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RESEARCH ARTICLE

Embryonic disruption of neuronal nitric oxide synthase alters hypothalamus and gonadal development in mice

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Abstract

The growing fetus is susceptible to changes in its environment during embryogenesis which can greatly affect its development. The neural circuitries in the brain, along with environmental, psychological, and genetic factors, are responsible for regulating the development of various systems of the body during embryogenesis. This regulation occurs via numerous neuromodulators and neurotransmitters, including nitric oxide, which 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 dimethyl sulfoxide:normal saline (DMSO:NS – 1:1) which acted as the control. Pups born to these pregnant female mice were sacrificed on postnatal days 0 (P0), 7 (P7), 14 (P14) and 21 (P21) to study the changes in the structure of the hypothalamic nuclei and gonad (testes and ovary) 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 has 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.

Keywords: Nitric oxide, neuronal circuitry, pre-natal mice, post-natal mice, embryonic development, hypothalamic nuclei, testes, ovary

1. 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, 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 the prenatal period, throughout childhood, and adolescence (Herbison et al., 2017). The central nervous system (CNS) of fetus and child is highly sensitive to persistent organic pollutants (POPs). Studies 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's 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 behaviors and NO-producing brain circuits implicating the control of these behaviors (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 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 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 negative impact on the development of 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 & Bale, 2007; 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; 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 (Hellemanset 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 showed 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 reduced 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, 2016). Primary culture of cerebellar neurons of rats by administering aluminum to pregnant female (prenatal exposure to aluminum – Al) 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 Al. 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 results 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) revealed 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, neuronal nitric oxide synthase (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 showed 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 showed that gestational (prenatal) exposure or perinatal exposure to various compounds and stressors have adverse effects on the behaviour and developmental processing the pups born to pregnant mothers exposed to external compounds or stressors. There is, however, a dearth of knowledge regarding the role that particular nNOS inhibitors play in the inhibition of nitric oxide synthase during embryonic and postnatal development, particularly during 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.

2. Materials and Methods

2.1. 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 control pregnant mice were treated with a solution of normal saline and dimethyl sulfoxide (1:1), which was used as a vehicle. Light dark cycle 12L:12D was maintained and food and water were available *ad libitum*. The pups born at postnatal days 0, 07, 14 and 21 were perfused through cardio-thoracic perfusion method ; brain and gonads (testes and ovary) were collected from the perfused animals. The excised organs, i.e. brain were processed for crystal violet staining while the gonads were processed for haematoxylin and eosin staining as described below. 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.

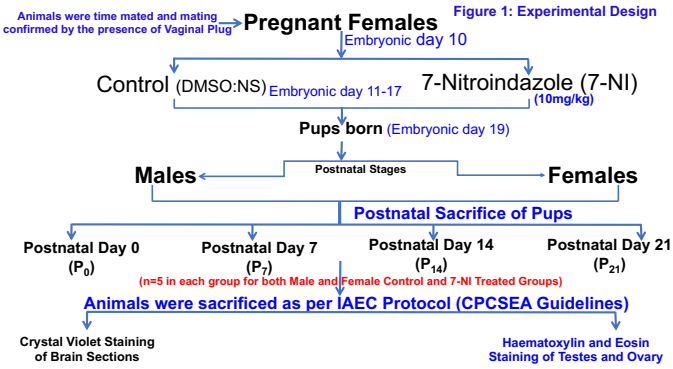

Figure 1: Flow chart showing the Experimental design.

2.2.Crystal Violet Staining of Brain Sections

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°C 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°C 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.

2.3.Histology of Gonads (Testes and Ovary)

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°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 37°C before hematoxylin-eosin staining of the sections.

2.4. 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; 5 minute each), 90% alcohol (2X; 5 minute each), 70% alcohol (2X; 5 minute each), 50% alcohol (1X; 5 minute) 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; 5 minute 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; 5 minute 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.

3. Result

3.1. Effect on the Different Nucleus of the Hypothalamus

Not much sex differences are observed in the distribution of neurons in the Pre-Optic area (POA) of 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)**.

