Role of Nitric Oxide in Anxiety and Depression-Like Behaviour in Mice

A Thesis Submitted to Rajiv Gandhi University for the Award of the Degree of Doctor of Philosophy in the Department of Zoology

Rajiv Gandhi University  **राजीव गांधी विश्वविद्यालय**

Year: 2022

Submitted By:
Hage Konya, M.Sc.
Enrollment No.: RGU/RS/559/2014
Department of Zoology
Rajiv Gandhi University
Rono Hills, Doimukh
Aruanchal Pradesh

Ph.D. Supervisor
Pankaj Kumar, Ph.D.
Associate Professor
Department of Zoology
Rajiv Gandhi University
Rono Hills, Doimukh
Itanagar - 791112
Aruanchal Pradesh

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(A Central University under M/o. Education, Govt. of India)

Rono Hills, Doimukh, Itanagar – 791112, Dist – Papumpare, Arunachal Pradesh

(www.rgu.ac.in)

Dr. Pankaj Kumar

M.Sc., Ph.D.
Associate Professor, Department of Zoology
pankaj.kumar@rgu.ac.in, Ph.: +91-9436271991

Supervisor's Certificate

This is to certify that the thesis/ dissertation entitled "**Role of Nitric Oxide in Anxiety and Depression-Like Behavior in Mice**" submitted for the partial fulfilment of the degree of **Doctor of Philosophy (Ph.D) in Zoology** to Rajiv Gandhi University, Rono Hills, Doimukh, Arunachal Pradesh, embodies the record of original research work carried out by **Ms. Hage Konya**, bearing **Enrolment No. - RGU/RS/559/2014**, under my/our supervision. The content of the thesis is consistent with the approved synopsis and is well within the realms of **Zoology**.

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Supervisor

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(Head, Department of Zoology)

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3. Date of Registration:	**12/09/2014**
4. Subject:	**Zoology**
5. Supervisor's Name:	**Dr. Pankaj Kumar**, Associate Professor, Dept. of Zoology
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5. Email id:	**pankuana@gmail.com**
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Hage Konya 27/09/2022

(Ms. Hage Konya)

Enrolment No.: RGU/RS/559/2014

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(Pankaj Kumar)

Supervisor

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Introduction

It is said that "All Birds have Anxiety" (Hoopmann, 2017). In a perfect world, everyone manages their life in one way or the other, and hence, none of us should be anxious or worried or depressed. However, the hard truth is that there is no perfect world and we all experience life in different ways and conditions. At times, on one hand, we all are anxious and worried while, on the other hand, we all are depressed about anything and everything. For example, we all become anxious when danger arrives. However, anxiety is not always harmful and bad, rather at times being anxious may motivate the individual and do things in a hurry, when it needs to be done. There are also times when we are not in danger but remain anxious while everything in life is normal and going on well. The thoughts of care, danger warnings and the urge of doing things in a hurry surprise us and gets out of control and become demons in our minds. Many of us have anxiety and this runs in our families. The same is true for depression as well. Thus, anxiety and depression often run in our families and these neuropsychiatric disorders affect how one thinks, feels and acts. The individuals suffering from anxiety and depression feel like nothing in this world is good enough for them. Whatever they do, say or try is not appropriate and in case, if they do well, it appears to them that it was a mistake. Thus, they keep thinking about their future, keep rewinding and replay about their past and somehow, stop enjoying the present moment. There's no "OFF" button. These individuals thus make their life complicated and do not sleep well. Days may go by when no work gets done and their job stack up around them. Anxiety and depression are much worse when the individual cannot control what happens and thus try to reorganize their lives so that there are no surprises around them (Hoopmann, 2017). Therefore, anxiety and depression, pertaining to current lifestyle have become major disorders in the population today.

Hagg Konya 27/09/2022 Pankaj Kumar 27/09/2022

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Rono Hills, Doimukh, Itanagar – 791112, Dist – Papumpare, Arunachal Pradesh

(www.rgu.ac.in)

Dr. Daniel Mize

M.Sc., Ph.D.
Associate Professor, Department of Zoology
Chairman, Department Research Committee (Zoology)

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2. Enrolment No:	**RGU/RS/559/2014**
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4. Subject:	**Zoology**
5. Supervisor's Name:	**Dr. Pankaj Kumar**, Associate Professor, Dept. of Zoology
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(A Central University under M/o. Education, Govt. of India)

Rono Hills, Doimukh, Itanagar – 791112, Dist – Papumpare, Arunachal Pradesh

(www.rgu.ac.in)

Dr. Pankaj Kumar

M.Sc., Ph.D.
Associate Professor, Department of Zoology
pankaj.kumar@rgu.ac.in, Ph.: +91-9436271991

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Supervisor

27.09.2022

(Head, Department of Zoology)

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(Ms. Hage Konya)

Enrolment No.: RGU/RS/559/2014

Pankaj Kumar
27/09/2022

(Pankaj Kumar)

Supervisor

DECLARATION

I, **Ms. Hage Konya**, bearing **Enrolment No. - RGU/RS/559/2014,** solemnly declare that the work embodied in this thesis entitled "**ROLE OF NITRIC OXIDE IN ANXIETY AND DEPRESSION-LIKE BEHAVIOR IN MICE**" is my original research work carried out by me under the supervision of **Dr. Pankaj Kumar**, Associate Professor, Department of Zoology, Rajiv Gandhi University. The contents embodied in this thesis, partially or fully, have not been previously submitted to any other university or institution for the award of any other degree/diploma.

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27/09/2022

(Ms. Hage Konya)

Enrolment No.: RGU/RS/559/2014

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Hage Konya
27/09/2022

(Ms. Hage Konya)

CONTENTS

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Abstract of Ph.D. Thesis

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Department of Zoology
Rajiv Gandhi University
Rono Hills, Doimukh
Aruanchal Pradesh

Ph.D. Supervisor
Pankaj Kumar, Ph.D.
Associate Professor
Department of Zoology
Rajiv Gandhi University
Rono Hills, Doimukh
Itanagar - 791112
Aruanchal Pradesh

Abstract

According to the literature anxiety and depression are one of the major disorders in today's population due to the demands and rigour of the fast-paced lifestyle of the individuals in today's society. 2017 WHO report on depression and other common mental health reports that 3.6% of the global population suffers from anxiety and 4.4% from depression. Anxiety and depression are comorbid conditions and are accompanied by many other factors such as alteration in the perception of social reality and the ability to adapt in an individual, affecting the quality of life of the people suffering from such disorders. In the general population, these mood disorders have been shown to be gender biased, being more prevalent in females than males. There could be various factors responsible for the development of such conditions in an individual, it could be environmental, genetic as well as epigenetic. To maintain homeostasis, the body has an elaborate interconnected system constantly being regulated by various molecules. Among such systems are the hypothalamo-pituitary-adrenal axis, responsible for the regulation of stress in the body and the hypothalamo-pituitary- gonadal axis, responsible for the reproductive functions. These axes again have an intricate system of neural circuitry comprising of neuromodulators and neurotransmitters modulating its functioning. One such molecule is the ubiquitously present nitric oxide. This nitric oxide is implicated to be involved in various physiological processes through cyclic guanosine 3′5′-monophosphate (cGMP), including the functions of the brain. Nitric oxide is produced as a byproduct in the enzymatic conversion of L-Arginine to L- Citrulline in the presence of NADPH, cofactors and the enzyme nitric oxide synthase (NOS), NOS has three isoforms in the body (neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), all playing different roles in the body physiology. Of the three isoforms, nNOS expression has been found to be distributed in various brain regions such as the cerebellar cortex, dorsal raphe, cerebral cortex, amygdala, hippocampus, preoptic area and also paraventricular, magnocellular, the supraoptic nucleus of the hypothalamus. These regions, especially the amygdala, hippocampus, and dorsal medial thalamus of the subcortical limbic regions have also been reported to be associated with mood disorders such as anxiety and depression. nNOS has been implicated in a varied range of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, anxiety, stroke and also learning and memory and neuropsychiatric disorders, including depression. NO produced in the brain is linked to be involved in regulating the HPA axis. On the other hand, NO is shown to be localized and expressed in the hypothalamus, hypophysis and gonads and

can act on the hypothalamo-hypophyseal-gonadal (HPG) axis to regulate the synthesis and release of GnRH and thus reproduction, as GnRH and NO-producing neurons occupy similar positions in the hypothalamus. NO has also been reported to regulate spermatogenesis, sperm motility, sperm capacitation, fertilization, oogenesis (follicle development/folliculogenesis), gonadal hormones and steroidogenesis. Nitric oxide is also involved in the embryonic development of the brain and gonads, affecting the overall development of the HPA and HPG axis. Various studies involving knock-out models of mice and also different inhibitors of NOS and specific nNOS inhibitors, as well as nitric oxide donor agents, have shown that nitric oxide is intricately involved in the modulation of both the HPA axis and HPG axis and thus therefore involved in the pathology of neurodegenerative disorders and mood disorders and consequently affects the reproductive behaviour and fertility. But still there exist many lacunas in understanding the concrete aetiology of these disorders. One such lacuna is the involvement of influences during embryonic development affecting the behaviour in adulthood.

Therefore, we hypothesize that the embryonic disruption of the nNOS signalling may alter the hypothalamic (POA, SCN, PVN and Arc. Nucleus) development and subsequently altered HPA axis activity/negative feedback leading to mood disorders such as anxiety- and depression-like behaviour and HPG axis (reproductive physiology) in mice.

Objectives:

- Does restricted embryonic and adult nitric oxide synthase blockade cause altered HPA axis-dependent behaviours and alteration in HPG axis?
- Does restricted embryonic and adult nitric oxide synthase blockade cause alteration in hypothalamic cytoarchitecture and HPA axis function?
- Does nitric oxide signalling regulate neuron development in the developing hypothalamus?

To answer these above objectives the doctoral work has been divided into three chapters:

Chapter 1

Embryonic Disruption of Neuronal Nitric Oxide Synthase Alters Anxiety- and Depression-like Behaviour and Reproductive Physiology of Mice in its Adulthood

Mood disorders like anxiety and depression are major contributors of the maladaptation of individuals to normal situations. To understand these disorders, anxiety and depression-like behaviour studies are done on animal models such as mice. There are many neuropeptides and neuromodulators known to influence the HPA axis, the stress axis, that are implicated in mood disorders like anxiety and depression. One such neuromodulator of the stress axis is nitric oxide. In the present study, the production of nitric oxide was inhibited by a specific inhibitor (7-Niroindazole, 7-NI) of its enzyme neuronal nitric oxide synthase (nNOS). Male and females were time mated in the evening hours (16:00 hrs) and a vaginal plug was observed the next day. Females showing vaginal plugs were considered pregnant. One group of pregnant females were kept in individual cages and were injected with 7-NI at a dose of 10mg/kg body weight from embryonic day 11 to embryonic day 17, while the other group was administered with vehicle control (Dimethlysulphuroxide:Normal Saline – DMSO:NS in 1:1 ratio) and served as the vehicle control group. The pups born to these pregnant females on day 19 were weaned on postnatal day 21, males and females were separated into different cages with individual ear punch markings for individual identification of mice. These male and females mice were raised to adulthood until 8 weeks when these animals were exposed to a battery of behavioural tests such as elevated plus maze (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT). All the behavioural tests were done in the light phase of the light-dark cycle. The behavioural tests in females were performed when the animals were in the diestrus phase. An interval of 4-7 days was maintained between each behavioural test. After the end of the behavioural tests, each animal was anaesthetized and sacrificed for studying the changes in hypothalamic structures of the brain and changes in gonad physiology. Results show that there were marked changes in anxiety- and depression-like behaviour (as displayed by EPM, OFT, FST and MBBT), the hypothalamic nuclei of the brain, nitric oxide production (total nitrate-nitrite concentration), gonad (testes and ovary) physiology and plasma testosterone levels and sperm count in males and plasma estradiol levels in females in both males and females born to 7-NI treated females when compared to males and females born to vehicle-treated pregnant female groups. The result shows that administration of nNOS inhibitor in-utero in mice alters both hypothalamo-hypophyseal-adrenal (HPA) axis as well as the hypothalamo-hypophyseal-gonadal (HPG) axis in the embryo itself which prolongs until adulthood by disturbing the neural circuitry responsible for its maintenance. Thus, nitric oxide acts as an anxiolytic and anti-gonadotrophic agent in mice, however, the molecular mechanism needs further elucidation.

Chapter 2

Nitric Oxide Modulates Anxiety- and Depression-like behaviour and Hypothalamo-Hypophyseal-Gonadal Axis in Mice

L-arginine (L-Arg)/nitric oxide (NO)/cGMP pathway is known to be involved in various physiological processes, the behavioural response being one of the many. In the present study nitric oxide donor sodium nitroprusside (SNP) (0.1mg/kg body weight), nitric oxide inhibitors NG-nitro-l-arginine-methyl-ester (L-NAME) (5mg/kg body weight), and selective neuronal nitric oxide synthase inhibitor 7-nitroindazole (7-NI) (10mg/kg body weight) was administered to 8-week-old adult male and female mice along with their respective controls, normal saline for SN and L-NAME treated animals while dimethyl sulphoxide:normal saline (DMSO:NS – 1:1) for 7-NI treated animals for 14 days. These male and female animals were then subjected to a battery of behavioural tests such as elevated plus maze (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT) to study the anxiety- and depression-like behaviour and changes in the hypothalamo-hypophyseal-gonadal (HPG) axis in these NO donor and NOS inhibitors administered animals. All the behavioural tests were performed in the light phase of the light-dark cycle and an interval of 4-7 days was maintained between each behavioural test. The behavioural tests in females were performed when they were in the diestrus stage of the estrous cycle. All the male and female animals were sacrificed as per approved animal ethics protocol following the completion of behavioural tests. The study shows that there are significant changes in the treatment groups, showing more anxiolytic and depression-inducing effects in the L-NAME and 7-NI treatment groups in both males and females. The sex difference was also observed in these behaviour tests. Crystal violet staining of the brain sections shows alteration in the distribution of neurons in the hypothalamic nuclei (preoptic area – POA, suprachiasmatic nucleus – SCN, paraventricular nucleus – PVN and arcuate nucleus) in the L-NAME and 7_NI treated male and female animals when compared to the control group. L-NAME and 7-NI administration to male and female animals also had inhibitory effects on the morphological and cellular organization of the testes and the ovaries when compared to SNP-treated and control groups. The level of plasma testosterone, sperm count and plasma estradiol along with the total nitrate-nitrite concentration in plasma, testes and ovaries were significantly reduced in L-NAME and 7-NI treated male and female animals when compared to control groups. Thus, it may be concluded that inhibition of nitric oxide synthase by L-NAME and 7-NI and supplementing the animals with NO donor-SNP has a

marked influence on the hypothalamo-hypophyseal-adrenal (HPA) axis as well as HPG axis in terms of alteration in anxiety and depression-like behaviour and gonad physiology. It appears that nitric oxide plays a major role in the interaction of the HPA and HPG axis for the maintenance of body homeostasis. However, the molecular mechanism and pathway need to be explored further.

Chapter-3

Embryonic Disruption of Neuronal Nitric Oxide Synthase Alters Hypothalamus and Gonadal Development in Mice

The growing fetus is susceptible to changes in its environment during embryogenesis, which can greatly affect its development. The neural circuitry in the brain along with environmental, psychological and genetic factors are responsible for the control of embryonic development of various systems of the body, which is regulated via numerous neuromodulators and neurotransmitters. Nitric oxide, one of the neurotransmitters has been shown to influence embryonic development in mammals. In the present study, male and female mice were time mated in the evening hours (16:00 hrs) and the next day female animals were observed for vaginal plugs in the early morning. Female animals with vaginal plugs were considered to be pregnant. Experiments were then performed to see the effects of inhibition of neuronal nitric oxide synthase (nNOS) by administering a specific nNOS inhibitor, 7-nitroindazole to these pregnant female mice from embryonic day 11 to embryonic day 17 along with pregnant female mice treated with dimethylsulphoxide:normal saline (DMSO:NS – 1:1) which acted as the control. Pups born to these pregnant female mice were sacrificed on postnatal day 0 (P0), postnatal 7 (P7), postnatal day 14 (P14) and postnatal day 21 for studying the changes in the structure of the hypothalamic nuclei and gonad (testes and ovaries) physiology. Results show that there were significant changes in the pattern and distribution of hypothalamic nuclei (preoptic area – POA, suprachiasmatic nuclei – SCN, paraventricular nuclei – PVN and arcuate nucleus – ARCN) and inhibition of the development of the morphological and cellular structures of testes and ovaries in the males and females respectively born to 7-NI treated pregnant female mice from postnatal day 0 to postnatal day 21. Thus, it may be concluded that inhibition of neuronal nitric oxide synthase during embryonic development alters the development of the brain and gonads in both males and females by inhibiting the neuronal circuitry responsible for the interaction between the hypothalamo-hypophyseal-gonadal (HPG) axis and have a major effect on HPG axis development and its consequent effects in adulthood.

However, the molecular mechanism responsible for the inhibition of neuronal circuitry during embryonic development needs to be further elucidated.

Thus, to maintain homeostasis in the body, the hypothalamus in the brain acts as the main coordinating centre. It also regulates the stress response by HPA axis and also maintains the reproductive behaviour through HPG axis. In the hypothalamus, there are various nuclei that coordinate the functions of the hypothalamus. Of such nuclei, the ones involved in the HPA axis are the paraventricular nucleus (PVN), which secretes corticotropin-releasing hormone (CRH), and the suprachiasmatic nucleus, the biological clock of the body that maintains the circadian rhythm by receiving inputs from the retina influences both the PVN as well as the POA. HPG axis has gonadotropin-releasing hormone (GnRH) as its main orchestrating hormone, this hormone is released from the preoptic area (POA) of the hypothalamus and stimulates the anterior pituitary to release gonadotropes. Arcuate nucleus due to its positioning has various terminals from the different nucleus of the hypothalamus and hence is involved in this hormonal crosstalk of the different regions of the hypothalamus and hence is implicated in the regulation of both CRH release from the PVN as well as GnRH release from the POA. These regions are interconnected to each other and helps regulate the anxiety and depression-like behaviour as well as the production of gonadal hormones and nitric oxide. When nitric oxide a key modulator of the brain function is disrupted by the in-utero inhibition of its enzyme nNOS by its specific inhibitor, 7-Nitroindazole, it is observed that there is a major disruption in the downstream functioning both HPA and HPG axis. Disrupting the neuronal nitric oxide synthase (nNOS) by specific nNOS inhibitor 7-Nitroindazole to pregnant mice, during the period of embryonic development has an anxiolytic and depressive effect on the pups born to the mother. The disruption during the embryonic stage has a long-term effect on the animals, evident from the significant change in the brain and gonad histology. The differences were also apparent in the behavioural pattern of the animals, showing more anxiety-like and depression-like behaviour. These results illustrate that the in-utero exposure to nNOS inhibitor had serious alterations in both HPA and HPG axis which prolongs until adulthood as well. Inhibition of nitric oxide synthase by L-NAME and 7-NI and supplementing the animals with NO donor-SNP has a marked influence on the hypothalamo-hypophyseal-adrenal axis as well as hypothalamo-hypophyseal-gonadal axis in terms of alteration in anxiety and depression-like behaviour and gonad physiology. It appears that nitric oxide plays a major role in the integration of the HPA and HPG axis for the maintenance of body homeostasis. In-utero exposure to specific nNOS inhibitors also led to an alteration in the structure of hypothalamic

(POA, SCN, PVN and Arcuate) nuclei and gonads (testes and ovaries). This further suggests that nitric oxide is important for neuronal and gonadal development and its inhibition during embryonic development may have serious implications in adulthood such as anxiety and depression and reproductive failure in both males and females. With the observations in this study, we can propose that in the crosstalk of various hypothalamic nuclei in the modulation of comorbid anxiety and depressive behaviour, nitric oxide is one of the key players having implications from embryonic development itself and also during adulthood.

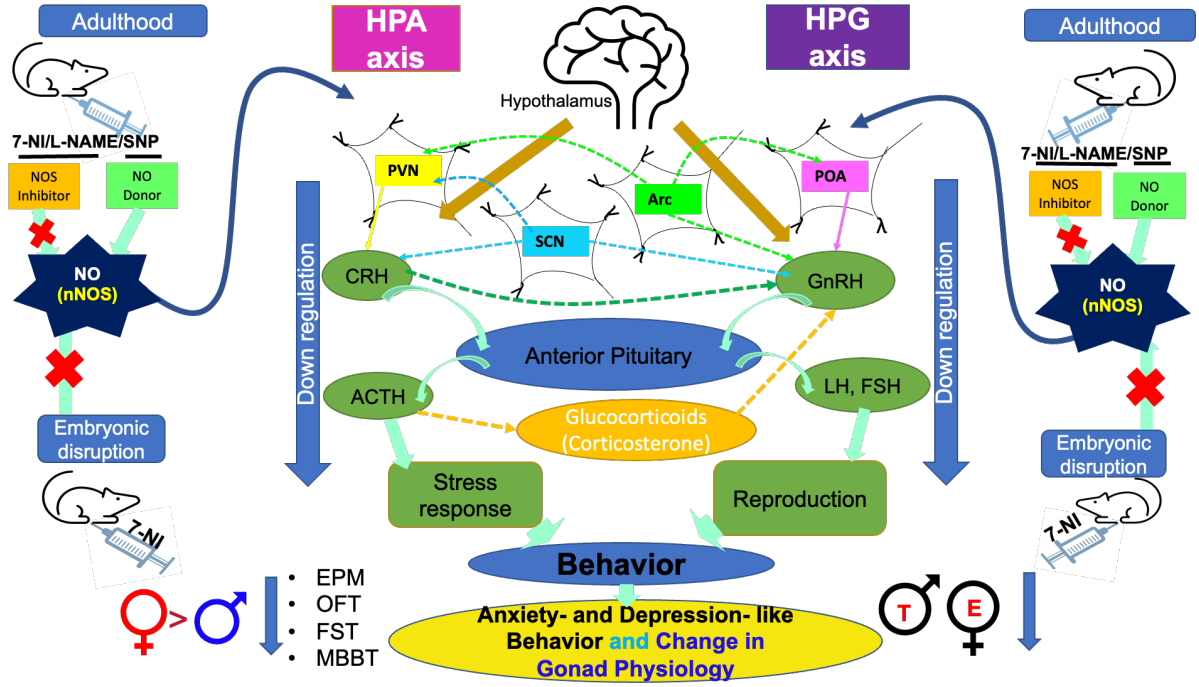


Figure: Image summarising the effects of neuronal nitric oxide synthase (nNOS) inhibition in-utero and in adults on anxiety and depression-like behaviour and gonadal physiology in adulthood via disruption in the Hypothalamo-Hypophyseal-Adrenal (HPA) axis and Hypothalamo-Hypophyseal-Gonadal (HPG) axis. 7-NI = 7-Nitroindazole; L-NAME = NG-nitro-l-arginine-methyl-ester; SNP = Sodium Nitroprusside; HPA = Hypothalamo-Hypophyseal-Adrenal Axis; HPG = Hypothalamo-Hypophyseal-Gonadal Axis; POA = Preoptic Area; SCN = Suprachiasmatic Nucleus; PVN = Paraventricular Nucleus; CRH = Corticotrophin-releasing Hormone; GnRH = Gonadotrophin-releasing Hormone; ACTH = Adrenocorticotropic Hormone; LH = Luteinizing Hormone; FSH = Follicle Stimulating Hormone; EPM = Elevated Plus Maze test; OFT = Open Field Test; FST = Forced Swim Test; MBBT = Marble Burying Behaviour Test; T = Testosterone; E = Estradiol

Introduction

It is said that "All Birds have Anxiety" (Hoopmann, 2017). In a perfect world, everyone manages their life in one way or the other, and hence, none of us should be anxious or worried or depressed. However, the hard truth is that there is no perfect world and we all experience life in different ways and conditions. At times, on one hand, we all are anxious and worried while, on the other hand, we all are depressed about anything and everything. For example, we all become anxious when danger arrives. However, anxiety is not always harmful and bad, rather at times being anxious may motivate the individual and do things in a hurry, when it needs to be done. There are also times when we are not in danger but remain anxious while everything in life is normal and going on well. The thoughts of care, danger warnings and the urge of doing things in a hurry surprise us and gets out of control and become demons in our minds. Many of us have anxiety and this runs in our families. The same is true for depression as well. Thus, anxiety and depression often run in our families and these neuropsychiatric disorders affect how one thinks, feels and acts. The individuals suffering from anxiety and depression feel like nothing in this world is good enough for them. Whatever they do, say or try is not appropriate and in case, if they do well, it appears to them that it was a mistake. Thus, they keep thinking about their future, keep rewinding and replay about their past and somehow, stop enjoying the present moment. There's no "OFF" button. These individuals thus make their life complicated and do not sleep well. Days may go by when no work gets done and their job stack up around them. Anxiety and depression are much worse when the individual cannot control what happens and thus try to reorganize their lives so that there are no surprises around them (Hoopmann, 2017). Therefore, anxiety and depression, pertaining to current lifestyle have become major disorders in the population today.

Anxiety disorders such as selective mutism, social anxiety disorder, separation anxiety disorder, specific phobia, generalized anxiety disorder and agoraphobia constitute the largest group of neuropsychiatric disorders in western societies and have been reported to be a leading cause of disability. People suffering from anxiety disorders tend to show excessive and enduring fear and avoidance of

perceived threats either in the external or internal environment. They also show symptoms of panic attacks as a type of abrupt fear response. Some anxiety disorders display avoidance behaviour by refusing to enter certain situations or showing some reliance on objects or people to cope. The DSM5 (The Diagnostic and Statistical Manual of Mental Disorders) and the international classification of diseases, tenth edition (ICD10) are the two common systems generally used apart from medical testing and differential diagnosis to diagnose anxiety disorders (Craske et al., 2017; Craske and Stein, 2017). Anxiety and depression often coexist, approximately 60% of patients with depression have anxiety, and 25% of those patients have panic attacks. Depressed patients often present with physical symptoms, particularly fatigue, insomnia, and unexplained pain. It is often difficult to differentiate anxiety from depression because many symptoms are common to both, that includes difficulty concentrating, somatic complaints, motor tension, and excessive worry and fatigue (Rakel, 1999). Depression also affects the cognitive and emotional processes in patients, in a meta-analysis of over 100 studies between depressed and control cases show a significant difference in performance on various neuropsychological tasks (Snyder, 2013). The Mental Health Survey Initiative of World Health Organization (WHO) in 2007 has estimated that the global 12-month prevalence of anxiety disorders is ~14%. Country-specific 12-month prevalence rates range from 2.4% in Italy to 29.8% in Mexico. There are also reports that low-income countries have a lower prevalence of anxiety disorders than high-income countries (Lewis-Fernández et al., 2010). In India, 14.3% of the country's population suffers from mental disorders, with one among every 7 people showing symptoms of either mild or severe mental disorders. Of the different ranges of mental disorders in India, anxiety and depression are the most prevalent with a 3.3% prevalence rate (Khambaty and Parikh, 2017). National Mental Health Survey of India reports that females are more prone to depressive disorders than males in the age group of 40-49, where urban metro females show depressive symptoms twice as that of rural area females (Gururaj et al., 2016). In a survey among college students, it was observed that there was a 19% occurrence of generalized anxiety disorders and about 12.1% that of clinical depression. After the covid-19 pandemic, studies on anxiety and depression in the south Asian population have reported a frequency of 31.9%

people suffering from anxiety and 33.7% from depression, more so in females than in males (Salari et al., 2020; Hossain et al., 2021 and Taylor, 2022). Pathogenesis of anxiety and depression disorders has been considerably attributed to heritability and genetic contribution of the disorder but there is also an involvement of environmental influences that account for variability not explained by genetic factors (Hettema et al., 2005). The field of epigenetics has been suggested to be helpful in deciphering the synergistic mechanistic links as well as the complexity of their interactions underlying risk in these disorders (Schiele and Domschke, 2017). Various studies show that mental disorders such as anxiety and depression are commonly prevalent today among the population and their etiology needs to be explored and understood. These disorders are of multifactorial origin, comprising environmental, psychological and genetic components. Though a vast array of research covering various aspects of these disorders has already been done and is still undergoing, there still is a lot that is not understood about the disorders and hence are unable to come up with a concrete therapeutic approach other than some cognitive behaviour therapies, antidepressants or drugs like benzodiazepines and buspirone. One such approach to understanding these disorders may be to look into the neural pathways and various cross talks going on between the neurotransmitters and neuromodulators in the brain. One such neurotransmitter / neuromodulator molecule is nitric oxide. Nitric oxide is a ubiquitous molecule involved in various functions of the body, including the brain in the regulation of neurotransmission, synaptic plasticity and development of hypothalamic nuclei (Tanda et al., 2009; Bellefentaine et al., 2011). Research concerning nitric oxide has yielded results, but still, their role in understanding the mood disorders like anxiety and depression is not completely explored and understood. Hence, an attempt has been made to explore the role of nitric oxide in the modulation of anxiety and depression-like behaviour in mice by inhibiting the neuronal nitric oxide synthase (nNOS) by a specific nNOS inhibitor, 7-Nitroindazole (7-NI) during embryonic development and adulthood. An attempt has also been made to integrate the functions of the hypothalamo-hypophyseal-adrenal axis with that of the hypothalamo-hypophyseal-gonadal axis with the modulation of anxiety and depression-like behaviour in mice.

The current lifestyle has led to one of the major disorders, i.e., anxiety and depression, in the human population. Anxiety and depression though considered to be two distinct entities according to the diagnostic criteria are co-morbid (Fawcett and Kravitz, 1983; Schoevers et al., 2005; Cairney et al., 2008; Goldberg and Fawcett, 2012; Choi et al., 2020). Humans having anxiety disorders commonly have depression while the vice-versa is true for patients with depression, i.e. they would often have anxiety disorders (Tiller, 2013). The anxiety disorder recognized clinically as mental disorders in humans include generalized anxiety disorder, acute and chronic posttraumatic stress disorder, panic disorder, and obsessive-compulsive disorder. This also includes various phobias such as social phobia, agoraphobia, and specific phobia (eg, fear of flying) (Hang et. al., 2015; Manchia and Fanos, 2017; Hyman, 2021). These disorders are sex-dependent, being increased in females than males in humans (Holden, 2005; Boyd et al., 2015; Riecher-Rössler, 2017; Yu, 2018; Rehm and Shield, 2019; Kokkosis and Tsirka, 2020). The above hypothesis is supported by the gonadic theory, suggesting that women have a wider range of fluctuations in hormone levels than men. This fluctuation in hormone levels affects the brain regions (hypothalamus, prefrontal cortex, hippocampus) that are known to be involved in the modulation of mood and behaviour (Faravelli et.al., 2013). Neuroimaging techniques like magnetic resonance imaging (MRI), positron emission tomography (PET), and single photon emission computed tomography (SPECT) have shown the brain regions namely cortical brain areas, and subcortical limbic brain regions to be associated with depression. The dorsal and ventral anterior cingulate cortex, the dorsal and medial prefrontal cortex, the orbital frontal cortex and the insula of the cortical brain areas are responsible for anxiety disorders while the hippocampus, amygdala and the dorsomedial thalamus of the sub-cortical limbic brain regions are said to be implicated in depression (Rigucci et al., 2009; Desmyte et al., 2011; Pandya et al., 2012). Further, the above neurocircuitry system comprising of the anterior cingulate cortex (ACC) (hypothalamus, amygdala, ventral striatum, insula, periaqueductal grey, and the ventral regions) and the pre-frontal cortex (PFC) (specifically ventromedial PFC and orbitofrontal cortex) are also reported to be

involved in fear and anxiety response (Quirk et al., 1995; Phelps and LeDoux, 2005; Phillips et al., 2008; Mah et al., 2016). During these psychiatric disorders, both structural and functional abnormalities have been found in these areas.

Moreover, there are reports that in India 14.3% of the total population of the country has mental disorders (Khambaty and Parikh, 2017). The report further reveals that one among every seven people in India has a mental disorder, which ranges from mild to severe. Among the mental disorders prevalent among the population, both anxiety and depressive disorders are of high prevalence (3.3% - 3.1-3.6 for depressive disorders and 3.0–3.5 for anxiety disorders) (Khambaty and Parikh, 2017). In another study by Sahoo and Khess (2010), symptoms of clinical depression and generalized anxiety disorder were 12.1% and 19.0% respectively in a stratified sample of college students. In the same study, of the total population analyzed, 18.5% population had depressive symptoms, while 24.4% had anxiety and 20% of the population had stress symptoms, which ranged from mild to severe conditions. There are also reports as per the National Mental Health Survey of India-2016 that females in India are more prone to depressive disorders, especially among the 40-49 age group (Gururaj et al., 2016). Further, depression disorders are more in females residing in urban metros than in rural areas. Among the Indian population, nearly 1 in 40 and 1 in 20 suffer from past and current depression (Gururaj et al., 2016). In the same survey, it was also reported that the prevalence of mental disorders was nearly twice as much in urban metros as compared to rural areas.

Similar to these reports, the recent global COVID-19 pandemic has also affected the global mental health of the population. In a study on the South Asian population, the COVID-19 pandemic resulted in anxiety and depression disorders at the rate of 34.1% to 41.3% in pooled patient samples (Taylor, 2022; Hossain et al., 2021). Salari et al. (2020) also reported a similar result with 31.9% for anxiety and 33.7% for depression in the population affected by the global effect of the COVID-19 pandemic, with females showing more anxiety and depression symptoms than males. This finding is in accordance with generalized findings before the occurrence of the pandemic, that anxiety and depression disorders are prevalent more in females than males (Markkula and

Suvisaari, 2017). In general, the pandemic impacted the general health of the population in low and middle-income categories (Kola et al., 2021), with anxiety and depression disorders in 25.6% and 27.6%, respectively in the general population, when the effect of the COVID-19 was adjusted in the said population of the study (Santomauro et al., 2021). Thus, the study shows that mental health disorders such as anxiety and depression are commonly prevalent today also among the population and their aetiology needs to be explored and understood. Like many other human diseases, most of the clinical studies of mood disorders have been performed on rodents because of their similarity to humans in anatomy, physiology, and genetics. Rodents are small and have a short generation time hence provide easy handling with controlled modification of variables, availability of different models, with the possibility of creating new models and also the availability of controls. They are cost-effective and have been proven to be an efficient tool to speed research and also the development of drug therapies (Vandamme, 2015). Behavioural and physiological responses to fear in rodents can be seen by the animal's tendency to show escape and avoidance behaviours from the potentially dangerous situation by displaying activities like flight, avoidance, freezing, defensive threat, defensive attack and risk assessment (Edmunds, 1974; Blanchard et al., 2003; Toth et al., 2013). They have also been observed to bury novel, unpleasant, or potentially dangerous stimuli (Treit et al., 1981; Dixit et al., 2020). Litvin et al., (2007) has reported that rats have around 22kHz ultrasonic alarm cries in their defense pattern in response to a predator threat. They also demonstrate cessation of ongoing behaviour (Estes and Skinner 1941; Brady and Hunt 1951). Behaviour changes are studied by observing the animals in various behaviour tests, like elevated plus-maze, light/ dark box and open-field tests are used to evaluate anxiety-like behaviours while forced-swim, tail suspension tests and marble burying tests are some tests for assessing depression. In these tests, the natural behaviors of rodents, such as exploratory behaviour, locomotor activity, rearing or food and water consumption, have been described to be decreased (Vuralli et al., 2019). Mental disorders such as anxiety and depression are of multifactorial origin, comprising environmental, psychological and genetic components. Research on these mental disorders in relation to neurotransmitters and small

peptide molecules has yielded specific results and has vastly contributed to the understanding of the disorder.

Hypothalamo-Pituitary-Adrenal (HPA) axis

In a normal circumstance, the endocrine response to stress is the secretion of glucocorticoids (Sapolski et al., 2000; Gjerstad et al., 2018; Scherholz et al., 2019), they are the systemic effector hormone of HPA axis and their secretion is dependent on environmental and experiential events (Herman et al., 2016; Joseph and Whirledge, 2017; Spencer and Deak, 2017). Apart from many pathological and biomedical disorders such as hypertension, chronic fatigue syndrome, etc., an altered HPA axis is associated with mental health disorders like depression, post-traumatic stress disorder (PTSD), and schizophrenia (Heim and Nemeroff, 2009; Dean and Keshavan, 2017). Cortisol is the principal circulating glucocorticoid hormone in humans, many other mammals and most fish, whereas in rats, mice, birds and most reptiles it is the corticosterone (Norris and James, 2013). Cortisol/corticosterone readily crosses the blood-brain barrier therefore this hormone can directly target the brain, and therefore is responsible for glucocorticoid negative feedback (Weil-Malherbe et al., 1959; Spiga et al., 2014). HPA axis encompasses a population of cells located within the hypothalamus, pituitary gland and adrenal gland, secreting hormonal signal that makes up this system (Joel and Baram, 2009). In the hypothalamus, the parvocellular cells of the paraventricular nucleus (PVN) produce a corticotropin-releasing hormone (CRH), which is the central player in the HPA axis. CRH-producing neurons receive neural input from various regions of the brain and their terminals project to the median eminence of the hypothalamus. CRH is a stimulator of the anterior pituitary for the secretion of adrenocorticotropic hormone (ACTH) (Herman et al., 2003). Mature ACTH after cleavage of prohormone Pro-opiomelanocortin (POMC) is stored in vesicles of the anterior pituitary for secretion upon receiving a signal from CRH for exocytosis (Cawley et al., 2016). ACTH further triggers the cells of zona fasciculata of the adrenal cortex to synthesize and secrete cortisol/corticosterone, which diffuses into circulation after secretion to give its

various physiological effector functions in the body in response to the stressor (Thrivikraman et al., 2000).

HPA axis activity and their corresponding effect on the cortisol/corticosterone activity has been reported to be in a rhythmic fashion, the amplitude of their release into the blood show modulation depending upon the daytime (ultradian) as well as circadian rhythm, some species even showing seasonal dependency (Dickmeis et al., 2013). Sex differences are also observed in the corticosterone level in rats, with females displaying higher corticosterone levels and higher HPA axis response when exposed to the same stressor, indicating the role of gonadal steroids. These sexual differences could be due to the sexual dimorphism in mammals resulting from genetic and hormonal events that begin early during development and may continue throughout the lifespan (Becker et al., 2005).

Hypothalamo-Pituitary-Gonadal (HPG) axis

As the HPA axis physiologically adapts by responding to the stressor, it also intercedes the activity of the Hypothalamo-Pituitary-Gonadal (HPG) axis- the reproductive axis of the body. There is an inhibitory response in reproductive physiology and behaviour in both sexes (Spencer and Deak, 2017). HPG axis constitutes the hypothalamus, pituitary and gonads (testis in males and ovary in females). The main player in the HPG axis is the gonadotropin-releasing hormone (GnRH) (Millar, 2005). GnRH stimulates the gonadotroph cells of the pituitary to synthesize and release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This FSH and LH further act on the ovary to regulate oocyte maturation, ovulation, and steroid hormone production (Richards and Pangas, 2010) and on testis for hormonal regulation of testicular functions, acting through their receptors in Sertoli and Leydig cells (Kaprara and Huhtaniemi, 2017). GnRH neurons originate outside the CNS, during development, GnRH neurons migrate from the medial olfactory placode of the developing nasal cavity across the nasal septum, together with vomeronasal axons, and enter into the forebrain with the nervus terminalis, arching into the septal-preoptic area and hypothalamus (Yoshida et al., 1995). Processes are then extended from the hypothalamus to the median eminence, and GnRH is released

into capillaries of the hypophysial portal system that reaches the anterior pituitary gland and modulates its gonadotropin synthesis and secretion (Millar, 2005). One way the GnRH secretion occurs is in a pulsatile way by regulating the ovarian cycle and normal folliculogenesis, this pulsatile secretion of GnRH occurs via Kiss1/NKB/Dyn (KNDy) neurons in the arcuate nucleus of the hypothalamus (Plant, 2020). Kisspeptin has been well accepted as the coordinator of secretion of reproductive hormones, acting at the apex of the hypothalamic-pituitary-gonadal (HPG) reproductive axis affecting puberty and fertility (Trevisan et al., 2018). Along with reproduction, kisspeptin also modulates behaviour, mood and emotions through its extensive distribution in the limbic system. Kisspeptin has also been identified as a hypothalamic anorexigenic factor (Mills et al., 2018; Bond and Smith, 2014). The other way GnRH secretion occurs is in the surge, particularly in females, this causes a preovulatory LH surge that leads to ovulation (Maeda et al., 2010). The pulsatile secretion of GnRH is dependent on various other neural inputs like norepinephrine, dopamine, serotonin, GABA, glutamate, neuropeptide Y (NPY), galanin and also the kisspeptin-neurokinin B-opioid pathway (Kaprara and Huhtaniemi, 2017). As a consequence of this pulsatile GnRH secretion, FSH and LH are secreted from the anterior pituitary and enter the circulation to reach their target organs testis and ovary. The testis is the male reproductive organ and it has two chief functions: the production of spermatozoa (spermatogenesis) and the production of steroids (steroidogenesis). The steroid hormone, testosterone, regulates the development of spermatozoa and the growth, development and maintenance of the accessory reproductive glands. It also influences the development of secondary sex characteristics and, to some extent, sexual behaviour. The testis is divided into pyramidal lobules, each containing numerous convoluted seminiferous tubules that produce the spermatozoa and loose connective tissue that contains Leydig cells the endocrine cells producing testosterone. The seminiferous epithelium consists of Sertoli cells and spermatogenic cells. Spermatogenic cells are proliferating populations of cells at various stages of the differentiative process of spermatogenesis. The cells are in the spermatogonial phase, spermatocyte phase (meiosis) and spermatid phase or spermiogenesis. The arrangement is such that the most immature cells are located near the basement membrane. As the cells

proliferate and undergo differentiation, they move towards the lumen. Leydig cells in response to LH release from the anterior pituitary produce an increasing amount of testosterone. This testosterone along with FSH from the anterior pituitary stimulates the process of sperm production within the seminiferous epithelium (Ross and Reith, 1989; Zirkin, 1998; Holdcraft and Braun, 2004; Hess and Renato de Franca, 2008). In females, the ovary is the reproductive organ responsible for the production of gametes by oogenesis and the production of steroids. The ovarian hormones are further in control of the regulation of oocytes and the growth and development of secondary sex organs and mammary glands. In the ovary, the peripheral portion, the cortex, contains the ovarian follicles, embedded in compact, richly cellular connective tissue. Each ovarian follicle contains an oocyte, the developmental state of the oocyte is determined by the size of the follicle. During the early embryonic developmental stage of the ovary, the primordial germ cells migrate to the genital ridges. In the early fetal life, these primordial germ cells develop into oogonia and undergo rapid proliferation by mitotic division. Oogonia enlarge to form primary oocytes. When the ovarian stromal cells form a flattened layer of follicular cells around each oocyte, primordial follicles are formed. The primary oocyte within the primordial follicles undergoes the first meiotic division before birth, but the process gets arrested at the diplotene stage and resumes the meiotic division only at puberty when a group of follicles begin their cyclic development. As the first meiotic division is completed in the mature follicle, each daughter cells of the primary oocyte receive an equal share of the chromatin, but one daughter cell receives a majority of the cytoplasm and becomes the secondary oocyte. As the secondary oocyte surrounded by a mass of cumulus cells leaves the follicle at ovulation, the second meiotic division is in progress. This division is arrested at the second metaphase and is not completed unless the secondary oocyte is penetrated by a spermatozoon. The polar body formed from the first and second division undergoes degeneration. During the development, a large number of primordial follicles are lost to atresia during the fetal, early postnatal and puberty, i.e., the follicles degenerate and disappear. After puberty, follicles can undergo atresia at any stage of its maturation and involves, invasion of granulosa cell layers, sloughing of the granulosa cells into the antrum of the follicle, hypertrophy of the theca interna

cells, the collapse of the follicle and invasion of the connective tissue into the cavity of the follicle. The oocyte undergoes degeneration by autolysis and disappears. The FSH and LH secreted by the anterior pituitary help the ovary in maintaining the ovarian cycle by secreting steroid hormones estrogen and progesterone from the granulosa cells and theca cells of the ovary (Ross and Reith, 1989; Sen and Caiazza, 2013; Bilinski et al., 2017).

Presence of a stressor, activation of HPA axis, causes suppression of HPG axis by inhibition of GnRH and GnRH receptor (GnRHR) synthesis, disruption in pituitary release of FSH and LH, and enhanced function of the gonadotropin-inhibitory hormone (GnIH; mammalian ortholog gene Rfrp3) neurons (Joseph and Whirledge, 2017).

The intricate functioning of various brain axis is under regulation by numerous neurotransmitters, hormones, neuropeptides and neuromodulators. One such neuromodulator is nitric oxide which has a ubiquitous presence and has been well documented to be involved in both the HPA axis and HPG axis along with its varied functions.

Nitric Oxide

Nitric oxide (NO), a biological messenger molecule, has various physiological functions in the brain i.e., regulation of neurotransmission, synaptic plasticity, development of hypothalamic nuclei etc. (McClellan et al., 2010; Tanda et al., 2009; Bellefentaine et al., 2011). In general, nitric oxide is derived from the enzymatic conversion of L-arginine to citrulline by nitric oxide synthase (NOS) in the presence of nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) and other co-factors (Bonthuis et al., 2010). NO mediates its signalling by interacting with soluble guanylyl cyclase (sGC) and stimulating its activity. There are three isoforms of nitric oxide synthases (NOS) including neuronal NOS (nNOS, or NOS1), endothelial NOS (eNOS, or NOS3), and inducible NOS (iNOS, or NOS2) (Alderton et al., 2001). iNOS is not expressed in the cell normally but its expression can be induced if there is some appropriate inducer like in macrophage the bacterial cytokines can illicit iNOS expression as its cytotoxic response, and once induced, it can remain active

without the regulation by intracellular Ca^{2+} (Nathan and Hibbs, 1991). In the brain expression of the inducible nitric oxide synthase (iNOS) in glia can lead to the killing of neurons by the astrocytes and microglial phagocytosis of neurons resulting in neurodegeneration (Brown and Neher, 2010). eNOS is expressed in the endothelial cells and Ca^{2+}-activated calmodulin is important for the regulation of its activity (Hemmens and Mayer, 1998). eNOS is a homeostatic regulator of numerous essential cardiovascular functions, dilating all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells, it also has a critical role in post-natal angiogenesis, is vasoprotective and also has an anti-atherosclerotic effect (Forstermann and Sessa, 2012). Of the three isoforms of nitric oxide synthase (NOS) (nNOS, eNOS, iNOS), NO derived from nNOS has been implicated in the modulation of neurophysiological functions (Kourosh-Arami, 2020), including neuroendocrine regulation of GnRH (Bellefontaine et al., 2011). In the brain, distribution of nNOS expression has been reported to be in the area of the hippocampus, the paraventricular, magnocellular and supraoptic nucleus of the hypothalamus, amygdala, cerebral cortex, dorsal raphe nucleus, striatum, olfactory bulb, basal ganglia, locus coeruleus, spinal cord and cerebellar cortex (Zhou et al., 2018). Numerous studies have suggested nNOS to be involved in the pathology of affective disorders such as major depressive disorder (MDD), borderline personality disorder (BPD) and anxiety disorder. In humans suffering from schizophrenia and depression, the nNOS-containing neurons were localized in several hypothalamic nuclei of the postmortem brains, with the vast majority of nNOS neurons in the paraventricular nucleus (PVN). Cell counts of immunoreactive (ir)-nNOS neurons revealed a significant reduction of cell density in the PVN of depressed and schizophrenic patients compared to the controls, while the total amount of ir-nNOS cells in the PVN was smaller in depressive and schizophrenic patients (Bernstein et al., 1998). It was either reduced or of abnormal size and branching pattern in the putamen of schizophrenic patients (Laurer et al., 2005). It was also shown that a small subset of ir-parvocellular PVN co-localize corticotrophin-releasing hormone (Bernstein et al., 1998; Costa et al., 1993; Egberongbe et al., 1994). Thus, it was postulated that the reduced expression of ir-nNOS in the PVN in depressive and schizophrenic patients may be due to the involvement of nitric oxide in the

release of CRH, arginine vasopressin and oxytocin, which have been shown to over-express in depressive-like conditions (Bernstein et al., 1998). Early genetic indications of nNOS function were provided by knockout mice with a deletion of exon 2 (Huang et al., 1993) and displayed aggressive behaviour and abnormal nocturnal motor coordination and cognitive performance (Weitzdoerfer et al., 2004; Nelson et al., 2006). Brueniq et al. (2017) have investigated the two genes of the nitric oxide pathway NOS1AP and NOS for their potential involvement in post-traumatic stress disorder (PTSD) and suggested that the genes from the nitric oxide pathway are likely to play a key role in PTSD, and other conditions related to it and in its resilience. Neurobiology of disorders such as anxiety, stress, and neuropsychiatric diseases has been observed to be associated with the dysfunction of the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) cascade. Hyperactivity of the HPA axis is one of the most consistent biological findings in anxiety- and depression-related disorders. Experiments involving the administration of nitric oxide donors or inhibitors also further add to prove the role of nitric oxide in anxiety and depression behaviour such as social isolation stress as well as nitrite levels in the cortex of isolated conditioned mice (Amiri et al., 2015). Moreover, disorders like anxiety- and depression-like behaviour lead to dysfunction of the hypothalamo-hypophyseal-adrenal axis, the axis responsible for the endocrine stress response. Nitric oxide has been reported to be involved in the neural network linking GnRH reproductive neuroendocrine axis during food deprivation and stress-related disorders. Administration of a NO donor like 3-morpholinosydonimine (SIN-1) reverses the inhibitory GnRH and LH responses and normalises the KISS1 and RF-amide related peptide 3 (RFRP-3) mRNA profiles indicating the nitric oxide signalling interaction with the neurotransmitters (Shakya et al., 2018).

