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# IRON AND POSTPARTUM DEPRESSION: A PRECLINICAL EVALUATION IN SPRAGUE-DAWLEY RATS

**BY**

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## DECLARATION

I hereby declare that this is the product of my own research undertaken under supervision and has neither been presented in whole nor in part for another degree elsewhere. I am solely responsible for any residual flaws in the work.

Signature…….. Date...../……. /……..

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## DECLARATION BY SUPERVISORS

We hereby declare that the principal work and presentation of the thesis was supervised by us in accordance with guideline on supervision of thesis laid down by the University of Ghana.

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## ABSTRACT

**Background:** Postpartum depression (PPD) is a mood disorder that affects 10 - 20 % of women after child birth. It has been observed that gestational iron deficiency which affects mostly mothers and their infants causes a deficit in behavioural, cognitive and affective functions precipitating depressive symptoms in both mothers and their infants during the postpartum period. The present work examined the role of iron in depression during the postpartum period in animal models.

**Method:** Female Sprague-Dawley rats (200-250 g) were crossed. Pregnant rats received iron (0.005 $mgkg^{-1}$ – 8.0 $mgkg^{-1}$) or fluoxetine (3 $mgkg^{-1}$ – 30 $mgkg^{-1}$) or desferrioxamine (50 $mgkg^{-1}$) or vehicle throughout the period of gestation (21-23 days). During the postpartum period, mothers from all groups were taken through the open field test (OFT) on postnatal day (PND) 2, forced swim test (FST) from PND 3 to PND 16 and novelty-induced hypophagia (NIH) from PND 18 to PND 22 and sacrificed on PND 28 for histological examination of the brains. After weaning the litter were taken through OFT on PND 35, FST from PND 36 – to PND 49, NIH from PND 51 to PND 55 and sacrificed on PND 57 for histological examination of the brains.

**Results:** Results showed that rats treated with iron chelator desferrioxamine and vehicle during gestation together with their litter had exhibited increased immobility scores in FST, increased latency scores with reduced feeding in NIH and a decreased number of neurons and dendritic branches in the cortex of the brain. These depression-related effects were attenuated by iron supplementation which caused decreased immobility scores in FST comparable to rats treated with fluoxetine, a clinically effective antidepressant. Iron treatment decreased latency scores with increased feeding in NIH. Iron treated rats and their litter had a higher

number of neurons with dendritic connections in the cortex similar to the effects of fluoxetine which has been associated with proliferation of neurons.

**Conclusion:** These results together suggest that, iron supplementation during gestation exerts an antidepressant-like effect on depressive behaviour in postpartum rats and their litter as well as attenuates the neuronal loss associated with depressive conditions.

## DEDICATION

I dedicate this thesis to God Almighty, for seeing me through from the beginning of this program until this point.

I also dedicate this work to my family and all the wonderful people I met during the period of my study.

God bless you all.

