance, onset, and outcomes of depressive illnesses vary significantly, as evidenced by the variety of scientific explanations for their genesis. These justifications include genetics (Kendler & Prescott, 1999), endocrine, biochemical processes, and neurophysiological aspects (Malhi et al., 2005), as well as emotional processes, and social circumstances (Brown & Harris, 1978). There is now more focus on physiological explanations for triggers, particularly endocrine theories, as a result of observations that certain medical conditions raise the prevalence of depression such as hypothyroidism. (Cassiano and Fava, 2002). The assumption that depression is a disorder found in brain

structure and function has been bolstered by developments in neuroimaging, even while psychology research stresses the significance of cognitive and emotional processes (Drevets et al., 2008; Beck, 2008).

Nevertheless, an individual's susceptibility to depressive disorders can be impacted by a number of variables, such as gender, adverse childhood events, the impacts of genetics and family, and interpersonal circumstances and temperamental characteristics. The stress-vulnerability model (Nuechterlein, 1984: Harris, 2000) states that adverse life circumstances or medical conditions are instances of stimuli that combine with vulnerability traits that lead to the occurrence of a bout of depression (Kendler et al., 2001). Moreover, early life events that include neglect, abusive, physical or sexual abuse, unstable marriages, divorce, bad parent-child connections, increase a person's risk of getting depression in future life (Fava & Kendler, 2000). Personality qualities like neuroticism raise the risk of depression when confronted with stressful life situations (Fava & Kendler, 2000).

1.7.4 Model of Depression Based on Maternal Separation

With the widespread practice of separating moms from newborns in hospital settings as soon as they are born, changes in society have fundamentally changed the relationship between mothers and their children (Odhong et al., 2019). There is mounting evidence to suggest that early-life adversity is positively correlated with the emergence of psychopathologies like anxiety and depression (Coplan et al., 2014). The utilization of a rodent model of childhood adversity is advantageous in understanding the neurobiological and behavioral changes associated with early-life neglect. Many protocols for experiments aimed at eliciting behavioral changes have been developed, most of them utilizing rats (Kaufman et al., 2010; Trent et al., 2019). Depression usually strikes during a person's developing years, stopping and lowering their quality of life and functioning. For instance, rodents that are separated from their mothers for three hours have altered HPA axis function as well as behavioral alterations such as anxiety, fearfulness, and attentiveness (Millstein et al., 2005). But the same routine also increases the amount of attention mothers give to their babies, lessening the impact of maternal deprivation. Because of these limitations in the outcomes of the 3-hour maternal separation model, the current study chose to combine

maternal separation with early weaning in an effort to reduce maternal care for infants (George et al., 2010).

Globally, infant adversity is omnipresent and takes the form of neglect. Neglect during infancy is expected to affect 24.4 out of 1000 children (Ogawa et al., 2004). It is uncertain how early life stress develops depression in living organisms due to a complex neurological process. An organism's interactions with its environment during the perinatal stage of life have a major impact on the structural and functional development of the organism, which is essential for optimal adaptations that have a tremendous effect on reproduction and succession. The structure and function of the organism may suffer from any departure from ideal conditions during the first year of growth (Silva et al., 2014; Colombo, 2019). Crucially, it has been noted that prenatal aggravations have long-lasting impacts on the organism as opposed to temporary ones.

Using a real animal model of depression turns out to be an effective way to test and develop treatments, as well as to understand the underlying causes of the illness. According to Lajud et al. (2012), HPA axis is affected by maternal separation, which can cause depressive symptoms after three hours of mother-infant separation. These symptoms are marked by hyperactivity in the HPA axis. These modifications to the rat model also have a strong resemblance to the consequences of maternal neglect and deprivation in humans. There is general agreement that children of neglectful mothers are more likely to experience depressive symptoms in the future (Belzung et al., 2011).

1.7.5 Depression triggered due to early weaning

Early life disruptions to the mother-pup bond are a powerful illustration of how childhood adversity affects neurobiology, physiology, and emotional reactions. According to research, this disruption prevents the neuroendocrine system from maturing, which has long-lasting impacts on the organism (Ellenbroek et al., 1998). A large body of research uses mother separation paradigms to study the long-term effects of early life adversity in rodents. According to Holmes and Mathews (2005), these ideologies sabotage parent-child interactions, which results in deprivation and neglect of the child during their formative years. Mother deprivation is a part of maternal separation protocols, albeit the length and frequency of separations can vary beginning two weeks after delivery. Thus, contingent

upon these variances, specific mother-infant separation processes have been reported to induce enduring anxiety and depression symptoms in addition to modifications in the HPA axis reaction to stressful situations (Richardson et al., 2004; Deschesnes et al., 2008). Levine's pioneering research has shed light on how emotional behavior and stress reactions are shaped by maternal care during infancy (Levine, 1957; Marais et al., 2008). Since then, a significant body of research has been accumulated, describing the effects of dam-pup separation on rats and mice during their formative years. From postnatal day 2 to day 14, one of the most popular procedures for maternal separation entails separating the mother and her pups for three hours each day (Barna et al., 2003). However, depending on the length of the separation and other subtleties of the protocol used, other studies have documented separations lasting anywhere from three to eight hours and even up to twenty-four hours, leading to varied degrees of experienced stress (George et al., 2010).

When combined with early weaning, maternal separation refers to prolonged times of isolation that take place at different postnatal ages, and then there is the added component of early weaning. In comparison to conventional maternal separation models, this separation model entails noticeably longer periods of segregation. Additionally, early weaning of the children from the mother limits the amount of time that they spend with their parents. An inventive approach that enables thorough an investigation of the behavioral and neurobiological effects of parental deprivation in mouse models is the separation of the mother and early weaning. Schmidt (2011) pointed out that the effectiveness of maternal deprivation models depends on specific environmental factors and genetic predisposition. Given its vital role, no depressive model of early childhood stress can be considered robust if it does not take the adult environment into account. In a demanding adult setting, early adversity may have adaptive effects; yet, in a non-aversive environment, it may have dysfunctional effects.

1.8 Neuronal proteins association with depression

Neurexin (NRXN1), nucleobindin-1 (NUCB1), NR2A, and neurofilament light chain (NFL) are some of the neuronal proteins which are found to be associated with depression and other neurodegenerative diseases.

1.8.1 Neurexin

NRXN1 is extensively expressed throughout the brain because it is vital to the neurological system. Presynaptic adhesive proteins known as neurexins are involved in the synapse-mediated attachment of neurons (Wiśniowiecka et al., 2010; Dachtler et al., 2016). Because of their postsynaptic connections to neuroligins, neurexins are involved in synaptic function. According to Dachtler et al., (2016) and Wiśniowiecka et al., (2010), mice with heterozygous deletion of neurexin I and neurexin II exhibit symptoms related to schizophrenia, autism, behavioral and cognitive impairments, depression, and anxiety. Research revealed a substantial difference between the mRNA and protein expressions of the NRXN1 gene in patients with recurrent depressive disorders and the healthy control group. In the group of patients suffering from depressive disorders, expression was reduced (Skiba et al., 2021). Nevertheless, a further investigation demonstrates elevated expression of NRXN1-α and β in bipolar illness and schizophrenia. Furthermore, there is a connection between hypothyroidism and NRXN1 function since both conditions cause the cerebellum to downregulate neurexin 1 (Wang et al., 2016).

1.8.2 NUCB1

Since NUCB1 is a multidomain eukaryotic protein that binds calcium and DNA, it is abundantly expressed in nerve cells. NUCB1 protein has been linked to tauopathy pathogenesis, Alzheimer's disease, and other neurodegenerative disorders. These processes include neuronal plasticity, the functioning of mitochondria, proteostasis and glucose metabolism, all of which are known to be dysregulated early in the process (Mikhaylina et al., 2023). Research indicates that people with mental illnesses associated with malfunction in the temporal cortex, like sadness and anxiety, have altered neuronal expressions of NUCB1. Patients with other illnesses, like HIV, frequently experience symptoms of depression. NUCB1 may be a possible target for depression since depressed symptoms in these patients are linked to increased NUCB1 expression.

1.8.3 NR2A

Two essential NR1 subunits in addition to a minimum of two NR2 (A-D) or NR3 (A-B) subunits make up the NMDA receptor11. As NR2A usually modulates synaptic

transmission, it is relevant to cognitive impairment. The presence of NR1 is prenatal in rodents; it is low at birth but gradually increases over the next two to three weeks. Throughout life, the NR2A subunit gradually rises. Fast neurotransmission is mediated by NR1/NR2A receptors, which are mostly found at synaptic sites (Sun et al., 2013). According to one study, depressed individuals' NR2A protein levels were noticeably and considerably higher (+115%) than those of controls. Western immunoblotting was used to determine the concentrations of NR1 and NR2A (Karolewicz et al., 2009). Moreover, a limited amount of literature also revealed that thyroxine rapidly decreases the NMDA concentration in the hippocampus of rat brains (Losi et al., 2008).

1.8.4 NFL

NFL is a subunit of neurofilaments that is found in axons, dendrites, and neuronal soma. It contributes to neuronal structural stability. NFL is a novel biomarker of neuroaxonal injury and other illnesses which include neurodegenerative disorders. Major depressive disorder is linked to neuroaxonal injury. NFL levels were substantially greater in individuals presented with depressive mental illness with respect to the control group (Chen et al., 2022). Moreover, there is evidence linking hypothyroidism to the NFL. In the developing rat brain, hypothyroid neurons exhibit an abnormal build-up of NFL in addition to increased oxidative stress due to thyroid hormone deprivation. This abnormal build-up of NFL aligns with additional past findings (Rahaman et al., 2001).

1.9 Aims and Objectives

Depression is a prevalent mental health issue in the modern era that requires a thorough investigation of its underlying mechanisms to develop effective therapeutic strategies. There hasn't been much research done on the connection between depression and ANP, the complex interaction between ANP, cortisol, and depression is acknowledged as a focus of the current study. This emphasizes how intricate neuroendocrine relationships play a role in the pathophysiology of depression. My research addresses the widespread problem of depression by examining the less-studied link between thyroid hormones, ANP, and depression, especially when LT4 medication is involved. Considering the established connections between ANP and the HPA axis, as well as its possible impact on thyroid function, it is imperative to examine how LT4 medication affects this complex network.

The thyroid hormone system, which is linked to ANP, is a fascinating research topic since it may have an impact on regulating the physiological reactions linked to depression. The objectives of the current study are as follows:

1. The generation of animal models of depression through maternal separation is the primary objective.
2. The second goal is to utilize *in silico* analysis to evaluate the interaction between neuronal hormones associated with depression and LT4.
3. The assessment of the impact of LT4 treatment to reverse the ANP concentration using behavioral testing is the third goal of this investigation.
4. The evaluation of morphological and histological alterations in the various brain regions using H&E staining is the fourth goal.
5. The final goal is to summarize the findings by utilizing RT-PCR to measure the ANP levels and provide evaluations of the numerous ways in which ANP influences the anxiolytic effects of LT4 in the depression induced mice model.

CHAPTER 2: RESEARCH METHODOLOGY

2.1 Ethical statement

Every experiment was carried out in accordance with Institute of Laboratory Animal Research regulations. To preserve the safety, rights, honor, and well-being of the subjects of research, all methods employing animals as participants have been cleared by NUST Institutional Review Board (IRB) research ethics committee.