NO, involved in various physiological processes implying pathophysiology of different conditions, may be connected and molecular mechanisms or pathways may be shared under certain conditions. In the stress physiology and stress-related disease processes, NO has a detrimental effect but also has ameliorating effect suggesting an important role in stress and adaptive response to it (Esch et al., 2002).

Central administration of inhibitors of NO, N_{ω}-Nitro-L-arginine methyl ester hydrochloride(L-NAME) attenuated the plasma ACTH response to intermittent electroshocks and concurrently decreased hypothalamic NOS activities. This suggests that NO exerts a stimulatory influence on the HPA response to physico-emotional stressors and that the hypothalamus is the critical site of their action. The study also confirmed the specificity of action of L-NAME at suppressing NOS (Rivier, 1998; Kim and Rivier, 2000). Further, it has been established that enkephalin and dynorphin systems of the rat hypothalamus exhibit expression of NOS by nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-diaphorase) activity or by labelling with antibodies such as methionine enkephalin (M-Enk) or dynorphin B (Dyn-B) in the paraventricular, arcuate and ventromedial nuclei (Murakami, 1994). A high ratio (37%-84%) of NADPH-diaphorase activity was reported in the Dyn-B neurons of the supraoptic and parvocellular and magnocellular PVN neurons in the rat hypothalamus. Hence, the study revealed that dynorphin and enkephalin have the ability to produce nitric oxide. The study also suggested that the increased expression of nitric oxide in magnocellular neurosecretory dynorphin-containing neurons may help the NO in the control of neurohypophyseal hormone secretion along with dynorphin (Murakami, 1994). In the Dyn-induced spinal cord injury (SCI), treatment with various NOS inhibitors (L-NAME, 7-NI and Aminoguanidine (AG), NO donor 3-propanediamine, N-{4-[l-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino] butyl} (Spermine NONOate, Sper/NO), and NOS substrate (L-arginine - L-Arg) show therapeutic effects, suggesting a differential role of NO/NOS in DYN induced SCI. Pretreatment with Sper/NO or L-Arg 10 min prior to i.t. Dyn A(l-17) not only prevented Dyn-induced elevations of nNOS and iNOS activities but also inhibited the basal nNOS and iNOS activities, implying that their neuroprotective mechanisms against Dyn-induced SCI may involve its inhibition of NOS after Dyn-induced paralysis has occurred as well as its initial vascular dilation (Hu et al., 1999). Further, the inhibition of NOS activity in heat stress attenuated the dynorphin immunoreaction and cell injury, indicating that dynorphin-induced neurotoxicity in hyperthermia is mediated via a mechanism involving nitric oxide (Sharma and Alm, 2001). Thus, it may be

speculated that the enkephalin and dynorphin systems may be involved in mood disorders such as anxiety and depression-like behaviour.

In the time-dependent sensitized model of a stressed animal, stress–restress evoked a long-lasting increase in hippocampal NOS activity that was accompanied by a reactive downregulation of hippocampal NMDA receptors and dysregulation of inhibitory GABA pathways. Also, treatment with iNOS inhibitor aminoguanidine blocked stress-induced NOS activation. This prominent role of NO in neuronal toxicity and the important regulatory role for glutamate and GABA, throws some light on the stress-related hippocampal degenerative pathology and cognitive deficits seen in patients with PTSD (Harvey et al., 2003).

Further, the NOS inhibitor, L-NAME has been reported to decrease the ACTH response to shock. L-NAME has also been shown to decrease the upregulation of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) peptides in the paraventricular nucleus of the hypothalamus due to exposure to neurogenic stressors. There have also been reports of an increase in the number of CRH and AVP transcripts following intracerebroventricular administration of NO in the paraventricular nucleus of rats (Nelson et al., 1997), suggesting that nitric oxide has a stimulatory role in the hypothalamus. Also, the role of nNOS in depression has been reported in chronic mild stress (CMS) mice (Zhou et al., 2007) using pharmacological and genetic approaches. CMS-exposed mice displayed impaired neurogenesis in the hippocampus and behavioural changes typical of depression. Deletion of the nNOS gene in the mice leads to the prevention and reversal of the effects of the CMS-exposed mice. Administration of nNOS inhibitor to the mice also had similar effects (Zhou et al., 2018). Moreover, nNOS knockout mice displayed antidepressant-like properties. This finding leads to the suggestion that overexpression of nNOS in the hippocampus of the brain is critical for CMS-induced depression. Further, the inhibition of nNOS signalling in the brain may lead to a novel approach to the etiology of depression-like disorders (Echeverry et al., 2004). It has also been reported that rats treated with 7-nitroindazole (7-NI), N_{ω}-Nitro-L-arginine (l-NOARG) and L-NAME produced anxiolytic-like behaviour in the elevated plus maze (EPM)

test (Volke et al., 2003). Further, when the light/dark exploration test was performed on the rats, which were treated with the NO donor, 3-morpholinosydnonimine (SIN-1), displayed an anxiolytic-like response (Perez-Nievas et al., 2007; Tsuchiya et al., 1997).

During fear exposure, NO concentration increases in some areas of the limbic system and is also involved in the modulation of aversive memory, increasing fear and anxiety-like behavior. Sodium nitroprusside administration has an anxiolytic effect in rats, contrastingly, administration of L-NAME also induces an anxiolytic effect in rats, indicating nitric oxide has a positive modulatory function (Medeiros et al., 2022). The characteristic feature of depressive illness is the hyperactivity of the HPA axis, which is a result of the decreased glucocorticoid receptor (GR) in the hippocampus that in turn is due to the glucocorticoid-induced hippocampal nNOS upregulation. Chronic mild stress (CMS) and corticosterone exposures cause hippocampal nNOS overexpression, suggesting hippocampal nNOS could be important for stress-related depression, also apparent because the inhibition of hippocampal nNOS almost abolishes both CMS- and corticosterone-induced depressive behaviours (Zhou et al., 2011). Pair housing increases anxiety-like responses in male mice, inhibition of nNOS by gene deletion or treatment with inhibitor 3-Br-7-NI affects the ability of the animal to respond behaviorally to social stimuli in pair-housed mice. It reduced open arm exploration in single-housed mice, but in pair-housed mice, nNOS inhibition increases open arm exploration indicating that nNOS inhibition is anxiolytic in group-housed male mice (Workman et al., 2008). Impairing NO and ROX pathway by creating double knockout mice by deleting the p47phox and nNOS genes in C57BL/6 mice show that deletion of these genes synergizes to impair cognitive function relevant to schizophrenia and social behaviours relevant to autism (Walton et al., 2013). Male mice with nNOS gene deleted also displayed a dramatic loss of behavioural inhibition resulting in elevated aggression and mating behaviour (Nelson et al., 2006).

Evidence indicates that anxiety and depression have always accompanied humans and animals. However, the concrete treatment regimen for the said disorder has been elusive to date. The research on anxiety and depression

disorders has improved the patient's quality of life, but with limiting results. At times, some of the clinical symptoms are misinterpreted or completely missed. Thus, studying molecules involved in controlling anxiety and depression disorders becomes essential, which may help improve understanding of the etiology of the mental disorder. There are still many lacunae in the research on such mood disorders. In the current work, we tried to undertake one such lacuna, where there has been no study yet reported on direct embryonic disruption of the nNOS during the critical developmental period and looking for its long-term effect postnatally, both on the anxiety- and depression-like behaviour of mice and also its effect in the hypothalamo-hypophyseal-gonadal axis level and integrate the HPA-HPG axis in the etiology of anxiety- and depression-like behaviour and reproductive functions. Thus, the present work will further add to the plethora of information available on anxiety- and depression-like behaviour and help add a connecting link in understanding the disorder better.

Animals

Adult Male (♂) and female (♀) albino mice (Swiss Albino Mice Strain) were time mated to maintain their colony in the animal house of the department. Both the sexes of mice, i.e., ♂ and ♀ mice were used to carry out the experiments for the present study. All the mice were housed in polypropylene cages of either size 29x22x14cms or 43x27x15cms and maintained under conditions of 12L:12D light-dark cycle throughout the experiment with a standard mice diet (VT/L/ANIMAL-FEED – Lab Animal Feed for Mice/Rat, Catalogue No. 23099090, VISHNU TRADERS, Roorkee, Uttrakhand) and water ad libitum. All the cages were provided with rice husk as the bed material and the rice husk was replaced in each cage on a biweekly basis. The experiments were carried out as per the Animal Ethics Committee of Rajiv Gandhi University and as per the guidelines within the framework of the revised Animals (Scientific Procedures) Act 2002 (CPCSEA Guidelines) of the Government of India. The body weights of all the animals were measured weekly.

Animal Identification

Each animal was identified by assigning them a specific number using the ear punching technique. This method is commonly used for the identification of rodents and involves using a punch to produce a small (0.5-2 mm) notch near the edge of the ear. The holes and notches are placed according to a predetermined code (Dickie, 1975; Ingalis, 1980; Stark and Ostrow, 1991). Ear Punching technique is the most preferred method to individually mark the male and female mice during weaning age or in adult mice, as the mice do not need to be anaesthetized. Before the ear punch was made on each individual mouse, the cutting edge was disinfected with 70% ethanol. The animal's right ear is used for single digits and the left ear for tens (Kasanen et al., 2011; Dalhborn et al., 2013) (**Figure 1**)

Chemicals:

7-Nitro-1H-indazole (7-NI)

7-NI was purchased from Alfa Aesar, Thermo Fisher Scientific India Pvt. Ltd., Mumbai (Catalogue No. L07970, Lot NO. 10118469, CAS: 2942-42-9). 7-NI is a specific neuronal nitric oxide synthase inhibitor, an enzyme that helps convert L-arginine to L-Citrulline and produce nitric oxide (NO) in neuronal tissue.

N-Omega-Nitro-L-arginine methyl ester hydrochloride (L-NAME)

L-NAME was purchased from Alfa Aesar, Thermo Fisher Scientific India Pvt. Ltd., Mumbai (Catalogue No. H63666, Lot NO. Y01D022, CAS: 51298-62-5). L-NAME is a non-selective inhibitor of nitric oxide synthase and is obtained by combining N(gamma)-nitro-L-arginine methyl ester with one equivalent of hydrochloric acid.

Sodium Nitroprusside (SNP)

SNP was purchased from Central Drug House (P) Ltd., New Delhi (Catalogue No. 010257, Batch NO. 130113, CAS: 13755-38-9). SNP is a NO donor, that reacts with sulfhydryl groups on blood cells specially erythrocytes (as well as albumin and other proteins) to produce NO. SNP is a hydrophilic molecule, i.e., it easily dissolves in water. Chemically, SNP is composed of ferrous iron complexed with NO and five cyanide ions. SNP acts upon vascular smooth muscle by triggering the intracellular cGMP-mediated activation of protein kinase G and thus, subsequent inactivation of myosin light chains that results in vascular smooth muscle relaxation. This signalling cascade results in peripheral vasodilation of both arteries and veins, however, the selectivity for veins is slightly more (Ivankovich et al., 1978; Fok et al., 2012).

Ketamine Hydrochloride Injections

Ketamine hydrochloride, a nonbarbiturate dissociative (Batch No. 201140, Neon Laboratories Limited, Mumbai and Batch No. KMI102, Themis Medicare Ltd., Uttrakhand) is an anaesthetic. It is a cyclohexane derivative that acts

rapidly and produces intense analgesia and anaesthesia. The structural formula of ketamine hydrochloride is CHClNO and is chemically known as±)-2-(o-chlorophenyl)-2-(methylamino). Ketamine acts as a non-competitive antagonist to N-methyl-D-aspartate (NMDA) and glutamate receptors and blocks HCN1 receptors. The unique property of dissociation action and partial agonism of ketamine hydrochloride on opiate mu-receptors helps it to perform the painful procedures in a consistent state of patient comfort and sedation. This anaesthetic may be used in procedures requiring short-term sedation/anaesthesia (approved by USA Food and Drug Administration) and has been shown to be safe when used for pain management (Rosenbaum et al., 2022)

Lignocaine Hydrochloride Injection

Lignocaine hydrochloride, a local anaesthetic (Batch No. V188085, Neon Laboratories Limited, Mumbai and Batch No. DV1022, German Remedies, Ahmedabad) is commonly used to numb an area of the body to help reduce pain or discomfort (numbing medication) caused by medical surgeries, insertion of catheter, needle punctures or insertion of breathing tube (invasive medial procedures). This local anaesthetic contains 1% or 2% of lignocaine hydrochloride as the active ingredient and water and sodium chloride as excipients. The function of this anaesthesia is to produce local anaesthetic effect and its action is rapid. Lignocaine hydrochloride stabilizes the potentially excitable membranes, thus preventing the initiation and transmission of nerve impulses. The effect of this anaesthetic may last for about 60-90 minutes.

Heparin Sodium Injections

Heparin (Batch No. T124597, Troikaa Pharmaceuticals Ltd., Uttrakhand) is an anticoagulant (blood thinner) that prevents the formation of blood clots. Heparin is an anticoagulant that is used for the prevention and thrombotic events such as pulmonary embolism, atrial fibrillation and deep vein thrombosis as well as for the prevention of excess coagulation during surgeries such as dialysis (continuous renal replacement therapy), extracorporeal circulation, cardiac surgery etc. Heparin upon administration binds to several proteins but its binding to antithrombin is especially important as this cause a surface change

and the molecule thrombin is inactivated. This step blocks different clotting factors but blockage of thrombin (Factor IIa) and Factor Xa is very important. Inactivation of thrombin by administration of heparin blocks the conversion of fibrinogen to fibrin which prevents the formation of clots and thus extends the time of blood clotting (Warnock and Huang, 2022).

All other chemicals and reagents for the experiments were of analytical grade and purchased from Merck Life Science Pvt. Ltd., Mumbai; SRL Pvt. Ltd. Taloja, Maharashtra; HiMedia Laboratory Pvt. Ltd., Mumbai; LOBA Chemie Pvt. Ltd., Mumbai and Thermo Fisher Scientific India Pvt. Ltd., Mumbai.

Estrous Cycle

Estrous cyclicity was assessed by collecting the vaginal smears from all-female mice. In rodents such as rat and mice, the estrous cycle is completed in 4-5 days and the four stages of this cycle, Proestrus, estrus, metestrus and diestrus (Figure 2) can easily be recognized and studied in the research laboratory with the help of a compound microscope from the vaginal smears. To check the estrous cycle, a vaginal smear test was generally done between 09:00 – 10:00 hrs. of the day. The vaginal smears were taken and evenly spread on a clean and dry glass slide with the help of earbuds soaked completely in 0.05M phosphate-buffered saline (PBS). The slides were then allowed to dry at room temperature (RT). Methanol (1-2 drops) was then added to the slides and again left to dry at RT. Few drops of Giemsa stain were then added to the slides and kept for 10 minutes. The extra stain was removed by washing the slides with the help of distilled water. The slides were allowed to dry again and observed under the microscope (**Figure 2**). All the stages were then recorded daily for each individual female mouse (McLean et al., 2012; Cora et al., 2015; Mohammed and Sundaram, 2018; Ajayi and Akhigbe, 2020).

Sperm Count

Before perfusion on the animals was performed, spermatozoa were obtained from the cauda epididymis. After exposing the epididymis, the cauda epididymis was transected at the point of origin of the vas deferens at the distal

end and at the boundary between the corpus and cauda epididymis at the proximal end. The tissue was placed in a watch glass containing 0.5ml of normal saline (NS) maintained at 37°C. The tissue was minced carefully with the help of fine forceps and scissors to ensure the extrusion of spermatozoa from the cauda epididymis. The tissue fraction was then removed by forceps or needles, and the suspension was used for sperm analysis according to Singh and Chakravarty (2003); WHO (1999).

Briefly, a haemocytometer (Fein optic, Jena, Germany) with an improved Newbauer chamber was employed for counting the spermatozoa. A 20-fold dilution was made by mixing the sperm suspension with the spermicidal solution ($NaHCO_3$: 4g + Phenol: 1 g in 100 ml of distilled water). The dilution was made using a white blood cell pipette; the sperm suspension was drawn to the 0.5 mark halfway up the stem and the spermicidal solution subsequently to the 11 mark at the top of the bubble chamber. The preparation was then thoroughly mixed, and one drop of it was added to both sides of the haemocytometer. The spermatozoa were allowed to settle optimally by keeping the haemocytometer in a humid (wet) chamber for 30 minutes. The humid chamber was constructed by placing a wet sponge inside a faintly airtight icebox. The number of spermatozoa was counted in the four corner squares of the haemocytometer under a microscope at 400X when spermatozoa crossed the lines of the joins. Only those at the top and right-hand sides of the squares were counted. Spermatozoa on both sides of the haemocytometer were counted, and the average number was recorded.

Concentration of spermatozoa = Average number of the spermatozoa counted (N) X multiplication factor (10,000) X dilution factor (20)

= N X 10,000 X 20 spermatozoa

= N X 0.2 X 10^6 Spermatozoa

Fixation of Tissues

After each experiment, the animals were sacrificed as per the approved Animal Ethics Committee Protocol and tissue was collected for histological studies. For the collection of tissue i.e., gonads (testis and ovary) and brain, the mice were anesthetized using ketamine hydrochloride injections (50mg/kg body weight) followed by Lignocaine hydrochloride injections (20mg/kg body weight). Once the animal was anaesthetized, its abdomen was dissected through two lateral incisions up to the diaphragm and the aorta was cross-clamped to excise the intra-abdominal organs, namely testes from males and ovaries from females, before perfusion. The left testis and left ovary of each animal were dissected and stored at -20ºC for biochemical analysis while the right testes and right ovaries were fixed in Bouin's Solution for 24 hours for the histological study of the tissues. The epididymis was separated out for sperm count in males. Blood was also withdrawn from the heart and collected in an EDTA-coated tube and plasma was separated by centrifuging the blood at 3000rpm at 4^0C for ELISA of steroid hormones (Testosterone and Estradiol) and biochemical analysis (total nitrate-nitrite estimation).

Perfusion of Animals

The animals were perfused with 4% Paraformaldehyde (4% PFA) for the fixation of brain tissue (Moore and Alejandro, 2021). Briefly, 4% PFA was prepared as per standard protocol. Each animal was anaesthetized with Ketamine/ Lignocaine hydrochloride solution and allowed to become unresponsive by 3-5 minutes to stimuli such as tail pinch or foot pinch. Each mouse was then placed on a dissecting wax tray by laying the animal dorsally and pinning each extremity with the help of a paper pin. The abdominal surface of the animal was then wiped with 70% ethanol and the abdominal cavity was exposed by grasping the skin of the abdomen with forceps and lifting away from the cavity. The abdomen was opened by making a small incision through the skin and the incision was then extended to the xiphoid process (cartilaginous section up to the lower of the sternum) using scissors. The anterior diaphragm was then cut by grasping the inferior portion of the sternum to enter the thoracic

cavity. The ribs were cut on either side without injuring the underlying pericardium and the vessels. Once the heart was exposed, a perfusion syringe with 25G needle having ice-cold 0.05M Phosphate Buffered Saline (0.05M PBS) with heparin solution was inserted into the left ventricle and pumped into the heart slowly and steadily to flush out the blood from the body. At the same time, the right atrium was pinched with a needle to allow the blood to extravasate from the lower body. Once the liver turned pale white/yellowish, another syringe with 25G needle having ice-cold 4% PFA was inserted into the left ventricle and pumped into the heart slowly and steadily till twitching of the muscles could be observed. The above procedure generally requires 20ml of both the solutions each to have a good fixation. Once the fixation process was completed, the mouse was decapitated with scissors. A cut along the midline of the scalp was made and all the skin was removed to expose the skull. At the posterior base of the skull, a midline incision and a cut across the lambdoid suture were made. Further, an incision was also made along the sagittal and metopic sutures as well as bilateral coronal sutures. The cranial nerves and the spinal cord help keep the brain intact. Both these structures were cut, and the brain was then dissected out and stored in the same fixative (4% PFA) for 24 hours at 4^0C and then changed to 0.1M phosphate buffer on a shaker at 4^0C. The tissues were stored in 0.1M PB at 4^0C until processing for sectioning and cresyl violet staining.

Crystal Violet staining

Crystal violet stain (SRL Pvt. Ltd. Mumbai, India, Cat. No. 074072, Batch No. T-837437, C.I. No. 42555) was used by dissolving 0.1g of crystal violet stain in 100 ml of 70% alcohol.

4% PFA fixed brains were processed through ascending series of alcohol, cleared in xylene and embedded in paraffin wax at 60^0C for the preparation of paraffin blocks. Paraffin blocks of the brain were then cut on a rotary microtome (Leica Histocore Multicut Rotary Microtome – Multicut 149MULTI0C1, Leica Biosystems, A Division of DHR Holding India Pvt. Ltd., Mumbai) at 6µm thickness. The brain sections were then spread on gelatin-coated slides and dried at 37^0C overnight.

The brain sections to be stained were dewaxed in xylene, rehydrated in descending series of alcohol followed by a rinse in distilled water and finally stained in the crystal violet stain (Fraser, 1982) for 15 minutes. The stained slides were then quickly rinsed in distilled water for a few seconds to wash off the excess stain and the sections were again dehydrated through ascending series of alcohol. The slides were cleared in xylene and then mounted with Dibutylphthalate Polystyrene Xylene (DPX). The DPX-mounted slides were left to air-dry and then examined under the microscope (Leica Upright Phase Contrast Microscope, Model No. DM2000 LED attached with Leica DFC450 C Digital Camera and SW Kit, Leica Microsystems, A Division of DHR Holding India Pvt. Ltd., Mumbai) for changes in the neuronal structures in the brain.

Tissue Processing

Bouin's fixed tissues (testes and ovaries) were first dehydrated through ascending series of alcohol followed by clearing in xylene. Xylene-cleared tissues were then embedded in paraffin wax (58-60^{0}C) to make paraffin blocks. The paraffin blocks of testes and ovaries were then trimmed and fixed to a block holder for microtomy (6μm thickness) as described for brain tissues.

Sections thus obtained were then stretched on clean glass slides coated with 5% gelatin and left overnight at 37ºC before hematoxylin-eosin staining of the sections.

Hematoxylin- Eosin (H&E) Staining

Hematoxylin-Eosin is a standard stain for histological examination of tissues. Much significant information about the structures and functions of cells can be deduced from this staining procedure (Chan, 2014).

In the present work, Harris hematoxylin staining solution from Stanbio reagents (Stanbio Reagents (P) Ltd., Kolkata, India C.No. 10228) was used. Eosin stain was prepared by dissolving 1g of Eosin Yellowish (Eosin Y) powder (Merck Life Science Pvt. Ltd., Mumbai, India, C.I. No. 45380) in 400 ml of 70% ethanol

and making up the volume to 500ml by adding 100 ml of distilled water. 0.25 ml acetic acid was added to the solution.

For the H&E staining of the sections (Bancroft and Layton, 2013), the sections on the slides were first dewaxed, using xylene as a clearing agent (3X; 5minute each), followed by a rehydration step involving passing the slide through decreasing concentrations of alcohol i.e., in the order absolute alcohol (2X; 5minute each), 90% alcohol (2X; 5minute each), 70% alcohol (2X; 5minute each), 50% alcohol (1X; 5minute) and one change of 30% alcohol for 5 minutes. The sections were then kept in distilled water for 5 minutes and then immersed in hematoxylin stain for 15 minutes. The hematoxylin-stained slides were then kept under running tap water for 15 minutes and then after passed through acid water for 10 seconds for differentiation. The slides were again allowed to wash under running tap water for 45 minutes, rinsed in distilled water and processed for dehydration again by passing through increasing concentrations of alcohol, 30% alcohol for 5 minutes, 50% alcohol for 5 minutes, 70% alcohol (2X; 5minute each). After the final change in 70% alcohol, the sections were stained with eosin stain for 2-4 minutes and then the dehydration process was continued 70% alcohol (2X; 5minute each), 90% alcohol (2X; 5minute each) and absolute alcohol (2X; 5minute each). The sections were finally cleared with xylene and the coverslip was mounted using DPX. The glass slides with stained sections and a coverslip were dried and then observed under a microscope (Leica Upright Phase Contrast Microscope, Model No. DM2000 LED attached with Leica DFC450 C Digital Camera and SW Kit, Leica Microsystems, A Division of DHR Holding India Pvt. Ltd., Mumbai) for histological examination.

ELISA of Testosterone and Estradiol

Blood, from the animals, was collected in ethylenediamine tetraacetic acid (EDTA)-coated tubes and refrigerated at 4^0C for separation of plasma. The blood, thus collected, was centrifuged at 3000rpm at 4^0C for 30 minutes and plasma was collected from each tube. The plasma thus collected was analysed for the presence of steroid hormones (testosterone in males and estradiol in females) using highly specific ELISA Kits of Testosterone and Estradiol (DiaMetra, Italy, Catalogue No. DKO002-12, Lot No. 5666A – Testosterone

and Catalogue No. DKO 003, Lot No. 5692A – Estradiol). The intra-assay and inter-assay precisions were calculated: the coefficients of variation were < 7.0% and <8.3% for testosterone and <9% and <10% for 17β-estradiol, respectively. An assay for both the steroids was performed as per the manufacturer's protocols. Cross-reactivity of the testosterone antibody with testosterone was 100% while it was less than 0.01% for androstenedione and 0.05% for androsterone. Similarly, cross-reactivity of the 17β-estradiol antibody with 17β-estradiol was reported to be 100% while with estrone and estriol, it was 2.0%.

Plasma collected was diluted 4 times using 0.05M phosphate buffer saline before the estimation of testosterone and estradiol. In the 96 well antibody (testosterone and estradiol) adsorbed microplates, provided with the kit, 25 µl of samples were loaded in duplicates. For the blank, phosphate buffer saline was used. Calibrators and control samples of different concentrations provided in the kit were also loaded in the microplate provided with the kit. Diluted plasma samples of the sacrificed animals were also added in to the microplate. This step was followed by the addition of conjugate solution (100µl in case of testosterone and 200 µl in case of estradiol) in all the wells and incubated at 37ºC for 2 hours. The content of each well was removed after incubation and the wells were washed three times with 300µl diluted wash solution with gently shaking the plate for a few seconds. The solution was removed by inverting the plate in one go. Once the washing was done, 100µl of TMB substrate was added to all the wells, and the plate was incubated at room temperature (25ºC) for 30 minutes in the dark. After 30 minutes of incubation, 100µl of stop solution was added to all the wells and absorbance was taken at 450 nm against blank in a Thermo Scientific Multiscan Go Spectrophotometer, Type 1510.

Total Nitrite and Nitrate concentration

The stable end products of nitric oxide oxidation are nitrite and nitrate. Estimation of these compounds is an indirect method to monitor nitric oxide levels in body fluids (Sastry et. al., 2002).

Total nitrite and nitrate concentration in plasma, testis and ovary were measured by the method of Sastry et al. (2002). Briefly, blood was collected in an EDTA-

coated tube and centrifuged at 3,000 rpm for 30 minutes to separate plasma for measuring the total nitrite-nitrate concentration. 5% tissue homogenate (w/v, testis and ovary) was prepared in 0.05M PBS (pH=7.4). The tissue (testis and ovary) homogenates were centrifuged at 15,000 rpm for 30 minutes at 4^0C. Supernatant from the homogenate after centrifugation was collected to determine the level of total nitrate and nitrite concentration in the testis and ovary.

100µl of each plasma sample, homogenate of testis and ovary and standards (KNO_3) were taken and 400 µl of carbonate buffer was added to it. To the mixture, a small amount (~ 0.15 g) of activated copper-cadmium alloy filings was also added. The mixture was then incubated with thorough shaking at room temperature. The reaction was stopped by the addition of 100 µl of 0.35M NaOH followed by 120 mM $ZnSO_4$ solution under vortex and allowed to stand for 10 minutes. The mixture was subsequently centrifuged at 5000 rpm for 15 minutes and the supernatant was collected for further processing.

In a 96-well microplate, 150µl of the supernatant of standard solution, as well as the samples (plasma, testis and ovary) to be tested, were taken in duplicates. To this Griess reagent (75µl of Sulphanilamide, prepared in 3N HCl and 75µl of N-naphthyl ethylenediamine, prepared in distilled water) was added to all the microplate wells. Finally, absorbance was taken at 545nm after 10 minutes of incubation using Thermo Scientific Multiscan Go Spectrophotometer, Type 1510. A standard graph was plotted against different concentrations (0, 20, 40, 60, 80 and 100µM concentrations) of KNO_3.

Statistical Analysis

All the data are represented as Mean ± SEM. Further, the data were either subjected to a t-test (if two groups) or Analysis of Variance (ANOVA) if more than two groups followed by a posthoc test (Bonferroni Test) with a confidence limit of 95%.

Animal Identification

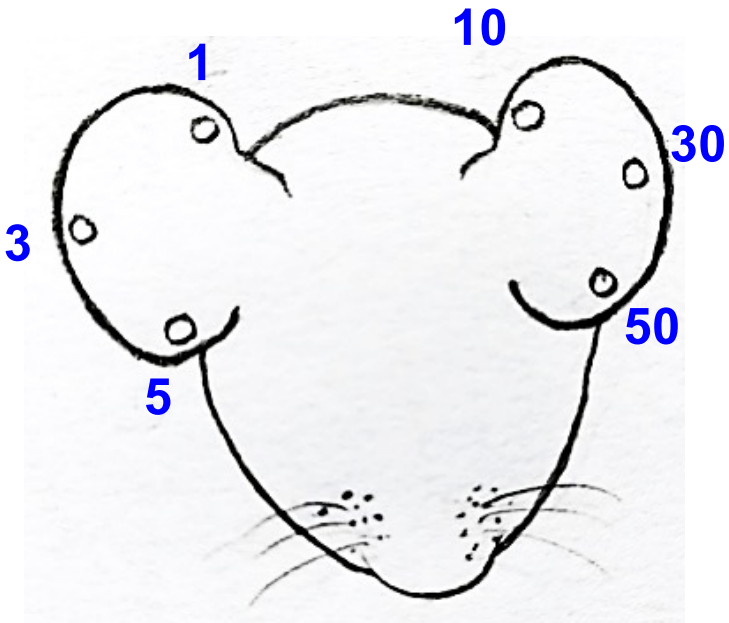


Figure 1: Diagrammatic representation of numbers used for individual mice identification (Drawing by Konya and Kumar, 2022). (Adapted from Macri et al., 2011)

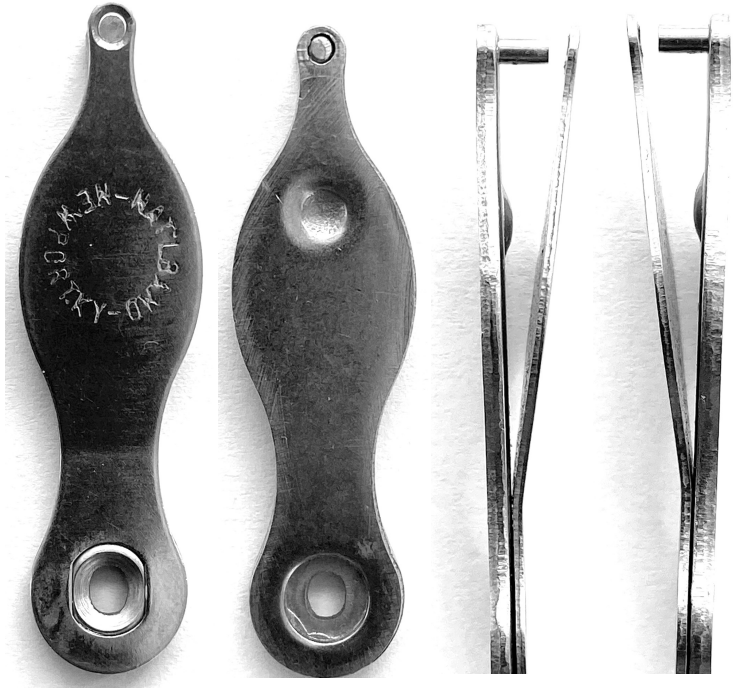


Figure 2: Ear Punch (Orchid Scientific & Innovative India Pvt. Ltd.)
(Photography by Konya and Kumar, 2022)

Determination of Estrous Cycle Stage by Giemsa Staining

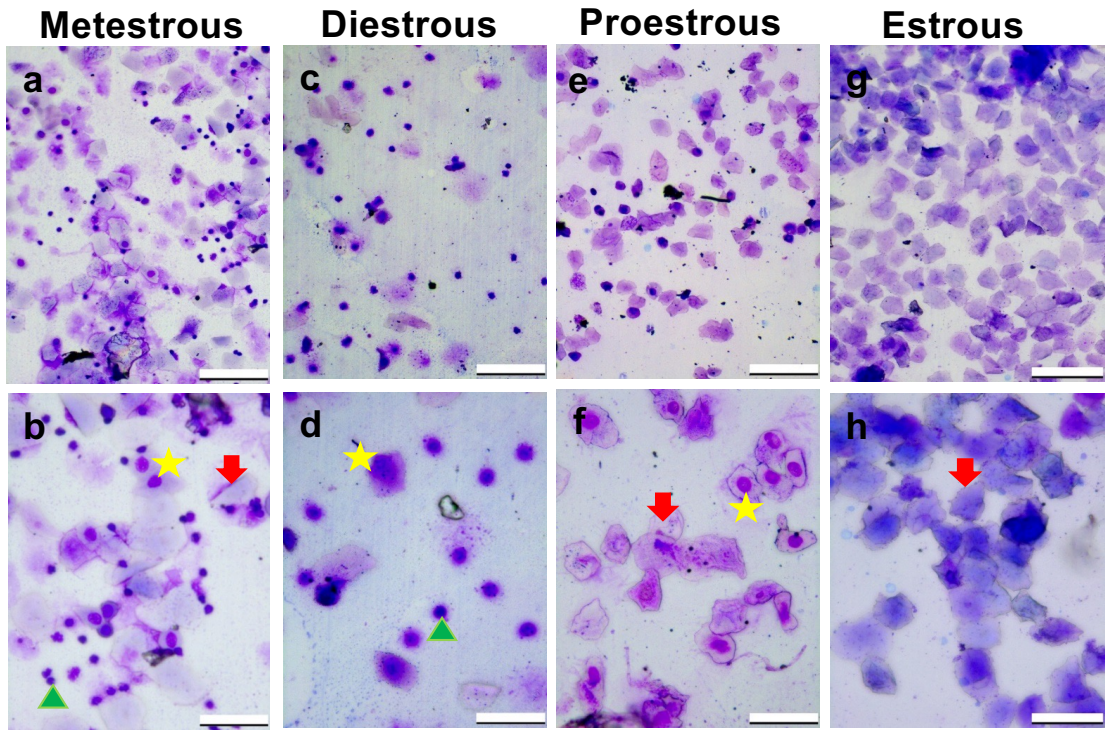


Figure 3: Representative images of different stages of Estrous Cycle. Images "a" and "b" show cells in Metestrous phase, images "c" and "d" are in Diestrous phase, images "e" and "f" are in Proestrous phase and images "g" and "h" are in Estrous phase. Images a, c, e and g have a scale bar = 100μm and images b, d, f and h have a scale bar = 50μm.

KEY: ★ Nucleated Epithelial Cells, ▲ Leucocytes, ↓ Cornified Epithelial Cells.

Chapter 1

Embryonic Disruption of Neuronal Nitric Oxide Synthase Alters Anxiety- and Depression-like Behaviour and Reproductive Physiology of Mice in its Adulthood

Abstract

Mood disorders like anxiety and depression are major contributors of the maladaptation of individuals to normal situations. To understand these disorders, anxiety and depression-like behaviour studies are done on animal models such as mice. There are many neuropeptides and neuromodulators known to influence the HPA axis, the stress axis, that are implicated in mood disorders like anxiety and depression. One such neuromodulator of the stress axis is nitric oxide. In the present study, the production of nitric oxide was inhibited by a specific inhibitor (7-Niroindazole, 7-NI) of its enzyme neuronal nitric oxide synthase (nNOS). Male and female mice were time mated in the evening hours (16:00 hrs) and a vaginal plug was observed the next day. Females showing vaginal plugs were considered pregnant. One group of pregnant females were kept in individual cages and were injected with 7-NI at a dose of 10mg/kg body weight from embryonic day 11 to embryonic day 17, while the other group was administered with vehicle control (Dimethlysulphoxide:Normal Saline – DMSO:NS in 1:1 ratio) and served as the vehicle control group. The pups born to these pregnant females on day 19 were weaned on postnatal day 21, males and females were separated into different cages with individual ear punch markings for individual identification of mice. These male and females mice were raised to adulthood until 8 weeks when these animals were exposed to a battery of behavioural tests such as elevated plus maze (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT). All the behavioural tests were done in the light phase of the light-dark cycle. The behavioural tests in females were performed when the animals were in the diestrus phase. An interval of 4-7 days was maintained between each behavioural test. After the end of the behavioural tests, each animal was anaesthetized and sacrificed for studying the changes in

hypothalamic structures of the brain and changes in gonad physiology. Results show that there were marked changes in anxiety- and depression-like behaviour (as displayed by EPM, OFT, FST and MBBT), the hypothalamic nuclei of the brain, nitric oxide production (total nitrate-nitrite concentration), gonad (testes and ovary) physiology and plasma testosterone levels and sperm count in males and plasma estradiol levels in females in both males and females born to 7-NI treated females when compared to males and females born to vehicle-treated pregnant female groups. The result shows that administration of nNOS inhibitor in-utero in mice alters both hypothalamo-hypophyseal-adrenal (HPA) axis as well as the hypothalamo-hypophyseal-gonadal (HPG) axis in the embryo itself which prolongs until adulthood by disturbing the neural circuitry responsible for its maintenance. Thus, nitric oxide acts as an anxiolytic and anti-gonadotrophic agent in mice, however, the molecular mechanism needs further elucidation.

Introduction

Body homeostasis, in general, is maintained by a complex interaction of neuroendocrine and autonomic nervous systems. This system in turn helps the higher brain centres, namely the central nervous system to generate complex communication mechanisms of autonomic/neuroendocrine outputs to regulate the various physiological functions and homeostasis of the body (Palkovits, 1999; Miller and O'Callaghan, 2002; Sladek et al., 2015; Geary, 2020; Yoo et al., 2021). Thus, this communication mechanism helps in establishing not only neuron-to-neuron interactions but also the complex neurovascular-glial interactions that contribute to the system to adapt to the change in the activity of peripheral inputs/signals (Laming et al., 2000; Miyata and Hatton, 2002). The hypothalamo-hypophyseal-adrenal (HPA) axis thus gets activated by the release of a cascade of molecules such as vasopressin (AVP), oxytocin (OT), neuropeptide Y (NPY), corticotrophin-releasing hormone (CRH) from the paraventricular nuclei which stimulates the adrenocorticotrophic cells of the pituitary to exert its effect on the adrenal, thus activating the stress axis. Likewise, the hypothalamo-hypophyseal-gonadal (HPG) axis gets activated by the synthesis and release of the gonadotrophin-releasing hormone (GnRH) from

the pre-optic area (POA) of the hypothalamus, which in turn activates the gonadotroph cells of the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to exert its effects on the gonads i.e., testes and ovary. However, disturbances in the HPA axis lead to stress disorders such as anxiety and depression (Demuyser et al., 2016; Wulsin et al., 2016; Kinlein et al., 2019) while alterations in the HPG axis lead to reproductive disorders such as dysfunctions in testicular and ovarian functions (Bailey and Silver, 2014; Iwasa et al., 2018; Patisaul, 2021).

Anxiety and depression, one of the major disorders in today's population are commonly prevalent due to the change in the lifestyle of the individual. Anxiety and depression, though considered to be two distinct entities according to the diagnostic criteria, are co-morbid (Choi et al., 2020). Humans having anxiety disorders commonly have depression, while the vice-versa is true for patients with depression, i.e. they would often have anxiety disorders (Tiller, 2013). The anxiety disorder recognized clinically as mental disorders in humans include generalized anxiety disorder, acute and chronic posttraumatic stress disorder, panic disorder, and obsessive-compulsive disorder. This also includes various phobias such as social phobia, agoraphobia, and specific phobia (e.g., fear of flying) (Hang et al., 2015). These disorders are sex-dependent, being increased in females than males in humans (Holden, 2005). The above hypothesis is supported by the gonadic theory, suggesting that women have a more comprehensive range of fluctuations in hormone levels than men. This fluctuation in hormone levels affects the brain regions (hypothalamus, prefrontal cortex, hippocampus) that are known to be involved in the modulation of mood and behaviour (Faravelli et al., 2013). Further, it is also established that stress, anxiety and depression, is a neurological or psychological problem that is widespread in today's population. This is proved by the fact that refugees showed higher anxiety and stress compared to the local Jordanians (Gammoh et al., 2016). Further, in these refugees when compared to local Jordanians, there was an increase in salivary nitric oxide content and glucose-6-phosphate dehydrogenase activity (Gammoh et al., 2016).

On the other hand, disturbances in the HPG axis may lead to reproductive dysfunctions. It is well established that the HPG axis regulates vertebrate reproduction by well-coordinated events in neuroendocrine physiology. The components of the HPG axis produce numerous neurotransmitters, neuromodulators and neuropeptides that help the system regulate diverse reproductive functions in an organism. These factors are also regulated by feedback mechanisms that help maintain the body's homeostasis.

Thus, it is evident that well-coordinated events in the neuroendocrine system such as intrinsic and extrinsic factors regulate both anxiety and depression as well as reproduction in an organism. Among these factors, nitric oxide (NO), a ubiquitous short-lived messenger molecule, plays an important role in both anxiety and depression as well as control of reproduction (Gregg, 2003; Spiacci et al., 2008; Nikkar et al., 2019; Luo et al., 2021; Coelho et al., 2022).

Nitric oxide (NO), an autocrine and paracrine cellular mediator, is synthesized by the conversion of L-arginine to L-Citrulline in the presence of nitric oxide synthase (NOS) enzymes, nicotinamide adenine dinucleotide phosphatases (NADPH) and co-factors and NO being released as a by-product of this reaction (Luo et al., 2021). NO, also referred to as a gaseous molecule, is a neurotransmitter in the central and peripheral nervous systems (Snyder 1992; Džoljić et al., 2015; Philippu, 2016; Akyuz et al., 2020) and acts through cyclic guanosine 3′5′-monophosphate (cGMP) by activating the enzyme guanylyl cyclase (Luo et al., 2021). Many of the cellular responses of NO on central and peripheral nervous systems are the result of guanylyl cyclase-mediated increase in cGMP (De Vente and Steinbusch, 1992; McDonald and Murad, 1995). NO is required for the maintenance of vascular tone (Nathan, 1992). The reaction of NO with cell surface thiols has been shown to be associated with modulation of ligand-receptor N-methyl d-aspartate (NMDA) activity (Lei et al., 1992) and alterations of smooth muscle function (Kowaluk and Fung, 1990, Bates et al., 1991). There are three isoforms of NOS enzymes, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), of which nNOS has been demonstrated to regulate stress such as anxiety and depression (Bielau et al., 2012; Akpinar et al., 2013; Barany et al., 2015; Cai et al., 2017; Zhou et al.,

2018) and reproduction (McCann et al., 1999; Ding et al., 2017; Nath and Maitra, 2018; O'Connor et al., 2018; Prashar et al., 2021; Delli et al., 2021). NO has a widespread role in neuromodulation as one of its enzymes, nNOS has been localized in diverse cell types of the various neuronal systems. The effects of NO on the vascular system and brain physiology form the basis for the diverse role NO displays on neuroendocrine function and behaviour. This short-lived molecule, with a half-life of seconds, is involved in various physiological functions in the brain, i.e., regulation of neurotransmission, synaptic plasticity, development of hypothalamic nuclei etc. (McClellan et al., 2010; Tanda et al., 2009; Bellefentaine et al., 2011); long-term potentiation, neuroprotection, neural degeneration and the regulation of peptidergic secretion (Dawson and Snyder, 1994; Dawson and Dawson, 1996; McCann et al., 2005); embryonic development, gonadal development and it's regulation (Kim et al., 2004; Huang et al., 2010; Basini and Grasselli, 2015; Gray and Cheung, 2014; Siamwala et al., 2019; Tiboni et al., 2021); various behavioural, cognitive and emotional processes (Pitsikas, 2015; Tewari et al., 2021) etc. NO has also been reported to influence signal transduction pathways that help in releasing adrenocorticosterone (Vulliemoz, 1998; Ridel, 2000). Further, NO is also demonstrated to influence the neuroendocrine and neuroimmune systems and their interactions in various physiological and pathological processes (Robbins and Grisham, 1997; Vallance, 2001; Garcia and Stein, 2006; Hannibal, 2016). The expression of nNOS in the PVN of the hypothalamus is decreased in sympathetic nerve-mediated cardiovascular diseases while in mice with spontaneous hypertension and vascular hypertension, the expression of nNOS is increased. This suggests that the expression of nNOS depends on excitation or inhibition of the sympathetic nerve system (McBryde et al., 2018).

Further, nNOS has been implicated in a varied range of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, anxiety, stroke etc.; learning and memory and neuropsychiatric disorders, including depression (Brown and Bal-Price, 2003; Wegener and Volke, 2010; Dawson and Dawson, 2018; Zhou et al., 2018). NO produced in stress response has been linked to behaviors similar to anxiety and depression. NO, produced by nNOS and iNOS in brain structures, is also involved in regulating the HPA axis (Keser et al.,

2011; Gadek-Michalska et al 2019; Yang et al., 2021; Han et al., 2021). NO has been shown to spread rapidly in the mammalian central nervous system membrane, which is one of the reasons why NO is more effective than other neurotransmitters (Forstermann and Sessa, 2012). Nitric oxide and glutamate neurotransmission in defensive reactions are modulated by dorsal periaqueductal grey - dlPAG (Aguiar and Guimaraes, 2009). Moreover, it has been demonstrated that reduced nitric oxide signalling shows a significant reduction in aggression and an increase in anxiety-like behaviour in both zebrafish and mice, without altered production of the stress hormone cortisol (Gutierrez et al., 2017). Evidence indicates an involvement of the NO pathway in mood disorders such as anxiety and depression (Boje, 2004; Guix et al., 2005; Gulati et al., 2017). All these functions of NO function via activation of cyclic guanosine monophosphate (cGMP). This outflow of cGMP can be inhibited by NOS inhibitors and soluble guanylate cyclase (Prast and Philippu, 2001). 7-Nitroindazole (7-NI) is one such specific nNOS inhibitor. 7-NI has been shown to disturb emotional memory, visual memory and olfactory memory, further suggesting the role of nNOS in the pathophysiology of memory (Akar et al., 2014). Measurement of total protein and gene expression in the hippocampus of rats treated chronically with 7-NI and subjected to Forced swim test (FST) has shown that inhibition of NO synthesis has antidepressant-like behaviour in mice suggesting the involvement of NO in the expression of genes related to oxidative stress, neuroplasticity and neurogenic processes (Ferreira et al., 2012). 7-NI also prevented the induction of LTP (long-term potentiation), which is a long-lasting enhancement of synaptic transmission induced by high-frequency afferent stimulation, thus supporting the theory that stimulation of NO production is necessary for the induction of LTP in vivo in the CA1 region of the hippocampus (Doyle et al., 1996). In a study involving anxiety-like response associated with pair housing in mice, it was reported that inhibition of nNOS either by gene deletion or treatment with 3-Br-7-NI reduced open arm exploration in single-housed mice but in pair-housed mice open arm exploration had increased in the elevated plus maze, showing that nNOS inhibition plays a role in the ability to respond behaviourally to social stimuli (Workman et al., 2008).