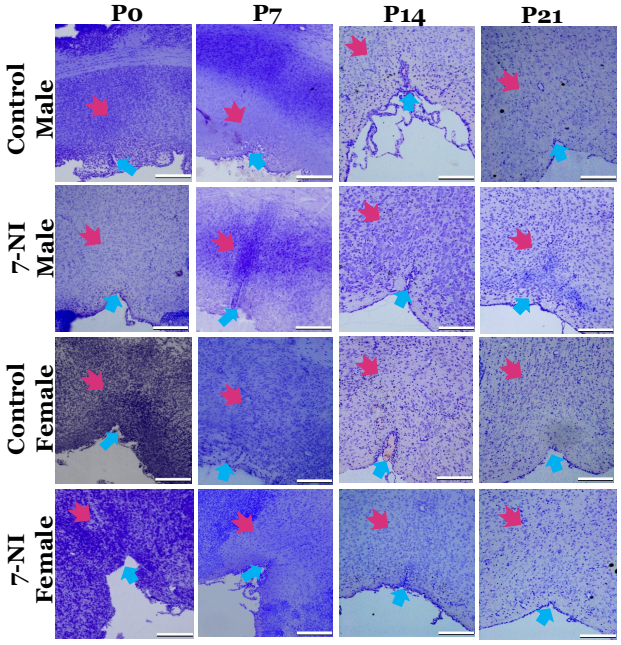

Figure 2: Representative images of brain sections showing preoptic area (POA) of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage during embryonic day 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 = 100mm.
Key: OVLT (Organum Vasculosum Lamina Terminalis); Preoptic Area (POA)

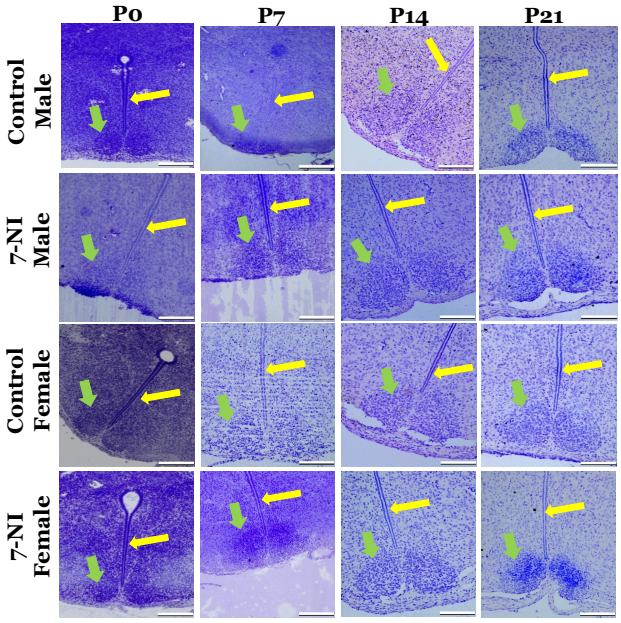

Figure 3: Representative images of brain sections of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal day P0, P7, P14 and P21 to study the changes in hypothalamic nuclei. Scale bar = 100mm.
Key: Third Ventricle ; Suprachiasmatic nucleus (SCN)6

3.2. Effects on Testes Histology

The histological section of the testes showed 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).

3.3. Effect on Ovarian Histology

P0 and P7 stage ovary showed 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).