2.2 Animals

Newly delivered female BALB/C mice and their pups were bought from the animal house of Atta ur Rehman School of Applied Biosciences in NUST. The mice along with their pups were kept individually in plastic cages at a room temperature of 23 ± 2°C with adequate ventilation. The mice were also kept in a 12-hour cycle of darkness and light, and they had unrestricted access to food and water.

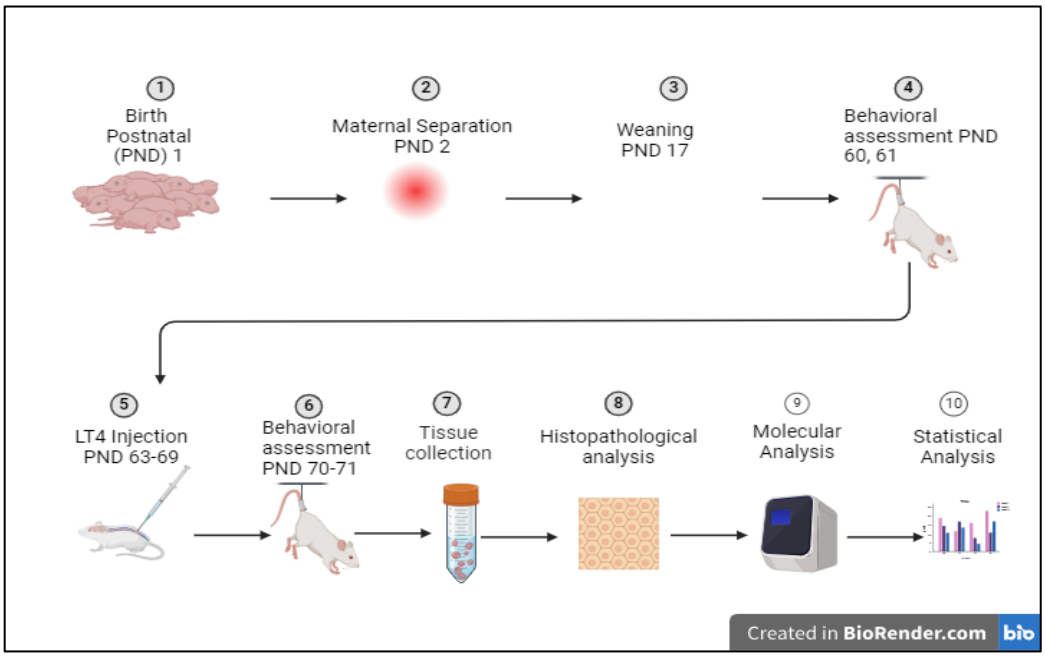

Figure 2.1 Proposed timeline of experimental study. Experimental timeline illustrating the 60-days process of developing the MSEW model, evaluation of behavior through anxiety related paradigms, LT4 treatment and again behavioral testing to assess the changes in behavior, followed by histopathological and molecular analysis.

2.3 Study model

2.3.1 Maternal separation paradigm

The protocol used in this study for the model of maternal separation along with early weaning (MSEW) was according to the guidelines established by George et al. (2010). Upon birth, the age of the pups was marked as P0. All the pups from one dam were randomly assigned to one of the groups. Littermates were housed together, up to two per cage, after weaning. A total of 15 mice were split into groups of two including control and experimental each consisting of 4 pups and a dam. The control group also contained 4 pups and a dam. The animals in the control group stayed unaltered except for when the bedding was changed. The participants in the control group received maternal separation from postnatal days 2 to 17 for 3 hours, then underwent weaning on day 17. The mice were subsequently put through tests related to depression to determine the accuracy of the MSEW model.

2.3.2 Behavioral test

Animals were tested for depressive-like phenotypes using recognized behavioral techniques in accordance with the depression-related behavioral paradigm. Prior to performing the tests, the mice had been adjusted to the experimental room and the testing environment for 48 hours. After each animal test, all the equipment was cleaned with ethanol. The digital video recording device was used for shooting videos to assess the test findings, identify the instrumentation being used, as well as recognize any behaviors that the observer was unaware of during the course of the test.

2.3.3 Tail suspension test

A crucial behavior test for evaluating the animal's reaction to depressive-like behavior is the tail suspension test (TST). Each mouse was separately suspended by its tail, which was affixed to a horizontal platform using adhesive tape, for the tail suspension test. The sticky tape was applied almost 1 cm from the tip of the tail to hold suspended mice 20 cm above the floor. The quantity of immobility duration will rise if the mice display more depressive-like behavior (Várkonyi et al., 2022). An expert observer kept track of the immobility time for 6 minutes. A six-minute session was examined and filmed. Following the testing phase, the behavioral parameters were assessed by examining the video footage. Mice were regarded as being immovable only when mice hung quietly and motionlessly. The total amount of time, when mice held by their tails remained motionless, was measured as suggested by Steru et al., (1985).

2.4 Dose preparation

Sodium hydroxide (4g) was dissolved in 100 ml of distilled water to create a 0.1M solution. LT4 15 µg was dissolved in 0.1M NaOH solution. Mice were injected with the solution at 11 a.m. every morning after it had been freshly prepared. The intraperitoneal mode of administration of LT4 injection was used on the mice for 7 days in a row, starting on Monday.

2.5 Dose administration

For seven days, 15 µg of the synthetic thyroxine known as LT4 was injected intraperitoneally into each mouse to alleviate depression brought on by MSEW (Gardener et al., 1987). For this, the two groups—the experimental group and the control group—were further separated in the manner described below:

- A control group without medication administration or maternal separation
- A study group that received MSEW and medication administration
- An experimental group without medication but with MSEW.

To demonstrate the efficiency of LT4 in raising ANP concentrations and to examine the effects on behavioral despondency and depression-like responses in mice models undergoing maternal deprivation combined with early weaning, the mice were evaluated once more using depression-associated paradigms.

2.6 Weight

The weight of mice can be an important parameter to monitor, as changes in weight may be indicative of physiological and behavioral alterations. Both weight reduction and weight gain have been reported in various examinations during the downturn. We have determined the weight of every mouse in the control group and experimental group after the completion of depression induction through maternal separation. Body weight measurements were also calculated after taking the drug for seven days.

2.7 Serum analysis

Serum analysis was done to evaluate serum protein values which includes the low-density lipoprotein (LDL), high density lipoprotein (HDL), uric acid, cholesterol, and C-

reactive protein (CRP). The blood pooling technique is used as there is not enough blood in one mouse to carry out the biochemical analysis. For this, the blood was collected through cardiac puncture. This method involves accessing the heart directly to collect for biochemical analyses. For the dissection of mice, a standard procedure was followed to collect blood samples directly from the heart. It's important to highlight that ethical guidelines and regulatory approvals were strictly adhered to throughout the process to ensure the well-being of the animals involved. The mice were anesthetized using chloroform to ensure proper depth and minimize stress during the procedure.

The anesthetized mice were positioned in a supine posture on a sterile surgical drape. To reduce the risk of infection, the chest area was thoroughly sterilized with an antiseptic solution. A small incision was made along the left side of the sternum using sterile scissors to expose the chest cavity. Carefully moving aside surrounding tissues revealed the beating heart, typically situated in the left thoracic cavity. The left ventricle of the heart was punctured using a sterile syringe and needle. The collected blood was then transferred to appropriate containers for further analysis. Blood samples of the control and experimental groups were analyzed through the pooling technique. Depending on the intended analysis, collected blood was placed in anticoagulant-treated tubes. This dissection procedure was conducted with the utmost care and adherence to ethical standards and institutional regulations.

2.8 Histopathological analysis:

Histopathological examination was conducted to scrutinize tissues at a microscopic level, aiming to investigate morphological alterations and histological patterns within the cellular structures of the brain.

2.8.1 Dissection and Fixation

The mice were euthanized under deep chloroform inhalation. For histopathological analysis, the transcardial perfusion was performed by using the fixative solution of 4% paraformaldehyde flushing through the circulatory system. By flushing through the bloodstream and displacing blood, the fixative ensured complete tissue fixation. After that the mice were carefully decapitated. The skull was then removed using scissors and a scalpel along the midline to expose the brain. By using little forceps, the brain from the skull was removed gently. To remove extra fixative and blood from the sample, the tissues were then washed with PBS. The brain tissue was then carefully immersed in the fixative solution of 4% paraformaldehyde.

2.8.2 H & E Staining

The brain was then dehydrated for H&E staining by gradually immersing the perfusion-fixed brain in ethanol at 100% concentrations. The samples were then shifted to xylene as a clearing agent to get rid of the ethanol. Subsequently, tiny pieces of tissue were removed. The cellular structures were then visible in the sections after they had been stained with H&E dye.

2.8.3 Microscopic assessment

After that, the light microscope was used to study the stained brain sections to analyze the cellular patterns, cell count, and tissue morphology. To examine the differences between the three groups and comprehend the impact of the treatment and disease processes, photomicrographs of the cerebral cortex were taken.

2.9 *In silico* analysis

Before starting the experimentation *in silico* analysis was carried out using different software and computational methods to gain insights and predict the hypothesis before conducting them physically. For that, the three-dimensional structure of multiple proteins listed in table 2.1 was downloaded from the RCSB Protein Data Bank. The chemical structures of these were obtained from the PubChem compound database. These structures were then cleaned by using the software Discovery Studio Visualizer 3.0.

2.9.1 Molecular docking

In order to appraise the binding energies, binding conformations, and binding affinities of noncovalent interaction between multiple proteins and a ligand, a methodology commonly referred to as molecular docking is employed. Using PyRx's integrated Vina Wizard module to dock ligands along with macromolecules, outcomes were found for the proteins and ligands listed in table 2.1. The accurateness and particularity of the binding molecules are increased by AutoDock Vina using multithreading on multiple core processors (Olson, 2010).

2.9.2 Interactions of Protein and Ligand

The bonding of the complex of a ligand and proteins was investigated by obtaining the complex using PyRx in the form of PDB and visualizing it in BIOVIA Discovery Studio for 2D picture illustration.

Table 2.1 Molecular docking of other proteins with LT4 that are associated with depression.

PROTEIN	LIGAND
NUCB1	LT4
NR2A	LT4
NRXN1	LT4
NFL	LT4

2.10 Reverse Transcription Polymerase Chain Reaction

2.10.1 Dissection

A medial incision that extended from the epigastrium towards the limbs was made to expose the skin of the chest, head, and neck of the mice following their administration of general anesthesia. The mice were then meticulously severed from their heads with sharp scissors. The brain was then made visible by making a midline cut to the skull with a scalpel and tiny scissors. The brain was carefully removed from the skull using tiny forceps and stored at -80°C for further analysis.

2.10.2 RNA extraction

The Trizol isolation reagent was used to separate the tissues' total RNA. The goal of the extraction process is to maintain the structural integrity of RNA. Following the extraction of a section of the cerebral cortex, 1000 ul of Trizol solution was added. The sample is homogenized and then allowed to sit at room temperature for five minutes to initiate the cell lysis process. The sample is then centrifuged for 10 minutes at 4°C at 12,000 rpm, and the resultant supernatant is carefully transferred to a new vessel. 200ul of chloroform is added to the solution to help with phase separation. The mixture is then violently agitated for 30 seconds and then centrifuged again under the same circumstances. After the supernatant has been discarded, 500ul of isopropanol is used to isolate the RNA. The sample was left to incubate at room temperature for ten minutes. Samples were then centrifuged once more while maintaining the same parameters. After the supernatant was removed, the particulate matter was once again suspended and then given a 100 µl rinse with 75% ethanol. After that, the sample was vortexed for a minute. The sample was centrifuged once more, but just for a brief two minutes under the same conditions. After dumping the supernatant, the pellet was allowed to air dry for five to ten minutes. Before further processing, the sample was kept at -80°C, and 20 µl to 50 µl of nuclease-free water was incorporated to protect the RNA.