On the other hand, NO levels are controlled by the nervous system as a regulator of the nitrogen energy system (Aban et al., 2018). NO is shown to be localized and expressed in the hypothalamus, hypophysis and gonads and can act on the hypothalamo-hypophyseal-gonadal axis to regulate the synthesis and release of GnRH and thus reproduction, as GnRH and NO-producing neurons occupy similar positions in the hypothalamus (Grossman et al., 1994; Herbison et al., 1996; Constantin et al., 2021). NO has also been reported to regulate spermatogenesis, sperm motility, sperm capacitation, fertilization, oogenesis (follicle development/folliculogenesis), gonadal hormones and steroidogenesis (Chun et al., 1995, Zackrisson et al., 1996, Yamauchi et al., 1997; Roselli et al., 1998; Ducsay and Myers, 2011; Guo et al., 2019; Dutta and Sengupta, 2022). For example, in the ovarian follicle stage, NO levels are upregulated by estrogen, progesterone downregulates NO secretion while estradiol (E_2) can protect blood vessels via NO (Kong et al., 2014). Further, it is also reported to regulate reproduction in mammals by activating the release of LHRH/GnRH in the brain including many other activities (McCann et al, 1998; Barnes et al., 2001; Bellefontaine et al., 2011). For example, NO aids in the rupture of follicles during ovulation and participates in the acrosome reaction of spermatozoa during capacitation (Revelli et al., 1999).

In view of the functional roles of nitric oxide in different aspects of mood disorders and reproductive regulation in mammals and the paucity of information during its inhibition in utero, i.e. disrupting the nNOS prenatally during pregnancy, an attempt was made to study the modulation of anxiety and depression-like behaviour and reproductive physiology of mice by prenatally disrupting the synthesis of nNOS during the pregnancy by administering an intraperitoneal dose of specific nNOS inhibitor, 7-Nitroindazole to the pregnant mice from embryonic day 11 to 17. Male and female mice born to these pregnant females were raised to adulthood for studying its effect on anxiety and depression-like behaviour, changes in the development of hypothalamic nuclei and the reproductive physiology of the animals.

Materials and Methods

Animals

Adult Male and female mice were time mated and pregnancy was confirmed by checking the vaginal plug. 5 pregnant female mice were administered with 7-Nitroindazole from embryonic day 11 to 17 while 5 pregnant mice were treated with a solution of normal saline and dimethyl sulfoxide (1:1), which was used as a control. The pups born were weaned at day postnatal day 21 and male and female mice were separated, then raised to adulthood i.e., 8 weeks old. Light dark cycle 12L: 12D was maintained and food and water were available ad libitum. The experimental design for the present study is as depicted in **Figure 1**. All the experiments were performed as per the Animal Ethics Committee of Rajiv Gandhi University and as per the guidelines within the framework of the revised Animals (Scientific Procedures) Act 2002 (CPCSEA Guidelines) of the Government of India.

Anxiety and Depression-like Behaviour Tests

Animals born to 7-NI treated and Vehicle-treated (DMSO: Normal Saline – 1:1) pregnant females were raised to adulthood (8-Weeks) and then subjected to a battery of behaviour tests such as elevated plus maze test (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT) as per the protocol described in General Material and Methods. All the behaviour tests in the female were carried out in the diestrous phase of the estrous cycle. All the behaviour tests for anxiety and depression-like behaviour tests were performed in the light phase. The interval between each test for each animal was maintained at 4-7 days.

Perfusion and Tissue Collection

After the behavior tests, all the animals were anaesthetized, blood was collected in EDTA-coated tubes to separate plasma and then animals were sacrificed by perfusion as per the protocol described in General Material and Methods and the required tissues were collected for histology and biochemical estimation.

Histology of Gonads (Testes and Ovaries)

6μm thick sections of the gonads were cut on a rotary microtome and processed for Hematoxylin and Eosin staining as per the protocol described in General Material and Methods.

Cresyl Violet Staining of Brain Sections

6μm thick sections of the brain (both males and females) were cut on a rotary microtome and processed for crystal violet staining as per the protocol described in General Material and Methods

Biochemical Estimation

Total nitrate-nitrite estimation was performed in 5% homogenates of gonads (Testes and Ovary) and plasma as per the protocol of Sastry et al. (2002) and as described in General Material and Methods.

Hormone Assay (ELISA)

ELISA for testosterone in males and estradiol in females was performed as per the protocol provided in the kit (Diametra) and as described in General Material and Methods.

Result

The gestation period in mice is in general 19 days. The pups were born to the 7-NI treated and vehicle-treated pregnant females on the 19^{th} day and the litter size was normal (2-4 pups per pregnant mice) in both groups. Each mouse was weighed individually per week and no significant difference was observed in male and female mice (data not shown) born to the 7-NI treated (referred to as Experimental males and Females) and vehicle-treated pregnant females (referred to as Control Males and Females).

Elevated Plus Maze

Elevated plus maze test was applied for observing anxiety-like behaviour in both male and female mice. No sex difference was observed in the time spent in the open arm of the EPM, however, time spent in the closed arm and the centre was significantly different in both male and female mice ($p<0.05$) born to the pregnant female treated with the nNOS inhibitor, 7-NI compared to the male and female mice born to the pregnant female administered with vehicle control (**Figure 2A**).

Open Field Test

In the open field test also, no sex difference was observed between males and females for the time spent in the outer area. However, male and female mice born to 7-NI-treated pregnant females showed a significantly higher time spent in the outer area ($p<0.05$) and lower time in the middle area and the centre ($p<0.05$) when compared to male and female mice born to the pregnant females administered with vehicle control (**Figure 2B**).

Forced Swim Test

A significant sex difference was observed in both the control and experimental group for the time spent in swimming and floating in the FST. Further, a significant decrease ($p<0.05$) in the amount of time in swimming activity in both male and female mice born to 7-NI treated pregnant females was observed when compared to male and female mice born to the pregnant females administered with vehicle control. Also, a significant difference in the amount of time spent swimming and floating between the groups was observed. Females, in general, show less time floating and more time swimming than the males ($p<0.05$) in the water tank irrespective of the mice born to pregnant females treated with vehicle control or 7-NI (**Figure 3A**).

Marble Burying Test

No sex difference in the number of marbles buried by the male and female mice was observed. However, male and female mice born to the pregnant females

treated with 7-NI showed less number of marbles being buried when compared to the male and female mice born to the pregnant females treated with vehicle control ($p<0.05$), thus signifying that more digging and burying activity was observed in the control group than the experimental group (**Figure 3B**).

Effect on Hypothalamic Regions

Crystal violet staining of the brain sections showed that the number of neurons in the male appeared to be more compact in the Preoptic area of the brain than in the female mice. Though the number of neurons has not been counted, it appears that animals (both male and female) born to 7-NI treated pregnant females had a reduced number of neurons when compared to their respective controls (**Figure 4**). Similarly, it appears that there is a difference in the distribution of neurons in the SCN and PVN also, with control groups showing greater distribution and compactness of neurons than the animals born to 7-NI treated pregnant females (**Figure 5** and **Figure 6**). In paraventricular nucleus (PVN), the neurons appear to be densely packed and more uniformly distributed in the mice born to the vehicle-control treated pregnant females (the control group) than in the mice born to the 7-Ni treated pregnant females (the experimental group) (**Figure 6**). The number and distribution of neurons in the arcuate nucleus appear to be more in females than in males. The neurons appear to be scattered in the arcuate nucleus in the animals born to 7-NI treated pregnant females than in the animals born to vehicular treated pregnant females (**Figure 7**).

Effect on Gonad Physiology

Male Gonad (Testes) and Testosterone

The transverse section of the testis showed that the normal intact arrangement of seminiferous tubules has been disturbed in the 7-NI treated mice with loosely arranged seminiferous tubules and less number of spermatozoa in lumen. Vacuoles have been formed between the spermatogonial cells; also, the number and distribution of Leydig cells in the interstitium have reduced drastically (**Figure 8** and **Figure 9**). Further, histological examination of the testes in males

showed a normal process of spermatogenesis along with normal sperm count (**Figure 13A**) and plasma testosterone levels (**Figure 13B**) in animals born to vehicular treated pregnant females while in animals born to 7-NI treated pregnant females, sperm count, and plasma testosterone levels were low ($p<0.05$). Further, the process of spermatogenesis appears to be inhibited as vacuolation and loosening of the seminiferous tubules are observed with few numbers of spermatozoa in the lumen and Leydig Cells appear to be very few in number.

Female Gonad (Ovary) and Estradiol

Histological examination of ovaries showed many atretic follicles in the female animals born to 7-NI-treated pregnant females with inhibition of the process of oogenesis (**Figure 8** and **Figure 11**). However, in the females born to vehicular treated animals, the follicular development of the ovary was normal with a number of follicles observed in the primordial follicle stage, primary, secondary and antral follicle stage. Graafian follicle stage was also observed in the ovary of these animals suggesting that the animals were ovulating and performing regular ovulatory cycles (**Figure 8** and **Figure 10**). This is justified by the observation of high plasma estradiol content in these animals compared to the female animals born to 7-NI-treated pregnant females ($p<0.05$) (**Figure 13C**).

Total Nitrate-Nitrite Concentration in Plasma and Gonads (Testes and Ovary)

The level of total nitrate-nitrite concentration in both plasma (**Figure 12A** and **Figure 12B**) and tissue homogenate (i.e., testis and ovary) (**Figure 12C** and **Figure 12D**) does not show any sex differences, however, inhibition of neuronal nitric oxide synthase in utero significantly reduced the levels of nitric oxide production in both male and females born to 7-NI treated pregnant females when compared to both male and females born to vehicular treated pregnant females. This suggests that nitric oxide may influence the levels of steroids in mice.

Discussion

There was a severe alteration in the Anxiety and depression-like behaviour and hypothalamo-hypophyseal-gonadal axis in adult male and female mice born to 7-Nitroindazole (designated as experimental male and experimental female) and vehicle Control (designated as control male and control female) administered for seven days to the pregnant female mice from embryonic day 11 to embryonic day 17. In mice, the embryonic day 11 to embryonic day 17 is the critical period of development i.e., the period when the critical hypothalamic regions are being formed (Stratton and Tobet, 2020) and altering the neuronal and gonadal structure by the administration of 7-NI in-utero has a profound effect on the development of the hypothalamus and gonads. These differences could be associated with differences in cognition, emotional control and neurological disorders between the two gender (Xin et al., 2019). Due to the combination of genetic and hormonal factors, differences begin early during development and anatomical, functional and biochemical variation and differences exist in every stage of life (Zaidi, 2010). The in-utero treatment of 7-NI has anxiolytic as well as depressive effects on both males and females in the adult. An elevated plus maze with two open and two closed arms has been commonly used as a means to establish the effects of both anxiolytic and anxiogenic agents (Lister, 1987). The mice born to 7-NI treated females (experimental male and female) show a significant decrease in the time spent in the open arm when measured against the control animals. A similar effect has been suggested in the study on nNOS knockout mice with a targeted disruption of the nNOS gene (Weitzdoerfer et. al, 2004). Another behaviour test suggestive of anxiety-like behavior in the rodents is the open field test that analyzes the locomotion, anxiety and stereotypical behavior such as grooming and rearing in rodents (Kraeuter et al., 2019). In the open field test apparatus, the rodents show a distaste for large and unknown spaces (Seibenhener et al., 2015). In this study, mice born to 7-NI treated pregnant female mice show less locomotory behaviour and tend to move close towards the corners and walls of the apparatus and also show less exploratory behaviour. They are significantly inclined to spending more time in the outer zone and do not move towards the centre when compared to the mice born to the control group of pregnant mice treated with

only the vehicle. Anxiety is often co-morbidly associated with depression (Tiller, 2013). To examine the depressive behaviour, mice were subjected to a forced swim test, a behavioural test utilizing the behaviour of an animal to escape a stressful situation, here being a cylindrical container filled with water and the inability to escape the situation leading to depression (Yankelevitch-Yahav et al., 2015). In the present study, the mice treated with 7-NI i.e. experimental male and females showed more period of immobility i.e, floating behaviour in which the animal does only the movement necessary for keeping its nose above water (Bogdanova et al., 2013). Within the experimental group of animals, the experimental males showed more periods of immobility i.e. floating behaviour when compared to females. The control male and female animals showed more swimming behaviour and a tendency to attempt to climb the walls of the container and also move horizontally across the test container. A sex difference was observed during the time of swimming and floating, signifying that anxiety and depression may be sex-dependent (Donner and Lowry, 2013; Borbélyová et al., 2017). An additional test used in the current study for inspecting anxiety and depression-like behaviour was the marble burying test which is an established test for depression and obsessive-compulsive-like behaviour (Brouwer et al., 2019; Lazic, 2015). At the end of the 30-minute test, the control male and female groups showed more digging behaviour and buried more number of marbles when compared to the marbles buried by experimental male and female groups. Thus, the alteration in anxiety and depression-like behaviour in mice may be attributed to the fact that inhibiting nNOS in-utero may alter the synthesis and secretion of neurotransmitters and hormones that influence the anxiety and depression-like symptoms (Hu et al., 2008; Alvik et al., 2013). Further, there were remarkable changes in the structural organization of the hypothalamic nuclei (POA, SCN, PVN and Arcuate Nuclei) in experimental males and females compared to their respective controls. Nitric oxide is closely associated with the hypothalamo - pituitary-adrenal (HPA) axis and is located (although not exclusively) within the paraventricular nucleus (PVN) (Nelson et al., 1997). The PVN, an important source of corticotrophin-releasing hormone stimulates the pituitary to release adrenocorticotrophic hormone (ACTH) which in turn stimulates the adrenal cortex to release cortisol and/or corticosterone (Owens et al., 1991). Further,

cortisol has been shown to exert its effect on the development of affective disorders, however, the central effects of CRH may in itself be crucial in anxiety and depression-like behaviour responses. (Heinrichs et al, 1997). The cell bodies, receptors and terminals of the CRH are well located throughout the brain and in the central nucleus of the amygdala (Van Pett et al., 2000), which plays an integral role in the autonomic and behavioural responses to fearful stimuli (LeDoux et al., 1988; Walker and Davis, 1997). Moreover, it is a known fact that the effects of NO inhibition on anxiety-like responses have been investigated, however, less focus has been on the changes in the brain areas associated with anxiety and depression. In addition, many of the studies do not consider the effect of in-utero inhibition of neuronal nitric oxide synthase on behaviour. Further, the CRH neurons also co-localize vasopressin, which mediates the effects of CRH. The CRH neurons project not only to the median eminence but also innervate other brain areas that regulate the adrenal innervation of the autonomic nervous system and affect mood disorders.

It is a well-known fact that the HPA axis mediates the stress response and the release of CRH from the hypothalamic PVN forms the basis of the HPA axis. Vasopressin is co-localized with CRH in the hypothalamic PVN which potentiates the effects of CRH. The innervations of CRH neurons are well established in the different brain regions, however, evidence also indicates its innervation in the median eminence that regulates the adrenal innervation of the autonomic nervous system and thus affects mood behaviour. The stress response is also influenced by the hypothalamo-neurohypophyseal system that releases vasopressin from the PVN and supraoptic nucleus (SON) and oxytocin (OT) from the PVN via the neurohypophysis into the bloodstream. The biological/circadian clock, the suprachiasmatic nucleus (SCN) is responsible for the rhythmic changes in the stress axis. Signs and symptoms of depression are influenced by the central release of CRH as well as increased levels of cortisol. It has been reported that intracerebroventricular injection of CRH induces symptoms of depression in experimental animals. Further, Cushing's disease as well as glucocorticoid treatment also leads to depression. The vasopressin neurons (PVN and supraoptic nuclei - SON) are also activated in the brain during depression and thus contribute to the increased release of

adrenocorticotrophic hormone (ACTH) from the anterior pituitary (Gomez et al., 1997; Keller-Wood, 2015; Bao and Swaab, 2018). This is justified in the present study as there are observational changes in the hypothalamic PVN and SCN nuclei in the male and female animals born to the 7-NI-treated pregnant females when compared to the male and female animals born to the vehicle-treated pregnant females. An increase in circulating vasopressin level is also responsible for the risk of suicide and it has been reported that the incidence, morbidity and prevalence risk of depression is more in females than in males. This may be due to the fluctuations in the level of sex hormone levels which is considered one of the major reasons for its etiology (Parker and Brotchie, 2010; Labaka et al., 2018; Serpytis et al., 2018). In the present study also, we find that the steroid levels in experimental males (plasma testosterone) and females (plasma estradiol) are low when compared to the control males and females. Nuclear estrogen receptor alpha (ERα) is colocalized with approximately 40% of the activated CRH neurons in the PVN during mood disorders. Further, it has been reported that estrogen stimulates the production of CRH and estrogen-responsive elements have been localized in the CRH promoter region. CRH gene promoter region also has an androgen-responsive element that initiates the inhibitory effect on CRH expression. Moreover, in neuropsychiatric disorders such as depression, it is reported that there is a decreased activity in the SCN that results in the disturbances of circadian and circannual fluctuations in sleep, mood and hormonal rhythms. In stressed or corticosteroid-administered rodents and primates, neuronal loss in the hippocampus has been reported. Hippocampus has inhibitory control on the HPA axis and thus damage to this structure was expected to disinhibit the HPA axis. This caused an increase in glucocorticoid levels by a positive feedforward mechanism. This 'glucocorticoid cascade hypothesis' of stress and hippocampal damage was proposed to be causally involved in the age-related accumulation of hippocampal damage in disorders like Alzheimer's disease and depression (Bao and Swaab, 2018). Preoptic area is known to be involved in the mating behaviour in mammals, thus influencing the HPG axis (Gibson, 1986; Charlton, 2008; Alshamrani et al., 2022). This is substantiated in the present study by the fact that there are marked differences in the pre-optic area of the hypothalamus

in the experimental group when compared to the control. This is also apparent by the disruption in the development of the gonads (testes and ovaries) in the experimental group when compared to the control group. Further, the sperm count is significantly reduced in the experimental male group, which may be influenced by significantly low testosterone levels in the group and major disruption in the number of Leydig cells and seminiferous tubules. The compact arrangement of the seminiferous tubules is completely lost in the experimental group, thus affecting the process of spermatogenesis when compared to the control group. This downstream differences in the reproductive axis may be due to the alteration in the neuronal structure of the POA (Gibson and Silverman, 1989; Gerecke et al., 2012; Joglekar et al., 2022). The levels of the estradiol vary significantly in the experimental female group when compared to the control female group, thus signifying that inhibition of nitric oxide synthase has profound effect on the production of estradiol in mice, which may influence follicular development. This implies that the selection window for the follicular development in the experimental female mice groups appears to be inactivated, which may lead to uneven follicular development in the group, i.e., primordial follicle, primary follicle, some secondary follicle and mostly atretic follicles when compared to the respective female group which displays a normal development of follicles in the ovary i.e., primordial follicle, primary follicle, secondary follicle and Graafian follicle. This is further substantiated by the change in the structure of the suprachiasmatic nucleus which is involved in the circadian rhythm and inevitably in the reproductive cycles may have an influence on the HPG axis (Miller et al., 2004; Williams 3rd and Kriegsfeld, 2012; Gotlieb et al., 2018). SCN receives signals from the retina and is light sensitive and has been shown to influence HPA axis, also affecting mood disorders like anxiety and depression (Bao and Swaab, 2018). Likewise, the paraventricular nucleus, a major region containing CRH releasing neurons of the HPA axis show a sex difference in the cellular arrangement in both control and 7-NI group. It appears that males of both group have more cells and larger sizes than females. The arcuate nucleus, that has terminals of major secretory neurons regulating the anterior pituitary and its subsequent downstream releases is known to influence PVN, POA and even directly the release of GnRH (Pompolo et al., 2001; Rivalland et al., 2006; Smith et al., 2009; Bromek et al.,

2013; Smith, 2013; Guo et al 2018; Metz et al., 2021). These differences in the biochemical, behavioural and histological analysis could be due to the differences in nitric oxide, a major neuromodulator in the development (Contestabile, 2012; Basini and Grasselli, 2015; Wei et al., 2021).

The testicular and ovarian structures were also altered in the experimental males and females, respectively compared to their respective controls. The total nitrate and nitrite concentration, an indirect measurement of NO production, in plasma, testes and ovary were also low in experimental males and females when compared to their respective controls. It appears that NO may regulate the activity of the gonads at the hypothalamo-hypophyseal-gonadal level by regulating the synthesis and release of hypothalamic GnRH and associated neuropeptide molecules involved in the control of GnRH secretion such as kisspeptin, neurokinin B and Dynorphin as well as autocrine and paracrine level i.e. at the testicular and ovarian level. Surprisingly, NO production in terms of total nitrite-nitrate concentration is altered with the change in the morphological and cellular organization of the testes and ovary as well as plasma steroid levels (testosterone and estradiol). It is reported that an increased concentration of NO in the blood helps in vasodilation of the blood vessels and thus is helpful in penile erection in mammals (Burnett et al., 1992).

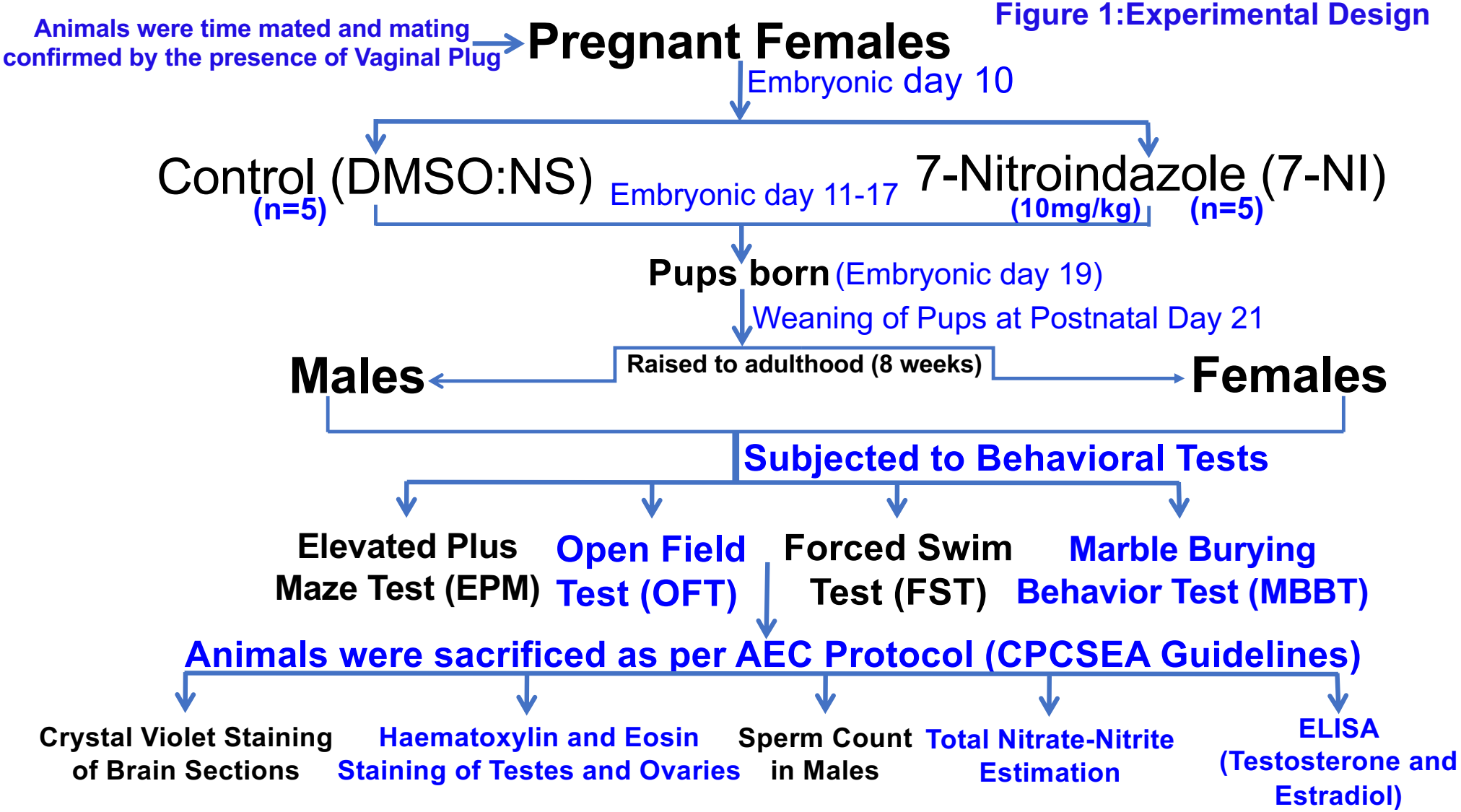
Further, it is an established fact that the neuroendocrine control of reproduction is under the control of the hypothalamic GnRH synthesis and release. The secretion of hypothalamic GnRH stimulates the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that supports the development of gonads, gametogenesis (spermatogenesis and oogenesis) and synthesis and release of sex steroids. This is under the influence of both external (light, temperature, humidity etc.) as well as intrinsic factors (neuropeptides, neurotransmitters, neuromodulators etc.) that regulate the HPG axis. However, the exact mechanism that activates the hypothalamic GnRH in response to extrinsic as well intrinsic factors is not yet elucidated. Possible candidates for the GnRH regulation in the hypothalamus have recently been suggested in the form of neuropeptides (Goodman et al., 2007; Merkley et al., 2012; Chehab, 2014; Grachev et al., 2014; Nakahara et al., 2014; Prashar et al., 2021; Xie et

al., 2022) such as kisspeptin, neurokinin B and dynorphin (KND Neurons). These KND neurons also have steroid-dependent and independent stimulation of the HPG axis (Pinilla et al., 2012; Moore et al, 2018; Uenoyama et al., 2021). Moreover, it has also been reported that NO controls the reproductive behaviour in rats which is influenced by the level of gonadal steroids and interactions of various neurotransmitters such as dopamine in males and noradrenaline in females (Hull et al. 1997, 1999, 2002; Chu and Etgen, 1997). Further, it has been shown that the gonadal steroid, testosterone, acts by increasing the nNOS immunoreactivity in male rats and the release of dopamine in the medial preoptic area is stimulated by NO (Lorrain and Hull, 1993; Du and Hull, 1999; Dominguez et al., 2004; Will et al., 2014; Sanna et al., 2017). It was also evident that castration in male hamsters lead to a decrease in the number of medial preoptic area neurons as visualized by nNOS immunoreactivity in the brain and NADPH-d activity (Hadeishi and Wood, 1996). In rats, naloxone blocked the inhibitory effect of β-endorphin on LHRH release and the activity of NOS while, in an vitro study, there was an increased NOS activity when medio-basal hypothalamus was incubated with β-endorphin. In this study itself, it was demonstrated that the activation and consequent synthesis of NOS in the mediobasal hypothalamus by the β-endorphin induced stimulation of μ-opioid receptors on NO neurons. β-endorphin also blocks the action of NO on PGE_2 release and consequently on LHRH release, by stimulating the GABAergic inhibitory input to LHRH terminals that blocks NO-induced activation of cycloxygenases (Cox) and subsequent PGE_2 release (Faletti et al., 1999). It is thus suggested that inhibition of NOS in the hypothalamic neurons and the gonads during embryonic development may lead to disruption in the production of NO, as evident in the present study by NO levels in the plasma and gonads. This may lead to inhibiting the receptors on the adjacent GnRH neurons to inhibit the synthesis and release of GnRH from the hypothalamus. Further, NO may also influence other nearby neural circuitry that may be responsible for the inhibitory cascade of the HPG axis.

Thus, it may be concluded that inhibition of neuronal nitric oxide synthase by administering a specific nNOS inhibitor, 7-Nitroindazole may alter hypothalamo-hypophyseal-adrenal and hypothalamo-hypophyseal-gonadal

axis by the change in the neuronal circuitry i.e. POA, SCN, PVN along with arcuate nucleus involved in the maintenance of both the axis. It appears from the present study that the neurons in these hypothalamic regions are interconnected to each other which help regulate the anxiety and depression-like behaviour as well as the production of gonadal hormones and nitric oxide, which may act in an either neuroendocrine, autocrine or paracrine manner. Thus, we propose that disrupting the neuronal nitric oxide synthase (nNOS) by specific nNOS inhibitor 7-Nitroindazole to pregnant mice, during the period of embryonic development has an anxiolytic and depressive effect on the pups born to the mother. The disruption during the embryonic stage has a long-term effect on the animals, evident from the significant change in the brain and gonad histology. The differences were also apparent in the behavioural pattern of the animals, showing more anxiety-like and depression-like behaviour. These results illustrate that the in-utero exposure of nNOS inhibitor had serious alterations in both HPA and HPG axis which prolongs until adulthood as well.

Figure 1:Experimental Design



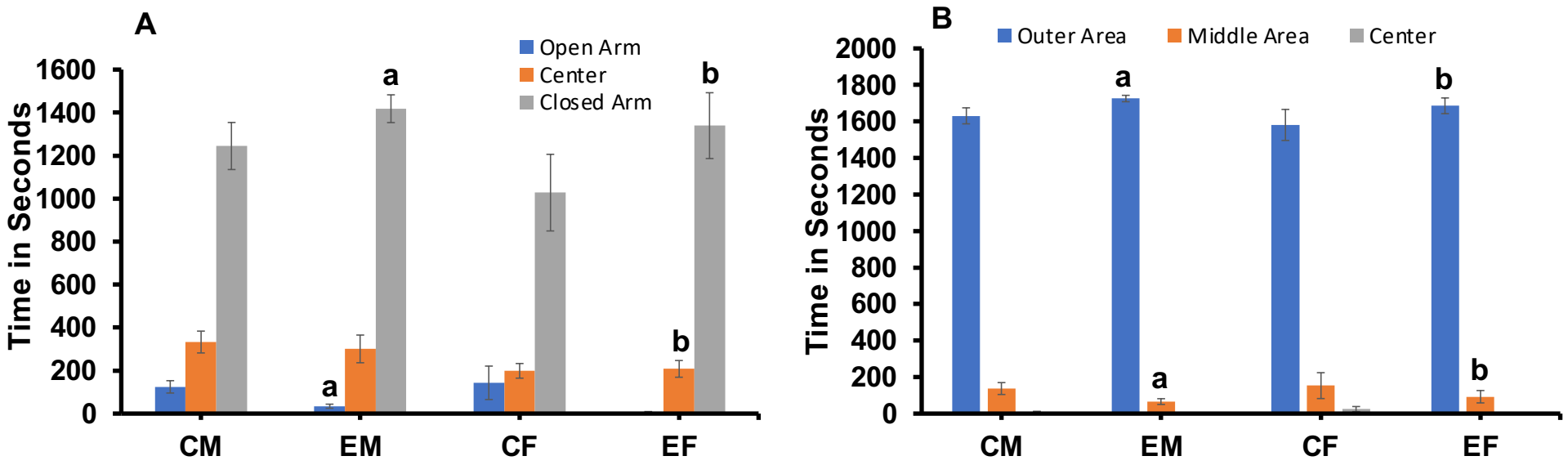


Figure 2: Histogram depicting changes in Anxiety and depression-like behavior in adult male and female mice born to pregnant female mice treated with 7-Nitroindazole (7-NI) [Designated as Experimental Male (EM) and Experimental Female (EF)] and vehicle control (DMSO: NS 1:1) [Designated as Control Male (CM) and Control Female (CF)] from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on anxiety and depression- like behavior. A. Elevated Plus Maze; B. Open Field Test. Means bearing superscript "a" and "b" differ from their respective controls ($p<0.05$).

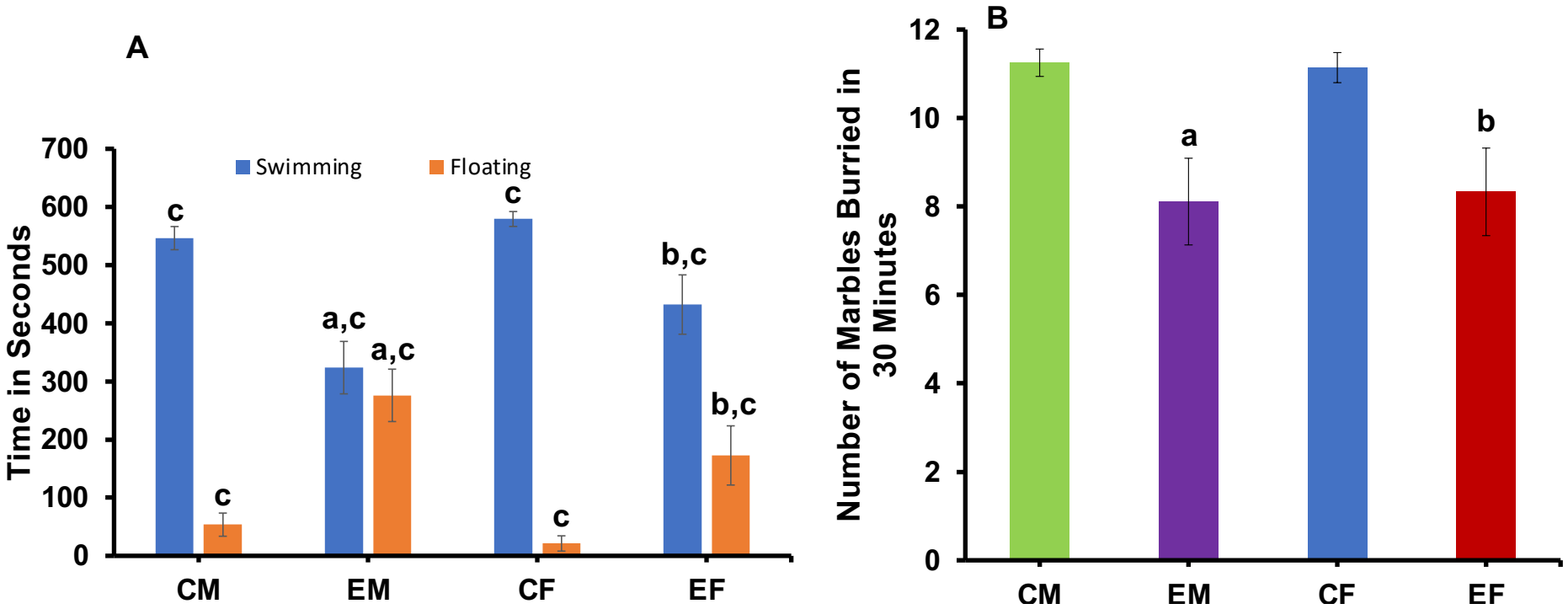


Figure 3: Histogram depicting changes in Anxiety and depression-like behavior in adult male and female mice born to pregnant female mice treated with 7-Nitroindazole (7-NI) [Designated as Experimental Male (EM) and Experimental Female (EF)] and vehicle control (DMSO: NS 1:1) [Designated as Control Male (CM) and Control Female (CF)] from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on anxiety and depression-like behavior. A. Forced Swim Test B. Marble Burying Behavior Test. Means bearing superscript "a" and "b" differ from their respective controls ($p<0.05$) while means bearing superscript "c" differ from each other ($p<0.05$).

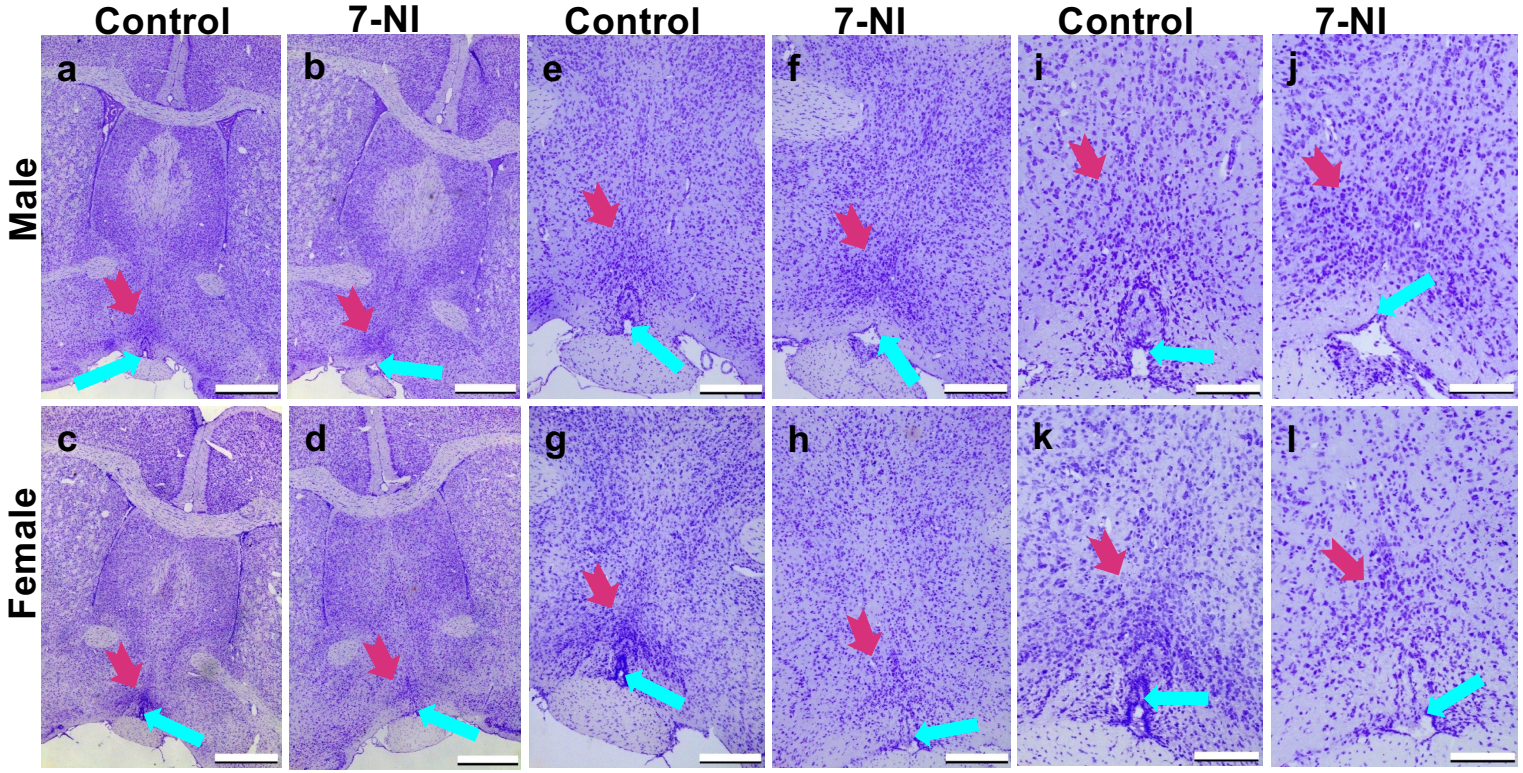


Figure 4: Representative images of transverse sections of adult male and female mice brain (Preoptic Area – POA) born to pregnant female mice treated with 7- Nitroindazole (7-NI) and vehicle control (DMSO: NS 1:1) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on changes in hypothalamic nuclei. Images a, b, c and d have a scale bar = 500μm, images e, f, g, and h have a scale bar = 200μm and images i, j, k and l have a scale bar = 100μm.

Key: → OVLT (Organum Vasculosum Lamina Terminalis), → Preoptic Area (POA)

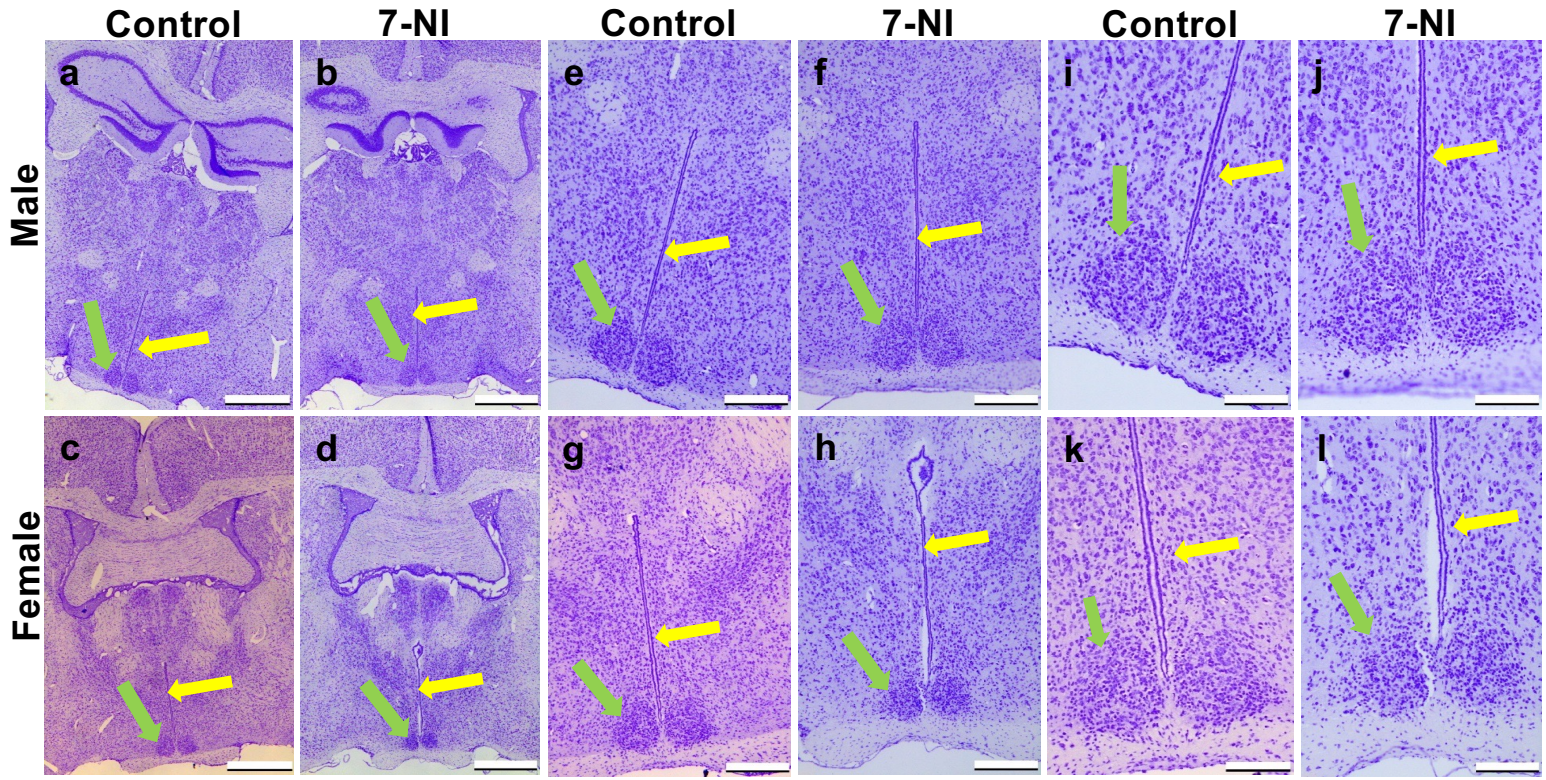


Figure 5: Representative images of transverse sections of adult male and female mice brain (Suprachiasmatic Nucleus - SCN) born to pregnant female mice treated with 7-Nitroindazole (7-NI) and vehicle control (DMSO: NS 1:1) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on changes in hypothalamic nuclei. Images a, b, c and d have a scale bar = 500μm, images e, f, g, and h have a scale bar = 200μm and images i, j, k and l have a scale bar = 100μm.

Key: Third Ventricle, Suprachiasmatic Nucleus (SCN)

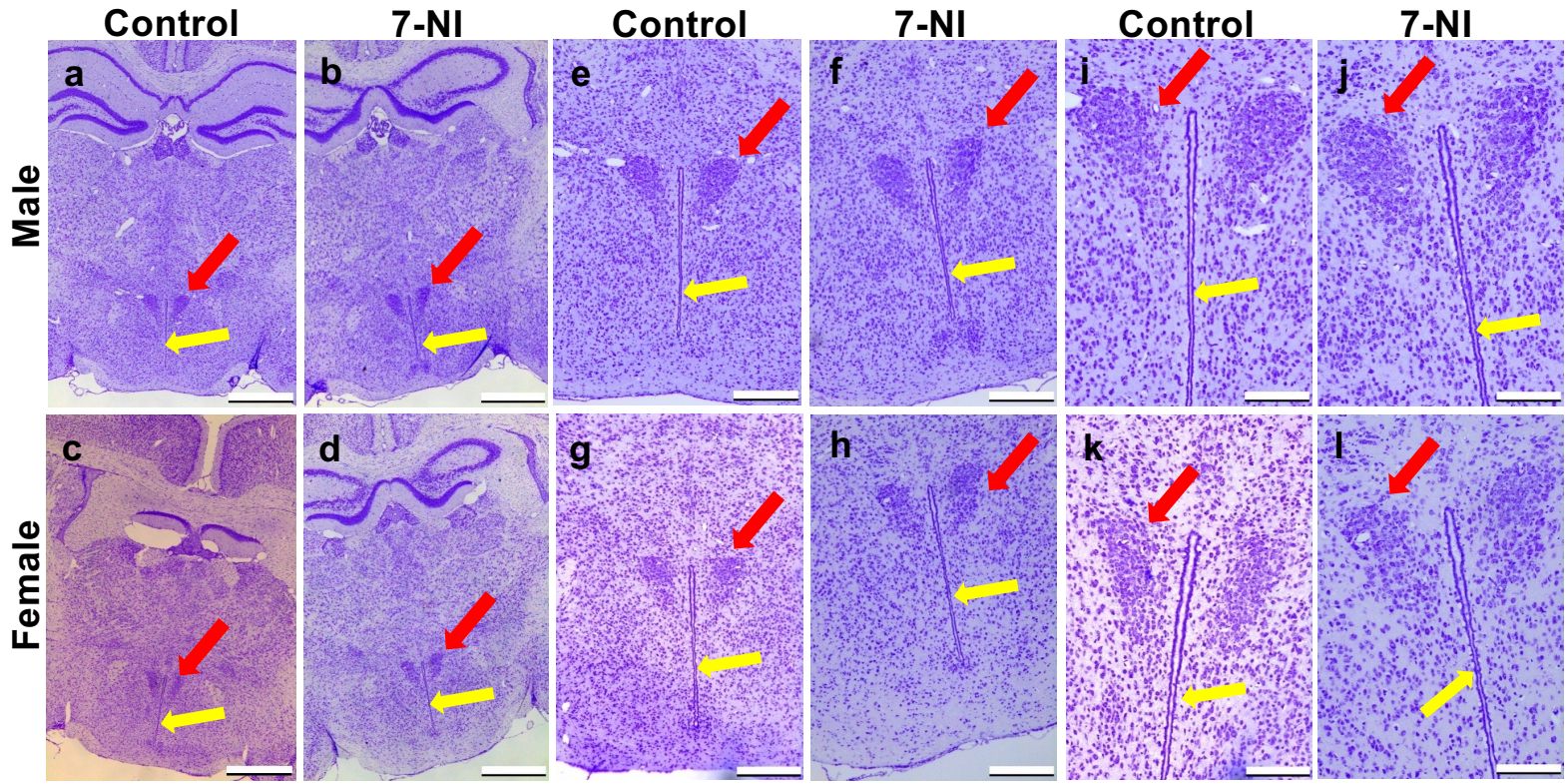


Figure 6: Representative images of transverse sections of adult male and female mice brain (Paraventricular Nucleus - PVN) born to pregnant female mice treated with 7-Nitroindazole (7-NI) and vehicle control (DMSO: NS 1:1) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on changes in hypothalamic nuclei. Images a, b, c and d have a scale bar = 500μm, images e, f, g, and h have a scale bar = 200μm and images i, j, k and l have a scale bar = 100μm.