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## ABBREVIATIONS

**5-HT**…….5-hydroxytyptamine

**AAP**……..American Academy of Paediatrics

**ACC/SCN**……....Administrative Committee on Coordination Sub-Committee on Nutrtion

**ACOG**……. American College of Obstetricians and Gynaecologists

**ACTH** …….Adrenocorticotropic Hormone

**ADHD**……..Attention Deficit Hyperactivity Disorder

**APA**…….American Psychiatric Association

**ATP**…….Adenosine Triphosphate

**BBB**…….Blood–Brain Barrier

**BDNF**……..Brain-Derived Neurotrophic Factor

**BDI**……..Beck Depression Inventory

**CBT**…….Cognitive-Behavioural Therapy

**CES-D**…….Centre for Epidemiological Studies Depression Scale

**CIS-R**……..Revised Clinical Interview Schedule

**CNS**…….Central nervous system

**COMT**……Catechol-O-methyltransferase

**CRF**……Corticotrophin-releasing factor

**CRH**……...Corticotrophin-Releasing Hormone

**CytOx**…….Cytochrome c oxidase

**DA**……...Dopamine

**DFx**…….Desferrioxamine

**DNA**……Deoxyribonucleic acid

**DMT**……...Divalent Metal Transporter

**DSM**……...Diagnostic and Statistical Manual of Mental Disorders

**EPDS**……..Edinburgh Postnatal Depression Scale

**Flx**……...Fluoxetine

**FST**…….Forced Swim Test

**GABA**……..…γ −aminobutyric acid

**HPA**……Hypothalamic-Pituitary-Adrenal axis

**ID**……Iron Deficiency

**IDA**……Iron Deficiency Anaemia

**IL**……...Interleukin

**LMICs**……..Low and Middle-Income Countries

**MAOIs**…….Monoamine Oxidase Inhibitors

**MAO-A/B**……Monoamine Oxidases A and B

**NA**……Noradrenaline

**NARIs**……..Noradrenaline Re-uptake Inhibitors

**NIH**……..Novelty-Induced Hypophagia

**OFT**…….Open Field Test

**PHQ**…….Patient Health Questionnaire

***p.o***…….*Per os*

**PPB**……..Postpartum Blues

**PPD**……..Postpartum Depression

**PPOCD**……Postpartum Obsessive Compulsive Disorder

**PPP**……..Postpartum Psychosis

**PPPD**……...Postpartum Panic Disorder

**PPPTSD**……..Postpartum Post Traumatic Stress Disorder

**PRIME-MD**……Primary Care Evaluation of Mental Disorders

**ROS**…..Reactive oxygen species

**SARIs**……..Serotonin Antagonist and Re-uptake Inhibitors

***s.c***.....Subcutaneous

**SCID**……..…...Structured clinical interview for DSM-IV disorders

**SRQ**……Self-Reporting Questionnaire

**SNRIs**…….Serotonin-Noradrenaline Reuptake Inhibitors

**SSRIs**…….…….Selective Serotonin Re-uptake Inhibitors

**TBG**……Thyroxin-Binding Globulin

**TCAs**……..Tricyclic Antidepressants

**Tf**……....Transferrin

**Tfr**…..Transferrin receptor

**TNF-α**……Tumour Necrosis Factor - alpha

**WHO**…….World Health Organization

# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGROUND

Neuropsychiatric disorders are among the most prevalent conditions gaining global attention. (Leung *et al.*, 2009; Javitt and Javitt, 2018). Mood disorders are prominent among the neuropsychiatric disorders and the commonest worldwide is major depression. A World Health Organization (WHO) report indicated that depression especially when long-lasting and with moderate or severe intensity, can become a serious health concern (WHO, 2018). Depression is currently the largest contributor to disability globally among individuals of reproductive age with women at a greater risk than men (Leung *et al.*, 2009 ; Moussavi *et al.*, 2007; Rechenberg, 2016; WHO, 2018). The risks associated with the disorder in women is far greater during pregnancy termed antenatal depression or after pregnancy termed postpartum depression (PPD) with long lasting effects on the offspring (Brummelte *et al.*, 2016; Leung *et al.*, 2009 ; Robertson *et al.*, 2003). PPD belongs to a spectrum of postpartum affective disorders. Apart from PPD, there are two other forms of postpartum affective disorders which include postpartum blues (baby blues, maternity blues) and puerperal (postpartum or postnatal) psychosis each of which differs in its prevalence, clinical presentation, and management (Brockington, 2003; Robertson *et al.*, 2003). Postpartum blues (baby blues, maternity blues) occurs in about 85 % of women after birth and resolves within a few days after delivery (Norhayati *et al.*, 2015). Symptoms may include brief episodes of crying spells, anxiety, sadness, poor sleep, confusion, and irritability. However, suicidal tendencies are absent, and no specific treatment is required (Cohen *et al.*, 2010). Postpartum psychosis on the other hand is rare, between 0.1–0.2% and associated with infanticide and suicide (Norhayati *et al.*, 2015) unlike the blues. Symptoms may include restlessness, agitation, sleep disturbance, extreme fear, confusion, impulsivity, hallucinations, and

delusions (Norhayati *et al.*, 2015; Robertson *et al.*, 2003). Symptoms peak two weeks after delivery and is common in new deliveries in women above 30 years (Cohen *et al.*, 2010; Robertson *et al.*, 2003).

PPD is the commonest postpartum affective disorder of childbearing affecting approximately 10-15% of women but can be as high as 30% depending on the location and criteria of diagnosis (Darcy *et al.*, 2008; Gavin *et al.*, 2005). PPD is a depressive episode with a postpartum onset (four weeks after delivery) with moderate to severe depressive symptoms (APA, 2013) including tearfulness, despondency, emotional lability, feelings of guilt and/or worthlessness, loss of appetite, feelings of inadequacy and inability to cope with infant, poor concentration and memory, fatigue and irritability, loss of interest in hobbies and usual activities, sleep disturbances and rarely suicidal ideations (Brummelte *et al.*, 2016; Patel *et al.*, 2012; Robertson *et al.*, 2003). According to the diagnostic and statistical manual of mental disorder fifth edition (DSM-5), depression is diagnosed when these symptoms recur for at least two weeks such that they interfere with a person's normal activities. Untreated PPD can have long-term adverse effects on the mother and deleterious effects on the infant including long-term emotional, intellectual and cognitive impairments (Giallo *et al.*, 2014; Goodman *et al.*, 2011; Grace *et al.*, 2003; Hay *et al.*, 2001). Despite the huge burden depressive disorders have on society, their aetiology is not completely understood (Brummelte *et al.*, 2010). However, it is generally accepted that depressive disorders including PPD occur as a result of dysregulation of neurotransmitter function, genetic factors, hormonal imbalances, psychosocial factors and deficiencies in nutrients (Brummelte *et al.*, 2010; Kaila, 2005; Nestler *et al.*, 2002). The risk factors for PPD are not different from those of major depression and the one biological risk factor gaining global attention is nutrient deficiencies (Leung *et al.*, 2009). Association studies by various authors have reported credible links between nutrient deficiencies and mood for micronutrients such as iron,

selenium, zinc, magnesium, copper vitamin B-12 among others (Beard, 2003; Mlyniec *et al.*, 2014; Singewald *et al.*, 2004). Of all the nutrient deficiencies, Iron deficiency (ID) is the single most common micronutrient deficiency globally with more than 20 % of women experiencing it during pregnancy (Percy *et al.*, 2016).