2.10.3 Evaluation of the quantity and quality of RNA

Colibri Nanodrop (Titertek- Berthold, Germany) and gel electrophoresis were utilized for the quantity and visualization of RNA.

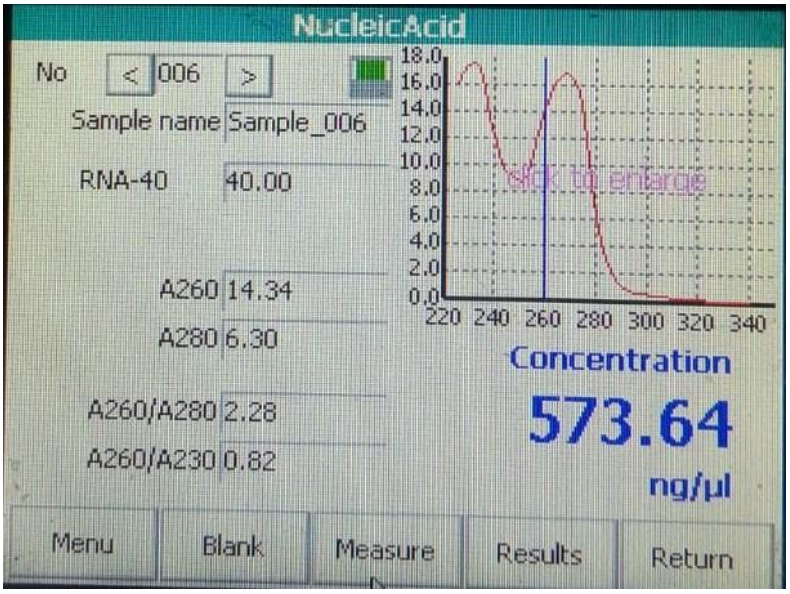

Figure 2.2 Quantity and quality of RNA. This figure shows the quality and quantity of RNA sample extracted through the Trizol method.

2.10.4 Formation of cDNA

RevertAid Reverse Transcriptase was used to perform cDNA formation after RNA extraction. An equal amount of the isolated RNA was transformed into cDNA. A reaction mixture was created by adding 6 ul of dNTPs, 1.5 ul of oligodT, and the 2 ul RNA sample. The mixture undergone incubation for 5 minutes at 55°C. After the mixture was incubated, 2 ul of dithiothreitol (DTT), 4 ul of 5x RT buffer, and 1 ul of RT enzyme were mixed. The reaction mixture's total volume was raised to 20ul by adding nuclease-free water.

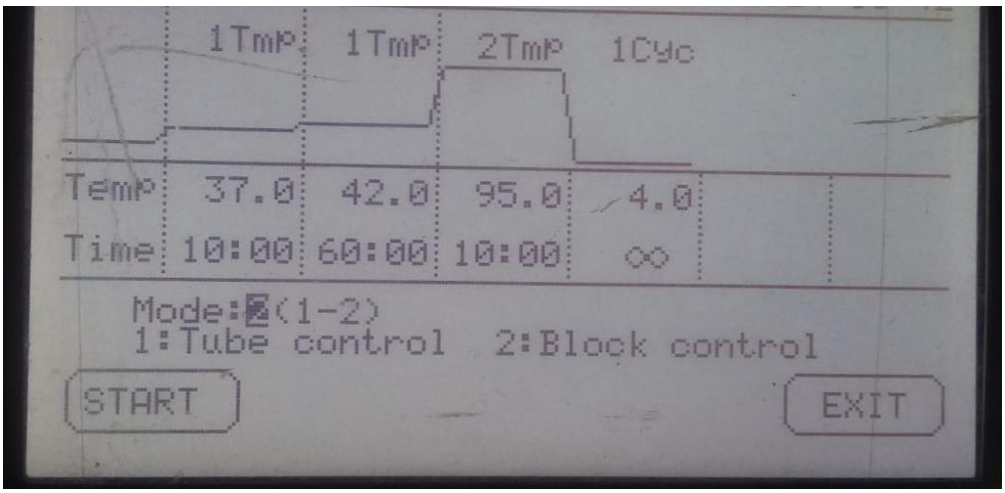

Figure 2.3 Temperature settings for cDNA formation. The figure shows the temperature range for cDNA preparation.

2.11 Polymerase chain reaction

2.11.1 Selection of Primer

Primer was selected due to its practical relevance after a thorough review of many research papers. Accurate binding and precise results require the right temperature and base-pair size. Before utilizing them for Polymerase Chain Reaction (PCR), primer BLAST was performed in the NCBI to confirm the high degree of specificity and precision of the chosen primer with the target gene. The measured annealing temperature for the primers was 60.5.

Name	Primer sequence	Product length bp	Temp
Beta Actin F1	GCCTTCCTTCTTGGGATGG	358	61.5
Beta Actin R1	CAGCTCAGTAACAGTCCGC		
ANP F2	GGCTTCTTCCTCGTCTTGG	81	60.5
ANP R2	ATCTGTGTTGGACACCGCA		

Figure 2.4 Table of primers used in this study. The primers utilized in this study, in conjunction with their precise sequence, length, and ideal binding temperature are displayed in the table.

We purchased the primers from a local vendor.

2.11.2 Primer Blast

Primer BLAST was performed in the NCBI to confirm the high degree of specificity and precision of the chosen primer with the target gene. Primers were developed especially for the genome of *Mus musculus*. The length of the PCR product was ascertained using nucleotide blast. The mRNA with the highest level of homology and specificity was selected using nucleotide BLAST.

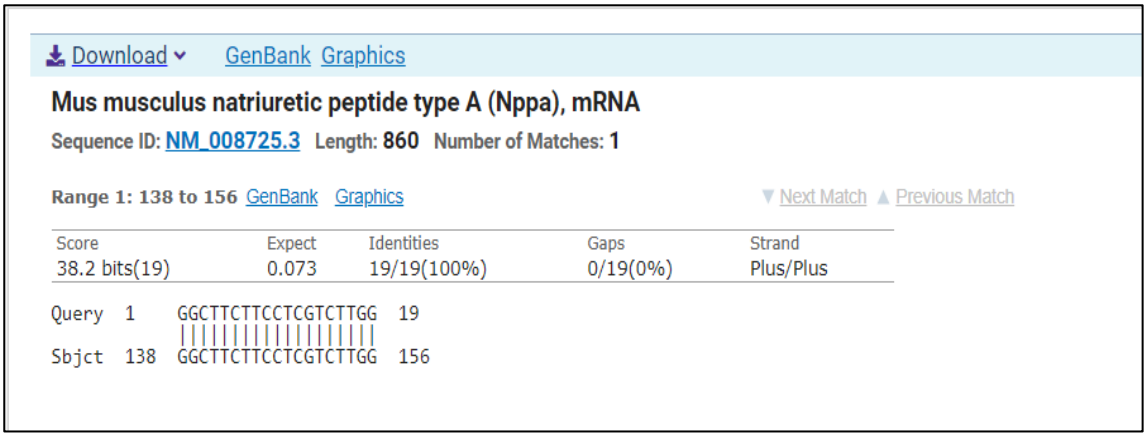

Figure 2.5 Blast for ANP forward primer. The details of the primer BLAST were done in NCBI to verify the specificity of the forward primer.

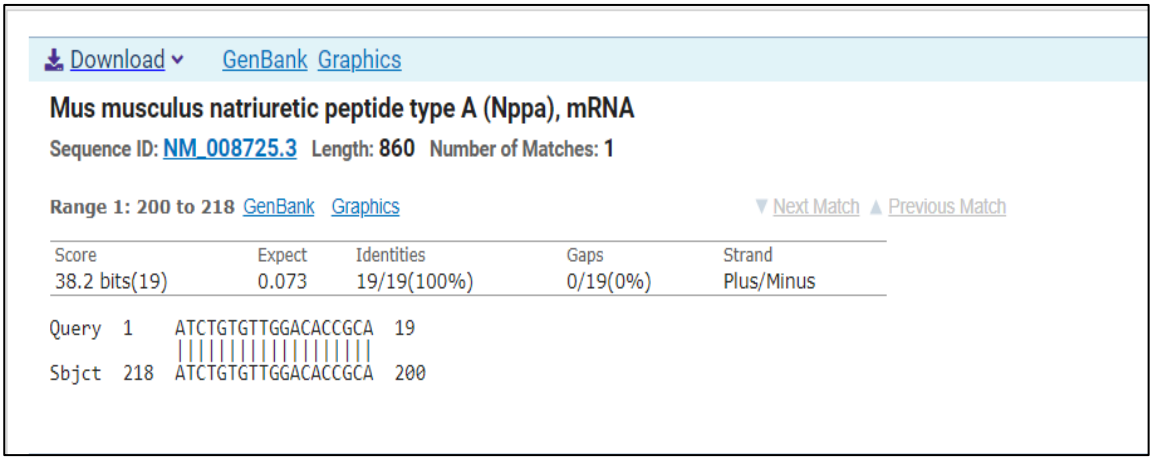

Figure 2.6 BLAST for ANP reverse primer. The details of the primer BLAST were done in NCBI to verify the specificity of the reverse primer.

2.11.3 Gradient Polymerase Chain Reaction (PCR) for primer optimizations

PCR was carried out using a Labnet PCR thermocycler (Labnet International Inc.). The DNase-free water was used to increase the amount of the reaction mixture, which contained 12.5ul of PCR master mix (wizpure), 1ul forward and reverse primers, and 2ul of cDNA template. The temperature gradient annealing temperatures were 58.5, 59.5, 60.5, 61.5, 62.5, and 63.5°C for 1 minute each, followed by 1 minute at 72°C, with a final dissociation

step. The gradient PCR profile is as follows. A 3-minute initial denaturation step at 94°C, followed by 35 cycles at 94°C for 30 seconds, and an annealing step at temperatures between 58.5 and 63.5°C for 30 seconds. Gradient temperatures were then followed by an extension step lasting 45 seconds at 72°C and a final extension lasting 7 minutes at 72°C. To ensure the reliability of the reaction, each sample underwent the amplification process twice. The purity of the PCR product was assessed using agarose gel electrophoresis.