Key: Third Ventricle, Paraventricular nucleus (PVN)

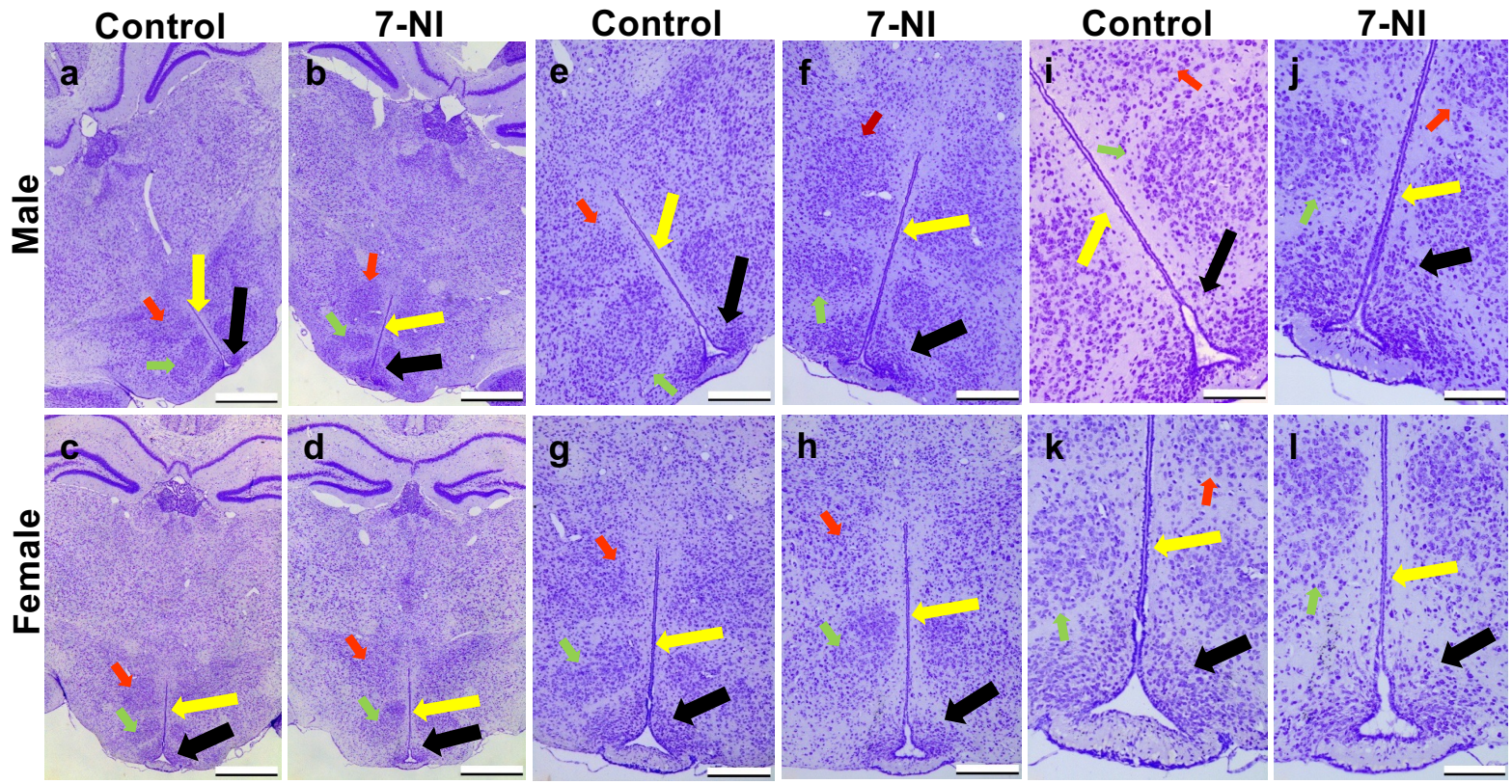


Figure 7: Representative images of transverse sections of adult male and female mice brain (Arcuate nucleus – Arc.; Dorsomedial Hypothalamic Nuclei – DMH; and Ventromedial Hypothalamic Nuclei – VMH) born to pregnant female mice treated with 7-Nitroindazole (7-NI) and vehicle control (DMSO: NS 1:1) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on changes in hypothalamic nuclei. Images a, b, c and d have a scale bar = 500μm, images e, f, g, and h have a scale bar = 200μm and images i, j, k and l have a scale bar = 100μm.

Key: Third Ventricle, Arcuate Nucleus (Arc.), Dorsomedial Hypothalamic Nucleus (DMH), Ventromedial Hypothalamic Nucleus (VMH)

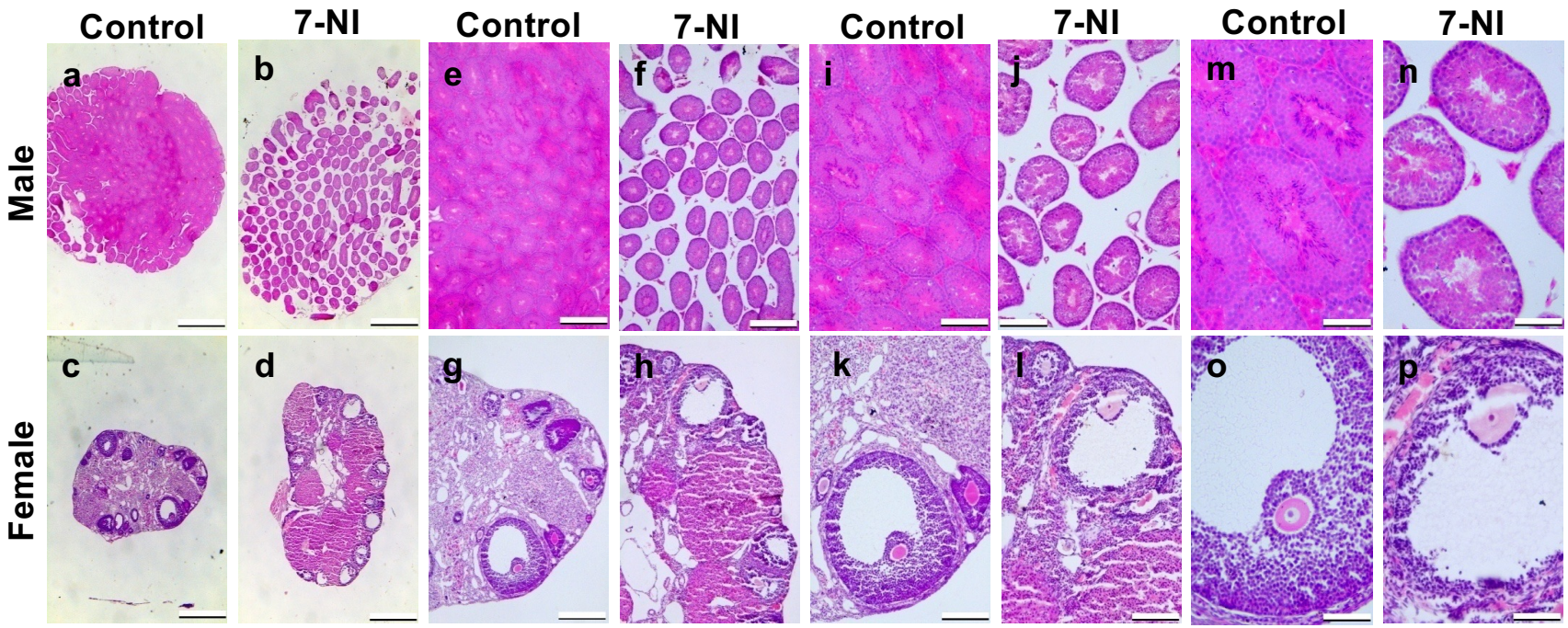


Figure 8: Representative images of transverse sections of adult male and female mice testes and ovaries, respectively born to pregnant female mice treated with 7-Nitroindazole (7-NI) and vehicle control (DMSO: NS 1:1) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on changes in gonadal (Testis and Ovary) physiology. Images a, b, c and d have a scale bar = 500μm, images e, f, g, and h have a scale bar = 200μm and images i, j, k and l have a scale bar = 100μm and images m, n, o and p have a scale bar =50μm.

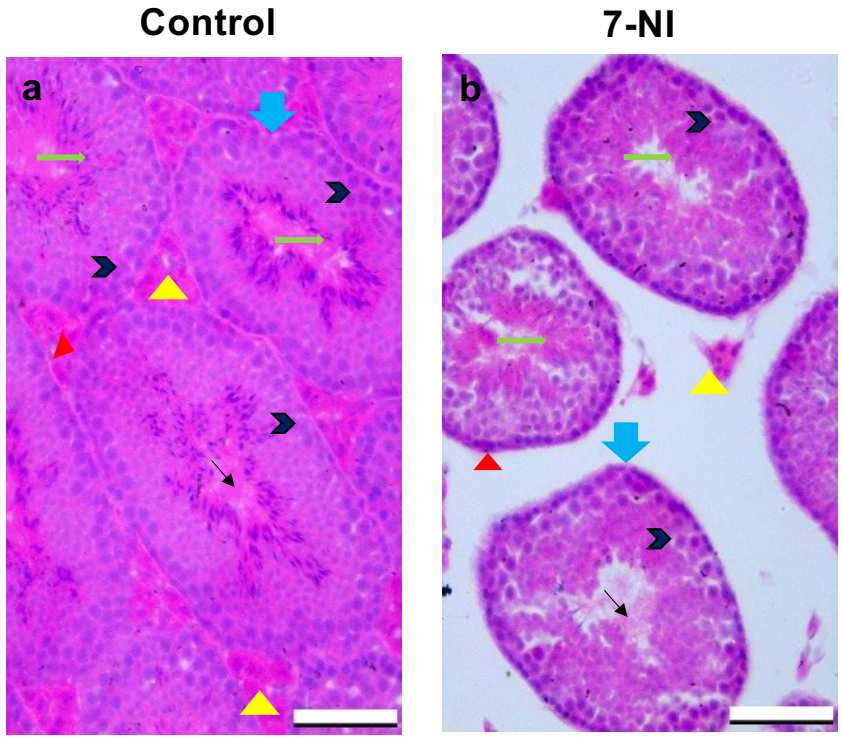


Figure 9: Representative images of transverse sections of adult male mice testes born to pregnant female mice treated with 7- Nitroindazole (7-NI) and vehicle control (DMSO: Normal Saline 1:1) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on changes in testicular structure. Scale bar = 50μm.

Key: Seminiferous Tubules, Spermatogonial Cells, Spermatids, Leydig Cells, Basement Membrane, Spermatozoa

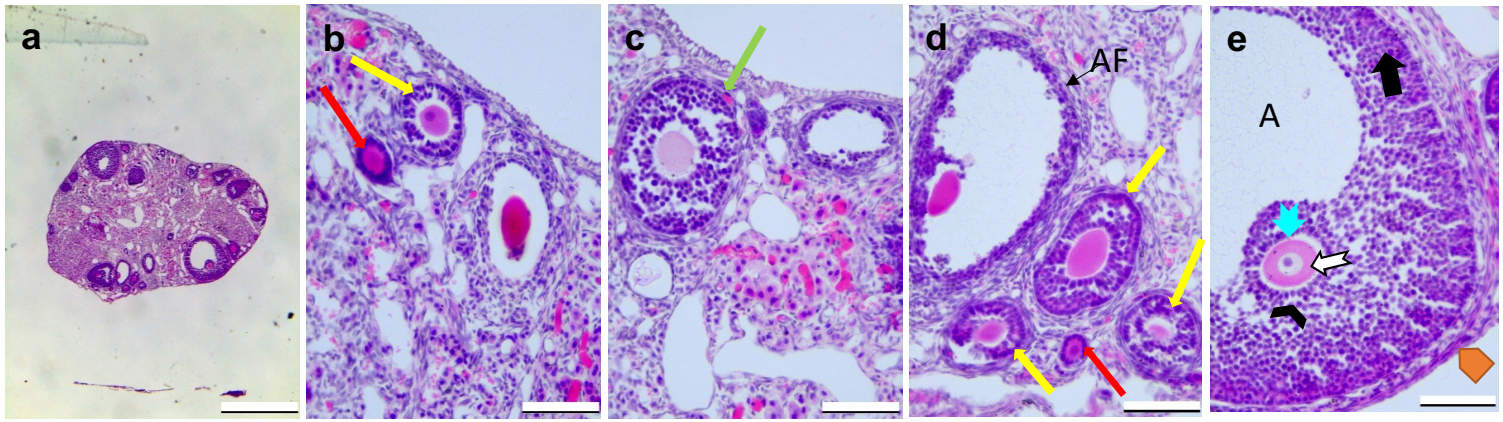


Figure 10: Representative images of transverse sections of adult female mice ovary, born to pregnant female mice treated with vehicle control (DMSO: Normal Saline 1:1) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on follicular development in ovaries. Scale bar = 50μm except image a, where scale bar = 500μm.

Key: **A,** Antrum, Primordial follicle, Primary follicle, Secondary follicle, **AF,** Atretic follicle, Graafian follicle, Granulosa cells, Zona pellucida, Ovum, Cumulus oophorus granulosa cells

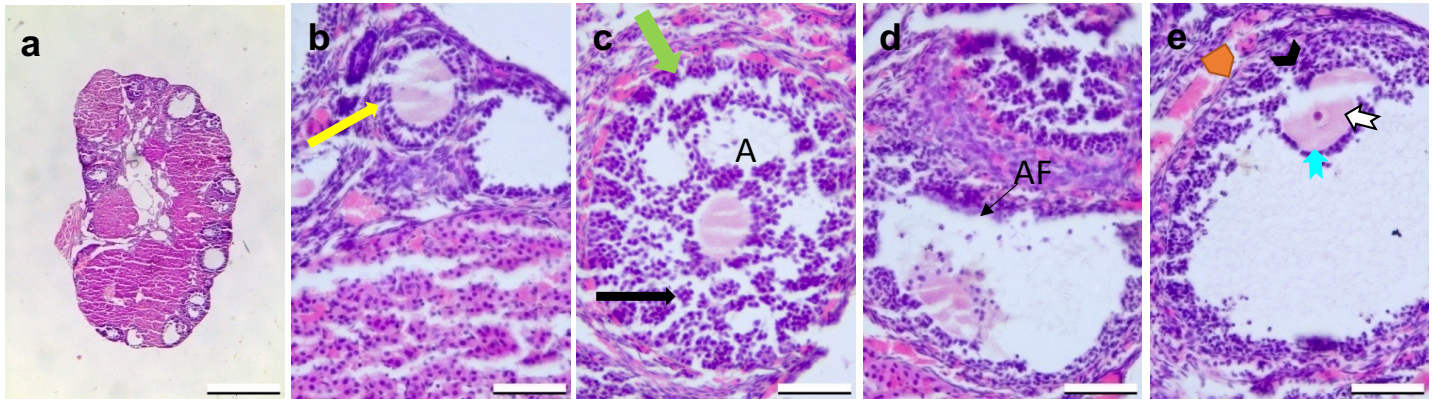


Figure 11: Representative images of transverse sections of adult female mice ovary born to pregnant female mice treated with 7-Nitroindazole (7-NI) (DMSO: Normal Saline 1:1) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on follicular development in ovaries. Scale bar = 50μm except image a, where scale bar = 500μm.

KEY: **A,** Antrum, Primary follicle, Secondary follicle, **AF,** Atretic follicle, Graafian follicle, Granulosa cells, Zona pellucida, Ovum, Cumulus oophorus granulosa cells

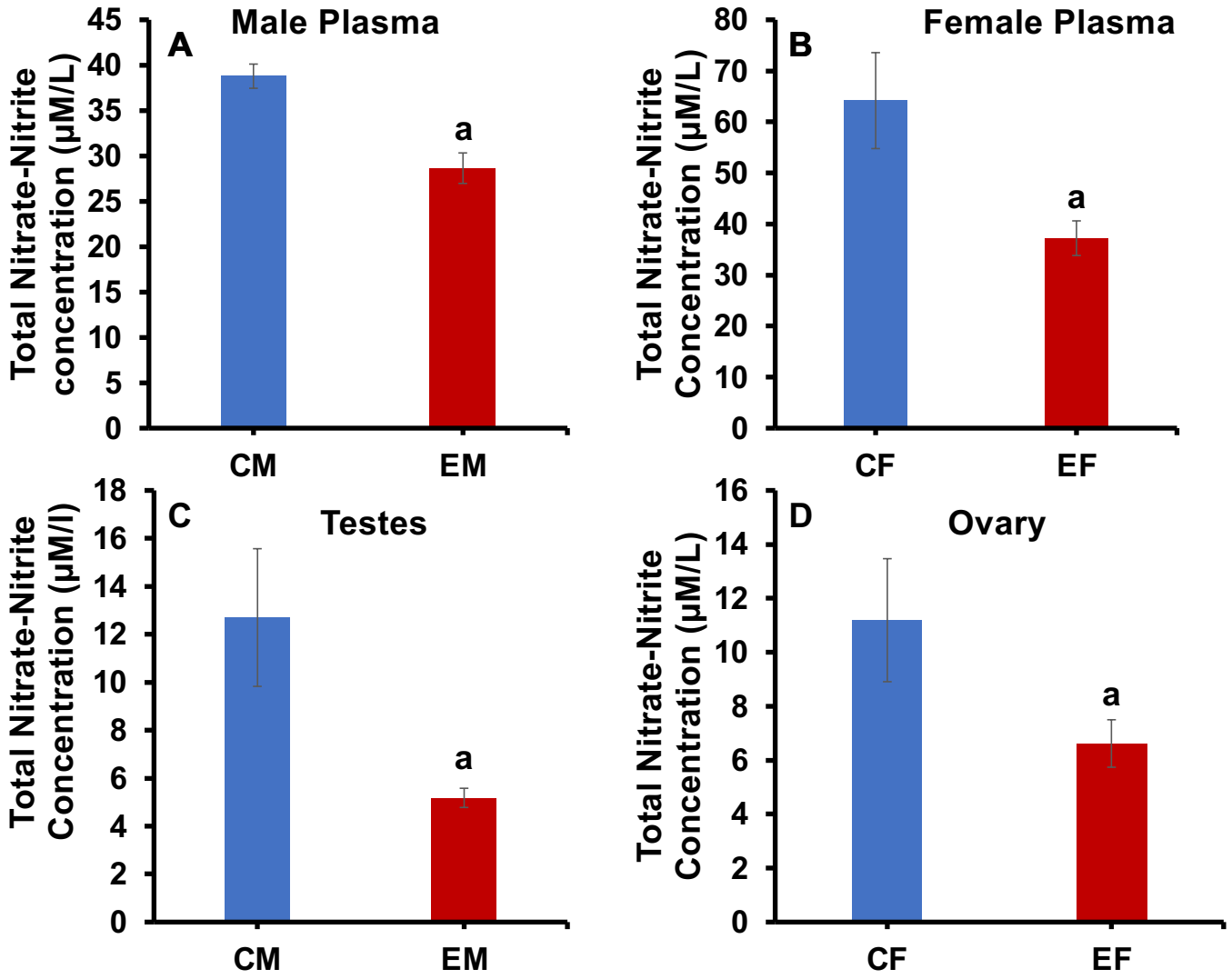


Figure 12: Histogram depicting changes in total nitrate-nitrite concentration in plasma of adult **(A)**- male and **(B)**- female mice and gonads (testes and Ovary) in adult **(C)**- male and **(D)**- female mice born to pregnant female mice treated with 7-Nitroindazole (7-NI) [Designated as Experimental Male (EM) and Experimental Female (EF)] and vehicle control (DMSO: NS 1:1) [Designated as Control Male (CM) and Control Female (CF)] from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on total nitrate-nitrite levels, an indirect method for measurement of NO production. Means bearing superscript "a" differ from their respective controls ($p<0.05$).

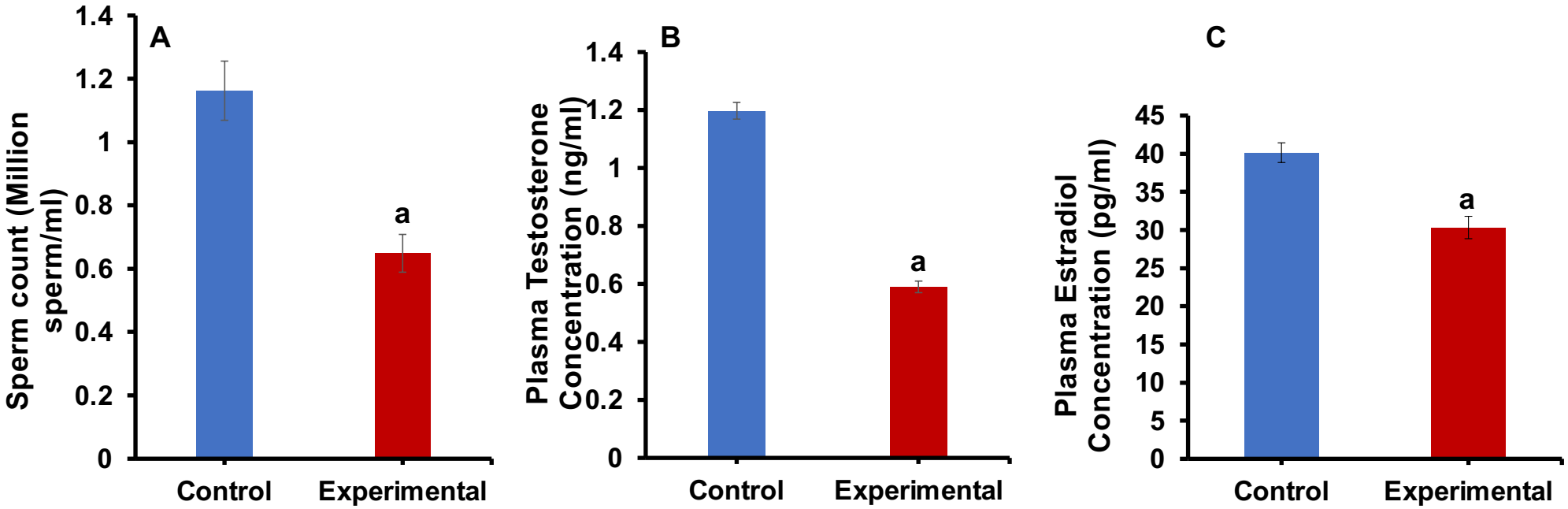


Figure 13: Histogram depicting changes in (A)- sperm count and (B)- plasma testosterone in adult male and (C)- plasma estradiol in adult female mice born to pregnant female mice treated with 7-Nitroindazole (7-NI) [Designated as Experimental Male (EM) and Experimental Female (EF)] and vehicle control (DMSO: NS 1:1) [Designated as Control Male (CM) and Control Female (CF)] from embryonic day 11 to embryonic day 17 (E-11 to E-17) and raised to adulthood (8 Weeks) for studying it's effect on concentration of sperm and production of steroid hormones. Means bearing superscript "a" differ from their respective controls ($p<0.05$).

Chapter 2

Nitric Oxide Modulates Anxiety- and Depression-like behaviour and Hypothalamo-Hypophyseal-Gonadal Axis in Mice

Abstract

L-arginine (L-Arg)/nitric oxide (NO)/cGMP pathway is known to be involved in various physiological processes, the behavioural response being one of the many. In the present study nitric oxide donor sodium nitroprusside (SNP) (0.1mg/kg body weight), nitric oxide inhibitors NG-nitro-l-arginine-methyl-ester (L-NAME) (5mg/kg body weight), and selective neuronal nitric oxide synthase inhibitor 7-nitroindazole (7-NI) (10mg/kg body weight) was administered to 8-week-old adult male and female mice along with their respective controls, normal saline for SNP and L-NAME treated animals while dimethyl sulphoxide:normal saline (DMSO:NS – 1:1) for 7-NI treated animals for 14 days. These male and female animals were then subjected to a battery of behavioural tests such as elevated plus maze (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT) to study the anxiety- and depression-like behaviour and changes in the hypothalamo-hypophyseal-gonadal (HPG) axis in these NO donor and NOS inhibitors administered animals. All the behavioural tests were performed in the light phase of the light-dark cycle and an interval of 4-7 days was maintained between each behavioural test. The behavioural tests in females were performed when they were in the diestrus stage of the estrous cycle. All the male and female animals were sacrificed as per approved animal ethics protocol following the completion of behavioural tests. The study shows that there are significant changes in the treatment groups, showing more anxiolytic and depression-inducing effects in the L-NAME and 7-NI treatment groups in both males and females. The sex difference was also observed in these behaviour tests. Crystal violet staining of the brain sections shows alteration in the distribution of neurons in the hypothalamic nuclei (preoptic area – POA, suprachiasmatic nucleus – SCN, paraventricular nucleus – PVN and arcuate nucleus) in the L-NAME and 7-NI treated male and female animals when

compared to the control group. L-NAME and 7-NI administration to male and female animals also had inhibitory effects on the morphological and cellular organization of the testes and the ovaries when compared to SNP-treated and control groups. The level of plasma testosterone, sperm count and plasma estradiol along with the total nitrate-nitrite concentration in plasma, testes and ovaries were significantly reduced in L-NAME and 7-NI treated male and female animals when compared to control groups. Thus, it may be concluded that inhibition of nitric oxide synthase by L-NAME and 7-NI and supplementing the animals with NO donor-SNP has a marked influence on the hypothalamo-hypophyseal-adrenal (HPA) axis as well as HPG axis in terms of alteration in anxiety and depression-like behaviour and gonad physiology. It appears that nitric oxide plays a major role in the interaction of the HPA and HPG axis for the maintenance of body homeostasis. However, the molecular mechanism and pathway need to be explored further.

Introduction

Maladaptive responses of the central nervous system lead to neuropsychiatric disorders that affect both cognition and behaviour (Petkovic and Chaudhury, 2022). Many million individuals suffered from mental disorders worldwide in 2017, of which depressive disorders account for 27% and anxiety disorders account for 29% of the total individuals (James et al., 2018). These mental disorders are accompanied by many other factors such as alteration in the perception of social reality and the ability to adapt in an individual (Ferreri et al., 2011; Milan et al., 2012; Weightman et al., 2014; Sole et al., 2017; Italia et al., 2020). However, there are no specific structural and molecular correlates that may be able to decipher and map these disorders (Nestler and Hyman, 2010) and one of the major issues with the treatment regimen of such disorders are relapse of the symptoms upon the withdrawal of the drug (Hyman, 2014). The cause of neuropsychiatric disorders such as major depressive disorder (MDD) may be diverse, but symptoms may remain more or less the same (Wallace et al., 2020). The rate of prevalence of these heterogenous disorders in today's population may be more than 50% of the total projected value (Toseeb et al., 2014). As per the report of WHO on depression and other common mental

disorders (2017), in India, the prevalence of depressive disorders is 4.5% while that of anxiety disorders is 3.0% of the total population. Depression is also reported to be a major contributor to suicide with the suicide rate more in males than in females with depressive disorders (WHO Global Health Estimates, 2017). Further, with the COVID-19 pandemic worldwide, there was an increase of 27.6% in anxiety and depression disorders (Santomauro et al., 2021). It is also reported that two-thirds of individuals committing suicide suffer from major depressive disorders (Van Dam et al., 2017). Further, it is predicted that the inheritance of depressive disorders is very high ~ 37% implying that there may be genetic factors involved that may drive stress vulnerability (Whiteford et al., 2013). However, the onset of neuropsychiatric disorders such as MDD requires a trigger from environmental factors. MDD is often accompanied by bidirectional disruptions (Alcantara et al., 2017). Thus, the heterogeneity of the disorder complicates the development of animal models for studying MDD such as anxiety and depression in adults. This is further complicated by the significant number of individuals' resistance to treatment for the disorder. Thus, no major breakthrough in the treatment of anxiety and depression disorders has been attained which may lead to the prevention and cure of the disorder (Czeh et al., 2016). Further, it is predicted that MDD exits as comorbid with other neuropsychiatric disorders is very high ~72.1% (Van Dam et al., 2017). This data assumes significance by the fact that depressive disorders and anxiety disorders are comorbid in themselves and lifetime comorbidity rates between these two disorders are 67.8% and 59.2%, respectively (Van Dam et al., 2017). Thus, studying the aetiology of anxiety and depression-like behaviour becomes very important in adults.

There have been various studies undertaken to understand the aetiology of mood disorders like anxiety and depression. One such approach has been to look into the role of nitric oxide in this behavioral disorder. Role of nitric oxide has been observed to be debatable, with some reports suggesting anxiogenic effect (Vale et al., 1998; Monzon et al., 2001), while others have reported it to be anxiolytic ((Volke et al., 1997; Dunn et al., 1998). In one study by Li and Quock (2002), administration of nitric oxide (NO) donor, 3-morpholinosyndnonimine (SIN-1) in mice show a significant increase in the amount of time spent in the light

compartment in the light/dark exploration test, implicating a role for NO in mediating anxiolytic effects. Studies on schizophrenia patients by treating them with NO donor sodium nitroprusside (SNP) improved the executive functions that are often impaired in patients with schizophrenia, suggesting an effect on cognitive function. Sodium nitroprusside (SNP), a NO donor, is a potent vasodilator (Bonaventura et al., 2007), when administered in ketamine-induced schizophrenia-like behavioural deficits in rats, has been shown to reverse the short-term recognition memory deficits (Trevlopoulou et al., 2015). SNP when administered in a range of low doses in repeated short-term has a dose-independent anti-anxiety behaviour in the rats (Papageorgoulis et al., 2020). In ethanol-induced anxious mice, intrahippocampal administration of SNP blocked the anxiolytic effect of ethanol (Ferreira et al., 1999). On investigating the effects of L-NAME, a non-selective inhibitor of NOS, 7-NI, a selective inhibitor of neuronal NOS and SNP, administered into the ventral hippocampus (VH) of rats and subjected to the elevated T-maze (ETM) test, a test to measure inhibitory avoidance and escape behaviour, show that L-NAME and 7-NI decreased inhibitory avoidance and prolonged escape latency in the ETM, suggesting an anxiolytic-like effect. On the other hand, SNP facilitated inhibitory avoidance without interfering with escape performance, suggesting an anxiogenic-like effect, indicating NO in the VH to be involved in the modulation of defensive behaviour of rats (Calixto et al., 2010). In anxiety-like and depression-like behaviour generated in mice by acute and chronic restraint stress, acute administration of L-NAME to inhibit NOS seems to prevent these acute and chronic stress-induced anxiogenesis and depression as observed in the elevated plus maze (EPM) test and forced swim test (FST) (Sevgi et al., 2006). Direct injection of L-NAME and 7-NI in the dorsal raphe nucleus (DRN) shows that low doses of both L-NAME and 7-NI caused anxiolytic-like effects while high doses of L-NAME and 7-NI show a decrease in locomotor activity in the EPM. Further, in FST low doses of L-NAME and 7-NI resulted in antidepressant-like effects, while high doses of L-NAME and 7-NI increased immobility time signifying disruption of NO-mediated neurotransmission in the DRN (Spiacci et al., 2008). In autism-like phenotype induced in male rats by intracerebroventricular infusion of propanoic acid, nordihydroguaretic acid (NDGA) show a neurotherapeutic property. Pre-treatment with L-NAME was

found to further potentiate the protective effect of NDGA but pretreatment with L-Arginine, a stimulant of nitric oxide was found to reverse NDGA's impact (Mehta et al., 2020). Inhibition of NOS by inhibitors like 1-(2-trifluoromethylphenyl) imidazole (TRIM) and 7-NI also demonstrate the anxiolytic effect in mice (Volke et al., 2003). 7-NI administration to the chronically ethanol intoxicated mice have a strong sedative effect, evident by a decrease in the number of entries into the open arm. Whereas administration of 7-NI to control mice significantly increased the percentage of entries made in the open arm and also the percentage of time spent in the open arm of the elevated maze (Pokk et al., 2001). Thus, alterations in anxiety and depression-like in animals activate the hypothalamo-hypophyseal-adrenal axis responsible for the stress axis. On the other hand, there are also reports that HPA and hypothalamo-hypophyseal-goandal axis well-coordinated (Bao et al., 2008). It is well known that stress has suppressive effects on the reproductive system by inhibiting the release of gonadotrophin-releasing hormone (GnRH) by corticotrophin-releasing hormone (CRH), which is responsible for a cascade of hormonal control of reproduction and sexual behaviour. Glucocorticoids, especially cortisol and corticosterone not only inhibit the release of GnRH but also inhibit LH-induced ovulation in females and sperm release in males (Calogero et al., 1999; Wade and Jones, 2004; Breen and Karsch, 2006; Bao et al., 2008; Whirledge and Cidlowski, 2010; Geraghty and Kaufer, 2015; Yip et al., 2021). Moreover, glucocorticoids are also known to directly inhibit the testes and ovary which in turns prevents the production of male and female sex hormones (Orr and Mann, 1992; Kalantaridou et al, 2004; Young and Altemus, 2004; Hardy et al., 2005; Zhang et al., 2019; Yang et al., 2019; Medar et al., 2021). It is also reported that CRH fibres from the paraventricular nuclei (PVN) innervate the portal capillaries as well as GnRH neurons in the infundibular nucleus which may help CRH in the control of reproductive functions (Dudas and Merchenthaler, 2003). On the other hand, the hypothalamic–pituitary–gonadal (HPG) axis also exerts extensive effects on the HPA axis. It is reported that gonadal sex hormones partially control the expression of CRH at the gene level. The nuclear protein estrogen receptor alpha (ERα) and androgen receptor (AR) is colocalized with CRH neurons in the PVN. Moreover, it has been

observed that in mood disorders, the level of CRH and ERα expression is upregulated in both males and females (Bao et al., 2005). It has also been reported that ovarian steroids influence the HPA axis by increasing its activity during psychological stress and thus activate the HPG axis to stress-induced inhibition in humans and rhesus monkeys (Kirschbaum et al., 1996; Roy et al., 1999). Thus, it is evident that well-coordinated events in the neuroendocrine system such as intrinsic and extrinsic factors regulate both anxiety and depression as well as reproduction in an organism. Among these factors, nitric oxide (NO), a ubiquitous short-lived messenger molecule, plays an important role in both anxiety and depression as well as control of reproduction (Tschugguel et al., 1998; Ji et al., 2007; Spiacci et al., 2008; Nikkar et al., 2019; Luo et al., 2021; Richards and Schonhoff, 2021) and as described in Chapter-1. Although there are reports of NO controlling anxiety and depression as well as reproduction in mammals, there is a paucity of information regarding the integration of HPA and HPG axis NO plays in coordinating the events during neuropsychiatric disorders and reproductive failure. Hence, in the present study, animals were administered with NO donor, SNP, universal NOS inhibitor, L-NAME and specific nNOS inhibitor – 7-NI on anxiety and depression-like behaviour and reproduction in 8-week-old adult male and female mice.

Materials and Methods

Animals

Adult Male and female mice (7 weeks old) were acclimatized in the animal house of the department for a week and then administered intraperitoneally with Nitric oxide donor – Sodium Nitroprusside (SNP), nitric oxide synthase inhibitor – L-NAME along with control (Normal Saline) and specific neuronal nitric oxide synthase inhibitor (nNOS) – 7-Nitroindazole (7-NI) dissolved in DMSO:Normal Saline (1:1) along with its vehicular control (DMSO:Normal Saline – 1:1) for 14 days. Light dark cycle 12L:12D was maintained and food and water were available ad libitum. The experimental design for the present study is depicted in **Figure 1**. All the experiments were performed as per the Animal Ethics Committee of Rajiv Gandhi University and as per the guidelines

within the framework of the revised Animals (Scientific Procedures) Act 2002 (CPCSEA Guidelines) of the Government of India.

Anxiety and Depression-like Behaviour Tests

All the animals (male and female mice) after the administration of the nitric oxide donor and NOS inhibitor, were subjected to a battery of behaviour tests such as elevated plus maze test (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT) as per the protocol described in General Material and Methods. All the behaviour tests in the female were carried out in the diestrous phase of the estrous cycle. All the behaviour tests for anxiety and depression-like behaviour tests were performed in the light phase. The interval between each test for each animal was maintained at 4-7 days.

Perfusion and Tissue Collection

After the behaviour tests, all the animals were anaesthetized, blood was collected in EDTA-coated tubes to separate plasma and then animals were sacrificed by perfusion as per the protocol described in General Material and Methods and the required tissues were collected for histology and biochemical estimation.

Histology of Gonads (Testes and Ovary)

6μm thick sections of the gonads were cut on a rotary microtome and processed for Hematoxylin and Eosin staining as per the protocol described in General Material and Methods.

Crystal Violet Staining of Brain Sections

6μm thick sections of the brain (both males and females) were cut on a rotary microtome and processed for crystal violet staining as per the protocol described in General Material and Methods.

Biochemical Estimation

Total nitrate-nitrite estimation was performed in 5% homogenates of gonads (Testes and Ovary) and plasma as per the protocol of Sastry et al., 2002 and as described in General Material and Methods.

Hormone Assay (ELISA)

ELISA for testosterone in males and estradiol in females was performed as per the protocol provided in the kit (Diametra) and as described in General Material and Methods.

Result

The administration of nitric oxide donor and NOS inhibitor along with their respective controls did not influence the body weight of the individual (data not shown) in either of the sex i.e., male, and female mice.

Behaviour Tests (EPM, OFT, FST and MBBT)

Sex differences in anxiety and depression-like behaviour were observed in almost all the behavioural tests. Further, L-NAME and 7-NI increased the time spent in the closed arm and open area in both the elevated plus maze (**Figure 2A** and **Figure 2B**) and open field test (**Figure 3A** and **Figure 3B**) while increased the time of floating and decreased the time of swimming in Forced swim test (**Figure 4A** and **Figure 4B**) signifying the high level of depression and low level of anxiousness by inhibition of nNOS, when compared to the control and SNP-treated animals. Sex differences in the number of marbles buried by both males (**Figure 5A**) and females (**Figure 5B**) were also significantly different in L-NAME and 7-NI treated animals when compared to control and SNP-treated animals.

Effect on the Hypothalamic Nucleus

Neuronal distribution in the POA region shows sex differences in all the groups, i.e., Control, SNP, L-NAME and 7-NI and a greater number in control than in

the treated groups. Among the treated groups, 7-NI treated animals showed a low number of cells in both males and females (**Figure 6** and **Figure 7**).

In the SCN, the compactness of neurons was lost along with loosely arranged neurons in both males and females in 7-NI treated groups while in the L-NAME treated group, the neurons in the SCN appeared to be scattered. However, a normal distribution pattern of neurons was observed in the SCN of both male and female control and SNP-treated groups (**Figure 6** and **Figure 7**).

The Y-Shaped structure of the neurons in the PVN is almost completely lost in the 7-NI and L-NAME treated groups while the compactness i.e. Y-shaped structure of the PVN neurons is maintained in the control and SNP-treated male and female mice (**Figure 6** and **Figure 7**).

Similar results were observed for the distribution of neurons in the Arcuate nucleus in the 7-NI and L-NAME treated animals than the control and SNP-treated male and female mice. The inhibition of nNOS leads to loosening and changes in the distribution of neurons (**Figure 6** and **Figure 7**).

Effect on Gonads

Male (Testes) and Testosterone Levels

Histological examination of testes reveals that the process of spermatogenesis along with production of male hormone, testosterone is maintained in the control and SNP-treated animals. However, 7-NI treatment to the mice drastically reduced the process of spermatogenesis along with the production of testosterone as observed by the levels of plasma testosterone levels when compared to their respective control group ($p<0.05$). Also, a significant loosening of seminiferous tubules was observed in the 7-NI treated males (**Figure 8** and **Figure 9**). The degree of loss of spermatogenesis was not as severe as that of 7-NI treated males, however, spermatogenesis and levels of testosterone (**Figure 15B**) were also reduced in L-NAME treated male mice compared to the control group ($p<0.05$). The sperm count was severely impaired in both L-NAME and 7-NI treated male animals compared to control and SNP-treated male mice ($p<0.05$) (**Figure 15A**).

Female (Ovaries) and Estradiol Levels

Ovaries of both the control groups (**Figure 10** and **Figure 13**) and SNP-treated (**Figure 11**) female mice show follicles in different follicular stages i.e., primordial, primary, secondary, antral follicles and Graafian follicle stage. Whereas the treatment groups have a lesser number of follicles in the secondary follicular stage and more number of follicles in the atretic stage (**Figure 12** and **Figure 14**). Plasma estradiol concentration is significantly low in the 7-NI and L-NAME treated females than in the control and SNP-treated male mice ($p<0.05$) (**Figure 15C**).

Total Nitrate-Nitrite Concentration in Plasma and Gonads (Testes and Ovaries)

Total nitrate-nitrite concentration is significantly low in the NOS inhibited groups (L-NAME and 7-NI) and more in the Nitric oxide donor group, SNP in the plasma (**Figure 16A** and **Figure 16B**) and gonads (**Figure 17A** and **Figure 17B**) of mice than their respective controls ($p<0.05$), thus suggesting that nitric oxide may be involved in the process of steroidogenesis.

Discussion

The present study demonstrates sex differences and alteration in HPA and HPG axis by the administration of NO donor – SNP, universal NOS inhibitor – L-NAME and specific nNOS inhibitor – 7-NI when compared to the control male and female adult mice. The preoptic area (POA) neurons responsible for GnRH secretion and thus control of reproduction (Clemens et al., 1976; Alexander and Leeman, 1992; Ciechanowska et al., 2007; Pereira et al., 2009; Glanowska and Moenter, 2014; Glanowska and Moenter, 2015; Uriarte et al., 2020) appear to be greatly reduced in number in the L-NAME and 7-NI treated male and female mice when compared to their control group. The arcuate nucleus, another site for the regulation of secretion of GnRH and further stimulation of the anterior pituitary for FSH and LH secretion (Plant et al., 1978; Lamperti, 1985; Pinilla et al., 2012; Skorupskaite et al., 2014; Melon and Maguire at al., 2016; Hu et al., 2019; Si et al., 2020), also show a reduction in the number of cells in the L-

NAME and 7-NI treatment groups when compared to the SNP-treated and control groups. Paraventricular nucleus , a site for corticotropin releasing hormone (CRH) release and secretion (Zhang et al., 2017; Yuan et al., 2019; Daviu et al., 2020), and suprachiasmatic nucleus responsible for maintaining circadian rhythm and consequentially influencing HPA axis as well as HPG axis (Swaab et al., 2005; Bailey and Silver, 2014; Gotlieb et al., 2018) show reduction in cell in the animals treated with L-NAME and 7-NI when compared to SNP-treated and control male and female mice demonstrating sex differences in the anxiety and depression-like behaviour in the adults. The alteration in anxiety and depression-like behaviour in L-NAME-treated adult male and female mice, besides the above reasons, may also be due to the fact that L-NAME is a universal NOS inhibitor. It may act on iNOS and/or eNOS to exert its effect. It has been reported that iNOS mutant mice exposed to stress by predator scent display a higher anxiety-like behaviour in EPM, increased acoustic startle responses and higher plasma corticosterone levels compared with their wild types. Further, when these iNOS mutant stress-exposed mice were systemically administered with a NOS inhibitor (L-NAME), the stress-related effects were completely reversed. Thus, these mice exhibit a behavioural phenotype with a characteristic susceptibility to stress. These findings are in agreement with previous reports showing a relationship between nitric oxide levels and neuropsychiatric disorders such as anxiety and depression (Abu-Ghanem et al., 2008).

On the other hand, the gonads are under the control of the hypothalamus, and it is reported that HPA and HPG axis are highly integrated (Kalantaridou et al., 2004; Bao et al., 2005; Swaab et al., 2005; Melon and Maguire, 2016; Oyola and Handa, 2017; Gotlieb et al., 2018; Ludwig et al., 2019). The PVN releases CRH in response to stress and this exerts an inhibitory signal on the GnRH secretion that exerts inhibition on gonad (testes and ovaries) physiology and sexual behaviour (Tsigos and Chrousos, 2002; Wade and Jones, 2004; Melon and Maguire, 2016; Phumsatitpong and Moenter, 2018; Padda et al., 2021; Yip et al., 2021). Moreover, the glucocorticoids (cortisol or corticosterone), also inhibit LH-induced ovulation in females and sperm release in males (Padmanabhan, et al., 1983; Rivier and Vale, 1984; Huang and Shirley, 2001;

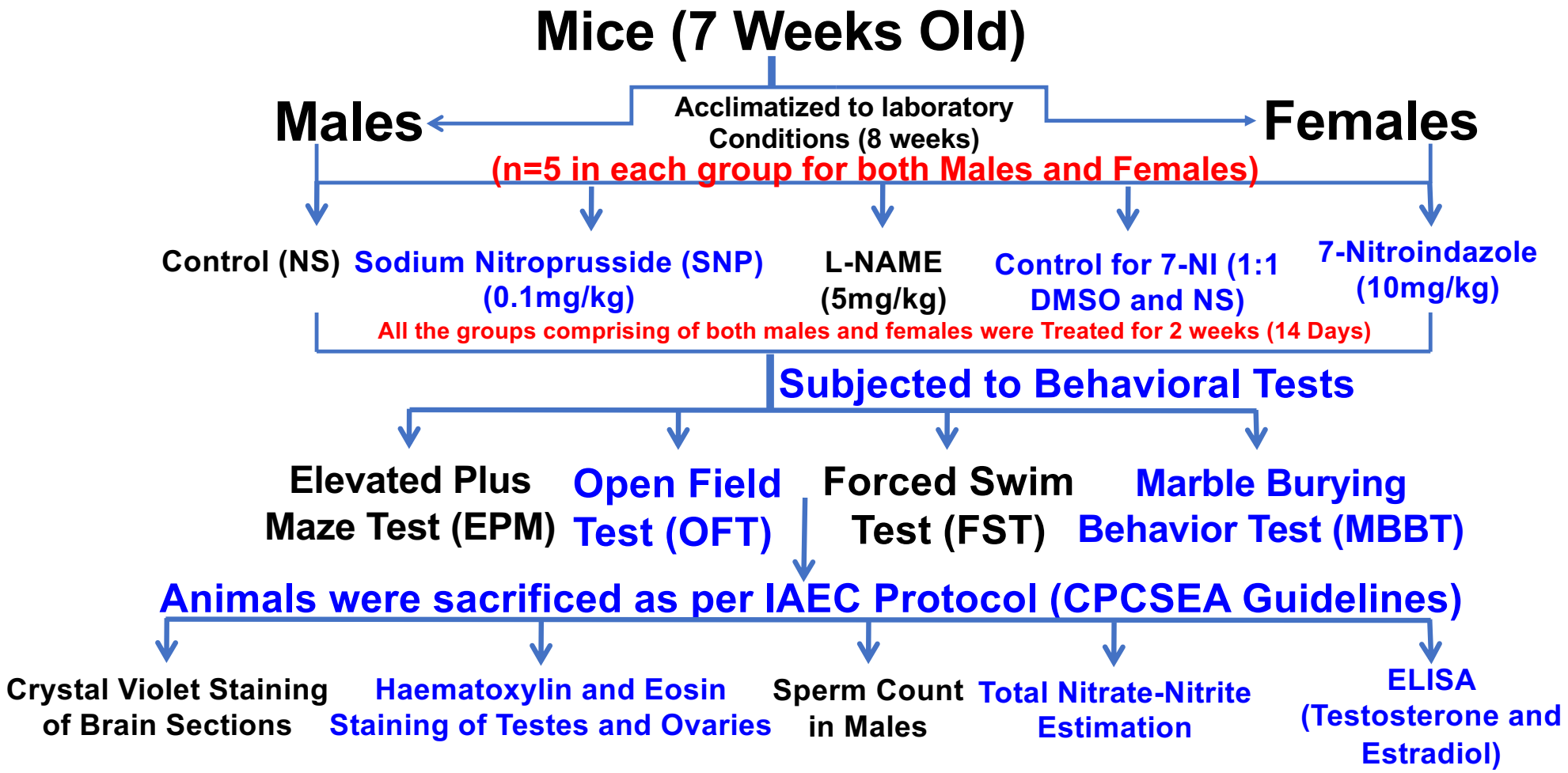
Bao et al., 2008). Moreover, gonads (testes and ovary) are directly inhibited by the secretion of glucocorticoids that further prevents the production of male and female sex hormones (Kalantaridou et al., 2004; Fanson and Parrott, 2015; Geraghty and Kaufer, 2015; Herman et al., 2016; Zhao et al., 2021). Further, it has also been demonstrated that the hypothalamo-hypophyseal portal system and the GnRH neurons in the infundibular nucleus in the brain are innervated by the CRH fibres from the PVN, which help CRH in the control of reproductive functions in male and females (Dudas and Merchenthaler, 2003). On the other hand, the hypothalamic–pituitary–gonadal (HPG) axis also exerts extensive effects on the HPA axis. It is reported that gonadal sex hormones partially control the expression of CRH at the gene level (Maeda et al., 1996; Heck and Handa, 2019). The nuclear protein estrogen receptor alpha (ERα) and androgen receptor (AR) is colocalized with CRH neurons in the PVN. Moreover, it has been observed that in mood disorders, the level of CRH and ERα expression is upregulated in both males and females (Bao et al., 2005). It has also been reported that ovarian steroids influence the HPA axis by increasing its activity during psychological stress and thus activate the HPG axis to stress-induced inhibition in humans and rhesus monkeys (Kirschbaum et al., 1996; Roy et al., 1999). Thus, it is evident that well-coordinated events in the neuroendocrine system such as intrinsic and extrinsic factors regulate both anxiety and depression as well as reproduction in an organism.