Iron is an important micronutrient required in the body for normal cell functions. It is involved in various functions including the synthesis of deoxyribonucleic acid (DNA), neurotransmitters and enzymes as well as the oxygenation of brain parenchyma (Dusek *et al.*, 2012). Considering the significant role of iron in tissues, its amount in the body is of critical importance especially with regards to brain function. Iron deficiency (ID) which is a reduction in the amount of total body iron (Camaschella, 2015; Pavord *et al.*, 2012) can result in impaired oxygenation and affect enzyme reactions involved in major metabolic pathways of the body including the brain (Percy *et al.*, 2016). ID is the commonest cause of anaemia globally (Johnson-Wimbley *et al.*, 2011; Kassebaum *et al.*, 2014) responsible for about 50 % of all anaemia cases (Camaschella, 2015). ID is capable of causing cognitive impairment in animals and humans through damage to brain mitochondria (Tamura *et al.*, 2002). The cognitive deficits caused affect emotions and behaviour, attention and intelligence as well as the sense of perception.

Although, the postpartum period has always been associated with lower risk of ID, studies have reported that postpartum ID is far more common than was previously known, making postpartum ID a health concern demanding global attention (Bodnar *et al.*, 2002; Perhrsson *et al.*, 2002). Several studies (Beard *et al.*, 2003; Corwin *et al.*, 2003; Pick *et al.*, 2005) have reported a strong relationship between maternal iron status and depression but there is limited literature on the exact role of iron in the postpartum period. Literature has indicated that gestational ID can result in cognitive and behavioural deficits in infants (Angulo-Kinzler *et al.*, 2002; Beard *et al.*, 2006), however, there are limited studies that characterize the exact

effects of gestational-maternal ID on the cognitive and behavioural functions of mother and offspring in the postpartum period. This study therefore investigated, using animal models, the role of gestational iron treatment on depression in postpartum rats and their first generation litter.

## 1.2 PROBLEM STATEMENT

There has been rising interest in neuropsychiatry research mainly depression due to the socio-economic burden it imposes on society. According to the WHO, major depression is the leading cause of long-term disability globally and a leading contributor to the global burden of disease (WHO, 2018). The estimated risk for depression-related suicide rates are between 5–8 % (Bradvik, 2018). Women have been shown to be at a greater risk of developing depression compared to men (Goldman *et al.*, 1999; Gutierrez-Lobos *et al.*, 2002) with the consequences impacting greatly during pregnancy (antenatal depression) and after pregnancy (PPD) (Leung et al., 2009). Even though depression is associated with high morbidity and mortality (Lee *et al.*, 2005), postpartum depression (PPD) also poses more serious and long lasting effects on both the mother and the offspring (Josefsson *et al.*, 2001; WHO, 2009). Its estimated that about 10 to 15% of women who go through labour experience PPD (Vesga-Lopez, 2008). PPD peaks around 12 months after delivery but may manifests from three months to one or more years (APA, 2013; Gavin *et al.*, 2005). Even though, the pathophysiology of PPD is still not clearly understood, just like major depression, several biological factors have been implicated including micronutrient deficiencies such as iron deficiency (Beard, 2003; Bodnar *et al.*, 2002; Corwin *et al.*, 2003; Leung *et al.*, 2009 ; Mlyniec *et al.*, 2014). Estimates indicate that iron deficiency anaemia (IDA) affects about 50 % of pregnant women in lower-middle income countries (LMICs) (Balarajan *et al.*, 2011; Stevens *et al.*, 2013; Daru *et al.*, 2018) such as Ghana. About 43% of children of children under 4 years are affected by iron deficiency and half is attributable to iron deficiency

anaemia (IDA) (Administrative Committee on Coordination Sub-Committee on Nutrtion (ACC/SCN) 2000; Habib *et al*., 2016). Iron has a very crucial role to play in the development and function of many systems and processes in the body including that of monoaminergic neurotransmitter systems, which has been the major system implicated in the pathophysiology of depression (Beard, 2003; Hulthén, 2003). Iron is implicated because it is a co-factor in the enzymes involved in the synthesis of these monoamines, their receptors, and their reuptake transporters (Beard *et al.*, 2003; Brunette *et al.*, 2010b; Georgieff, 2008).

Iron deficiency in women has been related to fatigue and poorer general health (Grondin *et al.*, 2008; Patterson *et al.*, 2000) as well as emotional and cognitive dysfunction. The exact role of iron and ID in cognitive and behavioural impairments remains largely unexplored in spite of the observation that ID precipitates depressive episodes, malaise, lethargy and poor concentration (Corwin *et al.*, 2003; Paterson *et al.*, 1994). Some studies have associated low Haemoglobin (Hb) levels with postnatal symptoms such as low energy, faintness/dizziness and tingling of fingers and toes but not with PPD (Paterson *et al.*, 1994). Others have found that anaemia and/or iron deficiency is associated with increased symptoms of postpartum depression (Beard *et al.*, 2006). Although PPD was assessed in these studies by the Edinburgh Postnatal Depression Scale (EPDS) (DeUngria *et al.*, 2000), disparity in iron measurements make direct comparisons difficult since some studies focused on anaemia (Corwin *et al.*, 2003; Paterson *et al.*, 1994) while others considered IDA (Beard *et al.*, 2006) or ID based using ferritin concentration (Albacar *et al.*, 2011).

This study sought to investigate the role of gestational iron treatment and ID on depressive-like behaviour in postpartum Sprague-Dawley rats as well as their litter using behavioural models and histological assessment.