Table 2.2: Gradient PCR temperatures for optimization of ANP

Temperatures for optimization of primer in ºC					
58.5	59.5	60.5	61.5	62.5	63.5

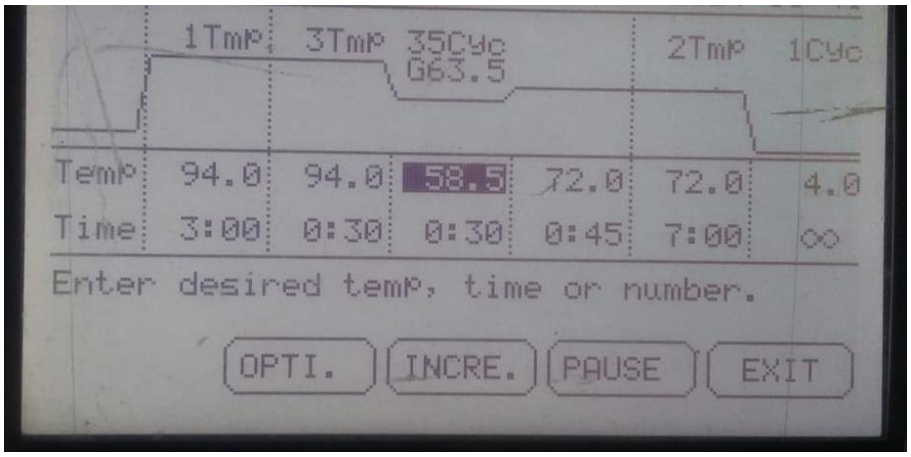

Figure 2.7 Gradient PCR settings for ANP optimization

2.11.4 Agarose gel electrophoresis

To validate whether annealing had occurred at the appropriate temperatures or not, gel electrophoresis was performed by using 2% agarose (Sigma Aldrich, catalog no: 39346, USA) and 10X TBE buffer (catalog no: T1051, Solarbio, China). The bands' locations were compared to the DNA ladder (ranging from 100 to 1500bp) to determine whether annealing had occurred or not. The gels were then analyzed using a Benchtop 2UV transilluminator (LM-20 | P/N 95044902, UVP Co., USA).

2.12 Gene Expression Analysis by Quantitative Real-time Polymerase Chain Reaction (qPCR)

Real-Time PCR, also known as the qPCR was used to measure the ANP expression levels in brain tissues on a real-time PCR detection system (Biorad) using ANP primers. Real-

time PCR cycling parameters: 35 cycles of denaturation at 94°C (3 min), annealing at 60.5°C (30 s), and elongation at 72°C (45 s). Mouse beta-actin (control) qPCR was also conducted employing the primers. Denaturation at 94°C for 30 seconds, annealing at 61.5°C for 30 seconds, and elongation at 72°C for 30 seconds. 35 cycles. The reaction mixture was made using WizPure™ qPCR Master (SYBR) (Catalogue No. W1711, Wizbio, Korea). The PCR reaction mix consists of a cDNA template, nuclease-free water, forward primer, reverse primer, and SYBR green master mix making a total of 20 µl of the reaction mixture. To assess the quality of the PCR product, amplification curves, and agarose gel electrophoresis were employed. The values obtained from these trials were analyzed about gene expression using their ΔCt values after all values were normalized to those obtained for β-actin.

2.13 Statistical analysis

Prior to conducting any statistical analysis, the normality test of each data set's distribution was assessed. To compare the differences between the diseased, treated, and control groups, statistical analysis was done. Statistical tests including the T-test and one-way ANOVA were used, followed by Tukey's test, to determine if there were any significant differences between the groups. Graph Pad Prism version 10.0 was used to construct the graphs, and $P < 0.05$ was chosen as the significance threshold. The standard deviation, or SD, was used to express the data and results.

CHAPTER 3: RESULTS

3.1 *In silico* analysis:

3.1.1 Interaction of NUCB1 with LT4

The protein structure of NUCB1 (PDB ID: 1SNL) is obtained from the RCSB protein data bank in PDB format, giving a thorough illustration of the protein's spatial organization. A ligand of interest, LT4, also had its chemical structure obtained in SDF format from PubChem providing comprehensive details on its molecular makeup and conformation. The ensuing *in silico* analyses, such as molecular docking simulations, are built on top of these molecular architectures. The results of this docking analysis reveal useful structural and energetic details regarding the potential affinities and binding mechanisms of LT4 with NUCB1. This *in silico* analysis demonstrated that LT4 interacts with NUCB1 at the molecular level, illuminating its function in neuroprotection pathways, and providing prospective directions for further study.

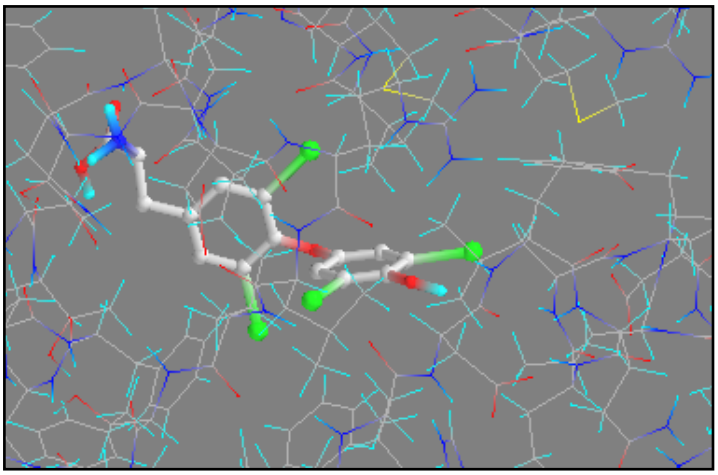

Figure 3.1 Molecular Docking of NUCB1 to Ligand LT4 using PyRx. The protein-ligand complex after being docked in Vina Wizard in PyRx was analyzed and visualized.

3.1.2 Interaction of NR2A with LT4

Protein structure of NR2A (PDB ID: 2A5S) in PDB format, giving a thorough illustration of the protein's spatial organization. A ligand of interest, LT4, also had its chemical structure obtained in SDF format from PubChem providing comprehensive details on its molecular makeup and conformation. The ensuing *in silico* analyses, such as molecular docking simulations, are built on top of these molecular architectures. Figure 3.3 displays the molecular complexes of the NR2A and LT4 that were examined utilizing the ligand-target docking method. The structural and functional information pertaining to the probable affinities and binding processes of LT4 with NR2A is revealed by the docking analysis results.

This *in silico* analysis demonstrated that LT4 interacts with NR2A at the molecular level and provided prospective directions for further study.

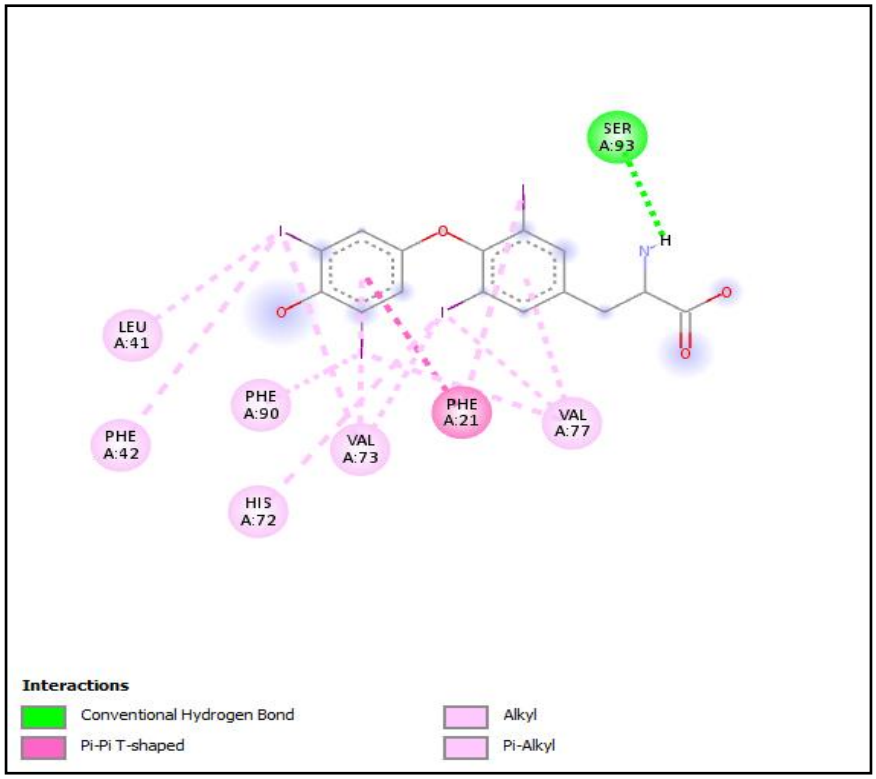

Figure 3.2 Potential binding sites of NUCB1: VAL A, PHE A, LEU A, HIS A The protein residues SER 93 forms conventional hydrogen bonds, PHE 21 forms Pi-Pi T-shaped bonds, while other residues form Alkyl and Pi-Alkyl bonds. Along with alkyl, pi-alkyl, and the Pi-Pi t shaped, hydrogen bonds are the most noticeable interactions that exist in conventional forms

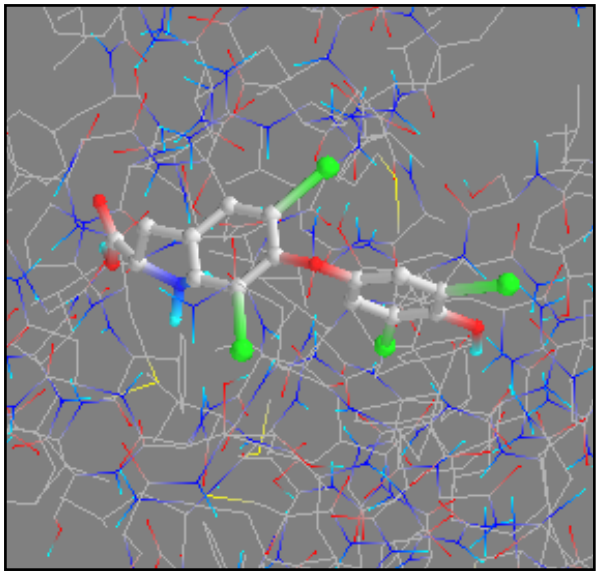

Figure 3.3 Molecular Docking of NR2A to Ligand LT4 using PyRx. The protein-ligand complex after being docked in Vina Wizard in PyRx was analyzed and visualized.

The 2D diagrams showing the interactions between the protein and the ligand for the protein-ligand complex were made by Discovery Studio. The protein-ligand complex after being docked in Vina Wizard in PyRx was analyzed and visualized in Discovery Studio to obtain a 2D image of interactions and bonding. Along with Pi-Pi, alkyl, pi-alkyl, and the unfavorable acceptor-acceptor contact, hydrogen bonds are the most noticeable interactions that exist in both carbon and conventional forms.

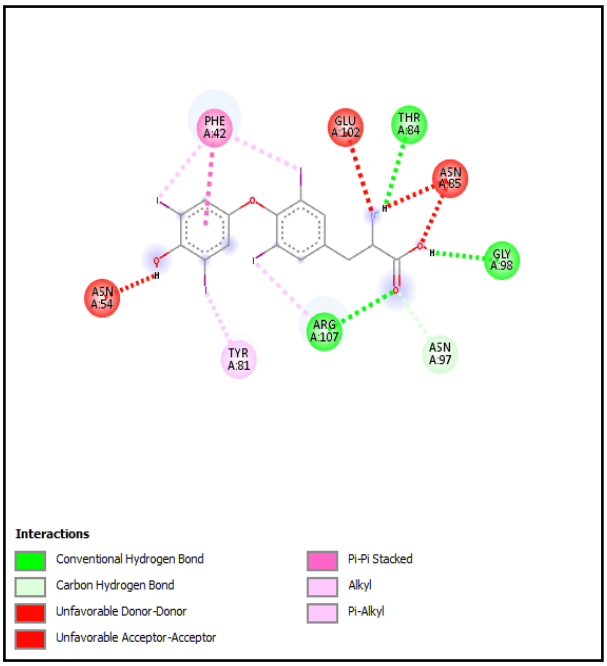

Figure 3.4 Potential binding sites of NR2A: ASN A, PHE A, ARG A, TYR A, GLY A, THR A The protein residues ARG 107, GLY 98 and THR 84 forms conventional hydrogen bond, PHE 42 form Pi-Pi stacked bond, TYR 81 form Alkyl and Pi-Alkyl bonds, ASN 97forms carbon hydrogen bonds while remaining residues forms unfavorable accepter and donor bonds.