Our findings with inhibition of nitric oxide synthase either by administration of L-NAME or 7-NI support the results of most of the studies done on inhibition. Like in the study on stress induced by water deprivation in rats, L-NAME administration to water-deprived rats shows suppression in the arginine vasopressin (AVP) in the hypothalamus and also a reduction in corticosterone levels in the plasma (Mornagui et al., 2010). When nNOS gene was deleted and 3-Bromo-7-Nitroindazole was used for inhibition of neuronal nitric oxide production in pair housed mice that showed anxiety like behaviour previously, taking nNOS out of the picture had anxiolytic-like effect by increasing open arm exploration in EPM (Workman et al., 2008). Supporting these, our results indicate an anxiolytic effect in the nitric oxide synthase inhibited group (L-NAME and 7-NI). The present findings are also supported by the fact that NO

stimulates the release of CRH and thus, activates the secretion of corticosterone, which in turn influences the HPA and HPG axis to have its effect on anxiety and depression-like behaviour and reproduction i.e., changes in the gonad (testes and ovaries) structure and sex hormone production in adult male and female mice (Rivier, 1995; Shi et al., 1998; Marchetti et al., 2001; Dixit and Parvizi, 2001; Mohn et al., 2005; Riedel, 2009; Gądek-Michalska et al., 2013; Zhou et al., 2018; Delli et al., 2021).

Thus, it may be concluded that inhibition of nitric oxide synthase by L-NAME and 7-NI and supplementing the animals with NO donor-SNP has a marked influence on the hypothalamo-hypophyseal-adrenal axis as well as hypothalamo-hypophyseal-gonadal axis in terms of alteration in anxiety and depression-like behaviour and gonad physiology. It appears that nitric oxide plays a major role in the interaction of the HPA and HPG axis for the maintenance of body homeostasis. However, the molecular mechanism and pathway need to be explored further.

Figure 1:Experimental Design



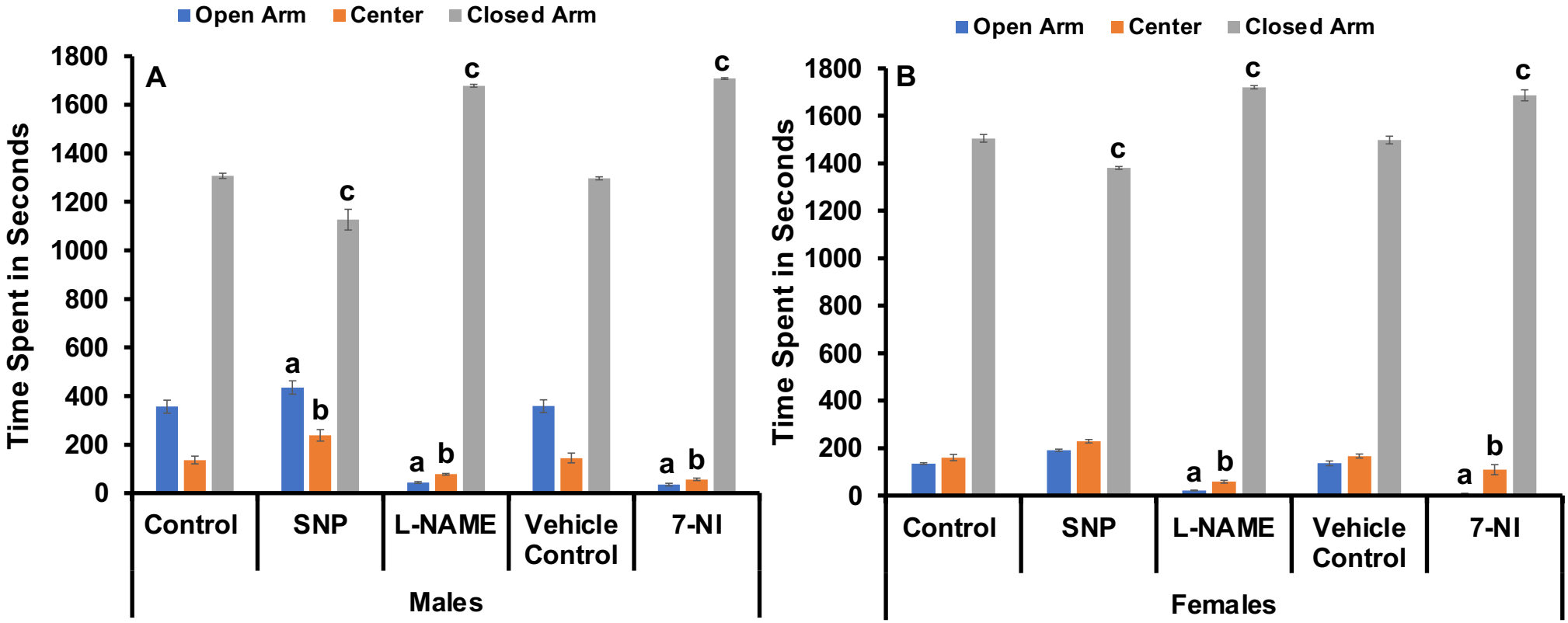


Figure 2: Histogram depicting time spent in seconds in open, centre and closed arms (Elevated Plus Maze Test – (A)-Males and (B)-Females) in adult 8-week-old male and female mice treated with Normal Saline (Control), SNP, L-NAME, Vehicle Control (DMSO:NS) and 7-Nitroindazole (7-NI) for 14 days and subjected to a battery of behavior test for studying its effect on anxiety and depression- like behavior. Means bearing superscript "a", "b" and "c" differ from their respective controls ($p<0.05$).

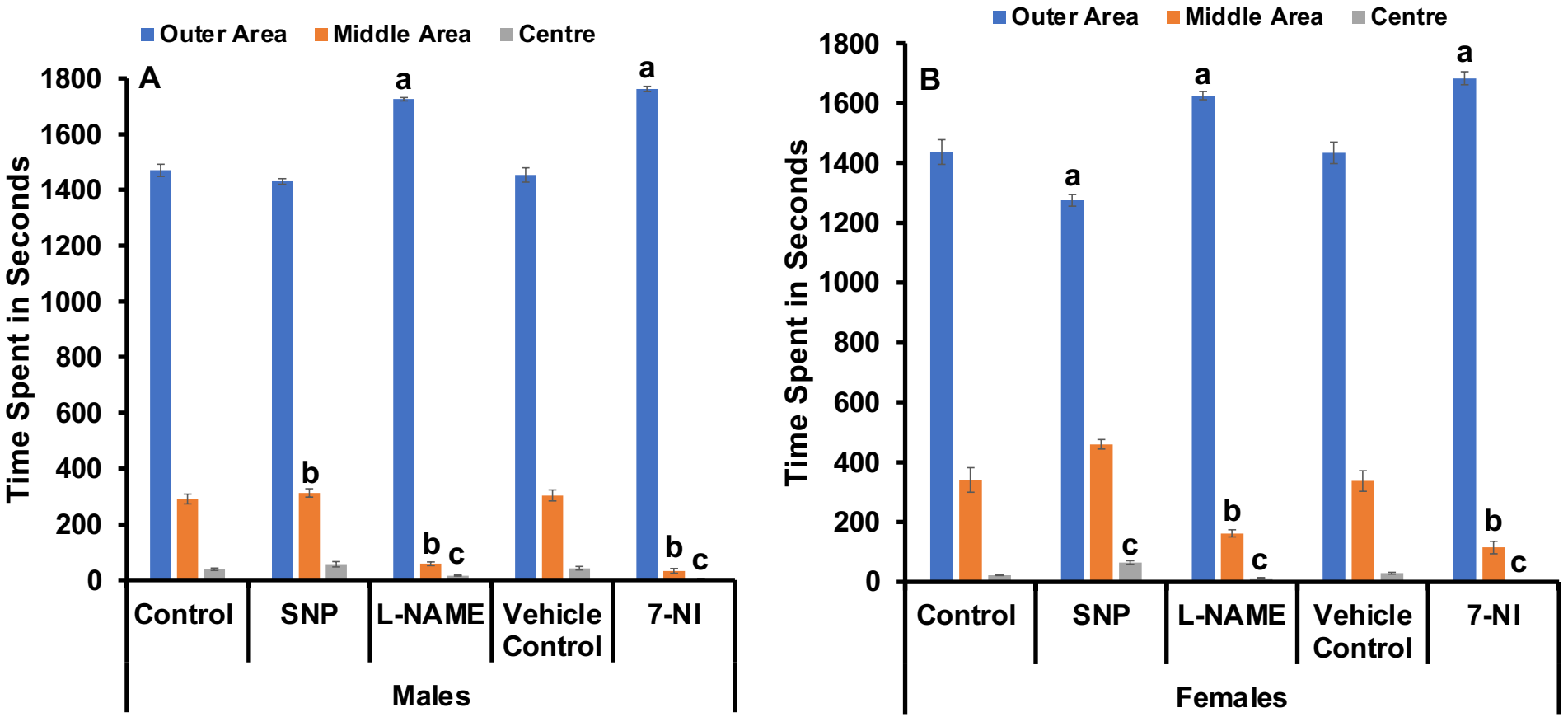


Figure 3: Histogram depicting time spent in seconds in the outer area, middle area and centre (Open Field Test – (A)-Males and (B)-Females) in adult 8-week-old male and female mice treated with Normal Saline (Control), SNP, L-NAME, Vehicle Control (DMSO:NS) and 7-Nitroindazole (7-NI) for 14 days and subjected to a battery of behavior test for studying its effect on anxiety and depression- like behavior. Means bearing superscript "a", "b" and "c" differ from their respective controls ($p<0.05$).

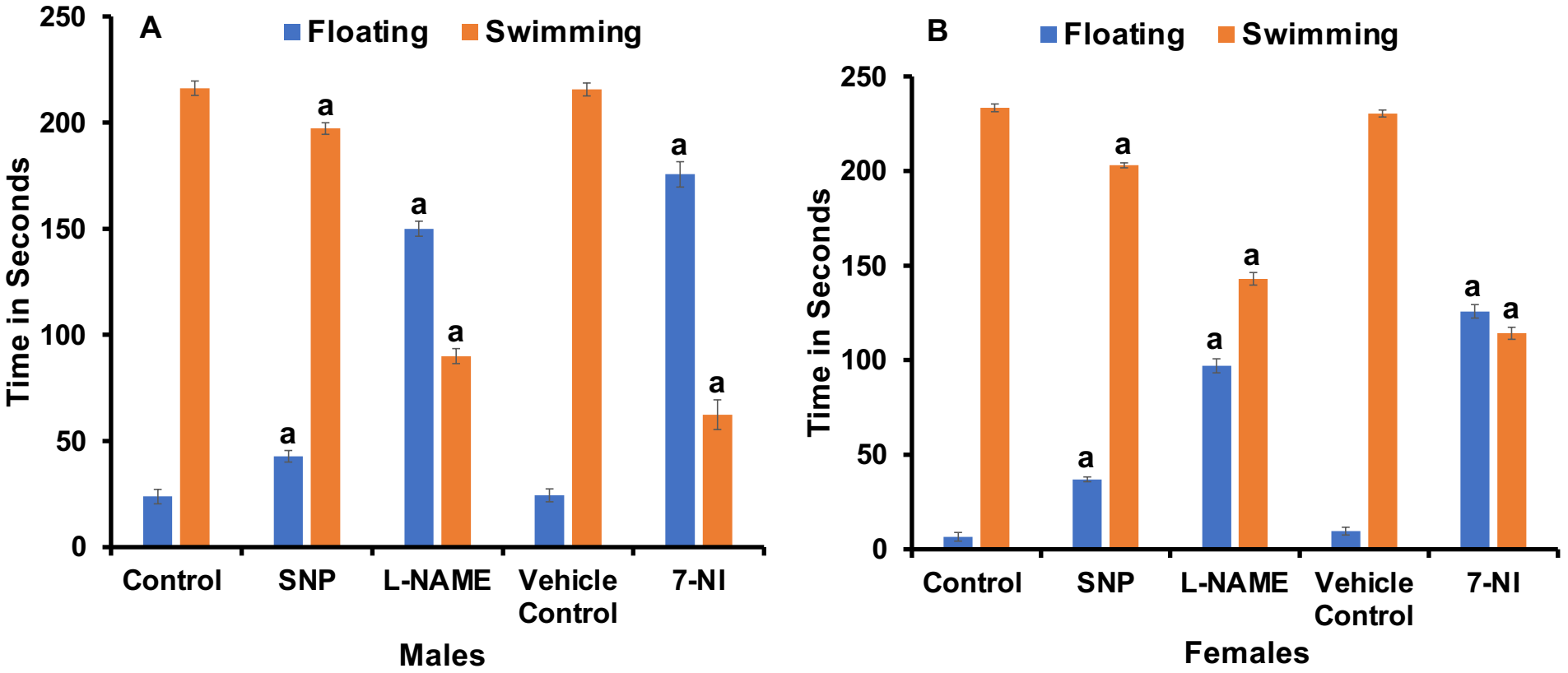


Figure 4: Histogram depicting time spent floating and swimming (Forced Swim Test- (A)-Males and (B)-Females) in adult 8-week old male and female mice treated with Normal Saline (Control), SNP, L-NAME, Vehicle Control (DMSO:NS) and 7-Nitroindazole (7-NI) for 14 days and subjected to a battery of behavior test for studying its effect on anxiety and depression- like behavior. Means bearing superscript "a" differ from their respective controls ($p<0.05$).

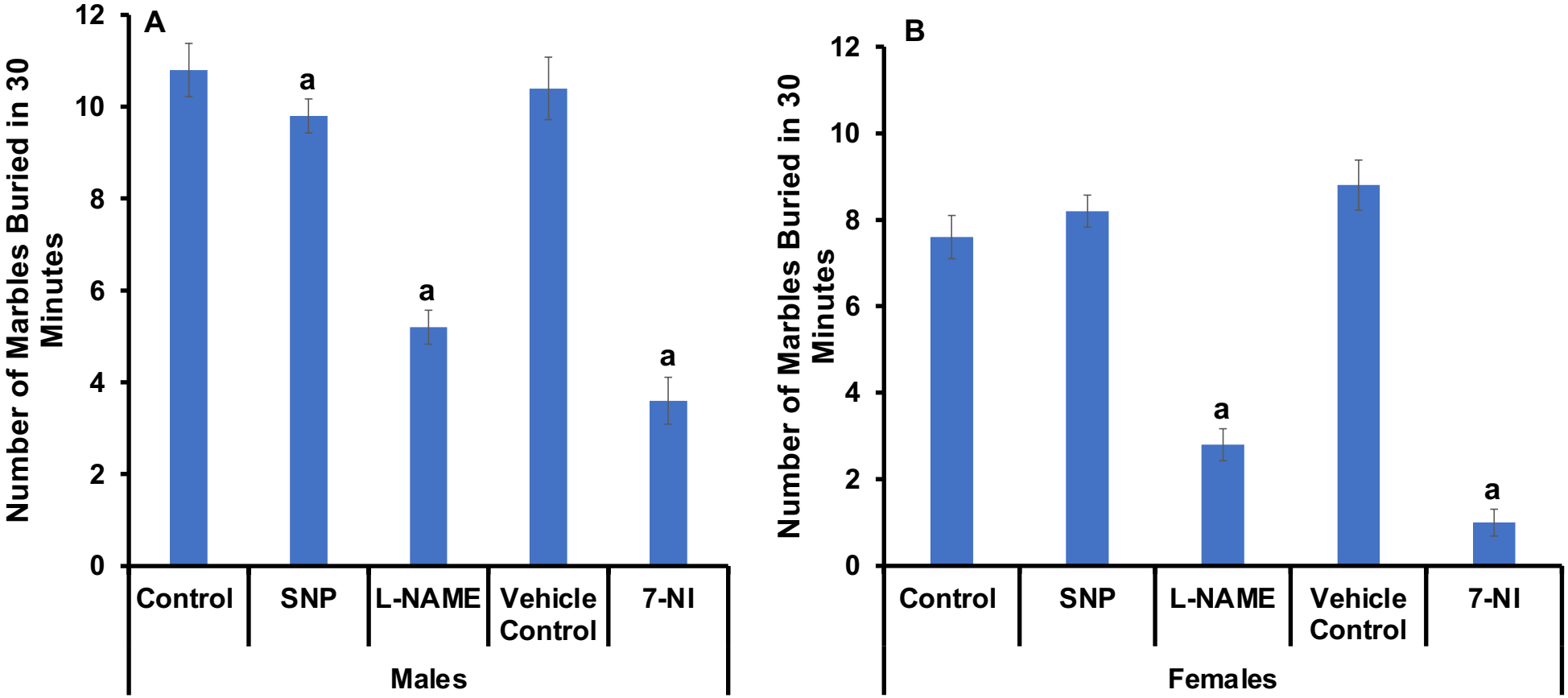


Figure 5: Histogram depicting number of marbles buried in 30 Minutes (Marble Burying Behavior Test– (A)- Males and (B)- Females) in adult 8-week old male and female mice treated with Normal Saline (Control), SNP, L-NAME, Vehicle Control (DMSO:NS) and 7-Nitroindazole (7-NI) for 14 days and subjected to a battery of behavior test for studying its effect on anxiety and depression- like behavior. Means bearing superscript "a" differ from their respective controls ($p<0.05$).

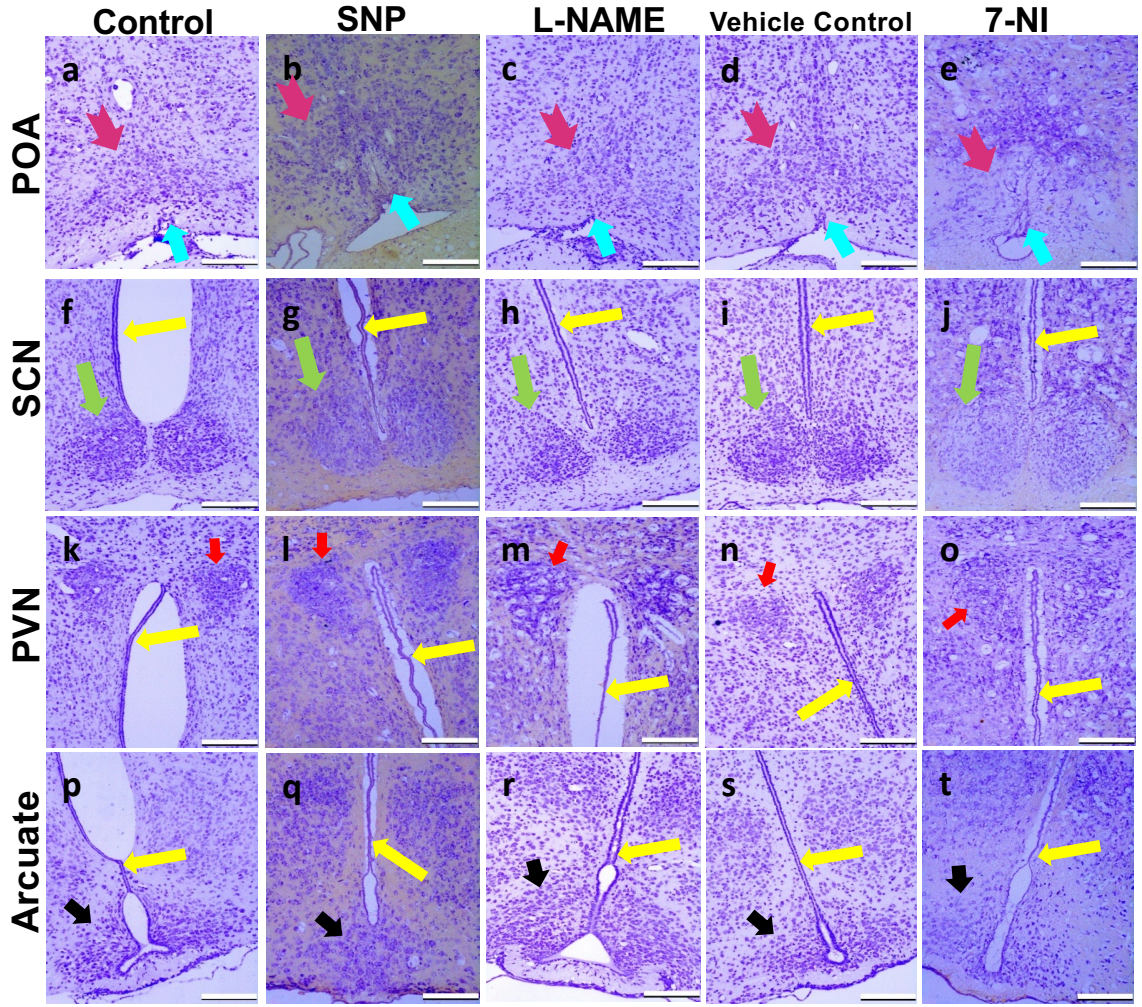


Figure 6: Representative images of transverse section of POA (images a, b, c, d and e), SCN (images f, g, h, I and j), PVN (images k, l, m, n and o) and Arcuate Nucleus (p, q, r, s and t) of the brain of adult male administered with Normal Saline (Control), NO Donor – SNP, NOS Inhibitor – L-NAME; Vehicle Control (DMSO:Normal Saline) and 7-Nitroindazole (7-NI) at the age of 8-weeks for 14 days for studying its effect on changes in hypothalamic nuclei. Scale bar = 100μm.

Key:

- OVLT (Organum Vasculosum Lamina Terminalis
- Preoptic Area (POA)
- Third ventricle
- Suprachiasmatic Nucleus (SCN)
- Paraventricular Nucleus (PVN)
- Arcuate Nucleus

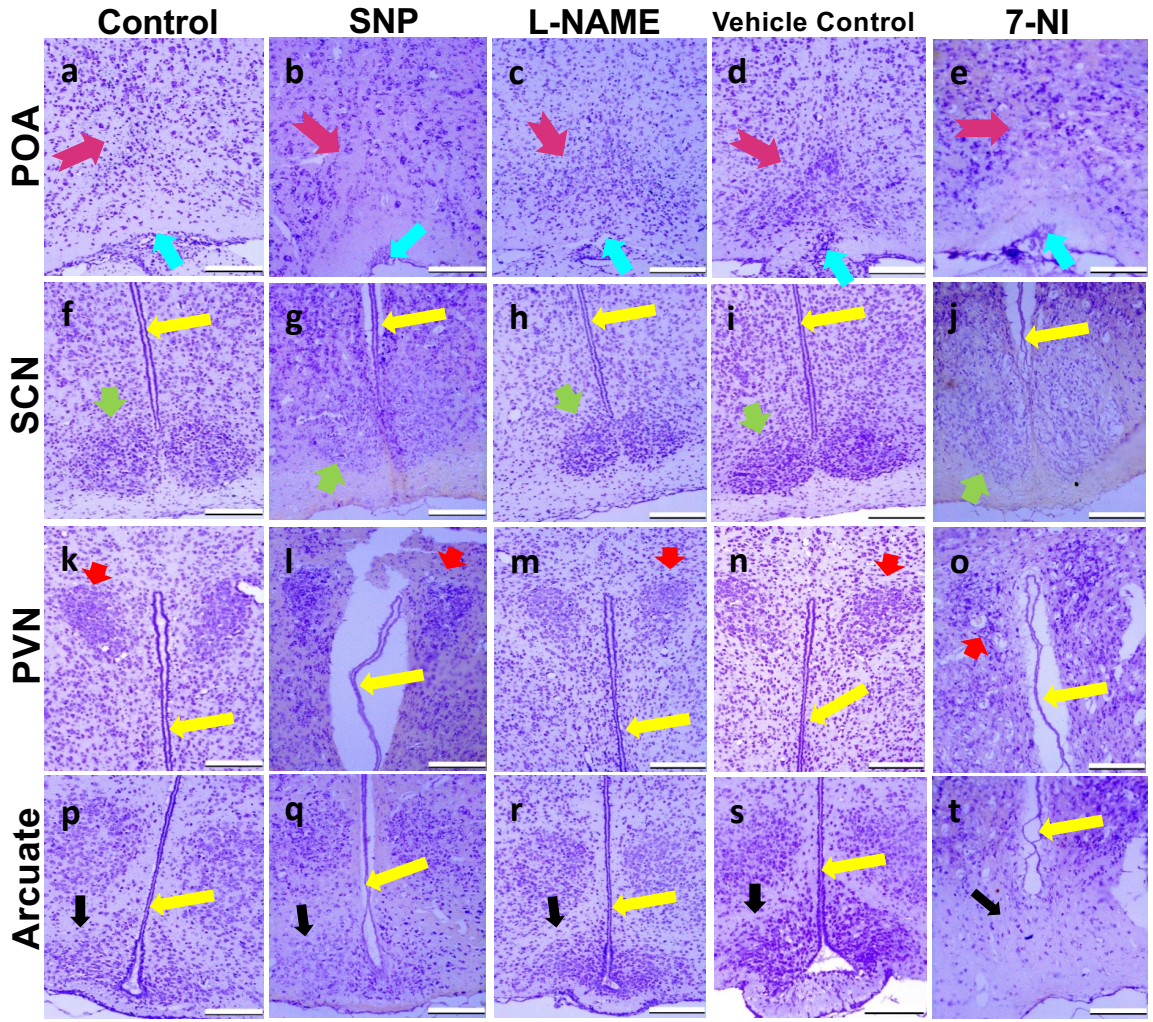


Figure 7: Representative images of transverse sections of POA (images a, b, c, d and e), SCN (images f, g, h, I and j), PVN (images k, l, m, n and o) and Arcuate Nucleus (p, q, r, s and t) of brain of adult female administered with Normal Saline (Control), NO Donor – SNP, NOS Inhibitor – L-NAME; Vehicle Control (DMSO : Normal Saline) and 7-Nitroindazole (7-NI) at the age of 8-weeks for 14 days for studying its effect on changes in hypothalamic nuclei. Scale bar = 100μm.

Key:

- OVLT (Organum Vasculosum Lamina Terminalis
- Preoptic Area (POA)
- Third ventricle
- Suprachiasmatic Nucleus (SCN)
- Paraventricular Nucleus (PVN)
- Arcuate Nucleus

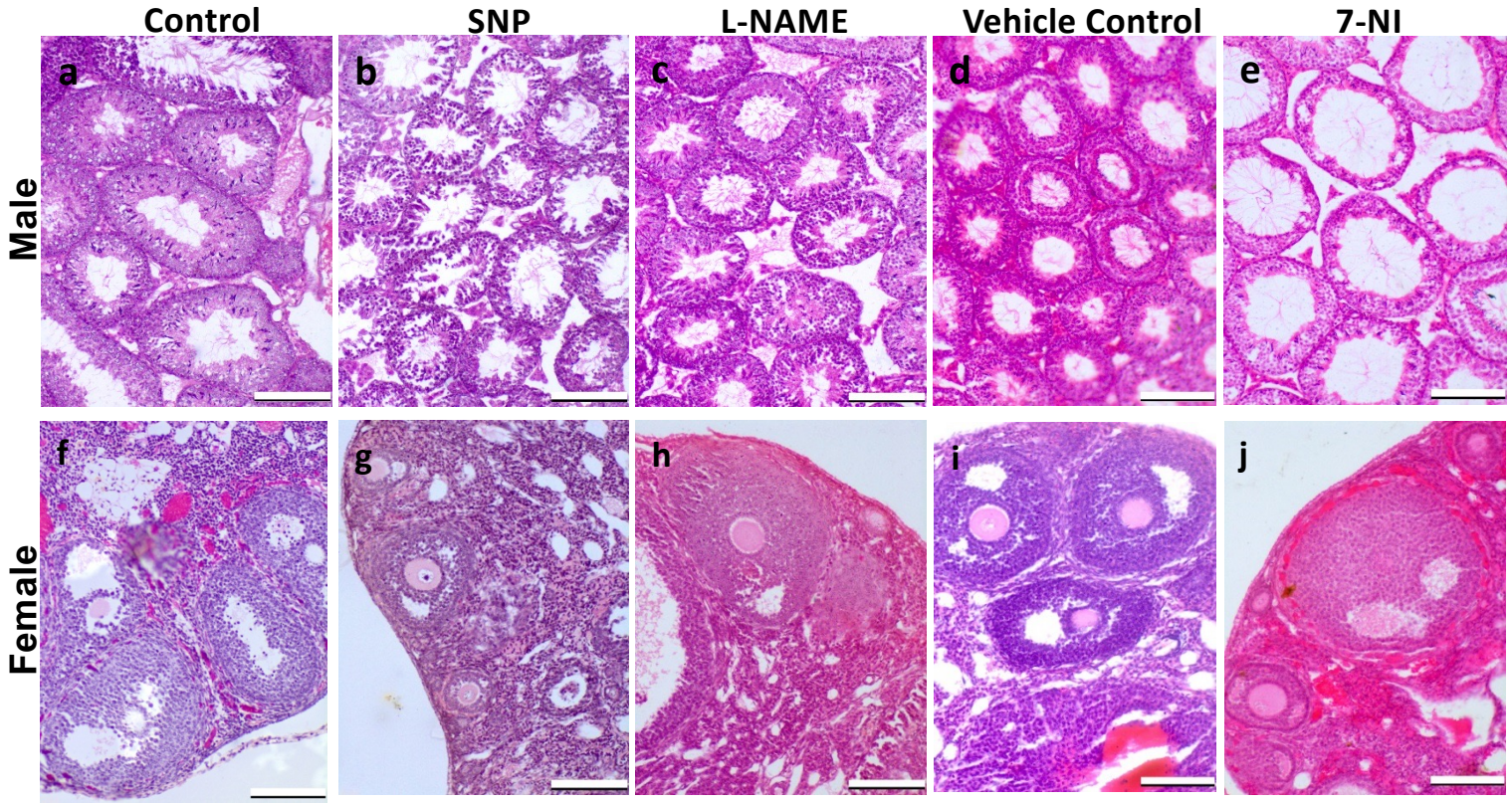


Figure 8: Representative images of transverse sections of testes and ovaries of adult mice administered with Normal Saline (Control), NO Donor – SNP, NOS Inhibitor – L-NAME; Vehicle Control (DMSO:Normal Saline) and 7-Nitroindazole (7-NI) at the age of 8-weeks for 14 days for studying its effect on gonadal (Testis and Ovary) physiology. Scale bar = 100μm.

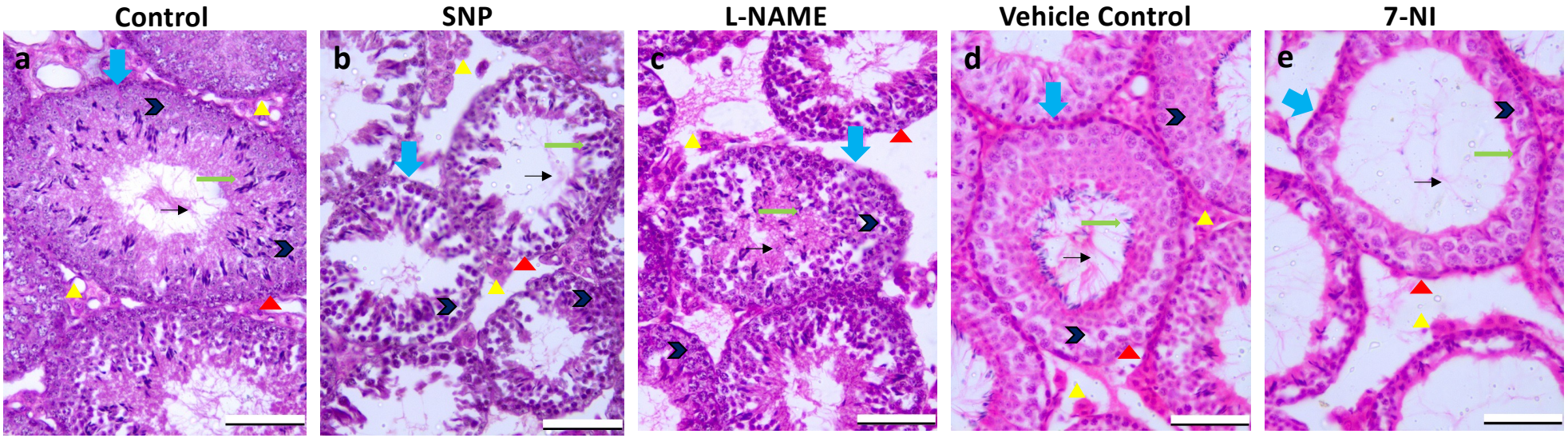


Figure 9: Representative images of transverse sections of testis of adult mice administered with Normal Saline (Control), NO Donor – SNP, NOS Inhibitor – L-NAME; Vehicle Control (DMSO:Normal Saline) and 7-Nitroindazole (7-NI) at the age of 8-weeks for 14 days for studying its effect on testicular structure. Image a, b, c, d and e showing seminiferous tubules of the testis have scale bar = 50μm.

Key: Seminiferous tubules, Spermatogonial cells, Spermatids, Leydig cells, Basement Membrane, Spermatozoa

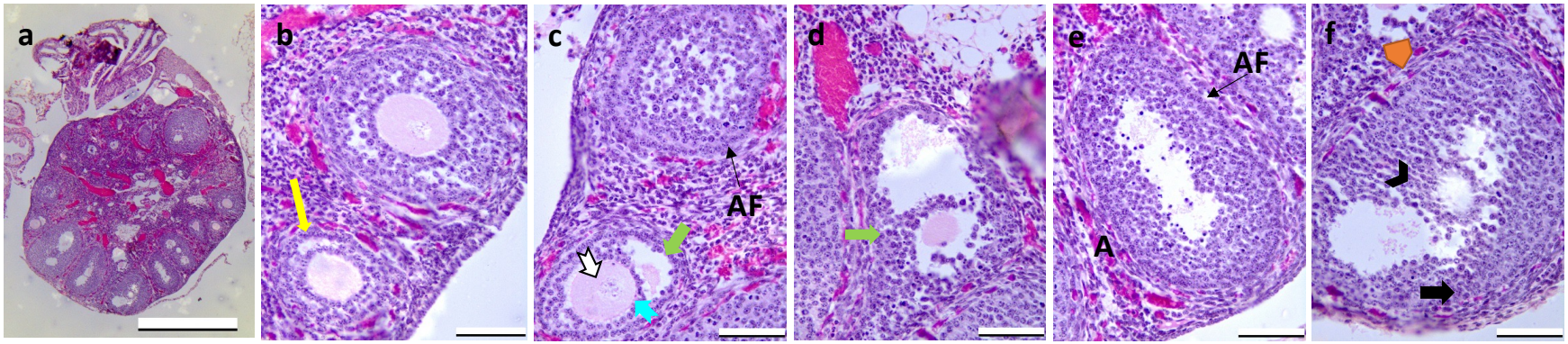


Figure 10: Representative images of transverse sections of ovaries of adult female mice administered with Normal Saline (Control) at the age of 8-weeks for 14 days for studying its effect on follicular development in ovary. Scale bar = 50μm, except image a, where scale bar = 500μm.

KEY: **A,** Antrum, Primary follicle, Secondary follicle, **AF,** Atretic follicle, Graafian follicle, Granulosa cells, Zona pellucida, Ovum, Cumulus oophorus granulosa cells

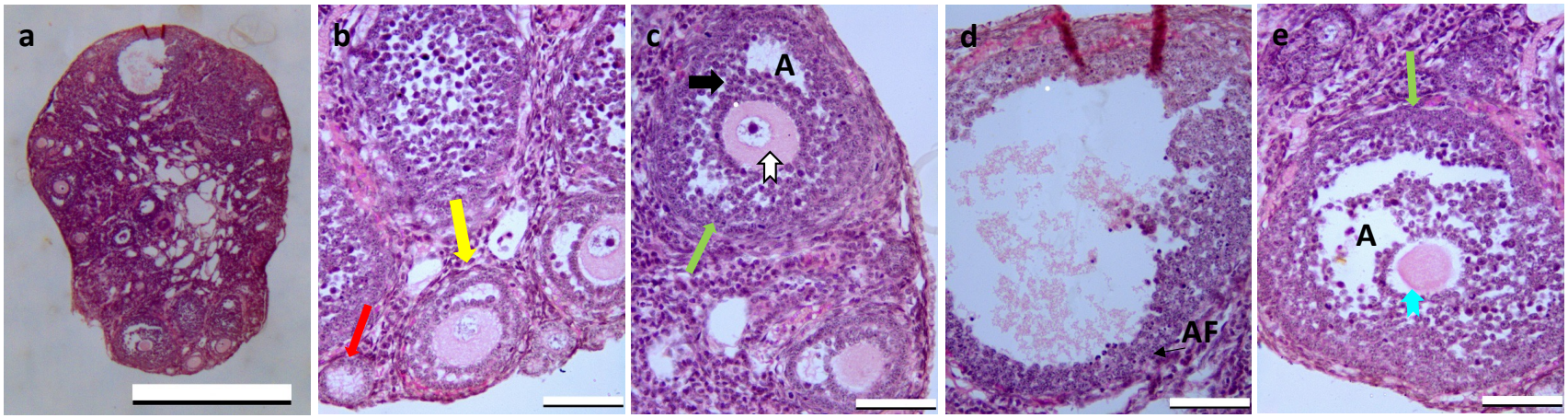


Figure 11: Representative images of transverse sections of the ovary of adult female mice administered with NO Donor – SNP at the age of 8 weeks for 14 days for studying its effect on follicular development in ovary. Scale bar = 50μm, except image a, where scale bar = 500μm.

KEY: A, Antrum, ➡ Primordial follicle, ➡ Primary follicle, ➡ Secondary follicle, **AF,** Atretic follicle, , ➡Granulosa cells, ⬆ Zona pellucida, ⇧ Ovum

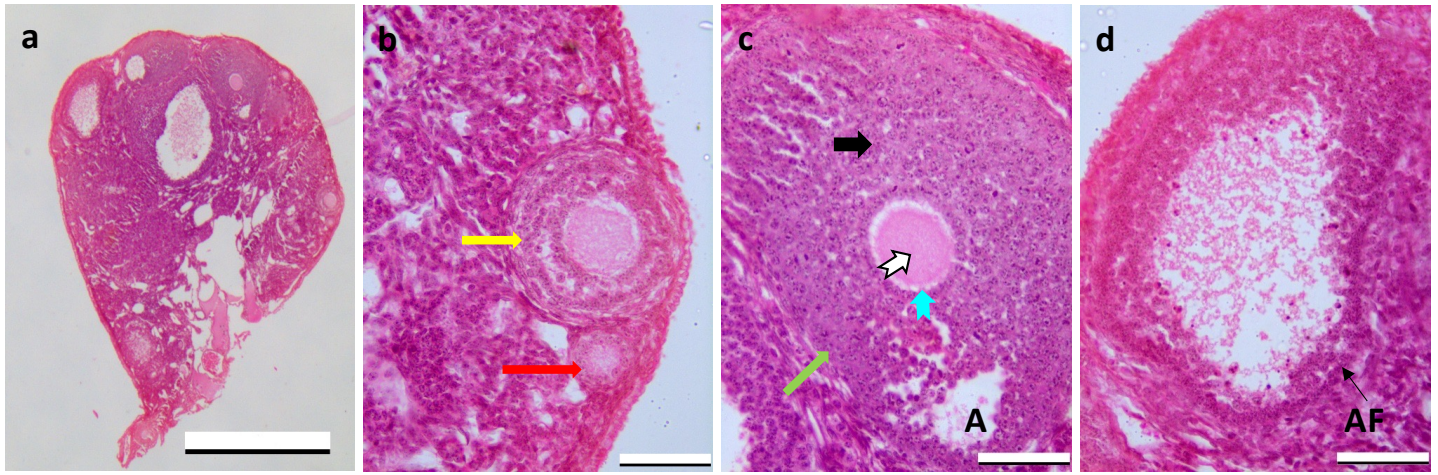


Figure 12: Representative images of transverse sections of ovary of adult female mice administered with NOS Inhibitor – L-NAME at the age of 8-weeks for 14 days for studying its effect on follicular development in ovary. Scale bar = 50μm, except image a, where, scale bar = 500μm. No graafian follicle was observed.

KEY: A, Antrum, Primordial follicle, Primary follicle, Secondary follicle, **AF,** Atretic follicle, Granulosa Cells, Zona pellucida, Ovum

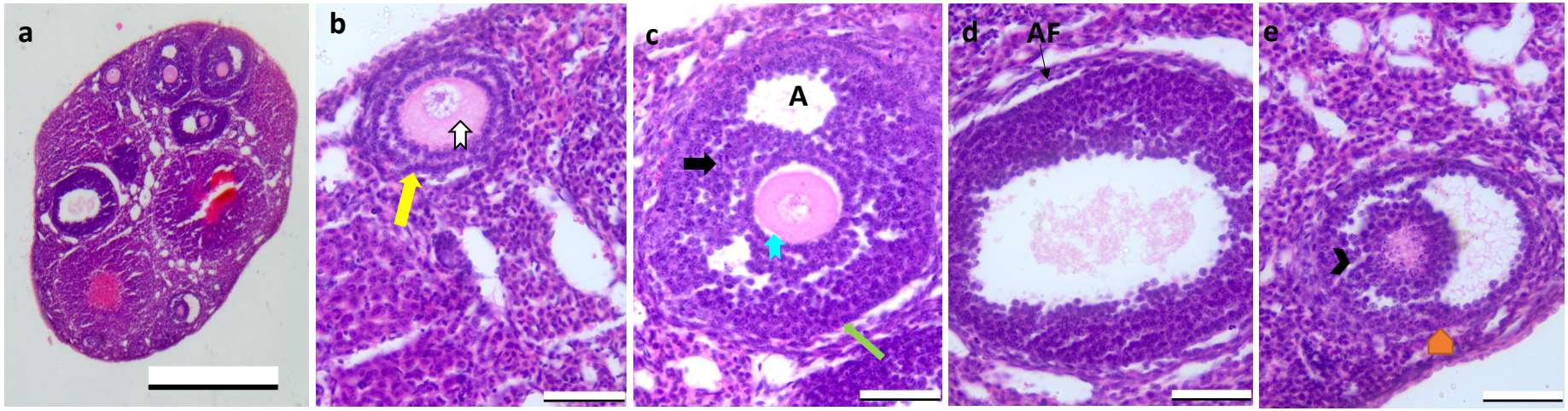


Figure 13: Representative images of transverse sections of ovary of adult female mice administered with Vehicle Control (DMSO : Normal Saline) at the age of 8 weeks for 14 days for studying its effect on follicular development in ovary. Image a, b, c, d, e, f, and g have scale bar = 50μm, showing growing follicles of the ovary.

KEY: A, Antrum, Primary follicle, Secondary follicle, **AF,** Atretic follicle, Graafian follicle, Granulosa cells, Zona pellucida, Ovum, Cumulus oophorus granulosa cells

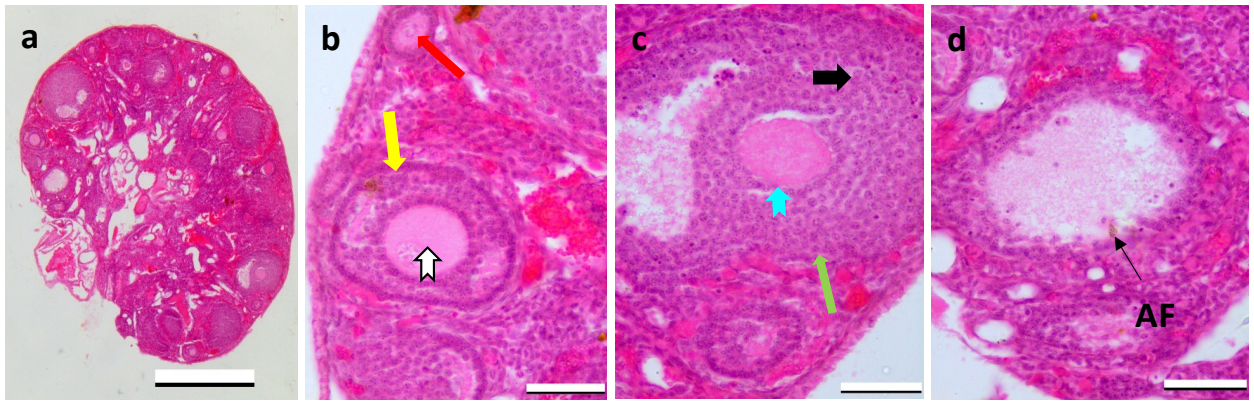
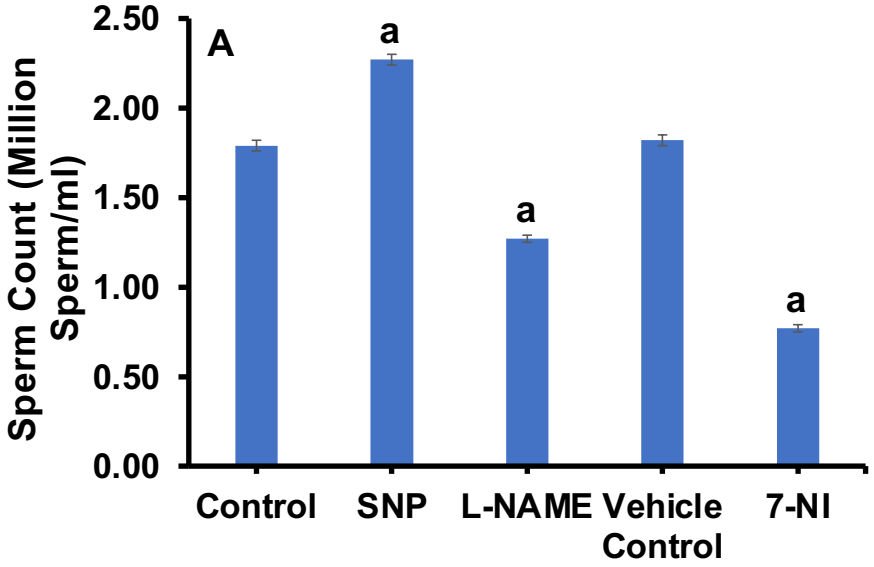
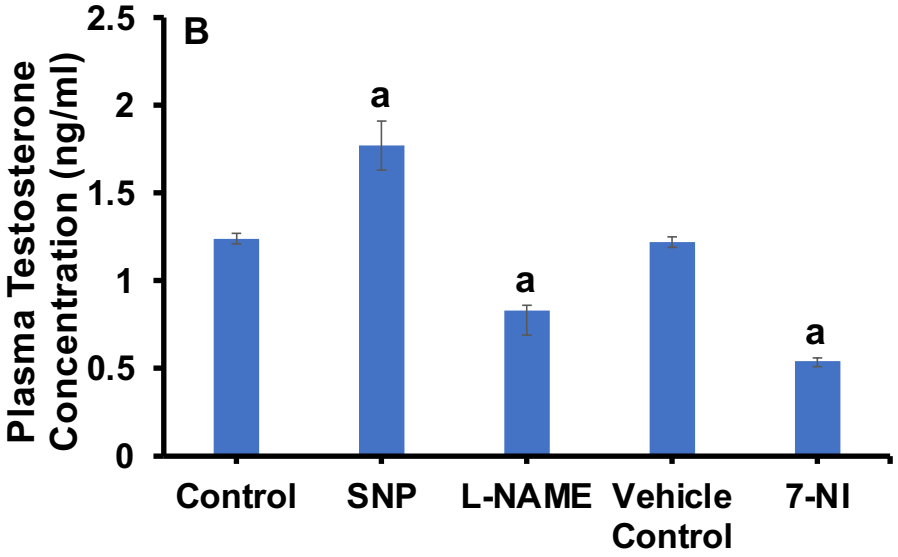


Figure 14: Representative images of transverse sections of the ovary of adult female mice administered with 7-Nitroindazole (7-NI) at the age of 8-weeks for 14 days for studying its effect on follicular development in ovary. Scale bar = 50μm, except in image a, where, scale bar = 500μm. No Graafian follicle was observed.