## 1.2 JUSTIFICATION

Depressive disorders pose enormous challenges to society. Over the past decades efforts have been aimed at improving the treatment of depression globally, yet it still remains a major cause of death and a leading medical cause of long-term disability (WHO, 2018). ID is a global challenge affecting especially pregnant women in developing countries such as Ghana. In most countries, iron supplements are given to pregnant women from their first antenatal visit till they deliver for a healthy development of the foetus. Iron is important for several neurological functions and processes (Beard *et al.*, 1993; Roncagliolo *et al.*, 1998; Weinberg *et al.*, 1980; Yehuda, 1990), including the development and function of the monoamine neurotransmitter systems, since iron is a co-factor for enzymes (tryptophan hydroxylase and tyrosine hydroxylase) involved in the synthetic pathways for the monoamines, their receptors, and their reuptake transporters (Beard, 2003; Brunette *et al.*, 2010b; Georgieff, 2008). Iron is also important in neuronal energy metabolism, potentially through its incorporation into cytochromes and its subsequent effect on electron transfer and adenosine triphosphate (ATP) generation. Iron is equally important in myelination, potentially through its role in enzymes that synthesize fatty acids (Beard *et al.*, 2003) and through glial (oligodendrocyte) energy metabolism (Rao *et al.*, 2001 ). Thus, iron is a critical nutrient for the brain function (Beard, 2003; Georgieff, 2006; Hulthén, 2003). Past research using rodent models has established several factors including the coexistence of dopaminergic neurons with iron in the brain, the elevation of dopamine and noradrenaline levels in brains of iron-deficient rats, the decrease in densities of the dopamine D1 and D2 receptors and dopamine transporters are all affected by dietary iron deficiency (Beard *et al.*, 2003). The degree of alteration in these and several other parameters is connected to the level of iron in the examined brain regions. Later studies also demonstrated alterations in the serotonin and noradrenaline transporter densities caused by dietary iron deficiency (Beard *et*

*al.*, 2006; Felt *et al.*, 2006) thus expanding the effect of iron deficiency to all monoamine transporters. The persistence of these alterations in the various brain regions implicated in PPD could be the biological basis for the behavioural and cognitive impairments observed in iron-deficient pregnant women and their infants (Beard *et al.*, 1993; Ben-Shachar *et al.*, 1985; Erikson *et al.*, 2001; Yehuda, 1990) but both past and present research have not investigated the effects of these alterations on postpartum depression. The effects of PPD are far reaching since it has been implicated in maternal mortality and morbidity with poor infant outcomes in terms of emotion, cognition and behaviour. With iron being implicated all these abnormalities, it is prudent to investigate the effects of gestational iron treatment and iron deficiency on postpartum depression and depression in the first generation litter.

## 1.4 HYPOTHESIS

It is hypothesised that perinatal iron treatment decreases vulnerability to depression in postpartum rats and their litter.

## 1.5 AIM

To investigate the effects of perinatal iron treatment on depression in postpartum rats and their litter.

## 1.6 SPECIFIC OBJECTIVES

1. Comparative assessment of the behavioural effects of supplemental iron versus desferrioxamine versus fluoxetine on depression in postpartum rats and their litter using the:

- forced swim test (FST)
- open field test (OFT)
- novelty-induced hypophagia (NIH).

2. Comparative assessment of histological effects of supplemental iron versus desferrioxamine versus fluoxetine on

- morphological changes
- neuronal density

in brain tissue of postpartum rats and their litter.