3.1.3 Interaction of NRXN1 with LT4

The protein structure of NRXN1 (PDB ID: 3BOD) is obtained from the RCSB protein data bank in PDB format, giving a thorough illustration of the protein's spatial organization. A ligand of interest, LT4, also had its chemical structure obtained in SDF format from PubChem providing comprehensive details on its molecular makeup and conformation. The NRXN1 and LT4 molecular complexes that were investigated using the ligand-target docking technique are shown in Figure (3.5). The results of the docking research provide structural and functional details on the likely affinities and binding mechanisms of LT4 with NRXN1. The results of this *in silico* investigation showed that LT4 interacts with NRXN1 at the molecular level and offers potential avenues for future research.

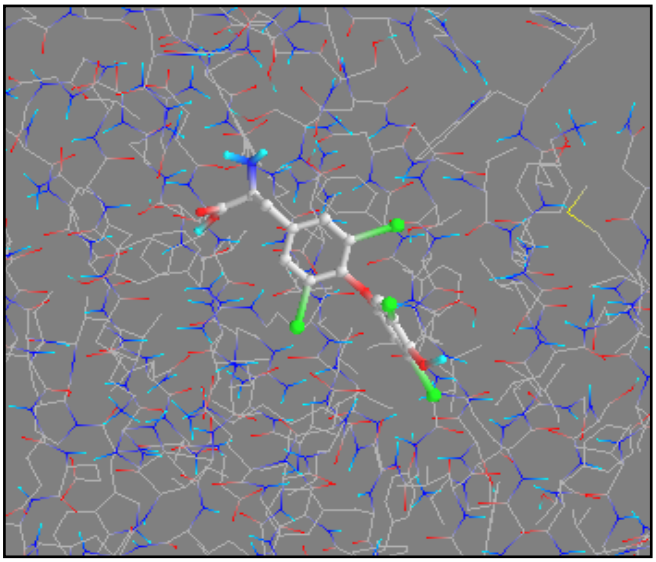

Figure 3.5 Molecular Docking of NRXN1 to Ligand LT4 using PyRx. The findings indicated that when a Pyrx program is executed to dock the corresponding molecules, the proteins neurexin and LT4 are binding.

The findings indicated that when a Pyrx program is executed to dock the corresponding molecules, the proteins neurexin and LT4 are binding. These results verify that the drug and the chosen target protein are bound together. Along with alkyl, pi-alkyl, and the unfavorable donor-donor contact, hydrogen bonds are the most noticeable interactions that exist in conventional forms.

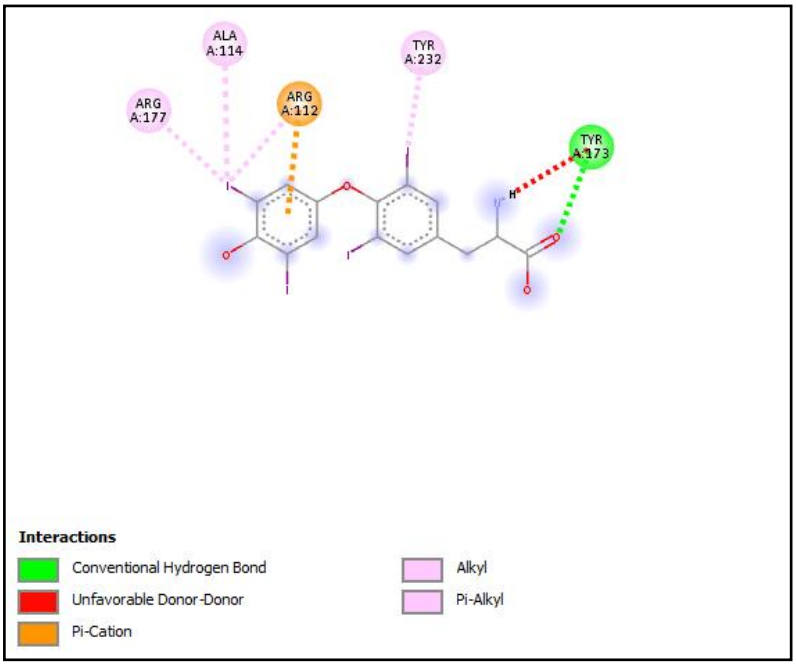

Figure 3.6 Potential binding sites of NR2A: ALA A, ARG A, TYR A. The protein residues TYR 173, form conventional hydrogen bonds, ARG 112 forms Pi-Cation bond, TYR 232, ALA 114, and ARG 117 form Alkyl and Pi-Alkyl bonds.

3.1.4 Interaction of NFL with LT4

NFL's protein structure can be found in PDB format at the RCSB Protein Data Bank. The chemical structure of LT4, a ligand of interest, was also received from PubChem in SDF format, offering detailed information on its molecular makeup and conformation. The FL and LT4 molecular complexes that were investigated using the ligand-target docking technique are shown in Figure (3.7). The results of the docking research provide structural and functional details on the likely affinities and binding mechanisms of LT4 with NFL. The results of this *in silico* investigation showed that LT4 interacts with NFL at the molecular level and offers potential avenues for future research.

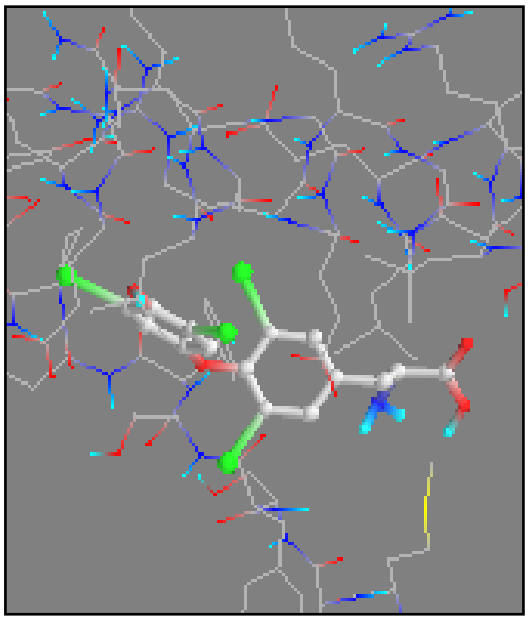

Figure 3.7 Molecular Docking of NFL to Ligand LT4 using PyRx. The protein-ligand complex for the compounds after being docked in Vina Wizard in PyRx was analyzed and visualized

The complicated 2D diagrams showing the interactions between the protein and the ligand for the protein-ligand complex were made by Discovery Studio. The protein-ligand complex for all the compounds after being docked in Vina Wizard in PyRx was analyzed and visualized in Discovery Studio to obtain a 2D image of interactions and bonding. Along with Pi-sigma, alkyl, and pi-alkyl are the most noticeable interactions.

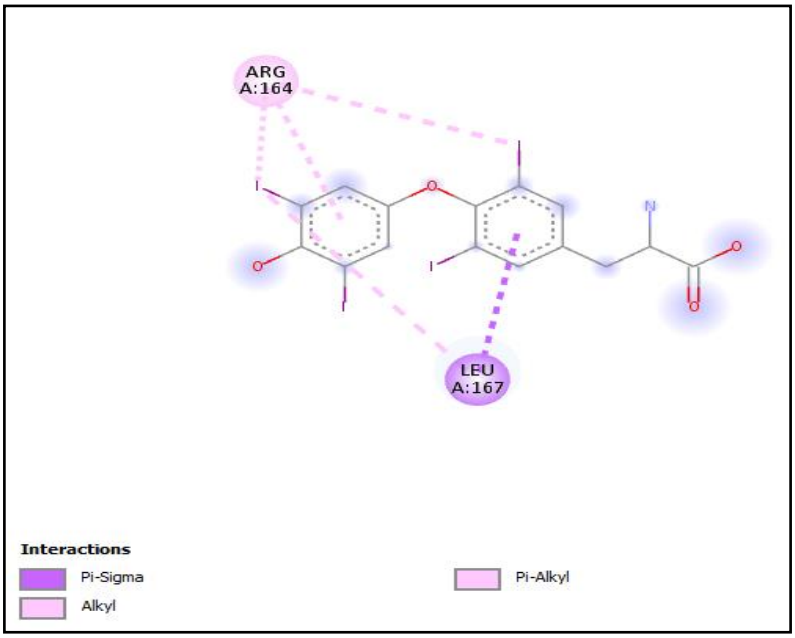

Figure 3.8 Potential binding sites of NFL: LEU 167, ARG 164 the protein residues ARG 164 forms Alkyl and Pi-Alkyl bonds, while LEU 167 forms Pi-Sigma bond.

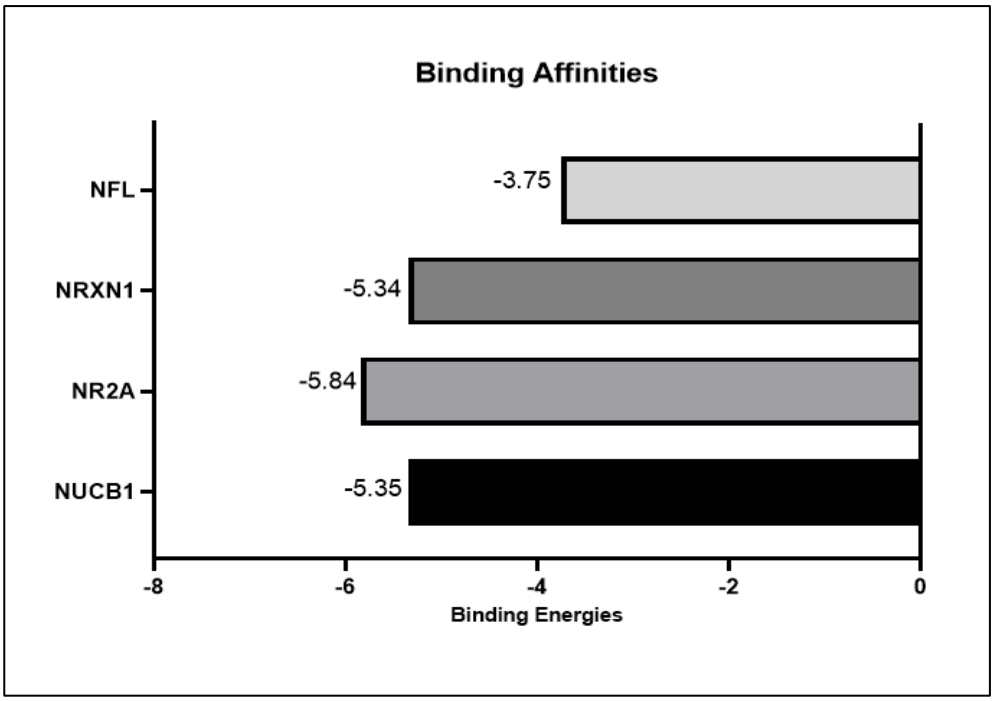

Figure 3.9 Binding energy graph of NUCB1, NR2A, NRXN1, NFL Binding energies of NUCB1, NR2A, NRXN1, and NFL calculated using pyrx are shown in the graph. NR2A exhibits the highest binding energy at -5.84 kcal/mol while NFL exhibits the lowest binding energy at -3.75 kcal/mol

3.2 Behavioral Tests

3.2.1 Tail suspension test

The graph illustrates how LT4 affects the tail suspension test. Each group contained four mice. The three groups' differences were found to be significantly different when the one-way ANOVA test was used. The test showed that the diseased group's duration of immobility was longer than that of the control group. Conversely, the group receiving treatment experienced a reduction in immobility time when compared to the disease group.