KEY: **A,** Antrum, Primordial follicle, Primary follicle, Secondary follicle, **AF,** Atretic follicle, Granulosa cells, Zona pellucida, Ovum





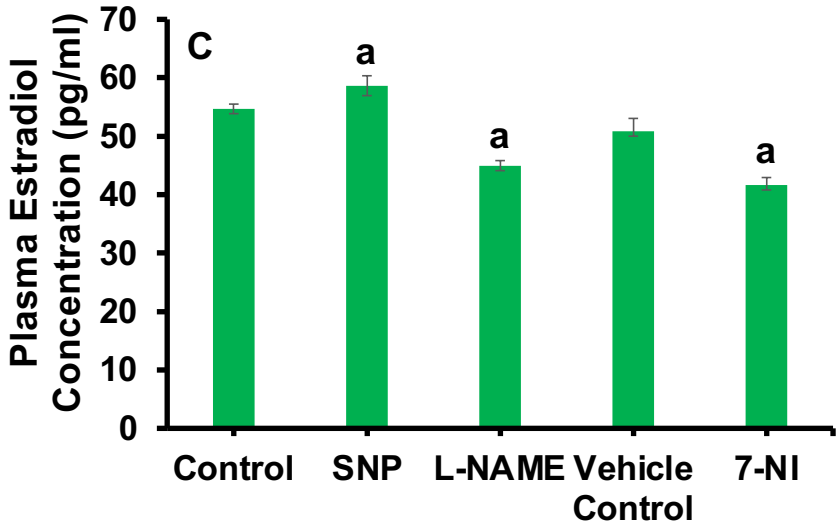


Figure 15: Histogram depicting changes in (A)- Sperm count and (B)- Plasma testosterone in adult male and (C)- Plasma estradiol in adult female mice administered with Normal Saline (Control), NO Donor – SNP, NOS Inhibitor – L-NAME; Vehicle Control (DMSO:Normal Saline) and 7-Nitroindazole (7-NI) at the age of 8-weeks for 14 days for studying its effect on concentration of sperm and steroid hormone production. Means bearing superscript "a" differ from their respective controls ($p<0.05$).

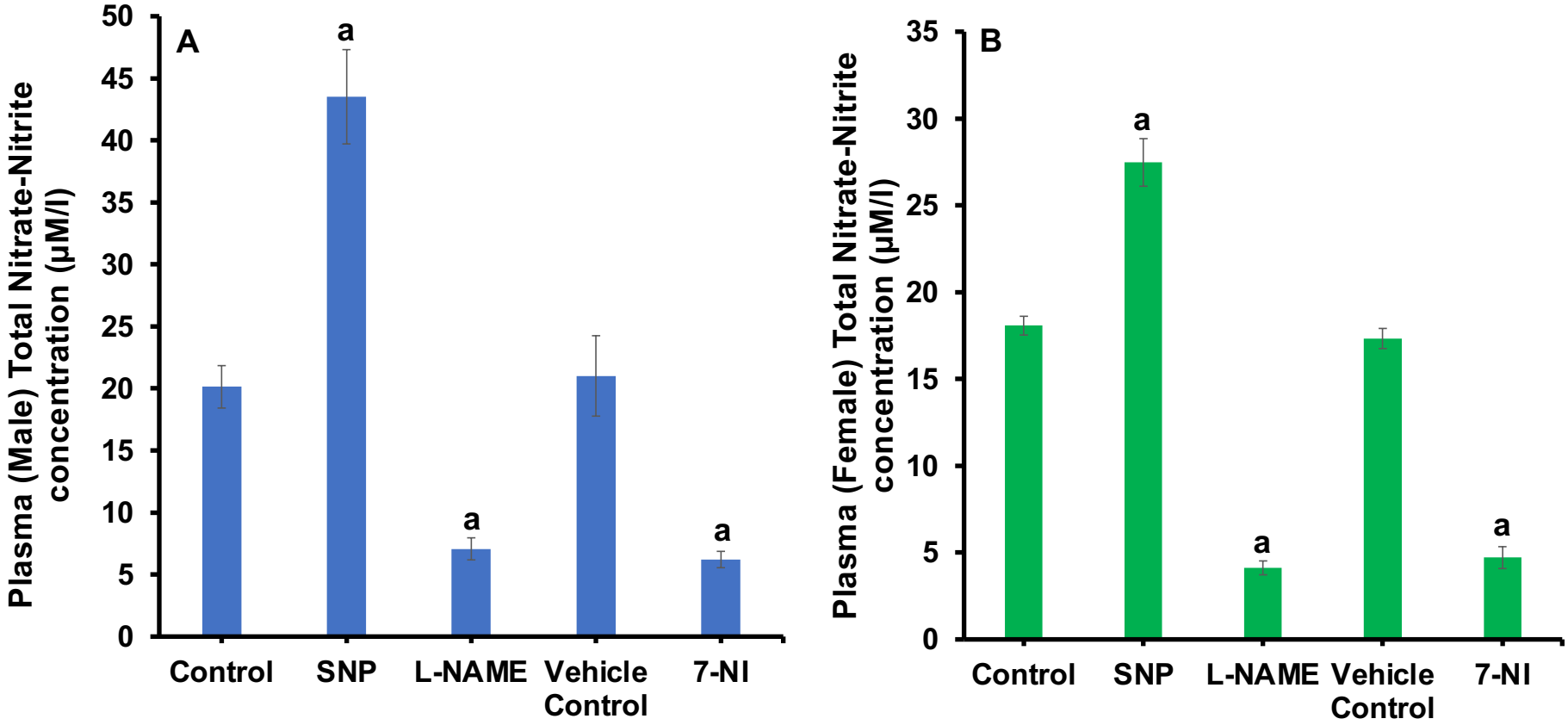


Figure 16: Histogram depicting changes in plasma total nitrate-nitrite concentration in plasma of adult (A)- Male and (B)- Female mice administered with Normal Saline (Control), NO Donor – SNP, NOS Inhibitor – L-NAME; Vehicle Control (DMSO:Normal Saline) and 7-Nitroindazole (7-NI) at the age of 8-weeks for 14 days for studying its effect on total nitrate-nitrite concentration, an indirect measure of NO production. Means bearing superscript "a" differ from their respective controls ($p<0.05$).

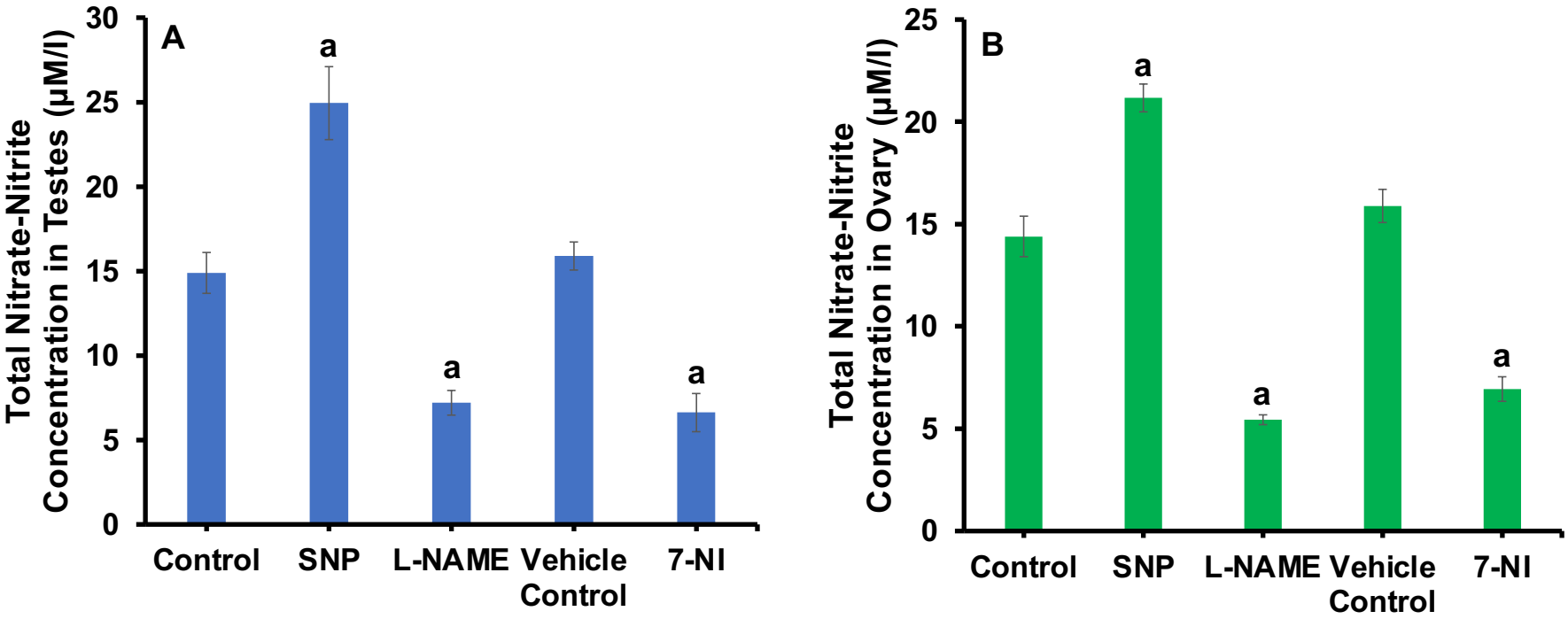


Figure 17: Histogram depicting changes in total nitrate-nitrite concentration in (A)- Testes of adult male and (B)- Ovary of female mice administered with Normal Saline (Control), NO Donor – SNP, NOS Inhibitor – L-NAME; Vehicle Control (DMSO:Normal Saline) and 7-Nitroindazole (7-NI) at the age of 8-weeks for 14 days for studying its effect on total nitrate-nitrite concentration, an indirect measure of NO production. Means bearing superscript "a" differ from their respective controls ($p<0.05$).

Chapter-3

Embryonic Disruption of Neuronal Nitric Oxide Synthase Alters Hypothalamus and Gonadal Development in Mice

Abstract

The growing fetus is susceptible to changes in its environment during embryogenesis, which can greatly affect its development. The neural circuitry in the brain along with environmental, psychological and genetic factors are responsible for the control of embryonic development of various systems of the body, which is regulated via numerous neuromodulators and neurotransmitters. Nitric oxide, one of the neurotransmitters has been shown to influence embryonic development in mammals. In the present study, male and female mice were time mated in the evening hours (16:00 hrs) and the next day female animals were observed for vaginal plugs in the early morning. Female animals with vaginal plugs were considered to be pregnant. Experiments were then performed to see the effects of inhibition of neuronal nitric oxide synthase (nNOS) by administering a specific nNOS inhibitor, 7-nitroindazole to these pregnant female mice from embryonic day 11 to embryonic day 17 along with pregnant female mice treated with dimethylsulphoxide:normal saline (DMSO:NS – 1:1) which acted as the control. Pups born to these pregnant female mice were sacrificed on postnatal day 0 (P0), postnatal 7 (P7), postnatal day 14 (P14) and postnatal day 21 for studying the changes in the structure of the hypothalamic nuclei and gonad (testes and ovaries) physiology. Results show that there were significant changes in the pattern and distribution of hypothalamic nuclei (preoptic area – POA, suprachiasmatic nuclei – SCN, paraventricular nuclei – PVN and arcuate nucleus – ARCN) and inhibition of the development of the morphological and cellular structures of testes and ovaries in the males and females respectively born to 7-NI treated pregnant female mice from postnatal day 0 to postnatal day 21. Thus, it may be concluded that inhibition of neuronal nitric oxide synthase during embryonic development alters the development of the brain and gonads in both males and females by inhibiting the neuronal circuitry responsible for the interaction between the

hypothalamo-hypophyseal-gonadal (HPG) axis and have a major effect on HPG axis development and its consequent effects in adulthood. However, the molecular mechanism responsible for the inhibition of neuronal circuitry during embryonic development needs to be further elucidated.

Introduction

The early embryonic development of the central nervous system is a precise network of events that are influenced by extrinsic (environment, toxic compounds, drugs, stress etc.) as well as intrinsic (Genetic, hormone etc.) factors (Chen et al., 2015). Disturbances in developmental processes in-utero may lead to anxiety and depression later in life and have been associated with exposure to stressful events during prenatal period, throughout childhood, and adolescence (Herbison et al., 2017). Central nervous system (CNS) of fetus and child is highly sensitive to persistent organic pollutants (POPs). Study on maternal exposure to one such POP, Nonylphenol (NP) during pregnancy and lactation activated microglia and increased the production of NO and prostaglandin E2 in the offspring hippocampus (Qui et al., 2019). Perinatal exposure to genistein, a phytosterone, during late pregnancy and early lactation has been shown to induce alterations in estrogen-dependent adult behaviours and NO-producing brain circuits implicating the control of these behaviours (Rodriguez-Gomez et al., 2014). Further, administration of a low dose of bisphenol A (BPA) during the perinatal period in animals hyperactivates the hypothalamo-hypophyseal-adrenal (HPA) axis, which leads to anxiety and depression-like behaviours in the adult, by inhibiting the HPA axis by the hippocampal glucocorticoid receptor (GR) mediated feedback and peripheral testosterone levels (Chen et al., 2015).

Further, in another study, Alvik et al. (2013), it has been reported that binge drinking in the early period of pregnancy causing high prenatal exposure of the fetus to high levels of alcohol consumption and may lead to neurobehavioral and cognitive problems (Alvik et al., 2013). Administration of alcohol in the postnatal days 4-10 to rat pups with higher blood alcohol concentrations (BACs) showed rapid brain growth resulting in significant microencephaly and cell loss in the brain (Bonthius and West, 1990). It has also been demonstrated that

maternal alcohol consumption during pregnancy can also have a negative impact on the development of the fetal brain (Burger et al., 2011; Hepper et al., 2012; Workman et al., 2015; Sanou et al., 2017; Wozniak et al., 2019; Tychkivska, et al., 2019). Alcohol exposure in-utero has been shown to negatively impact a multitude of cognitive domains including learning and memory, adaptive functioning, motor function, attention and activity levels, language development, visual perception and construction, executive function and overall general intelligence (Nayak and Murthy, 2008; Workman et al., 2015; Seleverstov et al., 2017). Further, it has also been demonstrated that there is a high comorbidity rate with other learning and behavioural processes when there is prenatal exposure to alcohol (Mattson et al., 2019). Thus, it has been proposed that alcohol acts as a teratogen during early embryonic development that may impact fetal development and thus negatively affect the fetus, leading to fetal alcohol spectrum disorders (FASD).

Further, Silveira et al. (2022), reported that supplementation of folic acid during pregnancy impaired memory, motricity, and deficient motor learning of the offspring. There was also an increase in anxiety- and depression-like behaviour in this group with a decrease in the total number of entries in the elevated plus maze and an increase in the latency for the first bite in the eating-related depression test (ERDT). Intake of folic acid by pregnant rats also resulted in an increase in nitrative stress and neuroinflammation in the cerebral cortex of the offspring, FASD throughout mating, pregnancy and lactation resulted in short-term memory impairment, decreased hippocampal size and decreased thickness of the dentate gyrus (Sittig et al., 2012; Mikael et al., 2013; McGarel, 2015; Bahous et al., 2017). It has also been shown that prenatal stress in pregnant female mice increases their vulnerability to neurodevelopmental disorders. Male pups born to early stress-exposed gestational females display maladaptive behavioural stress responsivity, anhedonia, and increased sensitivity to selective serotonin reuptake inhibitor treatment and also an elevated stress sensitivity (Cratty et al., 1995; Watson et al., 1999; Bale et al., 2000; Bale and Vale, 2004; Meaney et al., 2007; Mueller and Bale, 2007; Mueller and Bale, 2008; Darnaude´ry and Maccari, 2008). Alterations in corticotrophin-releasing hormone (CRH) and glucocorticoid receptor (GR) expression along with an

increase in hypothalamo-hypophyseal-adrenal axis were observed in these male mice. It was further shown that the male vulnerability to early prenatal stress may involve sex-specific placenta responsivity suggesting sex dependent response (Mueller and Bale, 2007; Mueller and Bale, 2008; Francis-Oliveira et al., 2013; Grundwald and Brunton, 2015; Verstraeten et al., 2019; Lam et al., 2019). Thus, sex-specific programming starts very early in the development process of the animals and stress and exposure to chemicals and compounds in-utero may have a negative impact on the overall development of the animal (Hellemans et al., 2010; Vedhara et al., 2012; Veru et al., 2014; Abuaish et al., 2021; Cusick et al., 2022). In humans, exposure to endocrine disrupting chemicals like bisphenol A, phthalates, triclosan, and perfluoroalkyl substance during potentially sensitive periods of development results in adverse neurobehavioral outcomes in children by disrupting hormonally mediated processes critical for growth and development during gestation, infancy, or childhood (Braun, 2017). Further, Di-n-butyl phthalate (DBP), an endocrine-disrupting compound has been reported to possibly suppress NOS/cGMP pathway in the penis of Sprague-Dawley rats prenatally exposed to this compound, even in a low dose. It resulted in penile fibrosis, decreased testosterone level, and endothelial dysfunction (Zhou et al., 2021). Prenatal exposure to polychlorinated biphenyls (PCBs), a class of endocrine-disrupting chemicals shows sexual differences where males show reduced anxiety-like behaviours and increased activity in the light:dark box in adulthood (Gillette et al., 2017). Prenatal lipopolysaccharide (LPS)-exposure led to increased anxiety and depressive-like behaviors in the adult offspring. There is also an indication of correlation of prenatal administration of LPS with the oxidative stress in adult lives shown by decreased levels of antioxidant enzymes such as superoxide dismutase, catalase and glutathione, while an increased level of lipid peroxidation, protein oxidation products, and NO in the adult lives. Increased level of toxic free radicals is also responsible for brain and liver damage (Al-amin et al., 2016). Primary culture of cerebellar neurons of rats with prenatal exposure to aluminum (Al) by administering aluminum to pregnant female in drinking water caused prevention of glutamate-induced proteolysis of the microtubule-associated protein-2, disaggregation of microtubules, and neuronal death, indicating an impairment of NMDA receptor-associated signal

transduction pathways (Llansola et al., 1999). It was also suggested that alterations in the expression of proteins of the glutamate–nitric oxide–cGMP pathway could be responsible for some of the neurotoxic effects of aluminum. In a study on perinatal cerebral ischemia, the developmental role of nitric oxide in the cerebral blood flow response to NMDA was investigated at mid- and late gestation in fetal sheep and the outcome suggested that NO contributes to the basal blood flow and increase in the neurovascular coupling to activation of NMDA receptors in neocortex during the last trimester in fetal sheep, indicating that the developmental increases in the role of NO in neurovascular regulation are specific for NMDA-receptor activation (Harris et al., 2008).

In the pregnant rats administered with tamoxifen one day prior to labour (E21) and on the childbirth day (E22), hippocampi of embryos at E22 and new-borns at postnatal days of 1, 7, and 21 (P1, P7, and P21) reveal that the cellular density was lower in early stages of development, however, cellular density and thickness gradually increased during the development, particularly in the third week. Also, nNOS expression was decreased in E22, P1, and P7 in animals treated with tamoxifen indicating that tamoxifen affects the development and differentiation of postnatal rat hippocampus, CA1 neurons, and nNOS expression (Nobakht et al., 2011). Further, female nNOS-CreER mice exposed to a single dose of the sodium salt of valproic acid (VPA) during pregnancy and offspring sacrificed and processed on the postnatal 35-day show a decrease in synapse-associated surface proteins of nNOS interneurons following VPA treatment, simultaneously there was a down expression of neuronal activity-regulated pentraxin (Narp), glutamate receptor 4 (GluA4) and protein kinase C gamma (PKCγ) surface protein in nNOS interneurons in VPA-treated mice suggesting an imbalance of synaptic transmission in autism spectrum disorder (ASD).

Thus, all these studies show that gestational (prenatal) exposure or perinatal exposure to various compounds and stressors have adverse effects on the behaviour and developmental process in the pups born to pregnant mothers exposed to external compounds or stressors. However, there is a paucity of information on the inhibition of nitric oxide synthase by specific nNOS

inhibitors in the process of the embryo and postnatal developmental processes, especially the development of the brain and gonads (testes and ovaries). Thus, in the present study, pregnant female mice were administered with a specific nNOS inhibitor at a dose of 10mg/kg body weight from embryonic day 11 to embryonic day 17 and its effect on the development of the brain and gonads (testes and ovary) were observed at postnatal day 0 (P0), postnatal day 7 (P7), postnatal 14 (P14) and postnatal 21 (P21).

Material and Methods

Animals

Adult Male and female mice were time mated and pregnancy was confirmed by checking the vaginal plug. 5 pregnant female mice were administered with 7-Nitroindazole from embryonic day 11 to 17 while five (5) control pregnant mice were treated with a solution of normal saline and dimethyl sulfoxide (1:1), which was used as a vehicle. The pups born at postnatal days 0, 07, 14 and 21 were perfused as described in General Material and Methods and brain and gonads (testes and ovaries) were collected from the perfused animals. Light dark cycle 12L:12D was maintained and food and water were available ad libitum. The experimental design for the present study is depicted in **Figure 1**. All the experiments were performed as per the Animal Ethics Committee of Rajiv Gandhi University and as per the guidelines within the framework of the revised Animals (Scientific Procedures) Act 2002 (CPCSEA Guidelines) of the Government of India.

Crystal Violet Staining of Brain Sections

6μm thick sections of the brain (both males and females) were cut on a rotary microtome and processed for crystal violet staining as per the protocol described in General Material and Methods

Histology of Gonads (Testes and Ovaries)

6μm thick sections of the gonads were cut on a rotary microtome and processed for Hematoxylin and Eosin staining as per the protocol described in General Material and Methods.

Result

Effect on the Different Nucleus of the Hypothalamus

Not much sex differences are observed in the distribution of neurons in the POA of the brain of the animals sacrificed at postnatal day P0 and P7, but between the control and 7-NI treated animals, there is a reduction in the number and distribution of cells (**Figure 2**). In the animals on postnatal days 14 and 21, sex differences begin to occur in the distribution of neurons in POA. In the SCN (**Figure 3**) and PVN (**Figure 4**), the sex difference and difference between the control and 7-NI treated group can be seen from postnatal day 0 onwards. The arcuate nucleus has more cells in female than in male animals (**Figure 5**).

Effect on Testes Histology

The histological section of the testes shows the intact arrangement of seminiferous tubules in control, whereas the arrangement is disturbed in the 7-NI treated males since P7. Even the arrangement and distribution of spermatogonial cells and Leydig cells are disturbed in 7-NI treated males in all the stages of development i.e., P0, P7, P14, P21 (**Figure 6** and **Figure 7**).

Effect on Ovarian Histology

P0 and P7 stage ovary show the uneven arrangement of the primordial cells in 7-NI treated females, whereas, in the p14 stage, there is a lesser number of follicles in the treatment group than in the control. In the P21 stage follicles can be seen in different growing stages i.e., primordial, primary and secondary stages with distinct antrum in the control animals, but in 7-NI treated animals, follicles are in either primordial or primary stages, suggesting a delay in development (**Figure 6** and **Figure 8**).

Discussion

Development as a whole is majorly influenced by genetic as well as environmental factors (Cetin et al., 2010; Nelissen et al., 2011; Hocher, 2014; Baldacci et al., 2018; Goyal et al., 2019; Besenfelder et al., 2020). Even a small change in biological processes during the critical period of development can have an adverse effect on the adulthood of the animal (Hoffman et al., 2021; Heindel et al., 2015). In the present study, an attempt was made to disrupt such processes by inhibiting neuronal nitric oxide synthase, an important neuromodulator involved in the development of the brain and gonad (Bredt and Snyder, 1994; Bertini and Bentivoglio, 1997; Gibbs, 2003; Bellefontaine et al., 2011; Ling et al., 2012; Xu et al., 2012). Histological results of different regions of the hypothalamus and also testis and ovary in the present study demonstrate that maternal inhibition of neuronal nitric oxide synthase (nNOS) during the critical period of development (E11 to E17) had a significant postnatal effect. In the rat hypothalamus, neurogenesis occurs in three waves, E13-E15 (corresponding to E11-E13 in mouse), the first wave, when the neurons form lateral hypothalamic structures, E15-E17, the second wave, when the neurons become part of the medial hypothalamus and E17-E19, the final wave, when the periventricular hypothalamic population is formed with exception of PVN formation which is nearly completed before E15 (Altman and Bayer, 1986; Bless et al., 2005; McClellan et al., 2008; McClellan et al., 2010; Stratton and Tobet, 2020).

Results in the preoptic area of the hypothalamus, containing GnRH neurons, formed between E10 to E13 (Miller and Nowakowski, 1988; Okamura et al., 1990; Schwanzel-Fukuda and Pfaff, 1989; Lee et al., 2008) a major regulator of the reproductive axis (Miller et al., 2002), show a significant reduction in the neuronal cell number during different developmental stages P0, P7, P14 and P21 in the pups exposed in-utero to 7-NI. Brain regions also show gender differences (Pakkenberg and Gundersen, 1997; Goldstein et al., 2001; Kaufmann et al., 2001; Cosgrove et al., 2007; Zaidi, 2010; Xin et al., 2019). POA show gender differences in P14 and P21, with males showing a greater number of cells than female. GnRH neuron terminals are also found in the

arcuate nucleus of the hypothalamus, making it a key regulator of the anterior pituitary and subsequently in the release of LH and FSH (Plant, 2019). Arcuate nucleus cell numbers were also observed to be more in the control than in the 7-NI treated groups. This discrepancy in the hypothalamic region controlling GnRH release is also reciprocated in the gonads. In the testis, it is observed that there is a significant reduction in the size of the seminiferous tubules from P0 onwards with considerable gaps between the otherwise intact tubules. In the P21 stage, the differences become more evident with spermatogonial cells in the testis of the 7-NI treated animal showing wide intracellular spaces and the germinal cells being detached from the basement membrane. The number of Leydig cells is also sparse, Leydig cell is the most important cell type for endocrine function of the testis (Svingen and Koopman, 2013), this reduction in the number of Leydig cells may be attributed to lower testosterone levels leading to further delay or alteration in the development of testis (Rolf et al., 2002). During the process of developmental events of the gonads, the primordial germ cells (PGC) migrates from the endoderm of yolksac to the developing gonad around days 7 to 11 (Anderson et al., 2000) and the ovary is first apparent in the day 10.5 in mice as a thickening of coelomic epithelium (Smith et al., 2014), and gonadal sex differentiation is considered to occur at day 12 (Menke et al., 2003). In the present study, in the ovary of P0 and P7 mice, primordial cells are seen to be uniformly arranged towards the periphery, but in the 7-NI treated mice, the distribution of primordial cells is disturbed. In the P14 control mice, more primary follicles are observed with distinctly arranged follicular cells than in experimental female mice. Follicles in different stages of development i.e., primary follicle and secondary follicle up to the late antral stage are observed in the mice born to pregnant females administered with vehicle control, whereas in pups born to 7-NI treated pregnant females, the number of follicles is very less, and all are in the primary follicular stage. This alteration in the ovary of 7-NI treated mice is indicative of abnormal or delayed folliculogenesis which could in adulthood be manifested to the disruption in estrous cycle and impaired fertility (Klein et al, 1998).

Neurons in the paraventricular nucleus is responsible for the release of corticotropin-releasing hormone (CRH) involved in the stress axis (Daviu et al.,

2020). In the current study, there appears to be an apparent decrease in the number and distribution of cells in the 7-NI treated animals in both males and females of all the stages when compared with their control. CRH is the chief hormone for activation of the HPA axis that triggers the secretion of glucocorticoids. Glucocorticoids further act on multiple organ systems to prepare the body to respond to the stressor (Herman et al., 2016). An increase in the nerve cells in PVN may be suggestive that there could be increased activity in the HPA axis, which is also responsible for various mood and cognitive disorders (Keller et al., 2017). As reported in earlier studies, animals with mood disorders have increased activity of the HPA axis (Swaab et al., 2005).

Cognitive and mood disorders greatly affect the cardiovascular regulation, respiration, appetite control and sleep pattern of the animal, implying SCN, the biological clock of the brain responsible for maintaining the circadian rhythm also has a role to play in the HPA axis (Swaab et al., 2005). Our study shows a reduction in the cell number in 7-NI treated animals than in the control group, indicative of the disruption of the biological rhythm of the animal. There is also a gender-dependent difference in the distribution of neural cells, being reduced in females than in males.

Thus, it may be concluded that in-utero exposure to a specific nNOS inhibitor leads to alteration in the structure of hypothalamic (POA, SCN, PVN and Arcuate) nuclei and gonads (testes and ovaries). This further suggests that nitric oxide is important for neuronal and gonadal development and its inhibition during embryonic development may have serious implications in adulthood such as anxiety and depression and reproductive failure in both males and females.

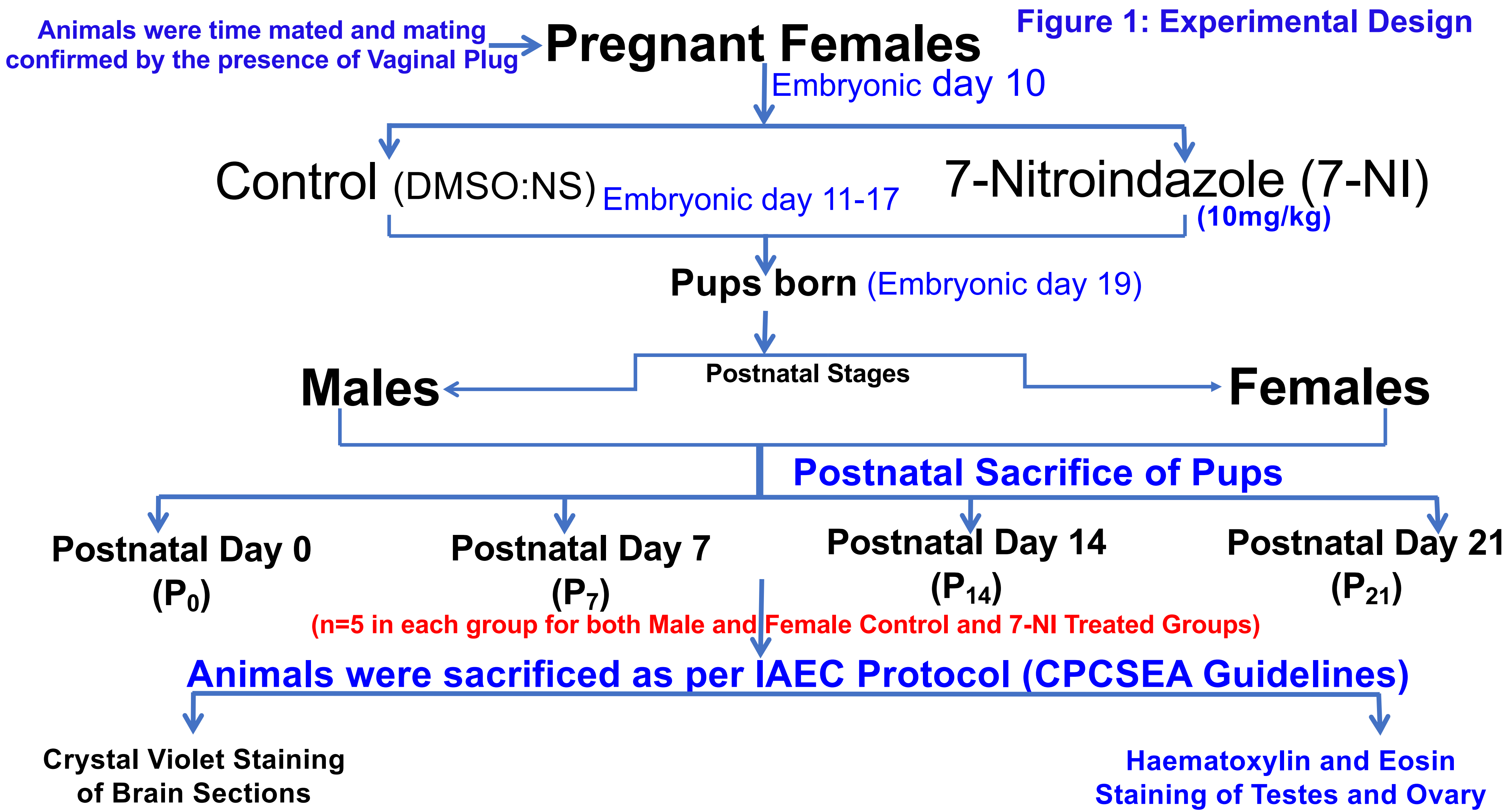


Figure 1: Experimental Design
Animals were time mated and mating confirmed by the presence of Vaginal Plug
Pregnant Females
Embryonic day 10
Control (DMSO:NS)
Embryonic day 11-17
7-Nitroindazole (7-NI)
(10mg/kg)
Pups born (Embryonic day 19)
Postnatal Stages
Males
Females
Postnatal Sacrifice of Pups
Postnatal Day 0
(P0)
Postnatal Day 7
(P7)
Postnatal Day 14
(P14)
Postnatal Day 21
(P21)
(n=5 in each group for both Male and Female Control and 7-NI Treated Groups)
Animals were sacrificed as per IAEC Protocol (CPCSEA Guidelines)
Crystal Violet Staining of Brain Sections
Haematoxylin and Eosin Staining of Testes and Ovary

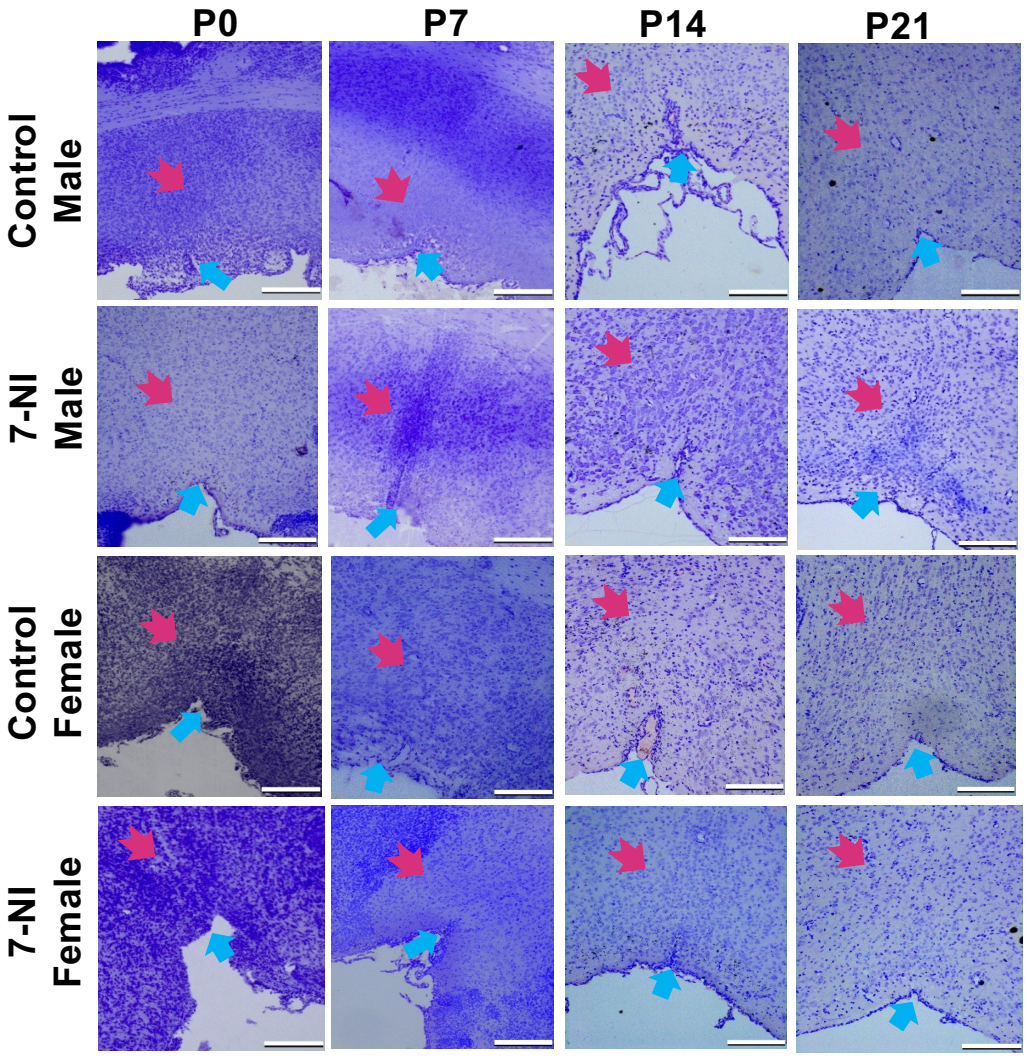


Figure 2: Representative images of brain sections showing preoptic area (POA) of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage during embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day P0, P7, P14 and P21, to study the changes in hypothalamic nuclei. Scale bar = 100μm.

Key: ⬇ OVLT (Organum Vasculosum Lamina Terminalis)

➧ Preoptic Area (POA)

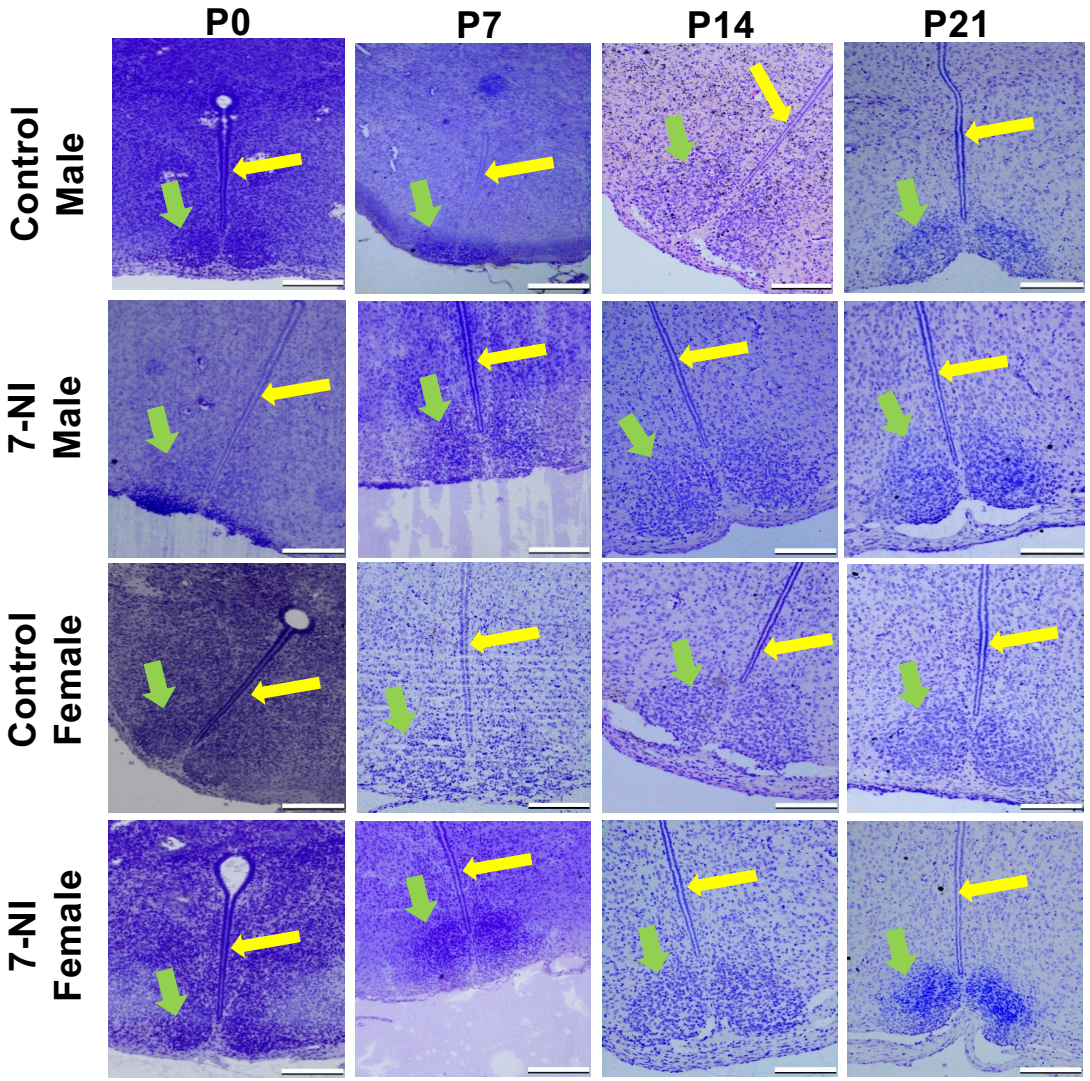


Figure 3: Representative images of brain sections of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day P0, P7, P14 and P21 to study the changes in hypothalamic nuclei. Scale bar = 100μm.

Key: Third Ventricle
Suprachiasmatic nucleus (SCN)

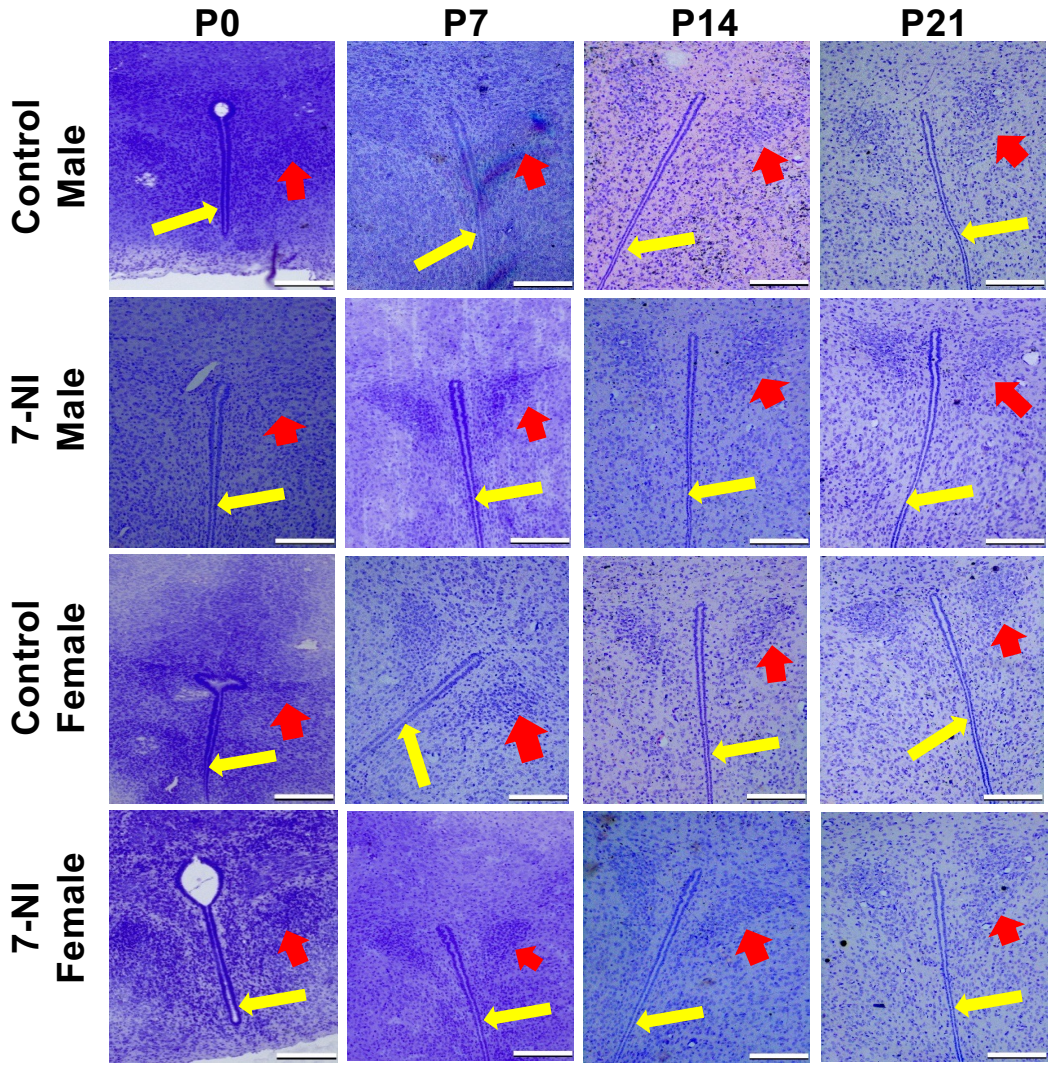


Figure 4: Representative images of brain sections of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day P0, P7, P14 and P21 to study the changes in hypothalamic nuclei. Scale bar = 100μm.

Key: Third Ventricle

Paraventricular nucleus (PVN)

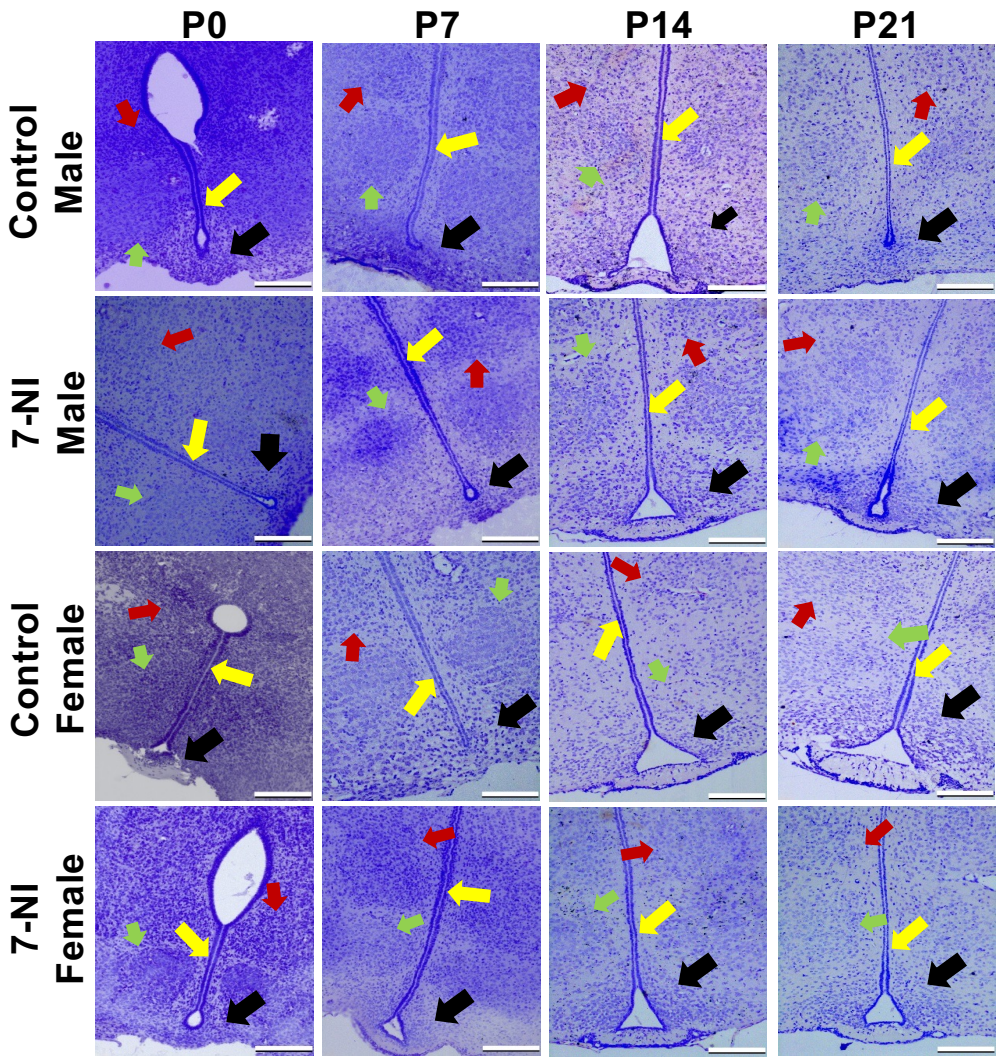


Figure 5: Representative images of brain sections of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at post natal day P0, P7, P14 and P21 to study the changes in hypothalamic nuclei. Scale bar = 100μm.