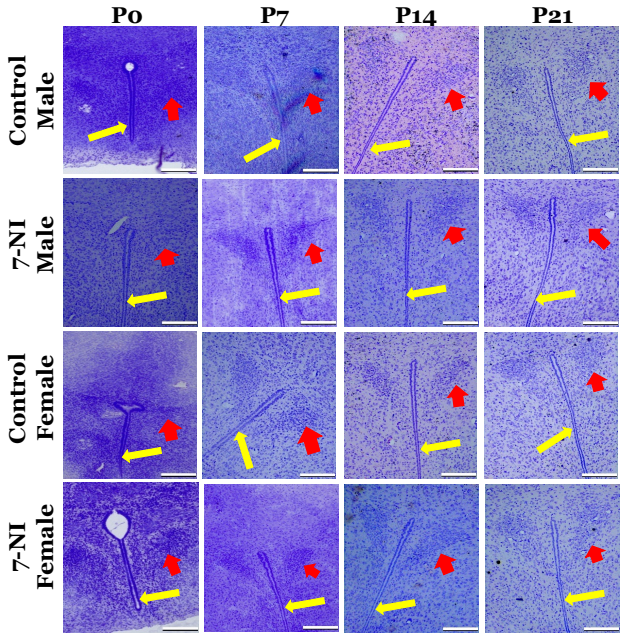

Figure 4: Representative images of brain sections of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to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

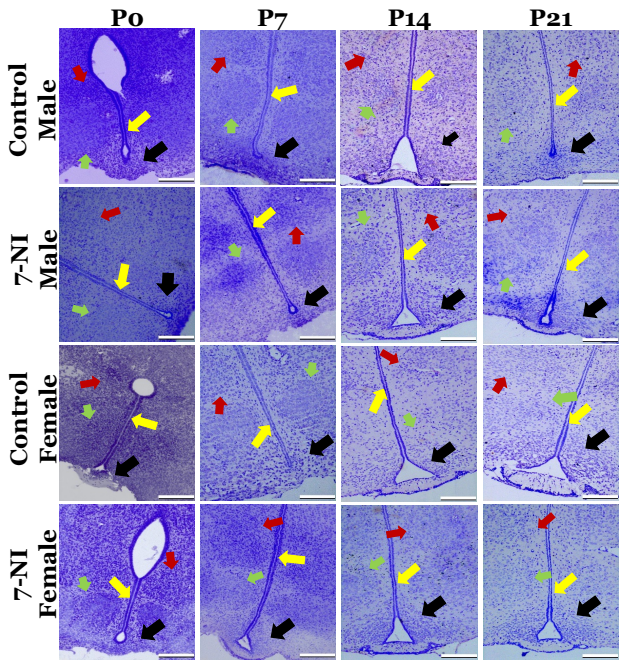

Figure 5: Representative images of brain sections of male and female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Male and Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to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

4. 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 (Heindel et al., 2015; Hoffman et al., 2021). 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, 1992; 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; Schwanzel-Fukuda and Pfaff, 1989; Okamura et al., 1990; Lee et al., 2008) a major regulator of the reproductive axis (Miller et al., 2002), showed 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, 2020). 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 yolk sac 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 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 are 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 at all stages when compared with their control. CRH is the chief hormone for activation of the HPA axis which 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 suggest 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 cell number in 7-NI treated animals compared to 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.