3.2.2 Weight

Within the constraints of our experimental setup, we aimed to investigate any possible relationship between the weight of the mice and treatment outcomes. Our results showed that, in contrast to predictions, treatment outcomes were not significantly impacted by the weight of the mice. Our rigorous data collecting, and comprehensive statistical analyses proved the insignificance of weight variations in predicting treatment response, contrary to the widespread idea that changes in body weight could serve as a reliable predictor of treatment efficacy.

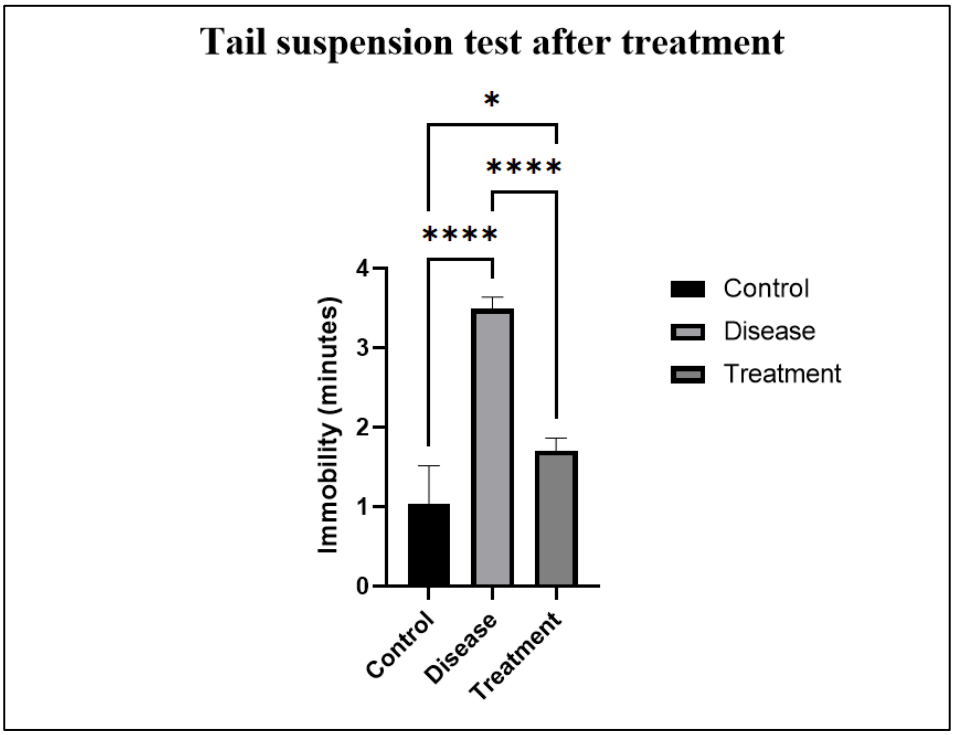

Figure 3.10 Graph showing Tail suspension test reading after treatment. This graph shows the effects of LT4 and the duration of immobility in the mice in the tail suspension test. Comparison with disease and control group using one-way ANOVA test; with n = 4 mice per group and *p<0.05.

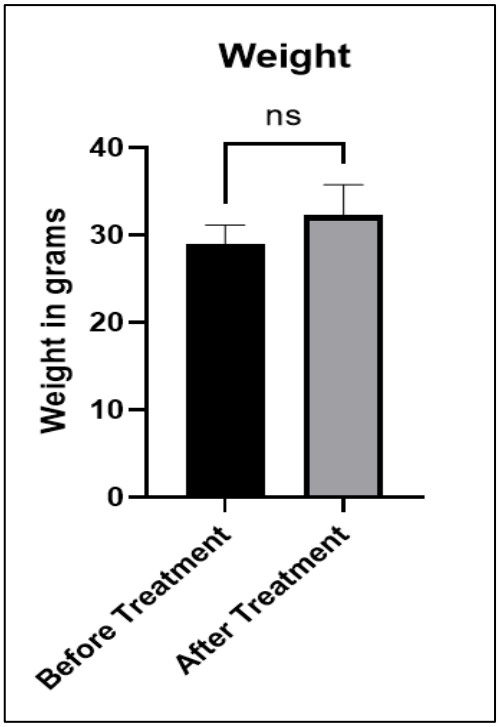

Figure 3.11 Weight of mice before and after treatment. The figure shows the body weight date of mice before and after treatment with no significant outcomes

3.3 Serum analysis

Serum analysis was done to examine the association between depression and the different serum proteins which include the LDL, HDL, cholesterol, uric acid, and CRP. For this, the blood was collected through cardiac puncture. This method involves accessing the heart directly to collect blood for various purposes, such as obtaining serum or plasma for biochemical analyses or collecting whole blood for specific assays. It is a common technique used in research for obtaining high-quality blood samples for various analyses. Since the mice don't have enough blood to examine the results, the blood pooling technique is employed in several research to evaluate the serum results. A heat map is made to evaluate the outcomes. We observed negative patterns in the lipid profile of depressed mice in our investigation. Furthermore, following a 7-day course of treatment, the medication LT4 somewhat improved these unfavorable trends. CRP and uric acid values are found to have upregulated in depressed mice while these values return to normal range after the administration of the drug for one week.

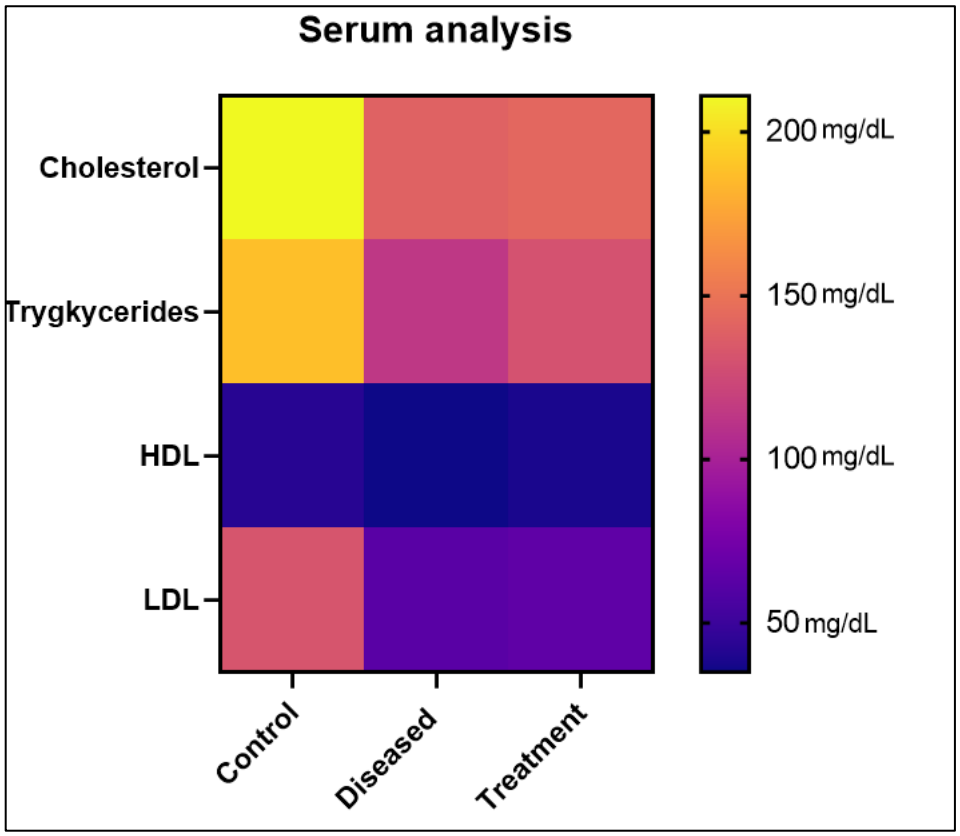

Figure 3.12 Heat map for serum analysis including lipid profile (mg/dl). Negative patterns in the lipid profile of depressed mice have been observed

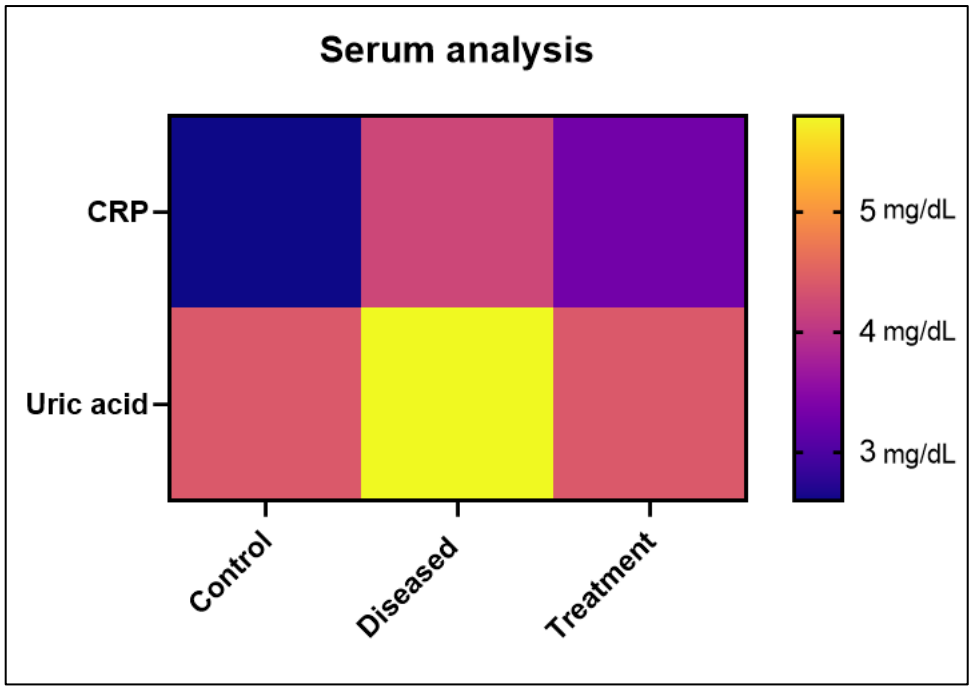

Figure 3.13 Heat map for serum analysis including CRP (mg/dl) and Uric acid (mg/dl) in mice. CRP and uric acid values are found to have upregulated in depressed mice while these values return to normal range after the administration of the drug for one week.

3.4 Improvement in cell survival post LT4 Treatment

The cerebral cortex has been examined in controlled, diseased and treatment groups under the 10X magnification for histopathological analysis. H & E staining has been done to visualize the cerebral cortex. In the controlled group the stained part of the cortex shows the healthy neurons with nuclei rounded in shape and no degeneration. While mice who undergo induction of depression depict abnormal and degenerative changes. The mice administered with LT4 for one week have low degeneration as compared to diseased ones.

3.4.1 Cell count graph

The cell count of the three groups was calculated after the microscopic assessment with the help of Image J. The graph revealed that there are significant differences between the control, diseased, and treated mice. The control group has a higher cell count compared to those of the diseased.