Key:
- Third Ventricle
- Arcuate nucleus (Arc.)
- Dorso medial hypothalamic nucleus (DMH)
- Ventro medial hypothalamic nucleus (VMH)

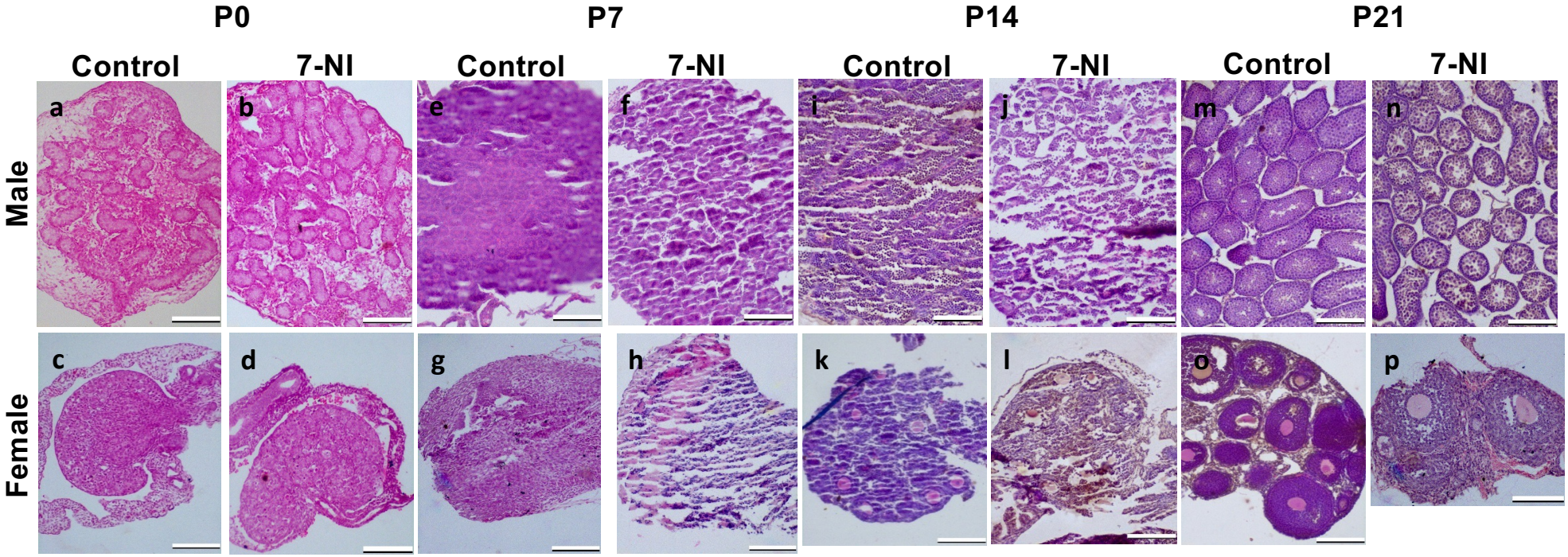


Figure 6: Representative images of transverse sections of adult male and female mice testes and ovaries, respectively born to pregnant female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO: NS 1:1) (designated as Control Male and Control Female) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day 0, 7, 14 and 21 to study its effects on gonadal (Testis and Ovary) development. Scale bar = 100μm.

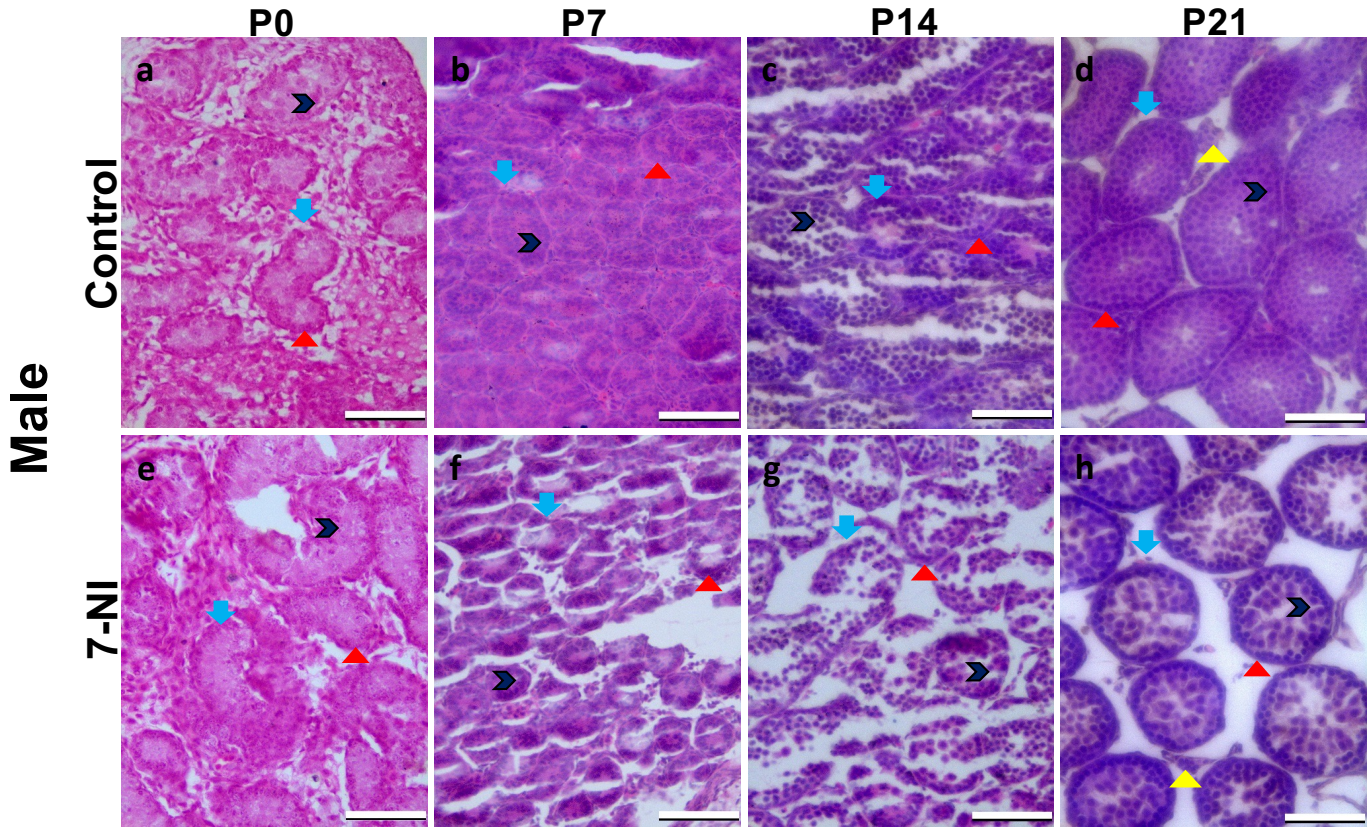


Figure 7: Representative images of transverse sections of testes of mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male) and vehicle control (DMSO:NS) (designated as Control Male) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day 0, 7, 14 and 21 for studying its effect on development of testis. Scale bar = 50μm.

Key: Seminiferous tubules, Spermatogonial cells, Leydig cells, Basement membrane

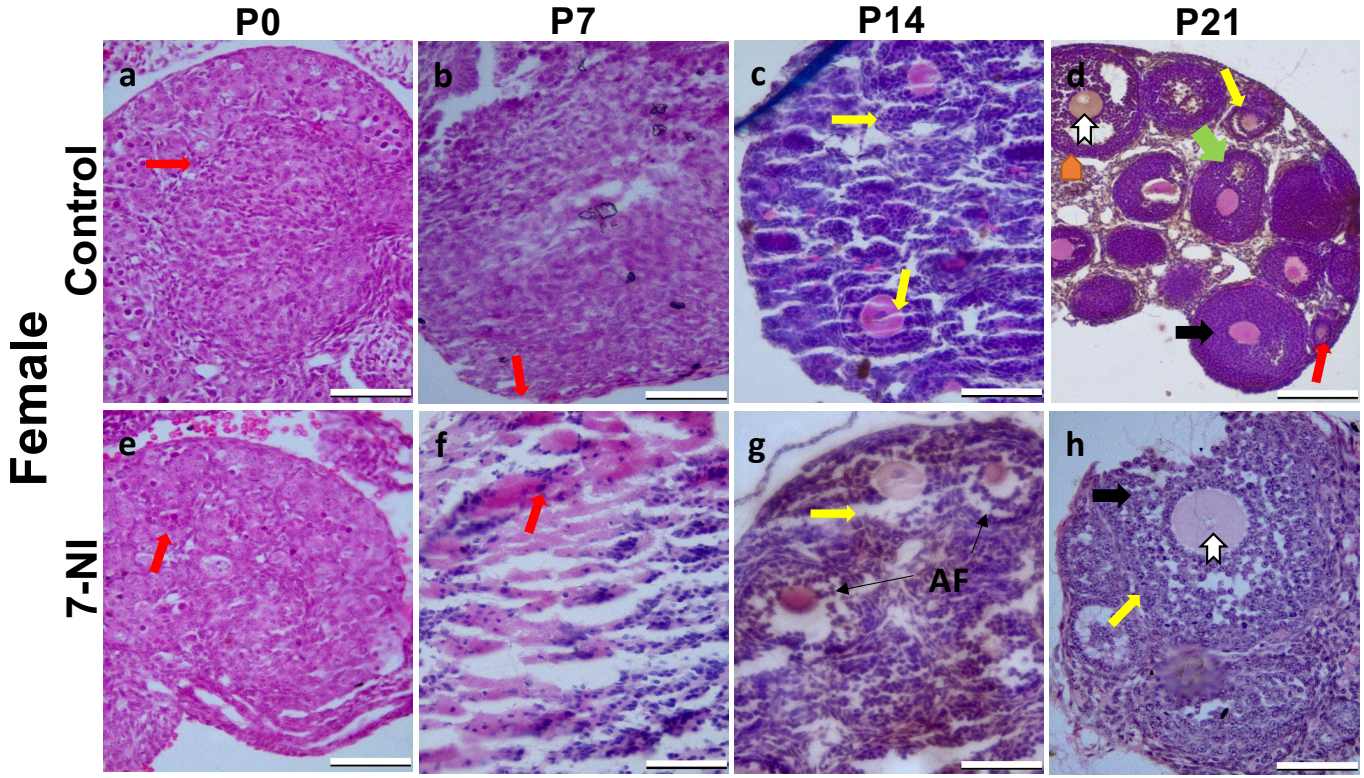


Figure 8: Representative images of sections of ovaries of mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at post natal day 0, 7, 14 and 21, for studying it's effects on follicular development in ovary. Scale bar = 50μm.

Key: **A,** Antrum, Primordial follicle, Primary follicle, Secondary follicle, **AF,** Atretic follicle, Graafian follicle, Granulosa cells, Ovum

Summary and Conclusion

According to the literature anxiety and depression are one of the major disorders in today's population due to the demands and rigour of the fast-paced lifestyle of the individuals in today's society. 2017 WHO report on depression and other common mental health reports that 3.6% of the global population suffers from anxiety and 4.4% from depression. Anxiety and depression are comorbid conditions and are accompanied by many other factors such as alteration in the perception of social reality and the ability to adapt in an individual, affecting the quality of life of the people suffering from such disorders. In the general population, these mood disorders have been shown to be gender biased, being more prevalent in females than males. There could be various factors responsible for the development of such conditions in an individual, it could be environmental, genetic as well as epigenetic. To maintain homeostasis, the body has an elaborate interconnected system constantly being regulated by various molecules. Among such systems are the hypothalamo-pituitary-adrenal axis, responsible for the regulation of stress in the body and the hypothalamo-pituitary- gonadal axis, responsible for the reproductive functions. These axes again have an intricate system of neural circuitry comprising of neuromodulators and neurotransmitters modulating its functioning. One such molecule is the ubiquitously present nitric oxide. This nitric oxide is implicated to be involved in various physiological processes through cyclic guanosine 3′5′-monophosphate (cGMP), including the functions of the brain. Nitric oxide is produced as a byproduct in the enzymatic conversion of L-Arginine to L-Citrulline in the presence of NADPH, cofactors and the enzyme nitric oxide synthase (NOS), NOS has three isoforms in the body (neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), all playing different roles in the body physiology. Of the three isoforms, nNOS expression has been found to be distributed in various brain regions such as the cerebellar cortex, dorsal raphe, cerebral cortex, amygdala, hippocampus, preoptic area and also paraventricular, magnocellular, the supraoptic nucleus of the hypothalamus. These regions, especially the amygdala, hippocampus, and dorsal medial thalamus of the subcortical limbic regions have also been reported to be associated with mood disorders such as anxiety and depression. nNOS has been

implicated in a varied range of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, anxiety, stroke and also learning and memory and neuropsychiatric disorders, including depression. NO produced in the brain is linked to be involved in regulating the HPA axis. On the other hand, NO is shown to be localized and expressed in the hypothalamus, hypophysis and gonads and can act on the hypothalamo-hypophyseal-gonadal (HPG) axis to regulate the synthesis and release of GnRH and thus reproduction, as GnRH and NO-producing neurons occupy similar positions in the hypothalamus. NO has also been reported to regulate spermatogenesis, sperm motility, sperm capacitation, fertilization, oogenesis (follicle development/folliculogenesis), gonadal hormones and steroidogenesis. Nitric oxide is also involved in the embryonic development of the brain and gonads, affecting the overall development of the HPA and HPG axis. Various studies involving knock-out models of mice and also different inhibitors of NOS and specific nNOS inhibitors, as well as nitric oxide donor agents, have shown that nitric oxide is intricately involved in the modulation of both the HPA axis and HPG axis and thus therefore involved in the pathology of neurodegenerative disorders and mood disorders and consequently affects the reproductive behaviour and fertility. But still there exist many lacunas in understanding the concrete aetiology of these disorders. One such lacuna is the involvement of influences during embryonic development affecting the behaviour in adulthood.

Therefore, we hypothesize that the embryonic disruption of the nNOS signalling may alter the hypothalamic (POA, SCN, PVN and Arc. Nucleus) development and subsequently altered HPA axis activity/negative feedback leading to mood disorders such as anxiety- and depression-like behaviour and HPG axis (reproductive physiology) in mice.

Objectives:

- Does restricted embryonic and adult nitric oxide synthase blockade cause altered HPA axis-dependent behaviours and alteration in HPG axis?

- Does restricted embryonic and adult nitric oxide synthase blockade cause alteration in hypothalamic cytoarchitecture and HPA axis function?

- Does nitric oxide signalling regulate neuron development in the developing hypothalamus?

To answer these above objectives the doctoral work has been divided into three chapters:

Chapter 1

Embryonic Disruption of Neuronal Nitric Oxide Synthase Alters Anxiety- and Depression-like Behaviour and Reproductive Physiology of Mice in its Adulthood

Abstract

Mood disorders like anxiety and depression are major contributors of the maladaptation of individuals to normal situations. To understand these disorders, anxiety and depression-like behaviour studies are done on animal models such as mice. There are many neuropeptides and neuromodulators known to influence the HPA axis, the stress axis, that are implicated in mood disorders like anxiety and depression. One such neuromodulator of the stress axis is nitric oxide. In the present study, the production of nitric oxide was inhibited by a specific inhibitor (7-Niroindazole, 7-NI) of its enzyme neuronal nitric oxide synthase (nNOS). Male and females were time mated in the evening hours (16:00 hrs) and a vaginal plug was observed the next day. Females showing vaginal plugs were considered pregnant. One group of pregnant females were kept in individual cages and were injected with 7-NI at a dose of 10mg/kg body weight from embryonic day 11 to embryonic day 17, while the other group was administered with vehicle control (Dimethlysulphuroxide:Normal Saline – DMSO:NS in 1:1 ratio) and served as the vehicle control group. The pups born to these pregnant females on day 19 were weaned on postnatal day 21, males and females were separated into different cages with individual ear punch markings for individual identification

of mice. These male and females mice were raised to adulthood until 8 weeks when these animals were exposed to a battery of behavioural tests such as elevated plus maze (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT). All the behavioural tests were done in the light phase of the light-dark cycle. The behavioural tests in females were performed when the animals were in the diestrus phase. An interval of 4-7 days was maintained between each behavioural test. After the end of the behavioural tests, each animal was anaesthetized and sacrificed for studying the changes in hypothalamic structures of the brain and changes in gonad physiology. Results show that there were marked changes in anxiety- and depression-like behaviour (as displayed by EPM, OFT, FST and MBBT), the hypothalamic nuclei of the brain, nitric oxide production (total nitrate-nitrite concentration), gonad (testes and ovary) physiology and plasma testosterone levels and sperm count in males and plasma estradiol levels in females in both males and females born to 7-NI treated females when compared to males and females born to vehicle-treated pregnant female groups. The result shows that administration of nNOS inhibitor in-utero in mice alters both hypothalamo-hypophyseal-adrenal (HPA) axis as well as the hypothalamo-hypophyseal-gonadal (HPG) axis in the embryo itself which prolongs until adulthood by disturbing the neural circuitry responsible for its maintenance. Thus, nitric oxide acts as an anxiolytic and anti-gonadotrophic agent in mice, however, the molecular mechanism needs further elucidation.

Chapter 2

Nitric Oxide Modulates Anxiety- and Depression-like behaviour and Hypothalamo-Hypophyseal-Gonadal Axis in Mice

Abstract

L-arginine (L-Arg)/nitric oxide (NO)/cGMP pathway is known to be involved in various physiological processes, the behavioural response being one of the many. In the present study nitric oxide donor sodium nitroprusside (SNP) (0.1mg/kg body weight), nitric oxide inhibitors NG-nitro-l-arginine-methyl-ester (L-NAME) (5mg/kg body weight), and selective neuronal nitric oxide

synthase inhibitor 7-nitroindazole (7-NI) (10mg/kg body weight) was administered to 8-week-old adult male and female mice along with their respective controls, normal saline for SN and L-NAME treated animals while dimethyl sulphoxide:normal saline (DMSO:NS – 1:1) for 7-NI treated animals for 14 days. These male and female animals were then subjected to a battery of behavioural tests such as elevated plus maze (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT) to study the anxiety- and depression-like behaviour and changes in the hypothalamo-hypophyseal-gonadal (HPG) axis in these NO donor and NOS inhibitors administered animals. All the behavioural tests were performed in the light phase of the light-dark cycle and an interval of 4-7 days was maintained between each behavioural test. The behavioural tests in females were performed when they were in the diestrus stage of the estrous cycle. All the male and female animals were sacrificed as per approved animal ethics protocol following the completion of behavioural tests. The study shows that there are significant changes in the treatment groups, showing more anxiolytic and depression-inducing effects in the L-NAME and 7-NI treatment groups in both males and females. The sex difference was also observed in these behaviour tests. Crystal violet staining of the brain sections shows alteration in the distribution of neurons in the hypothalamic nuclei (preoptic area – POA, suprachiasmatic nucleus – SCN, paraventricular nucleus – PVN and arcuate nucleus) in the L-NAME and 7_NI treated male and female animals when compared to the control group. L-NAME and 7-NI administration to male and female animals also had inhibitory effects on the morphological and cellular organization of the testes and the ovaries when compared to SNP-treated and control groups. The level of plasma testosterone, sperm count and plasma estradiol along with the total nitrate-nitrite concentration in plasma, testes and ovaries were significantly reduced in L-NAME and 7-NI treated male and female animals when compared to control groups. Thus, it may be concluded that inhibition of nitric oxide synthase by L-NAME and 7-NI and supplementing the animals with NO donor-SNP has a marked influence on the hypothalamo-hypophyseal-adrenal (HPA) axis as well as HPG axis in terms of alteration in anxiety and depression-like behaviour and gonad physiology. It appears that nitric oxide plays a major role in the interaction of the HPA and

HPG axis for the maintenance of body homeostasis. However, the molecular mechanism and pathway need to be explored further.

Chapter-3

Embryonic Disruption of Neuronal Nitric Oxide Synthase Alters Hypothalamus and Gonadal Development in Mice

Abstract

The growing fetus is susceptible to changes in its environment during embryogenesis, which can greatly affect its development. The neural circuitry in the brain along with environmental, psychological and genetic factors are responsible for the control of embryonic development of various systems of the body, which is regulated via numerous neuromodulators and neurotransmitters. Nitric oxide, one of the neurotransmitters has been shown to influence embryonic development in mammals. In the present study, male and female mice were time mated in the evening hours (16:00 hrs) and the next day female animals were observed for vaginal plugs in the early morning. Female animals with vaginal plugs were considered to be pregnant. Experiments were then performed to see the effects of inhibition of neuronal nitric oxide synthase (nNOS) by administering a specific nNOS inhibitor, 7-nitroindazole to these pregnant female mice from embryonic day 11 to embryonic day 17 along with pregnant female mice treated with dimethylsulphoxide:normal saline (DMSO:NS – 1:1) which acted as the control. Pups born to these pregnant female mice were sacrificed on postnatal day 0 (P0), postnatal 7 (P7), postnatal day 14 (P14) and postnatal day 21 for studying the changes in the structure of the hypothalamic nuclei and gonad (testes and ovaries) physiology. Results show that there were significant changes in the pattern and distribution of hypothalamic nuclei (preoptic area – POA, suprachiasmatic nuclei – SCN, paraventricular nuclei – PVN and arcuate nucleus – ARCN) and inhibition of the development of the morphological and cellular structures of testes and ovaries in the males and females respectively born to 7-NI treated pregnant female mice from postnatal day 0 to postnatal day 21. Thus, it may be concluded that inhibition of neuronal nitric oxide synthase during embryonic development

alters the development of the brain and gonads in both males and females by inhibiting the neuronal circuitry responsible for the interaction between the hypothalamo-hypophyseal-gonadal (HPG) axis and have a major effect on HPG axis development and its consequent effects in adulthood. However, the molecular mechanism responsible for the inhibition of neuronal circuitry during embryonic development needs to be further elucidated.

Thus, to maintain homeostasis in the body, the hypothalamus in the brain acts as the main coordinating centre. It also regulates the stress response by HPA axis and also maintains the reproductive behaviour through HPG axis. In the hypothalamus, there are various nuclei that coordinate the functions of the hypothalamus. Of such nuclei, the ones involved in the HPA axis are the paraventricular nucleus (PVN), which secretes corticotropin-releasing hormone (CRH), and the suprachiasmatic nucleus, the biological clock of the body that maintains the circadian rhythm by receiving inputs from the retina influences both the PVN as well as the POA. HPG axis has gonadotropin-releasing hormone (GnRH) as its main orchestrating hormone, this hormone is released from the preoptic area (POA) of the hypothalamus and stimulates the anterior pituitary to release gonadotropes. Arcuate nucleus due to its positioning has various terminals from the different nucleus of the hypothalamus and hence is involved in this hormonal crosstalk of the different regions of the hypothalamus and hence is implicated in the regulation of both CRH release from the PVN as well as GnRH release from the POA. These regions are interconnected to each other and helps regulate the anxiety and depression-like behaviour as well as the production of gonadal hormones and nitric oxide. When nitric oxide a key modulator of the brain function is disrupted by the in-utero inhibition of its enzyme nNOS by its specific inhibitor, 7-Nitroindazole, it is observed that there is a major disruption in the downstream functioning both HPA and HPG axis. Disrupting the neuronal nitric oxide synthase (nNOS) by specific nNOS inhibitor 7-Nitroindazole to pregnant mice, during the period of embryonic development has an anxiolytic and depressive effect on the pups born to the mother. The disruption during the embryonic stage has a long-term effect on the animals, evident from the significant change in the brain and gonad histology. The differences were also apparent in the behavioural pattern of the animals,

showing more anxiety-like and depression-like behaviour. These results illustrate that the in-utero exposure to nNOS inhibitor had serious alterations in both HPA and HPG axis which prolongs until adulthood as well. Inhibition of nitric oxide synthase by L-NAME and 7-NI and supplementing the animals with NO donor-SNP has a marked influence on the hypothalamo-hypophyseal-adrenal axis as well as hypothalamo-hypophyseal-gonadal axis in terms of alteration in anxiety and depression-like behaviour and gonad physiology. It appears that nitric oxide plays a major role in the integration of the HPA and HPG axis for the maintenance of body homeostasis. In-utero exposure to specific nNOS inhibitors also led to an alteration in the structure of hypothalamic (POA, SCN, PVN and Arcuate) nuclei and gonads (testes and ovaries). This further suggests that nitric oxide is important for neuronal and gonadal development and its inhibition during embryonic development may have serious implications in adulthood such as anxiety and depression and reproductive failure in both males and females. With the observations in this study we can propose that in the crosstalk of various hypothalamic nuclei in the modulation of comorbid anxiety and depressive behaviour, nitric oxide is one of the key players having implications from embryonic development itself and also during adulthood.

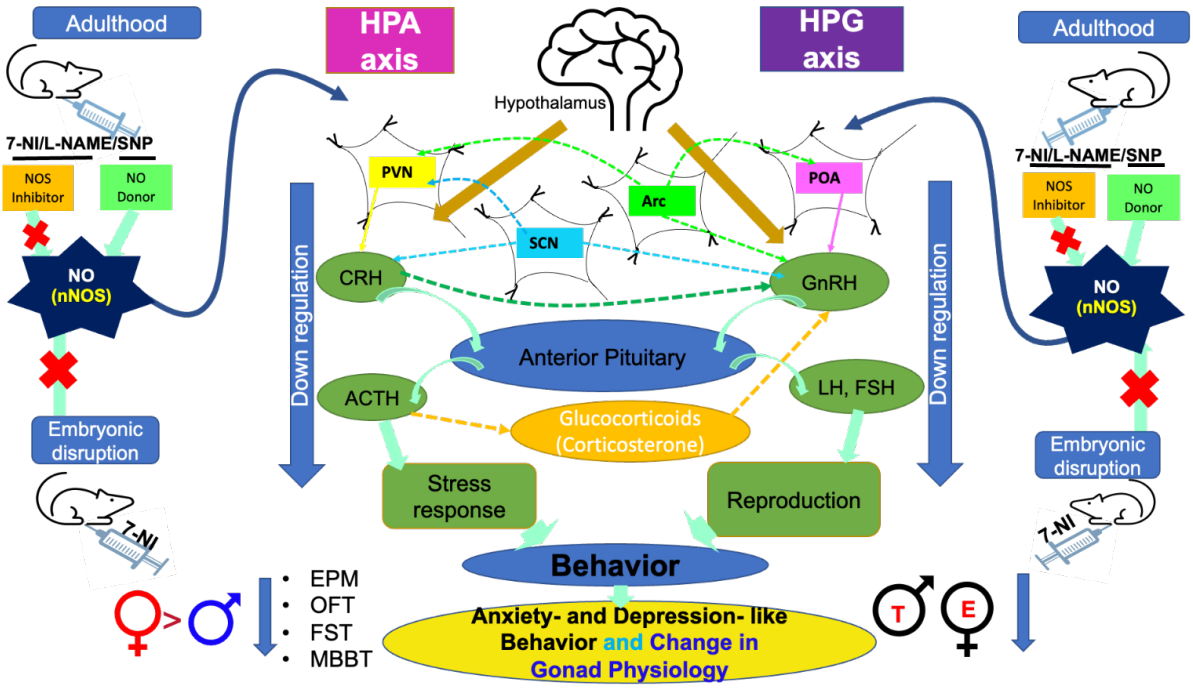


Figure: Image summarising the effects of neuronal nitric oxide synthase (nNOS) inhibition in-utero and in adults on anxiety and depression-like behaviour and gonadal physiology in adulthood via disruption in the Hypothalamo-Hypophyseal-Adrenal (HPA) axis and Hypothalamo-Hypophyseal-Gonadal (HPG) axis. 7-NI = 7-Nitroindazole; L-NAME = NG-nitro-l-arginine-methyl-ester; SNP = Sodium Nitroprusside; HPA = Hypothalamo-Hypophyseal-Adrenal Axis; HPG = Hypothalamo-Hypophyseal-Gonadal Axis; POA = Preoptic Area; SCN = Suprachiasmatic Nucleus; PVN = Paraventricular Nucleus; CRH = Corticotrophin-releasing Hormone; GnRH = Gonadotrophin-releasing Hormone; ACTH = Adrenocorticotropic Hormone; LH = Luteinizing Hormone; FSH = Follicle Stimulating Hormone; EPM = Elevated Plus Maze test; OFT = Open Field Test; FST = Forced Swim Test; MBBT = Marble Burying Behaviour Test; T = Testosterone; E = Estradiol

Role of Nitric Oxide in Anxiety and Depression-Like Behaviour in Mice

A Thesis Submitted to Rajiv Gandhi University for the Award of the Degree of Doctor of Philosophy in the Department of Zoology

Rajiv Gandhi University  **राजीव गांधी विश्वविद्यालय**

Year: 2022

Submitted By:
Hage Konya, M.Sc.
Enrollment No.: RGU/RS/559/2014
Department of Zoology
Rajiv Gandhi University
Rono Hills, Doimukh
Aruanchal Pradesh

Ph.D. Supervisor
Pankaj Kumar, Ph.D.
Associate Professor
Department of Zoology
Rajiv Gandhi University
Rono Hills, Doimukh
Itanagar - 791112
Aruanchal Pradesh

Chapter-3

Embryonic Disruption of Neuronal Nitric Oxide Synthase Alters Hypothalamus and Gonadal Development in Mice

Abstract

The growing fetus is susceptible to changes in its environment during embryogenesis, which can greatly affect its development. The neural circuitry in the brain along with environmental, psychological and genetic factors are responsible for the control of embryonic development of various systems of the body, which is regulated via numerous neuromodulators and neurotransmitters. Nitric oxide, one of the neurotransmitters has been shown to influence embryonic development in mammals. In the present study, male and female mice were time mated in the evening hours (16:00 hrs) and the next day female animals were observed for vaginal plugs in the early morning. Female animals with vaginal plugs were considered to be pregnant. Experiments were then performed to see the effects of inhibition of neuronal nitric oxide synthase (nNOS) by administering a specific nNOS inhibitor, 7-nitroindazole to these pregnant female mice from embryonic day 11 to embryonic day 17 along with pregnant female mice treated with dimethylsulphoxide:normal saline (DMSO:NS – 1:1) which acted as the control. Pups born to these pregnant female mice were sacrificed on postnatal day 0 (P0), postnatal 7 (P7), postnatal day 14 (P14) and postnatal day 21 for studying the changes in the structure of the hypothalamic nuclei and gonad (testes and ovaries) physiology. Results show that there were significant changes in the pattern and distribution of hypothalamic nuclei (preoptic area – POA, suprachiasmatic nuclei – SCN, paraventricular nuclei – PVN and arcuate nucleus – ARCN) and inhibition of the development of the morphological and cellular structures of testes and ovaries in the males and females respectively born to 7-NI treated pregnant female mice from postnatal day 0 to postnatal day 21. Thus, it may be concluded that inhibition of neuronal nitric oxide synthase during embryonic development alters the development of the brain and gonads in both males and females by inhibiting the neuronal circuitry responsible for the interaction between the

hypothalamo-hypophyseal-gonadal (HPG) axis and have a major effect on HPG axis development and its consequent effects in adulthood. However, the molecular mechanism responsible for the inhibition of neuronal circuitry during embryonic development needs to be further elucidated.

Introduction

The early embryonic development of the central nervous system is a precise network of events that are influenced by extrinsic (environment, toxic compounds, drugs, stress etc.) as well as intrinsic (Genetic, hormone etc.) factors (Chen et al., 2015). Disturbances in developmental processes in-utero may lead to anxiety and depression later in life and have been associated with exposure to stressful events during prenatal period, throughout childhood, and adolescence (Herbison et al., 2017). Central nervous system (CNS) of fetus and child is highly sensitive to persistent organic pollutants (POPs). Study on maternal exposure to one such POP, Nonylphenol (NP) during pregnancy and lactation activated microglia and increased the production of NO and prostaglandin E2 in the offspring hippocampus (Qui et al., 2019). Perinatal exposure to genistein, a phytosterone, during late pregnancy and early lactation has been shown to induce alterations in estrogen-dependent adult behaviours and NO-producing brain circuits implicating the control of these behaviours (Rodriguez-Gomez et al., 2014). Further, administration of a low dose of bisphenol A (BPA) during the perinatal period in animals hyperactivates the hypothalamo-hypophyseal-adrenal (HPA) axis, which leads to anxiety and depression-like behaviours in the adult, by inhibiting the HPA axis by the hippocampal glucocorticoid receptor (GR) mediated feedback and peripheral testosterone levels (Chen et al., 2015).

Further, in another study, Alvik et al. (2013), it has been reported that binge drinking in the early period of pregnancy causing high prenatal exposure of the fetus to high levels of alcohol consumption and may lead to neurobehavioral and cognitive problems (Alvik et al., 2013). Administration of alcohol in the postnatal days 4-10 to rat pups with higher blood alcohol concentrations (BACs) showed rapid brain growth resulting in significant microencephaly and cell loss in the brain (Bonthius and West, 1990). It has also been demonstrated that

maternal alcohol consumption during pregnancy can also have a negative impact on the development of the fetal brain (Burger et al., 2011; Hepper et al., 2012; Workman et al., 2015; Sanou et al., 2017; Wozniak et al., 2019; Tychkivska, et al., 2019). Alcohol exposure in-utero has been shown to negatively impact a multitude of cognitive domains including learning and memory, adaptive functioning, motor function, attention and activity levels, language development, visual perception and construction, executive function and overall general intelligence (Nayak and Murthy, 2008; Workman et al., 2015; Seleverstov et al., 2017). Further, it has also been demonstrated that there is a high comorbidity rate with other learning and behavioural processes when there is prenatal exposure to alcohol (Mattson et al., 2019). Thus, it has been proposed that alcohol acts as a teratogen during early embryonic development that may impact fetal development and thus negatively affect the fetus, leading to fetal alcohol spectrum disorders (FASD).

Further, Silveira et al. (2022), reported that supplementation of folic acid during pregnancy impaired memory, motricity, and deficient motor learning of the offspring. There was also an increase in anxiety- and depression-like behaviour in this group with a decrease in the total number of entries in the elevated plus maze and an increase in the latency for the first bite in the eating-related depression test (ERDT). Intake of folic acid by pregnant rats also resulted in an increase in nitrative stress and neuroinflammation in the cerebral cortex of the offspring, FASD throughout mating, pregnancy and lactation resulted in short-term memory impairment, decreased hippocampal size and decreased thickness of the dentate gyrus (Sittig et al., 2012; Mikael et al., 2013; McGarel, 2015; Bahous et al., 2017). It has also been shown that prenatal stress in pregnant female mice increases their vulnerability to neurodevelopmental disorders. Male pups born to early stress-exposed gestational females display maladaptive behavioural stress responsivity, anhedonia, and increased sensitivity to selective serotonin reuptake inhibitor treatment and also an elevated stress sensitivity (Cratty et al., 1995; Watson et al., 1999; Bale et al., 2000; Bale and Vale, 2004; Meaney et al., 2007; Mueller and Bale, 2007; Mueller and Bale, 2008; Darnaude´ry and Maccari, 2008). Alterations in corticotrophin-releasing hormone (CRH) and glucocorticoid receptor (GR) expression along with an

increase in hypothalamo-hypophyseal-adrenal axis were observed in these male mice. It was further shown that the male vulnerability to early prenatal stress may involve sex-specific placenta responsivity suggesting sex dependent response (Mueller and Bale, 2007; Mueller and Bale, 2008; Francis-Oliveira et al., 2013; Grundwald and Brunton, 2015; Verstraeten et al., 2019; Lam et al., 2019). Thus, sex-specific programming starts very early in the development process of the animals and stress and exposure to chemicals and compounds in-utero may have a negative impact on the overall development of the animal (Hellemans et al., 2010; Vedhara et al., 2012; Veru et al., 2014; Abuaish et al., 2021; Cusick et al., 2022). In humans, exposure to endocrine disrupting chemicals like bisphenol A, phthalates, triclosan, and perfluoroalkyl substance during potentially sensitive periods of development results in adverse neurobehavioral outcomes in children by disrupting hormonally mediated processes critical for growth and development during gestation, infancy, or childhood (Braun, 2017). Further, Di-n-butyl phthalate (DBP), an endocrine-disrupting compound has been reported to possibly suppress NOS/cGMP pathway in the penis of Sprague-Dawley rats prenatally exposed to this compound, even in a low dose. It resulted in penile fibrosis, decreased testosterone level, and endothelial dysfunction (Zhou et al., 2021). Prenatal exposure to polychlorinated biphenyls (PCBs), a class of endocrine-disrupting chemicals shows sexual differences where males show reduced anxiety-like behaviours and increased activity in the light:dark box in adulthood (Gillette et al., 2017). Prenatal lipopolysaccharide (LPS)-exposure led to increased anxiety and depressive-like behaviors in the adult offspring. There is also an indication of correlation of prenatal administration of LPS with the oxidative stress in adult lives shown by decreased levels of antioxidant enzymes such as superoxide dismutase, catalase and glutathione, while an increased level of lipid peroxidation, protein oxidation products, and NO in the adult lives. Increased level of toxic free radicals is also responsible for brain and liver damage (Al-amin et al., 2016). Primary culture of cerebellar neurons of rats with prenatal exposure to aluminum (Al) by administering aluminum to pregnant female in drinking water caused prevention of glutamate-induced proteolysis of the microtubule-associated protein-2, disaggregation of microtubules, and neuronal death, indicating an impairment of NMDA receptor-associated signal

transduction pathways (Llansola et al., 1999). It was also suggested that alterations in the expression of proteins of the glutamate–nitric oxide–cGMP pathway could be responsible for some of the neurotoxic effects of aluminum. In a study on perinatal cerebral ischemia, the developmental role of nitric oxide in the cerebral blood flow response to NMDA was investigated at mid- and late gestation in fetal sheep and the outcome suggested that NO contributes to the basal blood flow and increase in the neurovascular coupling to activation of NMDA receptors in neocortex during the last trimester in fetal sheep, indicating that the developmental increases in the role of NO in neurovascular regulation are specific for NMDA-receptor activation (Harris et al., 2008).

In the pregnant rats administered with tamoxifen one day prior to labour (E21) and on the childbirth day (E22), hippocampi of embryos at E22 and new-borns at postnatal days of 1, 7, and 21 (P1, P7, and P21) reveal that the cellular density was lower in early stages of development, however, cellular density and thickness gradually increased during the development, particularly in the third week. Also, nNOS expression was decreased in E22, P1, and P7 in animals treated with tamoxifen indicating that tamoxifen affects the development and differentiation of postnatal rat hippocampus, CA1 neurons, and nNOS expression (Nobakht et al., 2011). Further, female nNOS-CreER mice exposed to a single dose of the sodium salt of valproic acid (VPA) during pregnancy and offspring sacrificed and processed on the postnatal 35-day show a decrease in synapse-associated surface proteins of nNOS interneurons following VPA treatment, simultaneously there was a down expression of neuronal activity-regulated pentraxin (Narp), glutamate receptor 4 (GluA4) and protein kinase C gamma (PKCγ) surface protein in nNOS interneurons in VPA-treated mice suggesting an imbalance of synaptic transmission in autism spectrum disorder (ASD).

Thus, all these studies show that gestational (prenatal) exposure or perinatal exposure to various compounds and stressors have adverse effects on the behaviour and developmental process in the pups born to pregnant mothers exposed to external compounds or stressors. However, there is a paucity of information on the inhibition of nitric oxide synthase by specific nNOS

inhibitors in the process of the embryo and postnatal developmental processes, especially the development of the brain and gonads (testes and ovaries). Thus, in the present study, pregnant female mice were administered with a specific nNOS inhibitor at a dose of 10mg/kg body weight from embryonic day 11 to embryonic day 17 and its effect on the development of the brain and gonads (testes and ovary) were observed at postnatal day 0 (P0), postnatal day 7 (P7), postnatal 14 (P14) and postnatal 21 (P21).

Material and Methods

Animals

Adult Male and female mice were time mated and pregnancy was confirmed by checking the vaginal plug. 5 pregnant female mice were administered with 7-Nitroindazole from embryonic day 11 to 17 while five (5) control pregnant mice were treated with a solution of normal saline and dimethyl sulfoxide (1:1), which was used as a vehicle. The pups born at postnatal days 0, 07, 14 and 21 were perfused as described in General Material and Methods and brain and gonads (testes and ovaries) were collected from the perfused animals. Light dark cycle 12L:12D was maintained and food and water were available ad libitum. The experimental design for the present study is depicted in **Figure 1**. All the experiments were performed as per the Animal Ethics Committee of Rajiv Gandhi University and as per the guidelines within the framework of the revised Animals (Scientific Procedures) Act 2002 (CPCSEA Guidelines) of the Government of India.

Crystal Violet Staining of Brain Sections

6μm thick sections of the brain (both males and females) were cut on a rotary microtome and processed for crystal violet staining as per the protocol described in General Material and Methods

Histology of Gonads (Testes and Ovaries)

6μm thick sections of the gonads were cut on a rotary microtome and processed for Hematoxylin and Eosin staining as per the protocol described in General Material and Methods.

Result

Effect on the Different Nucleus of the Hypothalamus

Not much sex differences are observed in the distribution of neurons in the POA of the brain of the animals sacrificed at postnatal day P0 and P7, but between the control and 7-NI treated animals, there is a reduction in the number and distribution of cells (**Figure 2**). In the animals on postnatal days 14 and 21, sex differences begin to occur in the distribution of neurons in POA. In the SCN (**Figure 3**) and PVN (**Figure 4**), the sex difference and difference between the control and 7-NI treated group can be seen from postnatal day 0 onwards. The arcuate nucleus has more cells in female than in male animals (**Figure 5**).

Effect on Testes Histology

The histological section of the testes shows the intact arrangement of seminiferous tubules in control, whereas the arrangement is disturbed in the 7-NI treated males since P7. Even the arrangement and distribution of spermatogonial cells and Leydig cells are disturbed in 7-NI treated males in all the stages of development i.e., P0, P7, P14, P21 (**Figure 6** and **Figure 7**).

Effect on Ovarian Histology

P0 and P7 stage ovary show the uneven arrangement of the primordial cells in 7-NI treated females, whereas, in the p14 stage, there is a lesser number of follicles in the treatment group than in the control. In the P21 stage follicles can be seen in different growing stages i.e., primordial, primary and secondary stages with distinct antrum in the control animals, but in 7-NI treated animals, follicles are in either primordial or primary stages, suggesting a delay in development (**Figure 6** and **Figure 8**).

Discussion

Development as a whole is majorly influenced by genetic as well as environmental factors (Cetin et al., 2010; Nelissen et al., 2011; Hocher, 2014; Baldacci et al., 2018; Goyal et al., 2019; Besenfelder et al., 2020). Even a small change in biological processes during the critical period of development can have an adverse effect on the adulthood of the animal (Hoffman et al., 2021; Heindel et al., 2015). In the present study, an attempt was made to disrupt such processes by inhibiting neuronal nitric oxide synthase, an important neuromodulator involved in the development of the brain and gonad (Bredt and Snyder, 1994; Bertini and Bentivoglio, 1997; Gibbs, 2003; Bellefontaine et al., 2011; Ling et al., 2012; Xu et al., 2012). Histological results of different regions of the hypothalamus and also testis and ovary in the present study demonstrate that maternal inhibition of neuronal nitric oxide synthase (nNOS) during the critical period of development (E11 to E17) had a significant postnatal effect. In the rat hypothalamus, neurogenesis occurs in three waves, E13-E15 (corresponding to E11-E13 in mouse), the first wave, when the neurons form lateral hypothalamic structures, E15-E17, the second wave, when the neurons become part of the medial hypothalamus and E17-E19, the final wave, when the periventricular hypothalamic population is formed with exception of PVN formation which is nearly completed before E15 (Altman and Bayer, 1986; Bless et al., 2005; McClellan et al., 2008; McClellan et al., 2010; Stratton and Tobet, 2020).

Results in the preoptic area of the hypothalamus, containing GnRH neurons, formed between E10 to E13 (Miller and Nowakowski, 1988; Okamura et al., 1990; Schwanzel-Fukuda and Pfaff, 1989; Lee et al., 2008) a major regulator of the reproductive axis (Miller et al., 2002), show a significant reduction in the neuronal cell number during different developmental stages P0, P7, P14 and P21 in the pups exposed in-utero to 7-NI. Brain regions also show gender differences (Pakkenberg and Gundersen, 1997; Goldstein et al., 2001; Kaufmann et al., 2001; Cosgrove et al., 2007; Zaidi, 2010; Xin et al., 2019). POA show gender differences in P14 and P21, with males showing a greater number of cells than female. GnRH neuron terminals are also found in the

arcuate nucleus of the hypothalamus, making it a key regulator of the anterior pituitary and subsequently in the release of LH and FSH (Plant, 2019). Arcuate nucleus cell numbers were also observed to be more in the control than in the 7-NI treated groups. This discrepancy in the hypothalamic region controlling GnRH release is also reciprocated in the gonads. In the testis, it is observed that there is a significant reduction in the size of the seminiferous tubules from P0 onwards with considerable gaps between the otherwise intact tubules. In the P21 stage, the differences become more evident with spermatogonial cells in the testis of the 7-NI treated animal showing wide intracellular spaces and the germinal cells being detached from the basement membrane. The number of Leydig cells is also sparse, Leydig cell is the most important cell type for endocrine function of the testis (Svingen and Koopman, 2013), this reduction in the number of Leydig cells may be attributed to lower testosterone levels leading to further delay or alteration in the development of testis (Rolf et al., 2002). During the process of developmental events of the gonads, the primordial germ cells (PGC) migrates from the endoderm of yolksac to the developing gonad around days 7 to 11 (Anderson et al., 2000) and the ovary is first apparent in the day 10.5 in mice as a thickening of coelomic epithelium (Smith et al., 2014), and gonadal sex differentiation is considered to occur at day 12 (Menke et al., 2003). In the present study, in the ovary of P0 and P7 mice, primordial cells are seen to be uniformly arranged towards the periphery, but in the 7-NI treated mice, the distribution of primordial cells is disturbed. In the P14 control mice, more primary follicles are observed with distinctly arranged follicular cells than in experimental female mice. Follicles in different stages of development i.e., primary follicle and secondary follicle up to the late antral stage are observed in the mice born to pregnant females administered with vehicle control, whereas in pups born to 7-NI treated pregnant females, the number of follicles is very less, and all are in the primary follicular stage. This alteration in the ovary of 7-NI treated mice is indicative of abnormal or delayed folliculogenesis which could in adulthood be manifested to the disruption in estrous cycle and impaired fertility (Klein et al, 1998).

Neurons in the paraventricular nucleus is responsible for the release of corticotropin-releasing hormone (CRH) involved in the stress axis (Daviu et al.,

2020). In the current study, there appears to be an apparent decrease in the number and distribution of cells in the 7-NI treated animals in both males and females of all the stages when compared with their control. CRH is the chief hormone for activation of the HPA axis that triggers the secretion of glucocorticoids. Glucocorticoids further act on multiple organ systems to prepare the body to respond to the stressor (Herman et al., 2016). An increase in the nerve cells in PVN may be suggestive that there could be increased activity in the HPA axis, which is also responsible for various mood and cognitive disorders (Keller et al., 2017). As reported in earlier studies, animals with mood disorders have increased activity of the HPA axis (Swaab et al., 2005).