# CHAPTER TWO

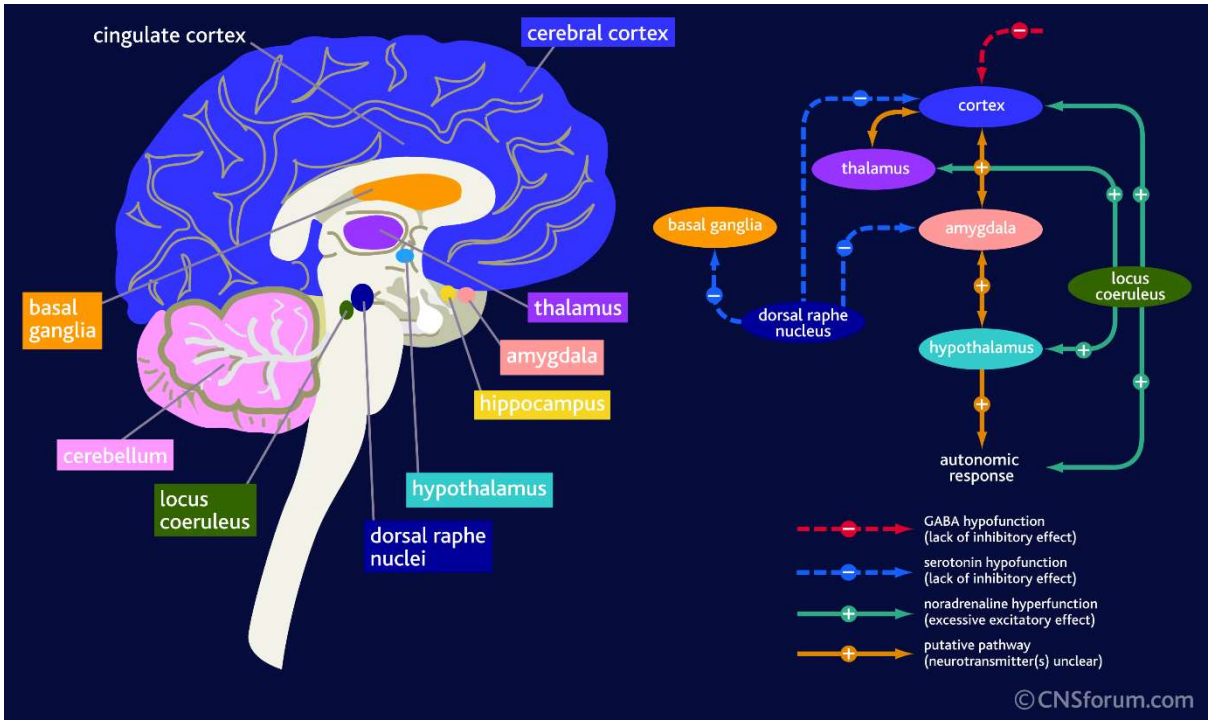
# LITERATURE REVIEW

## 2.1 DEPRESSION

### 2.1.1 Background

Depression is one of the oldest, well-recognized mood disorders having been clearly described in the earliest medical texts dating back to ancient Greece (Fava *et al.*, 2000). Depression can be described both as a temporary state of mood that can be experienced by almost all individuals at some stage in life as well as a clinical or behavioural condition known as major depressive disorder (MDD). As a mood disorder, it results in disturbances of affect and mood, neurovegetative functions, cognition, emotion and psychomotor activity mediated by certain brain regions including amygdala, hypothalamus, hippocampus and prefrontal cortex (PFC) (***Figure 2.1***). Depression in ancient Greece has been associated with immense sadness and its components such as sorrow, dejection, despondency, emptiness, anxiety, hopelessness, discouragement (Atindanbila *et al.*, 2011; Horowitz *et al.*, 2005), with the dominant emotional symptoms been anxiety, irritability and anhedonia (Drevets, 2001). The aetiology and pathophysiology of depression remain poorly understood but in recent times neuroimaging technology such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) has afforded the ability to investigate neurophysiological, neuroanatomical, neurochemical and genetic correlates of mood disorders *in vivo* (Albert *et al.*, 2013; Belmaker *et al.*, 2008). The results of these studies are being complimented by data from post-mortem tissues from depressed subjects, which have shown abnormalities in the cortical grey matter, prefrontal cortex and hippocampus (Albert *et al.*, 2013; Drevets, 2000; Rajkowska, 2003). Studies suggest that interactions between abnormalities in various brain structures and function underlie the pathophysiology of

depressive disorders. Diagnosis and treatment of depression of all forms is based on relatively subjective assessments of a wide array of symptoms.




**SOURCE: http://www.cnsforum.com**

Figure 2. 1: Brain areas implicated in depressive disorders

### 2.1.2 Depression during pregnancy and the postpartum

Depression is widely prevalent among women with a two to three times more likelihood to develop depression than men (Goldman *et al.*, 1999; Leung *et al.*, 2009 ). The effects of depression in women impacts greatly especially in child-bearing age (Shrivastava *et al.*, 2015). Depression during pregnancy is termed antenatal depression and after birth it is termed postpartum depression (PPD) (Parsons *et al.*, 2012; Shrivastava *et al.*, 2015). In fact, different studies across various several jurisdictions have reported presence of antenatal and postnatal depression among women, but this does not reveal the real situation because most of the cases are either not diagnosed, underdiagnosed or unreported due to the lack of global standard on the methods of screening (Mathisen *et al.*, 2013; Teissedre *et al.*, 2004). These forms of depression deserves more attention as they reflect periods of intense physiological,

social, mental change and transition for women, that essentially necessitates adaptation. Generally, the postpartum period which is the main focus of this study has been associated with symptoms such as fatigue, anxiety, disordered sleeping, changing mood, irritability, feelings of loss and sadness, and sometimes even loss of self-esteem (Babatunde *et al.*, 2012; Teissedre *et al.*, 2004) among others which are not different from those of major depression.

## 2.2 POSTPARTUM AFFECTIVE DISORDERS

The association between the postpartum period and mood disturbances has been noted since the time of Hippocrates (Bodnar *et al.*, 2002). Women are at increased risk of developing severe psychiatric illness during the first six weeks postpartum(Kendell *et al.*, 1987; Paffenbarger, 1982). However, mood disturbances following childbirth are not significantly different from affective illnesses that occur in women at other times since the clinical presentation of depression occurring in the postpartum period is similar to major depression occurring at other times, with symptoms such as depressed mood, anhedonia and low energy and suicidal ideation (Brummelte *et al.*, 2016).

### 2.2.1 Clinical Classification of Postpartum Illnesses

Childbirth as a general stressor, like any other life event can trigger an attack of illness across the whole spectrum of psychiatric disorders (Brockington, 2003). Postpartum affective disorders associated with childbirth are typically divided into three categories: postpartum blues, non-psychotic postpartum depression and puerperal psychosis with each differing in prevalence, onset and duration (Perfetti *et al.*, 2004; Robertson *et al.*, 2003). Other non-specific disorders include Postpartum Panic Disorder (PPPD), Postpartum Obsessive Compulsive Disorder (PPOCD), Postpartum Post Traumatic Stress Disorder (PPPTSD) (Beck, 2006; Horowitz *et al.*, 2005; Sara *et al.*, 2009).