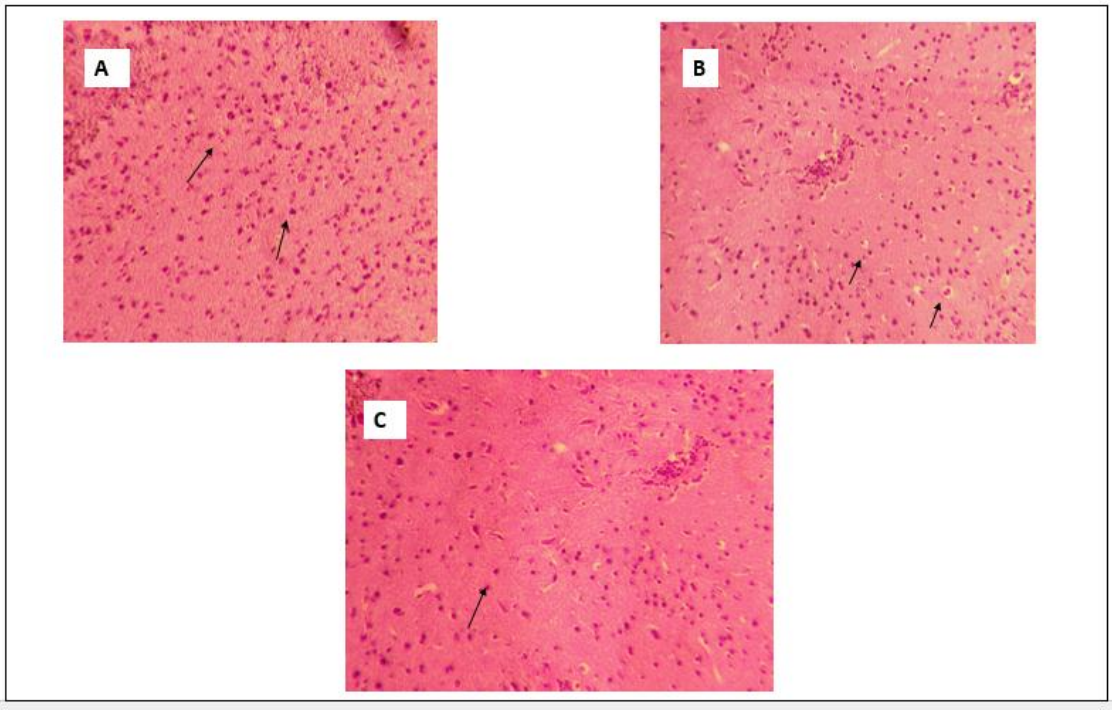

Figure 3.14 The section of the cortex stained with H&E. A) Control mice exhibited neurons with normal organization. B) The depressed group showed strong neuronal loss as well as swelling of neurons. C) The drug administered group showed that most neurons were like those in the control group

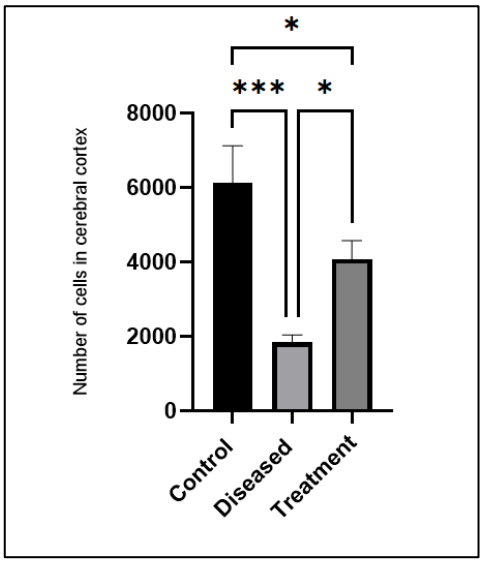

Figure 3.15 Effect of LT4 on stained tissue of cortex: Comparison with disease and control group using one-way ANOVA test; with n = 4 mice per group and *p<0.05**.**

3.5 Gradient PCR

The gel electrophoresis process has been done to optimize the best possible annealing temperature for ANP as well as confirm the presence of ANP in the brain tissue of mice. According to the literature, the amplicon size of ANP is 81bp which has been demonstrated on the gel electrophoresis figure along with the annealing temperature of 60.5˚C. Figure (3.16) showed that the ANP is surely expressed in the cortex of the brain.

3.5.1 Molecular evaluation of quantity of ANP through qPCR

To make meaningful inferences regarding gene levels, the quantitative polymerase chain reaction (qPCR) experiment's data must first be examined. ΔCt values compare the expression of the housekeeping gene β-actin to that of the gene of interest, ANP. The amplification curves represent the relative expression of ANP evaluated by qPCR.

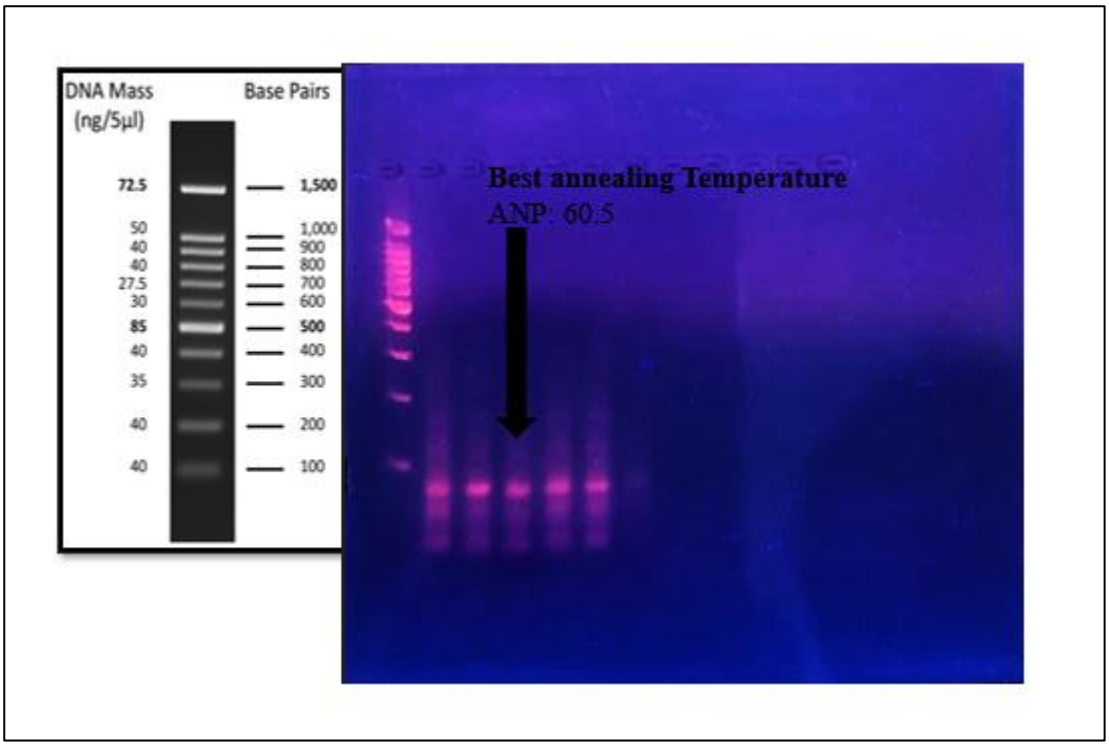

Figure 3.16 Gel Electrophoresis results for optimization. PCR analysis of ANP expression in the mice brain. The best band was noted at approximately 81bp at 60.5˚C.

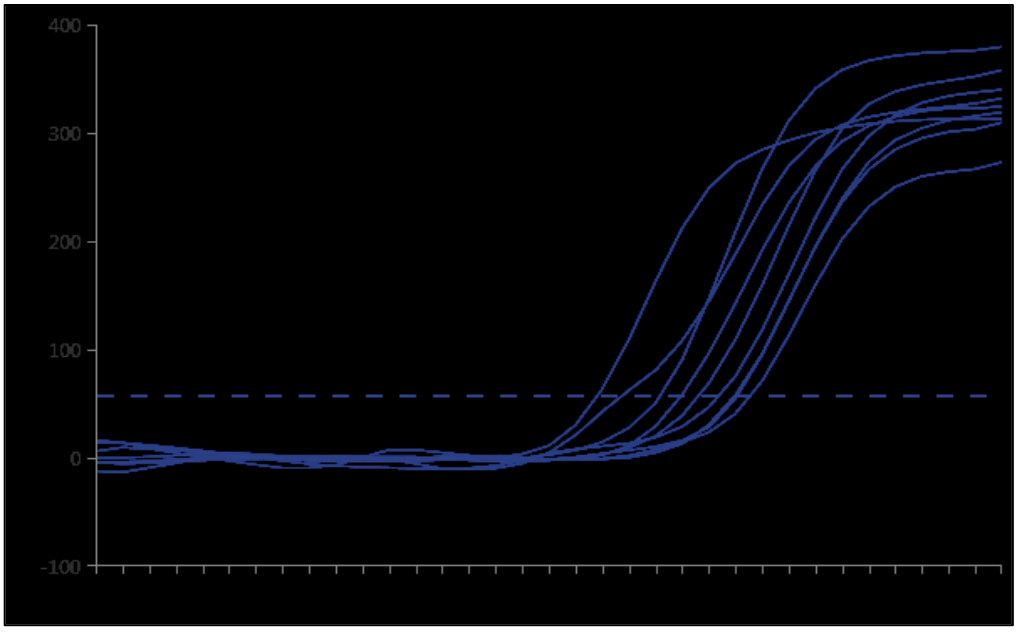

Figure 3.17 Standard curve. The representative amplification curves for ANP. The graph depicts the relative expression of ANP assessed by qPCR.

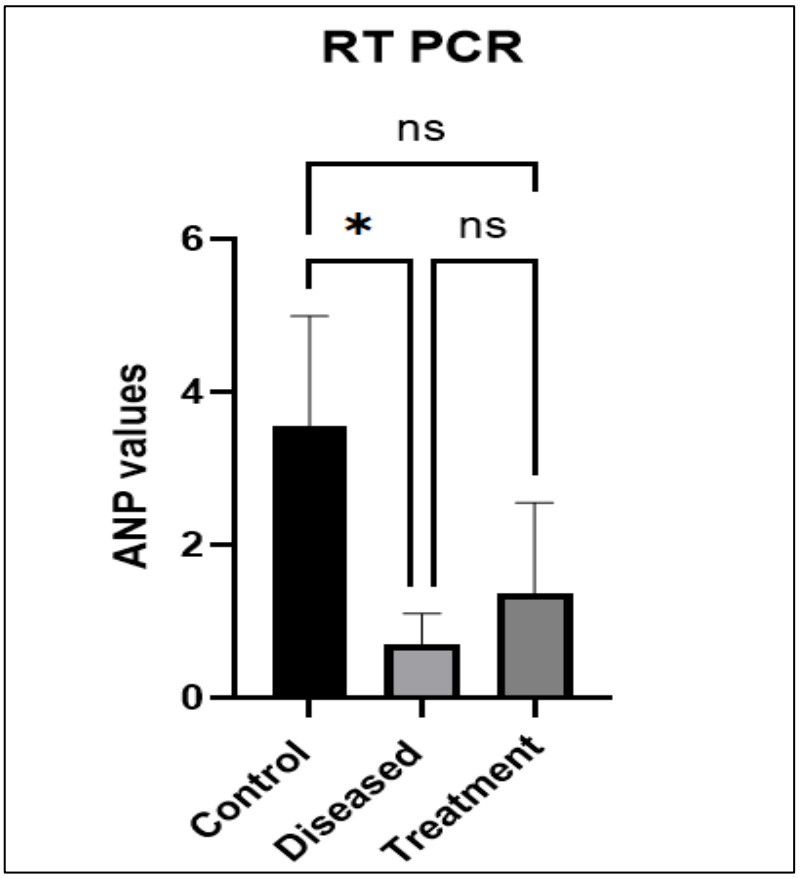

Figure 3.18 Relative expression of ANP evaluated by qPCR. The graph shows ΔCt values of all the groups after qPCR analysis. There is an important difference in the ANP protein expression of control and depressed mice. Treated groups have shown improvement in protein expression. For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test.