Cognitive and mood disorders greatly affect the cardiovascular regulation, respiration, appetite control and sleep pattern of the animal, implying SCN, the biological clock of the brain responsible for maintaining the circadian rhythm also has a role to play in the HPA axis (Swaab et al., 2005). Our study shows a reduction in the cell number in 7-NI treated animals than in the control group, indicative of the disruption of the biological rhythm of the animal. There is also a gender-dependent difference in the distribution of neural cells, being reduced in females than in males.

Thus, it may be concluded that in-utero exposure to a specific nNOS inhibitor leads to alteration in the structure of hypothalamic (POA, SCN, PVN and Arcuate) nuclei and gonads (testes and ovaries). This further suggests that nitric oxide is important for neuronal and gonadal development and its inhibition during embryonic development may have serious implications in adulthood such as anxiety and depression and reproductive failure in both males and females.

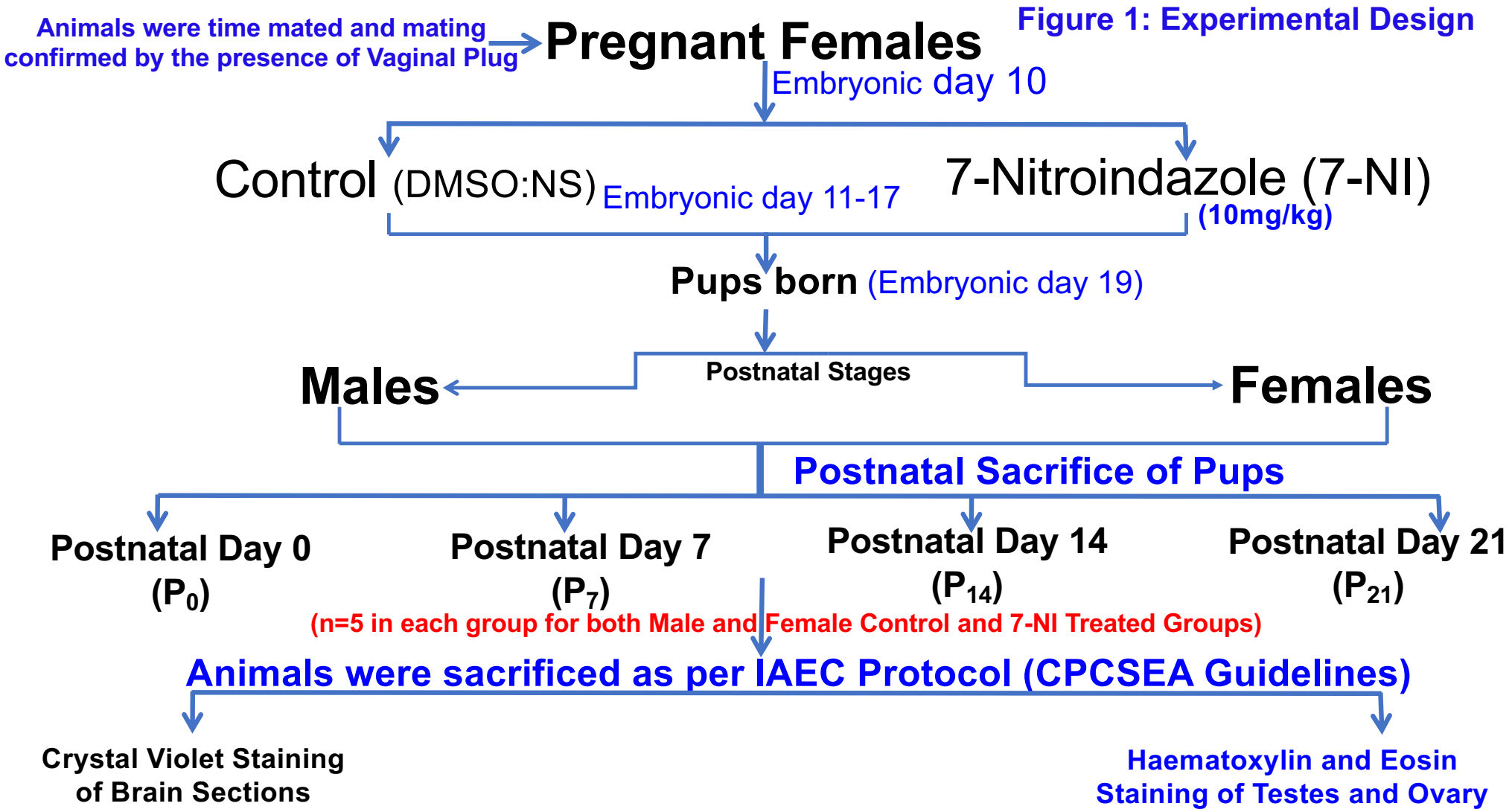


Figure 1: Experimental Design

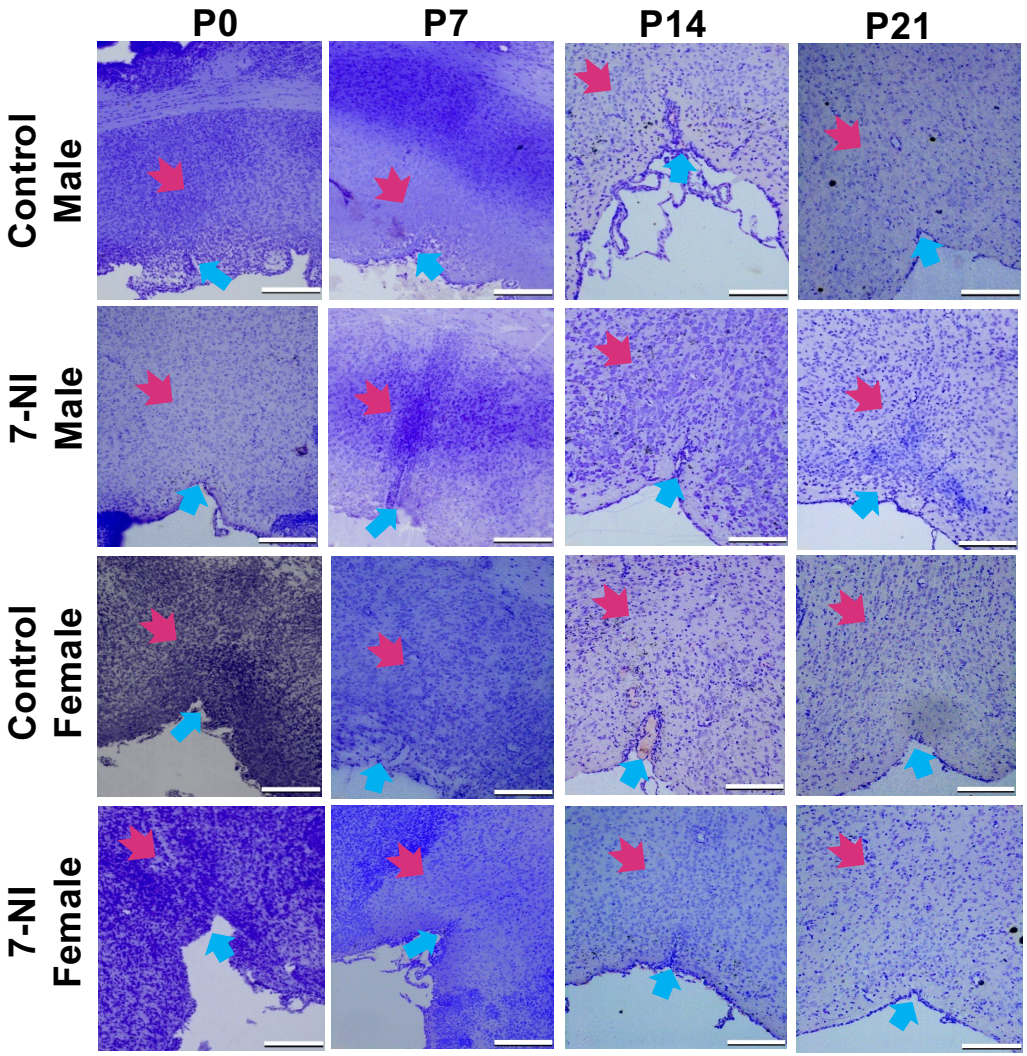


Figure 2: Representative images of brain sections showing preoptic area (POA) of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage during embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day P0, P7, P14 and P21, to study the changes in hypothalamic nuclei. Scale bar = 100μm.

Key: ⬇ OVLT (Organum Vasculosum Lamina Terminalis)

➧ Preoptic Area (POA)

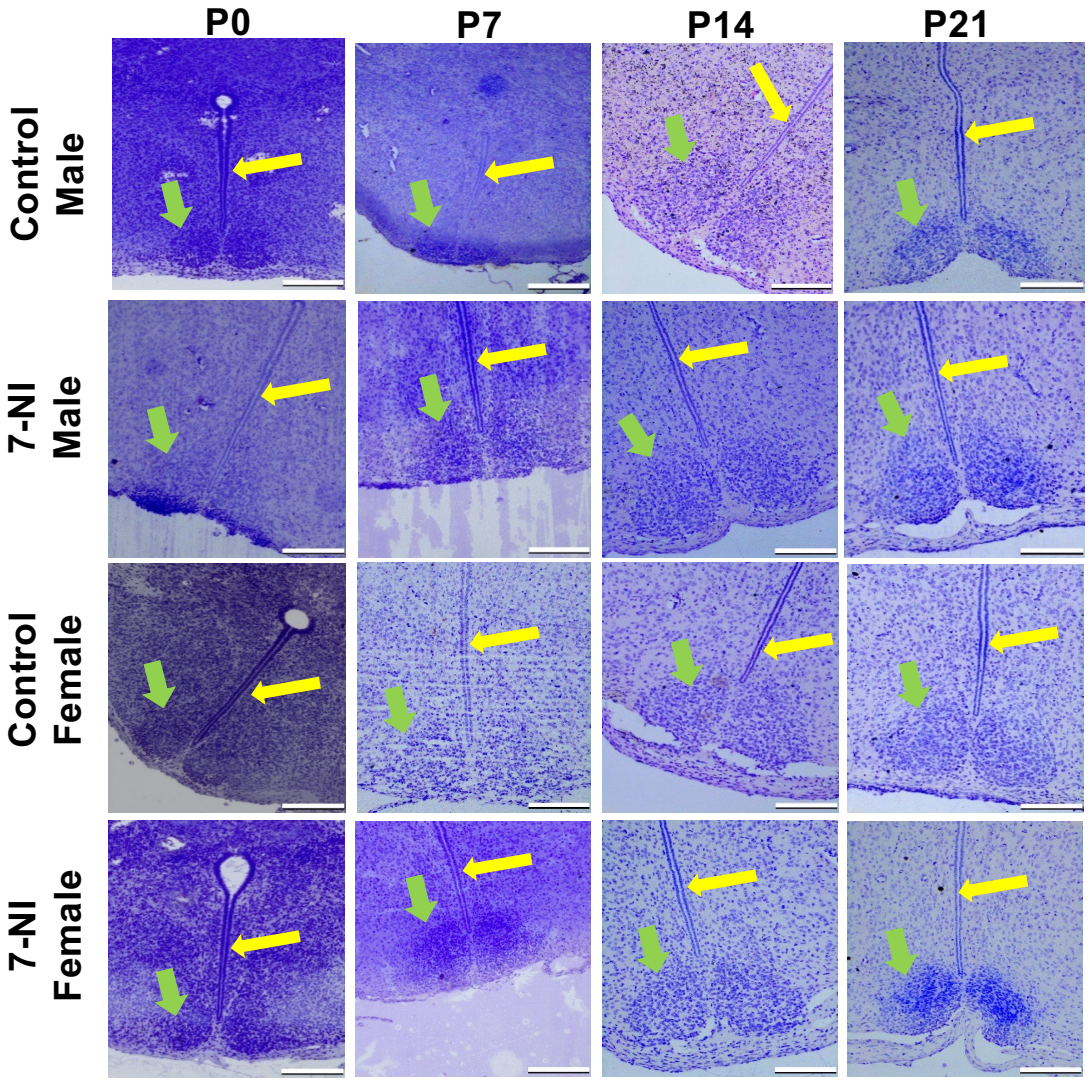


Figure 3: Representative images of brain sections of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day P0, P7, P14 and P21 to study the changes in hypothalamic nuclei. Scale bar = 100μm.

Key: Third Ventricle
Suprachiasmatic nucleus (SCN)

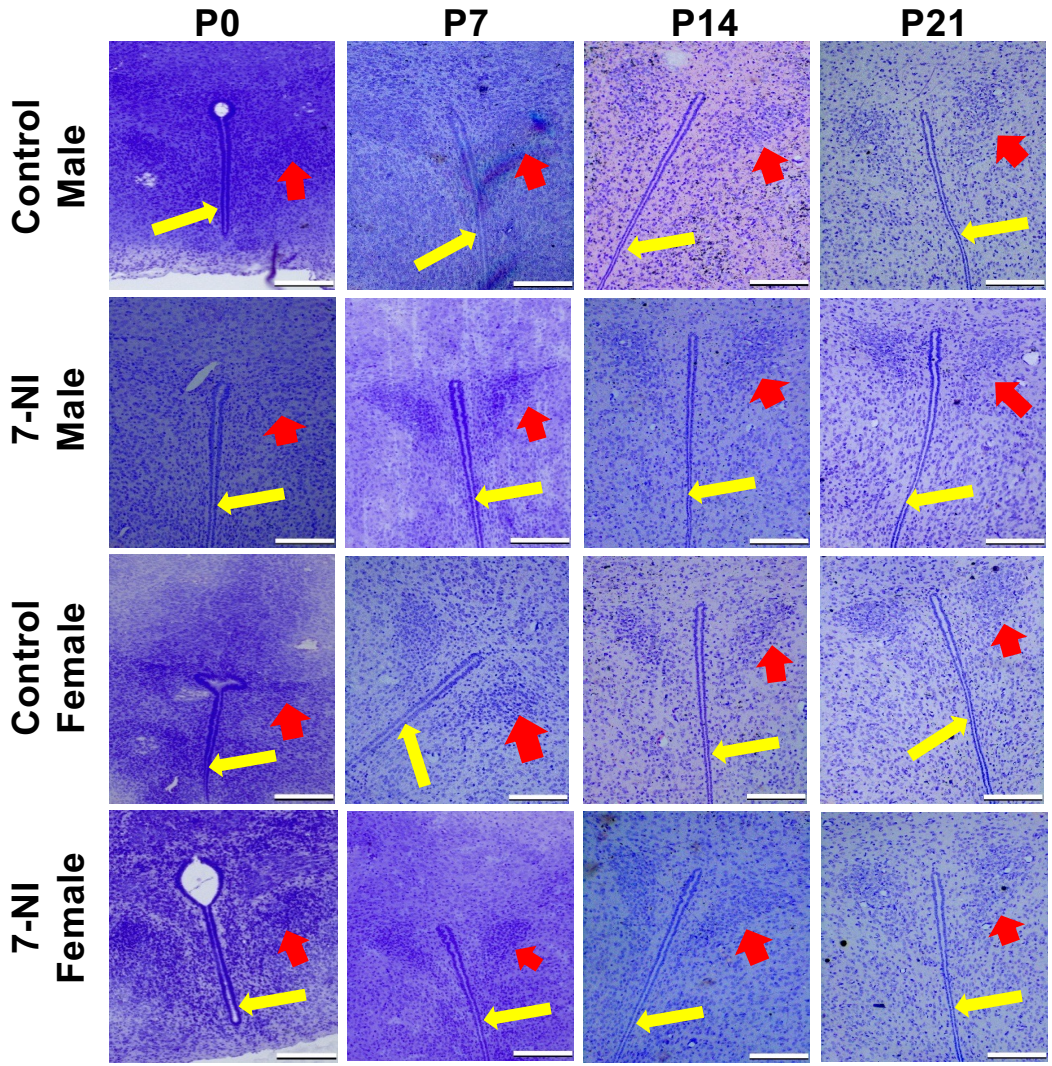


Figure 4: Representative images of brain sections of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day P0, P7, P14 and P21 to study the changes in hypothalamic nuclei. Scale bar = 100μm.

Key: Third Ventricle

Paraventricular nucleus (PVN)

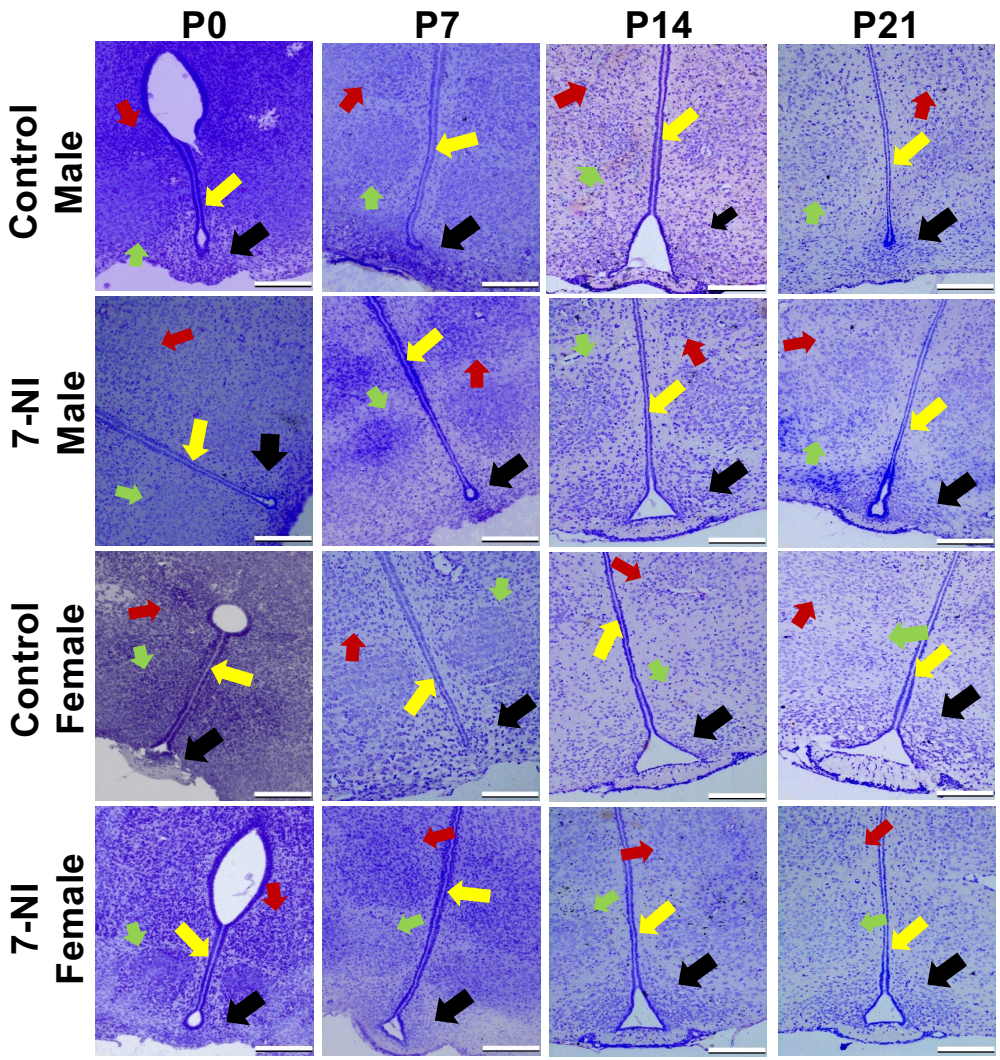


Figure 5: Representative images of brain sections of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at post natal day P0, P7, P14 and P21 to study the changes in hypothalamic nuclei. Scale bar = 100μm.

Key:
- Third Ventricle
- Arcuate nucleus (Arc.)
- Dorso medial hypothalamic nucleus (DMH)
- Ventro medial hypothalamic nucleus (VMH)

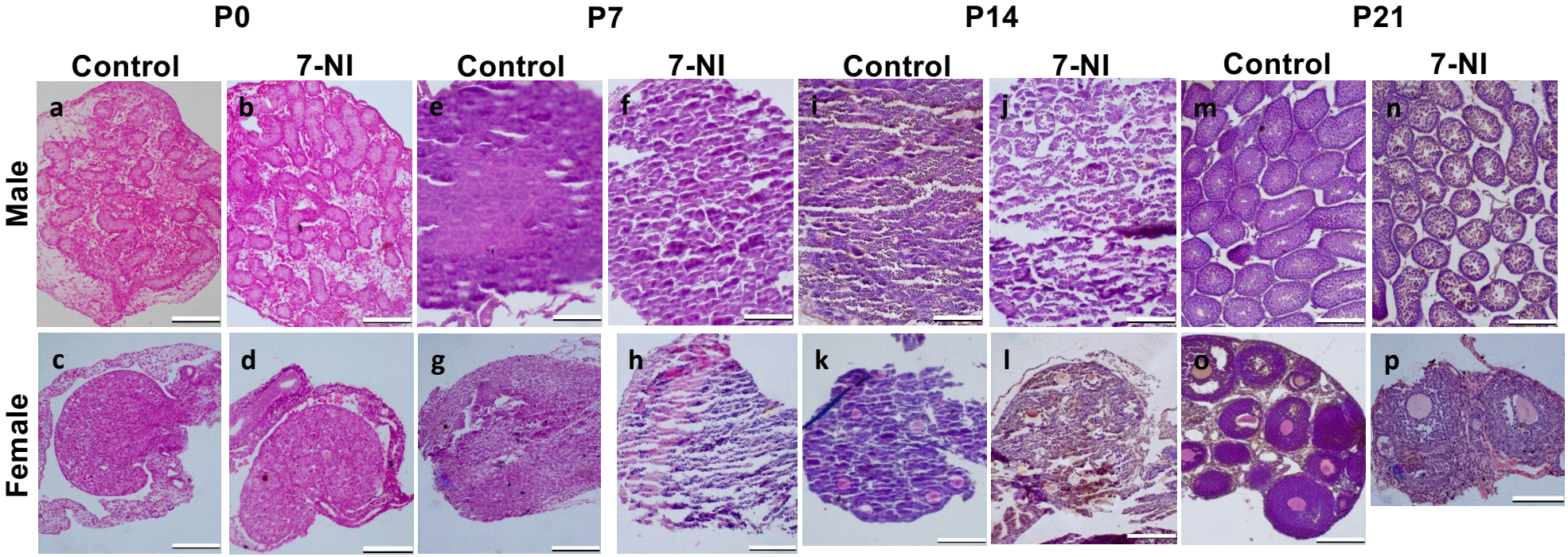


Figure 6: Representative images of transverse sections of adult male and female mice testes and ovaries, respectively born to pregnant female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO: NS 1:1) (designated as Control Male and Control Female) from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day 0, 7, 14 and 21 to study its effects on gonadal (Testis and Ovary) development. Scale bar = 100μm.

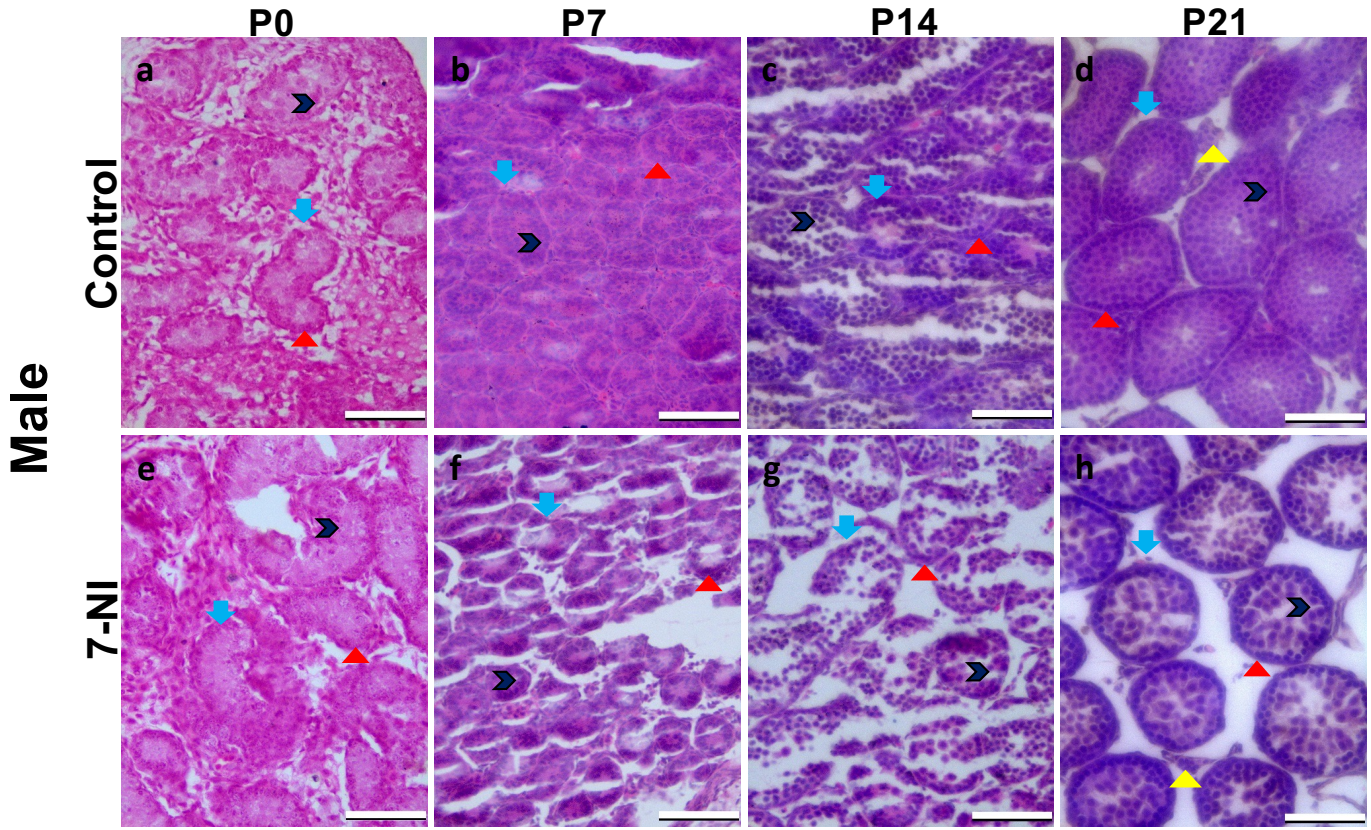


Figure 7: Representative images of transverse sections of testes of mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male) and vehicle control (DMSO:NS) (designated as Control Male) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day 0, 7, 14 and 21 for studying its effect on development of testis. Scale bar = 50μm.

Key: Seminiferous tubules, Spermatogonial cells, Leydig cells, Basement membrane

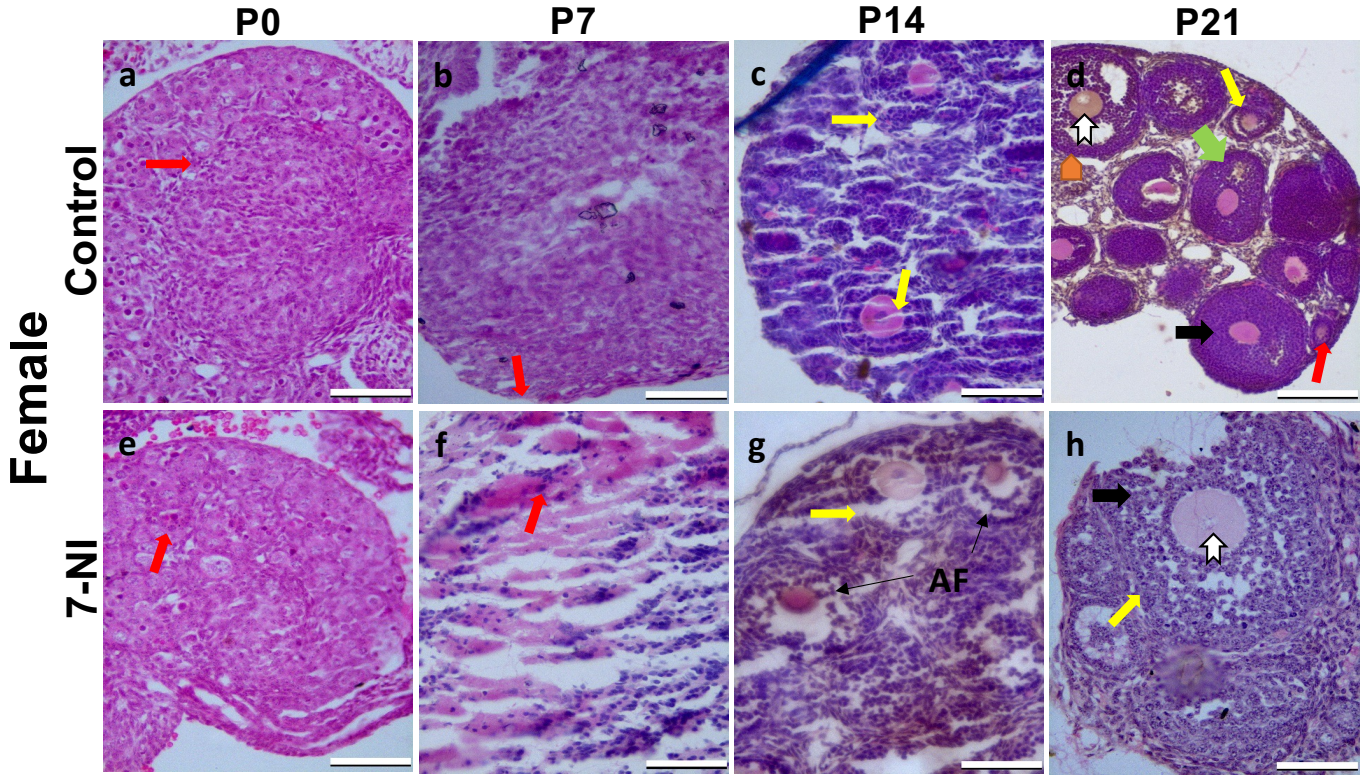


Figure 8: Representative images of sections of ovaries of mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at post natal day 0, 7, 14 and 21, for studying it's effects on follicular development in ovary. Scale bar = 50μm.

Key: **A,** Antrum, Primordial follicle, Primary follicle, Secondary follicle, **AF,** Atretic follicle, Graafian follicle, Granulosa cells, Ovum

Summary and Conclusion

According to the literature anxiety and depression are one of the major disorders in today's population due to the demands and rigour of the fast-paced lifestyle of the individuals in today's society. 2017 WHO report on depression and other common mental health reports that 3.6% of the global population suffers from anxiety and 4.4% from depression. Anxiety and depression are comorbid conditions and are accompanied by many other factors such as alteration in the perception of social reality and the ability to adapt in an individual, affecting the quality of life of the people suffering from such disorders. In the general population, these mood disorders have been shown to be gender biased, being more prevalent in females than males. There could be various factors responsible for the development of such conditions in an individual, it could be environmental, genetic as well as epigenetic. To maintain homeostasis, the body has an elaborate interconnected system constantly being regulated by various molecules. Among such systems are the hypothalamo-pituitary-adrenal axis, responsible for the regulation of stress in the body and the hypothalamo-pituitary- gonadal axis, responsible for the reproductive functions. These axes again have an intricate system of neural circuitry comprising of neuromodulators and neurotransmitters modulating its functioning. One such molecule is the ubiquitously present nitric oxide. This nitric oxide is implicated to be involved in various physiological processes through cyclic guanosine 3′5′-monophosphate (cGMP), including the functions of the brain. Nitric oxide is produced as a byproduct in the enzymatic conversion of L-Arginine to L-Citrulline in the presence of NADPH, cofactors and the enzyme nitric oxide synthase (NOS), NOS has three isoforms in the body (neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), all playing different roles in the body physiology. Of the three isoforms, nNOS expression has been found to be distributed in various brain regions such as the cerebellar cortex, dorsal raphe, cerebral cortex, amygdala, hippocampus, preoptic area and also paraventricular, magnocellular, the supraoptic nucleus of the hypothalamus. These regions, especially the amygdala, hippocampus, and dorsal medial thalamus of the subcortical limbic regions have also been reported to be associated with mood disorders such as anxiety and depression. nNOS has been

implicated in a varied range of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, anxiety, stroke and also learning and memory and neuropsychiatric disorders, including depression. NO produced in the brain is linked to be involved in regulating the HPA axis. On the other hand, NO is shown to be localized and expressed in the hypothalamus, hypophysis and gonads and can act on the hypothalamo-hypophyseal-gonadal (HPG) axis to regulate the synthesis and release of GnRH and thus reproduction, as GnRH and NO-producing neurons occupy similar positions in the hypothalamus. NO has also been reported to regulate spermatogenesis, sperm motility, sperm capacitation, fertilization, oogenesis (follicle development/folliculogenesis), gonadal hormones and steroidogenesis. Nitric oxide is also involved in the embryonic development of the brain and gonads, affecting the overall development of the HPA and HPG axis. Various studies involving knock-out models of mice and also different inhibitors of NOS and specific nNOS inhibitors, as well as nitric oxide donor agents, have shown that nitric oxide is intricately involved in the modulation of both the HPA axis and HPG axis and thus therefore involved in the pathology of neurodegenerative disorders and mood disorders and consequently affects the reproductive behaviour and fertility. But still there exist many lacunas in understanding the concrete aetiology of these disorders. One such lacuna is the involvement of influences during embryonic development affecting the behaviour in adulthood.

Therefore, we hypothesize that the embryonic disruption of the nNOS signalling may alter the hypothalamic (POA, SCN, PVN and Arc. Nucleus) development and subsequently altered HPA axis activity/negative feedback leading to mood disorders such as anxiety- and depression-like behaviour and HPG axis (reproductive physiology) in mice.

Objectives:

- Does restricted embryonic and adult nitric oxide synthase blockade cause altered HPA axis-dependent behaviours and alteration in HPG axis?

- Does restricted embryonic and adult nitric oxide synthase blockade cause alteration in hypothalamic cytoarchitecture and HPA axis function?

- Does nitric oxide signalling regulate neuron development in the developing hypothalamus?

To answer these above objectives the doctoral work has been divided into three chapters:

Chapter 1

Embryonic Disruption of Neuronal Nitric Oxide Synthase Alters Anxiety- and Depression-like Behaviour and Reproductive Physiology of Mice in its Adulthood

Abstract

Mood disorders like anxiety and depression are major contributors of the maladaptation of individuals to normal situations. To understand these disorders, anxiety and depression-like behaviour studies are done on animal models such as mice. There are many neuropeptides and neuromodulators known to influence the HPA axis, the stress axis, that are implicated in mood disorders like anxiety and depression. One such neuromodulator of the stress axis is nitric oxide. In the present study, the production of nitric oxide was inhibited by a specific inhibitor (7-Niroindazole, 7-NI) of its enzyme neuronal nitric oxide synthase (nNOS). Male and females were time mated in the evening hours (16:00 hrs) and a vaginal plug was observed the next day. Females showing vaginal plugs were considered pregnant. One group of pregnant females were kept in individual cages and were injected with 7-NI at a dose of 10mg/kg body weight from embryonic day 11 to embryonic day 17, while the other group was administered with vehicle control (Dimethlysulphuroxide:Normal Saline – DMSO:NS in 1:1 ratio) and served as the vehicle control group. The pups born to these pregnant females on day 19 were weaned on postnatal day 21, males and females were separated into different cages with individual ear punch markings for individual identification

of mice. These male and females mice were raised to adulthood until 8 weeks when these animals were exposed to a battery of behavioural tests such as elevated plus maze (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT). All the behavioural tests were done in the light phase of the light-dark cycle. The behavioural tests in females were performed when the animals were in the diestrus phase. An interval of 4-7 days was maintained between each behavioural test. After the end of the behavioural tests, each animal was anaesthetized and sacrificed for studying the changes in hypothalamic structures of the brain and changes in gonad physiology. Results show that there were marked changes in anxiety- and depression-like behaviour (as displayed by EPM, OFT, FST and MBBT), the hypothalamic nuclei of the brain, nitric oxide production (total nitrate-nitrite concentration), gonad (testes and ovary) physiology and plasma testosterone levels and sperm count in males and plasma estradiol levels in females in both males and females born to 7-NI treated females when compared to males and females born to vehicle-treated pregnant female groups. The result shows that administration of nNOS inhibitor in-utero in mice alters both hypothalamo-hypophyseal-adrenal (HPA) axis as well as the hypothalamo-hypophyseal-gonadal (HPG) axis in the embryo itself which prolongs until adulthood by disturbing the neural circuitry responsible for its maintenance. Thus, nitric oxide acts as an anxiolytic and anti-gonadotrophic agent in mice, however, the molecular mechanism needs further elucidation.

Chapter 2

Nitric Oxide Modulates Anxiety- and Depression-like behaviour and Hypothalamo-Hypophyseal-Gonadal Axis in Mice

Abstract

L-arginine (L-Arg)/nitric oxide (NO)/cGMP pathway is known to be involved in various physiological processes, the behavioural response being one of the many. In the present study nitric oxide donor sodium nitroprusside (SNP) (0.1mg/kg body weight), nitric oxide inhibitors NG-nitro-l-arginine-methyl-ester (L-NAME) (5mg/kg body weight), and selective neuronal nitric oxide

synthase inhibitor 7-nitroindazole (7-NI) (10mg/kg body weight) was administered to 8-week-old adult male and female mice along with their respective controls, normal saline for SN and L-NAME treated animals while dimethyl sulphoxide:normal saline (DMSO:NS – 1:1) for 7-NI treated animals for 14 days. These male and female animals were then subjected to a battery of behavioural tests such as elevated plus maze (EPM), open field test (OFT), forced swim test (FST) and marble burying behaviour test (MBBT) to study the anxiety- and depression-like behaviour and changes in the hypothalamo-hypophyseal-gonadal (HPG) axis in these NO donor and NOS inhibitors administered animals. All the behavioural tests were performed in the light phase of the light-dark cycle and an interval of 4-7 days was maintained between each behavioural test. The behavioural tests in females were performed when they were in the diestrus stage of the estrous cycle. All the male and female animals were sacrificed as per approved animal ethics protocol following the completion of behavioural tests. The study shows that there are significant changes in the treatment groups, showing more anxiolytic and depression-inducing effects in the L-NAME and 7-NI treatment groups in both males and females. The sex difference was also observed in these behaviour tests. Crystal violet staining of the brain sections shows alteration in the distribution of neurons in the hypothalamic nuclei (preoptic area – POA, suprachiasmatic nucleus – SCN, paraventricular nucleus – PVN and arcuate nucleus) in the L-NAME and 7_NI treated male and female animals when compared to the control group. L-NAME and 7-NI administration to male and female animals also had inhibitory effects on the morphological and cellular organization of the testes and the ovaries when compared to SNP-treated and control groups. The level of plasma testosterone, sperm count and plasma estradiol along with the total nitrate-nitrite concentration in plasma, testes and ovaries were significantly reduced in L-NAME and 7-NI treated male and female animals when compared to control groups. Thus, it may be concluded that inhibition of nitric oxide synthase by L-NAME and 7-NI and supplementing the animals with NO donor-SNP has a marked influence on the hypothalamo-hypophyseal-adrenal (HPA) axis as well as HPG axis in terms of alteration in anxiety and depression-like behaviour and gonad physiology. It appears that nitric oxide plays a major role in the interaction of the HPA and

HPG axis for the maintenance of body homeostasis. However, the molecular mechanism and pathway need to be explored further.

Chapter-3

Embryonic Disruption of Neuronal Nitric Oxide Synthase Alters Hypothalamus and Gonadal Development in Mice

Abstract

The growing fetus is susceptible to changes in its environment during embryogenesis, which can greatly affect its development. The neural circuitry in the brain along with environmental, psychological and genetic factors are responsible for the control of embryonic development of various systems of the body, which is regulated via numerous neuromodulators and neurotransmitters. Nitric oxide, one of the neurotransmitters has been shown to influence embryonic development in mammals. In the present study, male and female mice were time mated in the evening hours (16:00 hrs) and the next day female animals were observed for vaginal plugs in the early morning. Female animals with vaginal plugs were considered to be pregnant. Experiments were then performed to see the effects of inhibition of neuronal nitric oxide synthase (nNOS) by administering a specific nNOS inhibitor, 7-nitroindazole to these pregnant female mice from embryonic day 11 to embryonic day 17 along with pregnant female mice treated with dimethylsulphoxide:normal saline (DMSO:NS – 1:1) which acted as the control. Pups born to these pregnant female mice were sacrificed on postnatal day 0 (P0), postnatal 7 (P7), postnatal day 14 (P14) and postnatal day 21 for studying the changes in the structure of the hypothalamic nuclei and gonad (testes and ovaries) physiology. Results show that there were significant changes in the pattern and distribution of hypothalamic nuclei (preoptic area – POA, suprachiasmatic nuclei – SCN, paraventricular nuclei – PVN and arcuate nucleus – ARCN) and inhibition of the development of the morphological and cellular structures of testes and ovaries in the males and females respectively born to 7-NI treated pregnant female mice from postnatal day 0 to postnatal day 21. Thus, it may be concluded that inhibition of neuronal nitric oxide synthase during embryonic development

alters the development of the brain and gonads in both males and females by inhibiting the neuronal circuitry responsible for the interaction between the hypothalamo-hypophyseal-gonadal (HPG) axis and have a major effect on HPG axis development and its consequent effects in adulthood. However, the molecular mechanism responsible for the inhibition of neuronal circuitry during embryonic development needs to be further elucidated.

Thus, to maintain homeostasis in the body, the hypothalamus in the brain acts as the main coordinating centre. It also regulates the stress response by HPA axis and also maintains the reproductive behaviour through HPG axis. In the hypothalamus, there are various nuclei that coordinate the functions of the hypothalamus. Of such nuclei, the ones involved in the HPA axis are the paraventricular nucleus (PVN), which secretes corticotropin-releasing hormone (CRH), and the suprachiasmatic nucleus, the biological clock of the body that maintains the circadian rhythm by receiving inputs from the retina influences both the PVN as well as the POA. HPG axis has gonadotropin-releasing hormone (GnRH) as its main orchestrating hormone, this hormone is released from the preoptic area (POA) of the hypothalamus and stimulates the anterior pituitary to release gonadotropes. Arcuate nucleus due to its positioning has various terminals from the different nucleus of the hypothalamus and hence is involved in this hormonal crosstalk of the different regions of the hypothalamus and hence is implicated in the regulation of both CRH release from the PVN as well as GnRH release from the POA. These regions are interconnected to each other and helps regulate the anxiety and depression-like behaviour as well as the production of gonadal hormones and nitric oxide. When nitric oxide a key modulator of the brain function is disrupted by the in-utero inhibition of its enzyme nNOS by its specific inhibitor, 7-Nitroindazole, it is observed that there is a major disruption in the downstream functioning both HPA and HPG axis. Disrupting the neuronal nitric oxide synthase (nNOS) by specific nNOS inhibitor 7-Nitroindazole to pregnant mice, during the period of embryonic development has an anxiolytic and depressive effect on the pups born to the mother. The disruption during the embryonic stage has a long-term effect on the animals, evident from the significant change in the brain and gonad histology. The differences were also apparent in the behavioural pattern of the animals,

showing more anxiety-like and depression-like behaviour. These results illustrate that the in-utero exposure to nNOS inhibitor had serious alterations in both HPA and HPG axis which prolongs until adulthood as well. Inhibition of nitric oxide synthase by L-NAME and 7-NI and supplementing the animals with NO donor-SNP has a marked influence on the hypothalamo-hypophyseal-adrenal axis as well as hypothalamo-hypophyseal-gonadal axis in terms of alteration in anxiety and depression-like behaviour and gonad physiology. It appears that nitric oxide plays a major role in the integration of the HPA and HPG axis for the maintenance of body homeostasis. In-utero exposure to specific nNOS inhibitors also led to an alteration in the structure of hypothalamic (POA, SCN, PVN and Arcuate) nuclei and gonads (testes and ovaries). This further suggests that nitric oxide is important for neuronal and gonadal development and its inhibition during embryonic development may have serious implications in adulthood such as anxiety and depression and reproductive failure in both males and females. With the observations in this study we can propose that in the crosstalk of various hypothalamic nuclei in the modulation of comorbid anxiety and depressive behaviour, nitric oxide is one of the key players having implications from embryonic development itself and also during adulthood.

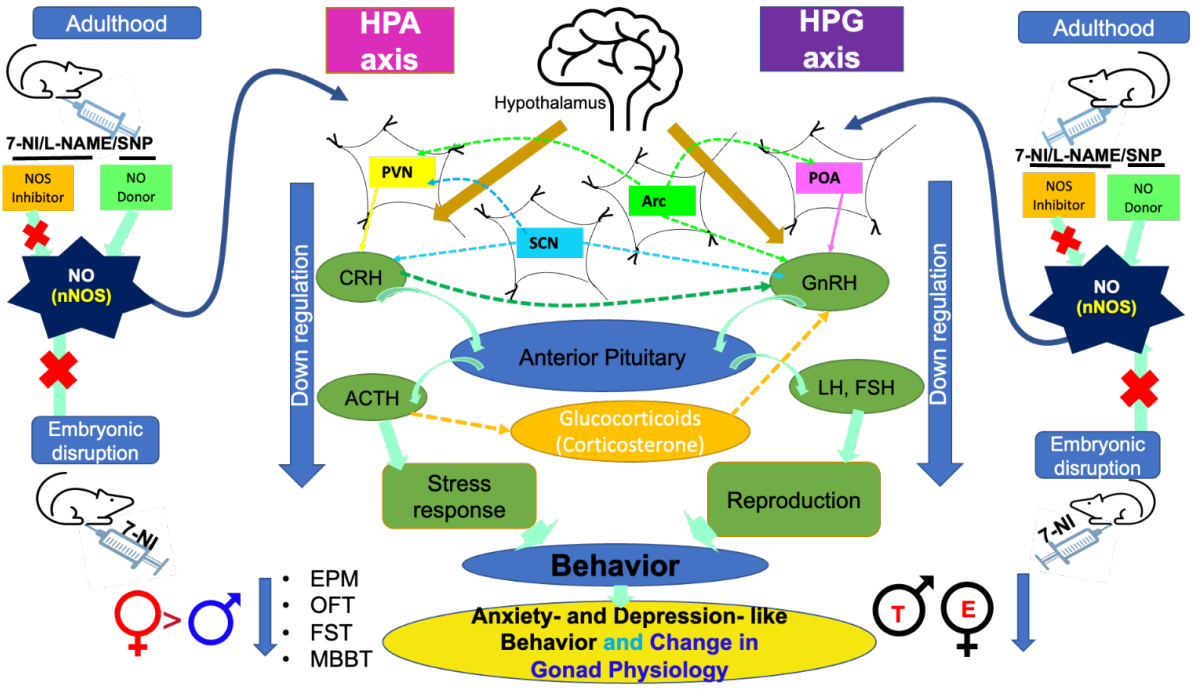


Figure: Image summarising the effects of neuronal nitric oxide synthase (nNOS) inhibition in-utero and in adults on anxiety and depression-like behaviour and gonadal physiology in adulthood via disruption in the Hypothalamo-Hypophyseal-Adrenal (HPA) axis and Hypothalamo-Hypophyseal-Gonadal (HPG) axis. 7-NI = 7-Nitroindazole; L-NAME = NG-nitro-l-arginine-methyl-ester; SNP = Sodium Nitroprusside; HPA = Hypothalamo-Hypophyseal-Adrenal Axis; HPG = Hypothalamo-Hypophyseal-Gonadal Axis; POA = Preoptic Area; SCN = Suprachiasmatic Nucleus; PVN = Paraventricular Nucleus; CRH = Corticotrophin-releasing Hormone; GnRH = Gonadotrophin-releasing Hormone; ACTH = Adrenocorticotropic Hormone; LH = Luteinizing Hormone; FSH = Follicle Stimulating Hormone; EPM = Elevated Plus Maze test; OFT = Open Field Test; FST = Forced Swim Test; MBBT = Marble Burying Behaviour Test; T = Testosterone; E = Estradiol