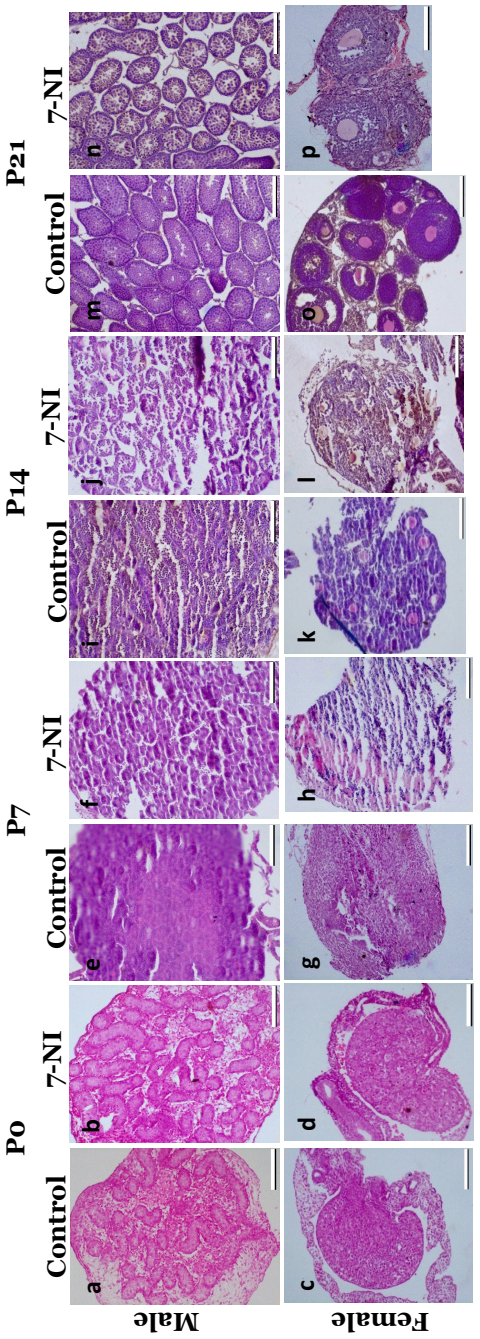

Figure 7: Representative images of transverse sections of adult male and female mice testes and ovaries, respectively born to pregnant female mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male and 7-NI Female) and vehicle control (DMSO: NS 1:1) (designated as Control Male and Control Female) 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 = 100mm.

Thus, it may be concluded that in-utero exposure to a specific nNOS inhibitor leads 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.

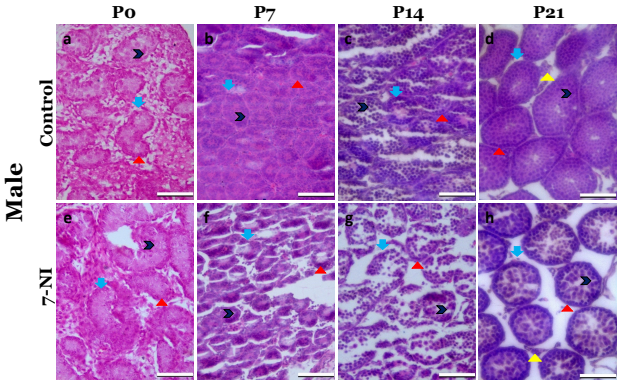

Figure 7: Representative images of transverse sections of testes of mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Male) and vehicle control (DMSO:NS) (designated as Control Male) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at postnatal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

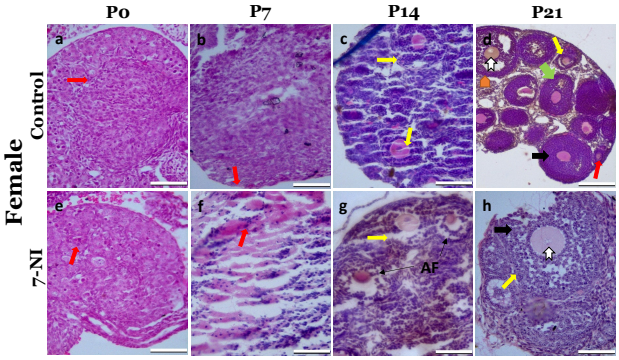

Figure 8: Representative images of sections of ovaries of mice treated with 7-Nitroindazole (7-NI) (designated as 7-NI Female) and vehicle control (DMSO:NS) (designated as Control Female) in the pregnant stage from embryonic day 11 to embryonic day 17 (E-11 to E-17) and sacrificed at post natal day 0, 7, 14 and 21, for studying it's effects on follicular development in ovary. Scale bar = 50mm.
Key: A, Antrum; Primordial follicle, Primary follicle; Secondary follicle, AF, Atretic follicle; Graafian follicle, Granulosa cells; Ovum

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Author's contributions

PK contributed in the concept, literature review, draft manuscript development, and manuscript finalization. HK performed literature review, experiments and contributed in the development of the manuscript. MY and BL contributed in intellectual approaches and concepts, literature review, proofreading and finalization of the manuscript.

Conflict of interests

The authors declare that they have no conflict of interest.

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