#### *2.2.1.1 Postpartum blues (PPB)*

PPB is the most common mood disturbance of childbirth affecting between 50 - 80 % of new mothers, with an early onset, peaking at day five, and showing full resolution between 10 - 14 days postpartum (O'Hara *et al.*, 2014; Sara *et al.*, 2009). Symptoms include emotional lability, crying spells, anxiety, fatigue, sleep disturbance, anger/irritability and sadness. PPB is normal with childbirth but can develop into PPD if symptoms persist for two weeks or more. PPB is considered a risk factor for PPD and can lead to women developing a chronic depressive course (Beck, 2006; Moses-Kolko *et al.*, 2004; Perfetti *et al.*, 2004).

#### *2.2.1.2 Postpartum depression (PPD)*

PPD is a disabling non-psychotic but treatable mental disorder that represents one of the most common complications of childbirth (Howard *et al.*, 2014; Stewart *et al.*, 2016). Postpartum depression is included in the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5), as a major depressive episode with peripartum onset if mood symptoms occurs during pregnancy or within 4 weeks postpartum (APA, 2013; Stewart *et al.*, 2016). The symptom profile of PPD include sad mood, excessive or inappropriate guilt, restlessness and/or agitation, impaired concentration, sleep disturbance, irritability, feelings of worthlessness, suicidal/infanticidal ideations have also been reported (Pawluski *et al.*, 2017; Stewart *et al.*, 2016; Wisner *et al.*, 2013) which are not different from the symptoms of major depression occurring at any time during adulthood.

#### *2.2.1.3 Postpartum Psychosis (PPP)*

Postpartum psychosis (PPP) is the most severe childbirth related mood disorder. Although the prevalence is as low as 0.1 – 0.5 %, it is associated with maternal suicide, infanticide (Munk-Olsen *et al.*, 2006; Spinelli, 2004; Veen *et al.*, 2016) and in a majority of cases, the onset is rapid within two weeks postpartum. Early symptoms include insomnia and severe mood

fluctuation such as cognitive symptoms, mania, depression, or a mixed state, as well as loss of touch with reality, auditory and visual hallucinations, rapid speech, paranoia, and suicidal and/or infanticidal ideations (Beck, 2006; Bergink *et al.*, 2011; Moses-Kolko *et al.*, 2004; Perfetti *et al.*, 2004; Sara *et al.*, 2009; Sit *et al.*, 2006; Spinelli, 2009; Veen *et al.*, 2016). Apart from changes in tryptophan metabolism during the physiological postpartum period, the downstream changes in the metabolites of tryptophan such as the profound decrease of neuroprotective KynA and increase of neurotoxic hydroxykynurenine (3-HK) in the physiological postpartum period is one recent biological factor implicated in the pathophysiology of PPP (Birner *et al.*, 2017; Veen *et al.*, 2016).

### 2.3 POSTPARTUM DEPRESSION (PPD)

PPD is defined as depression that occurs within 4 - 6 weeks, can last as long as 14 months (APA, 2013; Gaynes *et al.*, 2005; Goodman, 2004; Patel *et al.*, 2012; Wisner *et al.*, 2010) after childbirth. Apart from the fact that the onset of PPD is unique, it is unclear whether it has any other unique symptomatology (Pawluski *et al.*, 2017) from major depression. PPD is often comorbid with anxiety (Field, 1990; Figueira *et al.*, 2009; Le Strat *et al.*, 2011; O'Hara *et al.*, 2013) and is often predicted by a history of major depression, perinatal depression or anxiety (Bloch, 2003; Di Florio *et al.*, 2015; Fleming, 1988; Horowitz *et al.*, 2004). The consequences of PPD for both mother and infant are well established and women who suffer from PPD are twice as likely to experience future episodes of chronic or recurrent depression over a period of time (Jacobsen, 1999; O'Hara *et al.*, 2013). The effect of PPD on maternal–infant interactions leads to emotional, behavioural and cognitive difficulties as well as attachment insecurity, developmental delay and social interaction problems in affected children (Beck, 1998; Luoma *et al.*, 2001). Several factors have been implicated in the pathophysiology of PPD which range from social to biological factors as well as deficiencies in essential mineral elements especially iron. This study will explore the role of perinatal iron

supplementation and perinatal-induced iron deficiency (ID) on PPD and postpartum Sprague-Dawley rats and behavioural changes in the litter.

### 2.3.1 Prevalence of PPD

PPD is a major health concern for women from diverse cultures (Dennis *et al.*, 2004). The estimated prevalence of postpartum depression ranges from 6.5 to 12.9 % in developed countries and could go as high as 50 % in low and middle-income countries (LMICs) depending on the criteria and measure used (Gaynes *et al.*, 2005; Howard *et al.*, 2014; Munk-Olsen *et al.*, 2006; WHO, 2009). In Africa, a wide range of prevalence ranges have been reported with no clear differences emerging in prevalence rates between northern and sub-Saharan African countries even though low average rates have been reported in Uganda (7.1%) and high rates in Zimbabwe (33%) (Parsons *et al.*, 2012). Prevalence rates of PPD in most African countries are higher than rates reported in high-income countries. Mean prevalence of PPD has been reported to be 18 % among the studies reviewed (Gold *et al.*, 2013; Sawyer *et al.*, 2010). In Ghana a study that compared 3 screening instruments for PPD reported that 11 % of 160 respondents had scores representing clinically significant depression (Gold *et al.*, 2013; Weobong *et al.*, 2009). The variation in PPD prevalence are due to cultural variables, varied screening criteria, stigma, as well as socio-economic factors (Halbreich *et al.*, 2006; Lanes *et al.*, 2011).