CHAPTER 4: DISCUSSION

This study aimed at establishing a model for early childhood depression by using Balb/c mice. Subsequently, the mice were subjected to treatment involving the administration of LT4, a synthetic variant of thyroxine produced by the thyroid hormone, to elevate the level of ANP. The concentration of ANP was subsequently assessed through molecular analysis encompassing histopathology and qPCR techniques.

Based on previous studies it is indicated that a mother's separation from her offspring for a duration of three hours each day enhances her care for the child, thereby mitigating the repercussions of the separation (Millstein & Holmes., 2007). Contrary to earlier research on the effects of maternal separation, it has been found that such separation does not yield consistent behavioral changes or persistent despondency (Tsuda & Ogawa, 2012). Nonetheless, a total separation of the mother from her offspring at PND 17, following a period of permanent maternal separation, would enhance the efficacy of the maternal separation paradigm. Furthermore, past research has substantiated that early weaning of offspring from the mother induces neuroendocrine manipulations that lead to enduring behavioral despondency, hyperactivity, heightened aggressive responses, and anxiety (Carola et al., 2002; Kikusui et al., 2006).

The present study has verified that the intraperitoneal administration of LT4 induces an upsurge in the concentration of anxiolytic protein (ANP) in mice. This phenomenon is assessed through the mice's reactions to various examples, which are subjected to comprehensive evaluation and validated as clinical models of depression (Meaney et al., 1991). The results are further corroborated through molecular analysis techniques such as qPCR, histopathological assessments, and serum analysis. Previous experiments have indicated that intracerebroventricular injections of 200–500 ng ANP to rats in a variety of anxiety models, such as the tail suspension test, forced swim test, elevated plus maze test, and open field test, alleviate anxiety-like responses and behavioral despondency. The findings of the current investigation are in line with prior research that supports the anxiolytic function of ANP. The anxiolytic function of ANP has been the subject of previous investigations, and the outcomes align with the present study (Biro et al., 1995).

The fact that the concentration of ANP and thyroid were positively correlated was another significant finding of our experiment. Animals exposed to MSEW were given synthetic thyroxine, a hormone generated by the thyroid gland, which was found to significantly raise the levels of ANP in depressed animals. Previous research has demonstrated that giving

thyroid hormone in the form of LT4 to rats that had their thyroidectomies doubled the amount of ANP in the blood (Gardner et al., 1987). Another early study showed that LT4 enhanced ANP concentration in a dose-dependent manner at concentrations of (6-10M), indicating that LT4 has stimulating effects on ANP's cellular contents (Mori et al., 1990).

Furthermore, the *in silico* examination of additional neuronal proteins has shown that LT4 also has some effect on these other proteins, some of which are somewhat involved in depression. The *in silico* study of NRXN1, NUCB1, NR2A, NFL, and LT4 interaction showed the anxiolytic effects of LT4. It was found that LT4 can connect to these proteins in a number of different ways, each with a distinct binding affinity, using molecular docking simulations. The differences in binding affinities can be attributed to the different conformations and orientations of LT4 within the binding pocket. The most favorable binding site, denoted by the highest negative binding affinity, suggests a significant interaction between LT4 and these proteins. The substantial binding affinity between them suggests that these proteins might possess a role in LT4's anxiolytic action.

The relationship between serum lipid profile and depression has gathered significant attention, with studies reporting alterations in lipid metabolism in individuals with depressive disorders (Bartoli et al., 2017; McNamara et al., 2017). Our research findings support this relationship, as we have identified dysregulation in the lipid profile of depressed individuals. This dyslipidemia observed in our study may contribute to the development of depression by potentially impacting the functioning of neurons and synaptic plasticity. The role of CRP, which is a well-established marker of systemic inflammation, has been implicated in the pathophysiology of depression (Valkanova et al., 2013; Haapakoski et al., 2015).

Our results demonstrate a positive correlation between CRP levels and the severity of depressive symptoms, suggesting that neuroinflammation may play a role in the development or worsening of depression. Further investigations are necessary to fully understand the precise mechanisms to understand how CRP contributes to depressive pathophysiology and to determine if it can be targeted therapeutically. Uric acid, traditionally associated with gout, has recently gained attention due to its potential neuroprotective properties (Goldberg et al., 2021). Interestingly, our study indicates slightly elevated levels of uric acid in mice with depression, which links the connection between reduced uric acid levels and neuronal vulnerability. Future studies should focus on exploring the neuroprotective mechanisms of uric acid and investigating whether its elevated levels could offer therapeutic benefits in depression by reducing oxidative stress and inflammation. Additionally, there are previous

studies that suggest a link between hypothyroidism and lipid metabolism, as well as CRP and uric acid.

Thyroid hormones T3 and T4 influence lipid metabolism by affecting the synthesis, mobilization, and clearance of lipids in the body (Duntas, 2019). Hypothyroidism has been associated with elevated inflammatory marker levels, including CRP (Pearce & Farwell, 2003). The chronic low-grade inflammation observed in hypothyroidism may further contribute to the neuroinflammatory processes as seen in depression (Dantzer et al., 2008). The shared elevation of CRP in both hypothyroidism and depression suggests a potential synergistic effect, indicating the need to study if thyroid dysfunction could impact the inflammatory state in individuals with depression. In order to gain a more comprehensive understanding of the complex interactions between hypothyroidism, lipid metabolism, inflammation, and uric acid in depression, it is necessary to conduct further comprehensive studies that incorporate multi-omics approaches.

The current investigation aimed to explore the neuronal foundations of depression in mice by means of evaluating the expression of ANP using qPCR. The findings of this study unveil significant modifications in ANP expression in the mouse brain under conditions of chronic stress, thereby providing valuable insights into the potential contribution of ANP to the pathophysiology of depression. The results obtained through qPCR reveal a noticeable decrease in the ANP levels in the brain cortex region of depressed mice comparatively to control group. This observation is consistent with prior research implicating ANP in the regulation of stress responses and mood disorders (Smith et al., 2020; Amiri et al., 2020). The alterations observed in ANP expression suggest its potential applicability as a biomarker for depression and underscore the significance of further investigating its specific mechanisms in states of depression.

It is imperative to acknowledge the limitations inherent in this discourse, including the heterogeneity of depression and hypothyroidism. Future investigations should contemplate exploring the bidirectional association between thyroid function and the aforementioned factors by employing longitudinal designs to elucidate the causal relationships and potential therapeutic interventions. A comprehensive analysis of the enigmatic relationship between numerous cellular systems and ANP is imperative. The local production of ANP, in addition to its production by cardiomyocytes, is insufficiently documented in the existing literature, despite the widespread understanding of the peripheral release of ANP. This is particularly true for the brain. Further research is warranted to fully comprehend the mechanisms underlying the intricate interactions, the negative correlation between ANP and cortisol, and

the role played by ANP in modulating the HPA axis during the stress response. This will aid in rectifying the abnormal hormonal imbalances in the central nervous system that are induced by anxiety, stress, behavioral despair, depression, and substance abuse.

CHAPTER 5 : SUMMARY

Depression, a complex psychiatric disorder influenced by neurological, genetic, and environmental factors, frequently involves disturbances in hormonal regulation. The present investigation explores the underexplored association between ANP and depression, emphasizing its potential for ameliorating melancholic symptoms. By employing a mouse model of early weaning and maternal separation, the researchers examined the anxiolytic effects of LT4 in the context of ANP. To assess the impact of LT4 on ANP, the researchers conducted behavioral assessments and histological analyses. The study employs RT-PCR to investigate the distribution and expression of ANP in the central nervous system of mice, with a particular focus on the cortex. The findings reveal significant differences in the brain expression levels of ANP between treated mice and depressed mice, suggesting a potential therapeutic role for ANP in depression.

An *in silico* analysis of NUCB1, NRXN1, NR2A, and NFL is conducted, revealing varying binding energies. NR2A exhibits the highest binding energy at -5.84 kcal/mol, while NFL displays the lowest at -3.75 kcal/mol. These computational insights provide a molecular perspective to the study. Behavioral analysis, specifically the tail suspension test, indicates prolonged immobility in the group with depression compared to the control group. Serum analysis explores the associations between depression and serum proteins, demonstrating negative patterns in the lipid profile of depressed mice. There is an improvement in these unfavorable trends following a 7-day treatment with LT4. The values of CRP and uric acid, which are upregulated in depressed mice, return to normal levels after the administration of the drug.

Histopathological analysis of the cerebral cortex using H & E staining under 10X magnification reveals degenerative changes in depressed mice, whereas mice treated with LT4 for one week exhibit lower levels of degeneration compared to the group with depression. This highlights the potential neuroprotective effects of LT4 in the context of depression. The distribution and expression of ANP in the central nervous system of mice were examined using RT-PCR, with a particular emphasis on the cortex region of the brain. Our findings indicate that the brain expression levels of ANP in treated mice are significantly different from those in depressed mice. The result of our study implies the potential therapeutic applications of ANP in alleviating depression and offers intriguing avenues for further research in the context of mouse models of depression induced by parental separation.

In summary, this comprehensive study unveils a promising association between ANP and depression, suggesting potential therapeutic applications. The *in silico* analysis provides molecular insights into potential targets for drug development. Behavioral and serum analyses offer a comprehensive understanding of the impact of depression and the potential benefits of LT4. The histopathological and RT-PCR findings underscore the protective effects of LT4 on the cerebral cortex. This study not only contributes to the understanding of the neurobiological basis of depression but also proposes opportunities for future research and therapeutic interventions.

CHAPTER 6: CONCLUSION

In conclusion, our research illuminates the hitherto overlooked link between depression and ANP and reveals its potential as an effective means for reducing symptoms associated with depression. After conducting a thorough analysis using an early weaning mouse model in conjunction with mother separation, we concentrate on the anxiolytic effects of LT4 concerning ANP. The research we conducted has shed significant insight into the complex relationships between LT4, depression, and ANP. ANP's multifunctionality has led to the discovery of possible therapeutic use for treating depression in addition to its conventional role in maintaining diuresis and natriuresis.

Promising findings were observed when LT4 was administered to depressed mice, indicating a significant rise in ANP concentrations. This finding raises the possibility that ANP has therapeutic value for treating depression. Additionally, baseline ANP levels were lower in distressed mice than in healthy mice, supporting the idea that ANP is important in the pathophysiology of depression. These results add to the increasing amount of data demonstrating ANP's role in mental health, particularly its anxiolytic benefits. Investigating LT4 as an ANP modulator offers a fresh approach to possible treatment options for depression. In conclusion, our research emphasizes how critical it is to take ANP into account when diagnosing and treating depression. The prospective direction for future research and clinical applications targeted at utilizing the therapeutic effects of ANP in treating depressive disorders is shown by the positive outcomes observed with LT4 treatment.

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