### 2.3.2 Screening for PPD

There is no single method for detecting postpartum depression in clinical settings. Screening for PPD usually involves a sensitive clinical inquiry centred on mood during postpartum visits which facilitates the case finding process. This is achieved by the administration of the 10-item Edinburgh Postnatal Depression Scale (EPDS) (Cox *et al.*, 1987; Stewart *et al.*, 2016) which is recommended by both the American College of Obstetricians and

Gynaecologists (ACOG) and the American Academy of Paediatrics (AAP) (Earls, 2010; Stewart *et al.*, 2016) as a method of diagnosing possible postpartum depression or the most widely used screen currently being the Patient Health Questionnaire 2 (PHQ-2) questionnaire which covers depressive and dysphoric mood nearly every day for at least two weeks (Gjerdingen *et al.*, 2009). Patients who screen positive are further evaluated with the PHQ-9 to determine whether they meet criteria for a depressive disorder. Other modes of screening involves structured psychiatric interviews such as Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV)), or a range of self-report scales including the Beck Depression Inventory (BDI), revised Clinical Interview Schedule (CIS-R), the World Health Organization (WHO) Self-Reporting Questionnaire (SRQ), the Centre for Epidemiological Studies Depression Scale (CES-D), the mini international neuropsychiatric interview, the Hamilton depression rating scale, Zung's self-rating depression scale, and the Kessler scales (Parsons *et al.*, 2012). These different scales produce different estimates of PPD, even for the same women at the same time.

#### *2.3.2.1 Edinburgh Postnatal Depression Scale (EPDS)*

This is the most widely used instrument in postpartum depression studies and for population-based screening is the EPDS, a 10-item self-report scale specifically designed to screen for postpartum depression in community samples (Cox *et al.*, 1987). Each item is scored on a 4-point scale (from 0 - 3), with a total score ranging from 0 to 30. The items, written in the past tense, include questions related to maternal feelings during the past 7 days and refer to depressed mood, anhedonia, guilt, anxiety, and suicidal ideation. This scale does not include symptoms such as insomnia and appetite changes, which may occur naturally after birth.

#### *2.3.2.2 Patient Health Questionnaire (PHQ)*

The PHQ was developed in 1999 as a self-report version of the Primary Care Evaluation of Mental Disorders (PRIME-MD) for screening and diagnosis as well as monitoring the severity of depression (Kroenke *et al.*, 2010; Spitzer *et al.*, 1994). It is a criteria-based diagnosis of most commonly encountered mental disorders. (Wittkampf *et al.*, 2007). Although the EPDS is the widely used tool for the assessment of PPD, other studies have tried the PHQ. In head-to-head comparisons with the EPDS, the PHQ has been found in some instances to be more accurate in a study involving (Weobong *et al.*, 2009), comparable in others (Bennett *et al.*, 2008), or even slightly less accurate (Hanusa *et al.*, 2008) in varying subject populations. Studies have found the PHQ-2 to be highly sensitive and the PHQ-9 was highly specific for identifying PPD (Gjerdingen *et al.*, 2009). The first two items of PHQ-9 make up the PHQ-2. These pertain to depressed mood and loss of interest (anhedonia), of which at least one is required to establish a diagnosis of any DSM-V depressive disorder. These two items are comparable to many longer case-finding measures for depression In comparing the PHQ-9 with the reference standard, the DSM-IV criteria, one study that the PHQ-9 detects patients with a depressive episode, while the Structured Clinical Interview for DSM-IV Disorders (SCID) detects patients with a depressive disorder since the DSM-IV exclusion criteria for a depressive disorder are not included in the PHQ-9 (Wittkampf *et al.*, 2007).

### 2.3.3 Treatment of PPD

Treatment of PPD can be achieved using pharmacological agents, non-pharmacological techniques or both. Several factors such as distress levels, access to care, and previous treatment may dictate the choice of treatment, thus pharmacological or non-pharmacological treatment for the PPD (Fitelson *et al.*, 2011b). There is paucity of data comparing the

effectiveness of pharmacological against other treatment modalities for PPD, but the few suggest that medications are at least as effective as most psychological interventions based on effect size (Pearlstein *et al.*, 2006 ; Sit *et al.*, 2006; Turner *et al.*, 2008).

#### *2.3.3.1 Pharmacological treatment for PPD*

The prevalence of pharmacotherapy using antidepressants for the treatment of PPD is on the increase. These antidepressant drugs treat the symptoms of depression and are commonly used as the first treatment option for women with PPD (Molyneaux *et al.*, 2014). Antidepressants can be classified into the selective serotonin re-uptake inhibitors (SSRIs, e.g. fluoxetine, citalopram, escitalopram, paroxetine, sertraline, fluvoxamine), tricyclic antidepressants (TCAs, e.g. imipramine, clomipramine, desipramine, trimipramine, amitriptyline, nortriptyline, protriptyline), noradrenaline re-uptake inhibitors (NARIs, e.g. reboxetine), monoamine oxidase inhibitors (MAOIs: *irreversible*: isocarboxazid, phenelzine, tranylcypromine; *reversible*: brofaramine, moclobemide, tyrima), serotonin-noradrenaline reuptake inhibitors (SNRIs: duloxetine, milnacipram, venlafaxine, desvenlafaxine), noradrenaline-dopamine re-uptake inhibitors (NDRIs, e.g. amineptine, buproprion), noradrenergic and specific serotonergic antidepressants (NASSAs, e.g. mirtazapine), serotonin antagonist and re-uptake inhibitors (SARIs, e.g. trazodone), heterocyclic antidepressants (e.g. mianserin) (Molyneaux *et al.*, 2014). The SSRIs mostly fluoxetine and citalopram are the mainstay pharmacotherapy for PPD especially when the mother is suicidal (Molyneaux *et al.*, 2014) and also because they are not implicated in any gross teratogenic alterations (Salari *et al.*, 2015). SSRI's are either given for remission of symptoms of PPD and prevention of a depressive or to prevent the symptoms from recurring and achieve a normal quality of life (Sharma *et al.*, 2013). SSRI treatment may last up to 12 weeks or even 24 months or longer depending on the patient (Kennedy *et al.*, 2009).

Even though, treatment with SSRI's may decrease maternal depressive symptoms and alleviate its effects on the new born, its use during gestation and postpartum has been a major concern because antidepressants and their pharmacologically active metabolites are lipid soluble and are excreted in breast milk (Molyneaux *et al.*, 2014). According to Berle *et al.* (2011), exposure to antidepressants in breast fed infants is considerably lower between 5 and 10 fold than exposure in-utero, but immaturity or impairment of liver or kidneys especially in preterm babies could lead to higher concentrations of the drug. Non-specific adverse events in infants exposed through breastfeeding to fluoxetine and citalopram are poor feeding and poor sleep respectively (Berle *et al.*, 2011). Data from literature is however inconclusive on the safety of gestational use of antidepressants and long term breast feeding on child outcomes but some papers have reported preterm delivery (Chambers *et al.*, 1996; Hayes *et al.*, 2012; Lund *et al.*, 2009; Simon *et al.*, 2002), lower birth weight (Chambers *et al.*, 1996; Hayes *et al.*, 2012), heart defects (Hayes *et al.*, 2012; Kulin *et al.*, 1998), transient neonatal syndrome, pulmonary hypertension in the new born as well as risk of autism, attention deficit hyperactivity disorder (ADHD) with late motor development in children (Molyneaux *et al.*, 2014). Despite these side effects associated with the treatment of PPD, pharmacotherapy is still considered the effective way to alleviate PPD symptoms.

#### *2.3.3.2 Hormone therapy*

There is a drastic decrease in maternal levels of oestrogen and progesterone after child birth, and this change in hormonal levels has been postulated as one major factor for PPD onset in women (Fitelson *et al.*, 2011a). Some studies have reported improvement in the symptoms of PPD patients on oestrogen supplementation (Gregoire *et al.*, 1996) while others report progesterone as an effective therapy against recurrent PPD (Ahokas *et al.*, 2001; Dalton, 1985; Epperson *et al.*, 1999; Sichel *et al.*, 1995). However other studies that were designed to mimic maternal hormonal changes experienced around the postpartum period have only

proved true for women with a prior history of PPD but not new mothers (Bloch *et al.*, 2000) suggesting that the vulnerability to PPD as a result of decreasing levels of oestrogen and progesterone may only be true for a subset of the population (Fitelson *et al.*, 2011a). Despite the promising nature of Oestrogen supplementation in the treatment of postpartum depression (Gregoire *et al.*, 1996), it is not a recommended treatment option (Sharma *et al.*, 2013) because of methodological flaws (Moses-Kolko *et al.*, 2004).

#### *2.3.3.3 Non-pharmacological treatment of PPD*

In contrast to pharmacotherapy and hormone therapy, psychotherapy has been widely used for the management of PPD as reported by several publications. Psychotherapy involves interpersonal therapy, cognitive-behavioural therapy, and psychodynamic therapy, as well as psychosocial interventions such as nondirective counselling (Cooper *et al.*, 2003; Holden *et al.*, 1989; O'Hara, 2009). Some mothers with PPD prefer psychotherapy due to concerns about infant exposure and potential side effects of antidepressant medication, (Dennis *et al.*, 2006) and therefore often prefer psychological treatments which are equally effective interventions in decreasing depressive symptoms and are plausible treatment options for PPD (Buist *et al.*, 2005; Dennis *et al.*, 2006; Pearlstein *et al.*, 2006 ; Turner *et al.*, 2008).

Cognitive-behavioural therapy (CBT) has been used to treat PPD by focusing on evaluating and modifying dysfunctional thoughts, enhancing problem-solving abilities, and promoting adaptive behaviour (Beck *et al.*, 1979).

Other non-pharmacological treatment options such as exercise, massage, phototherapy, and acupuncture are used but data on their effectiveness is scarce (Sharma *et al.*, 2013).

### 2.3.4 Risk factors for PPD

There are several risk factors that have been described by many authors with varied views on their importance in relation to PPD. However, most of the authors agree that the factors associated with postpartum depression can be classified in five domains which include psychiatric risk factor (Davé *et al.*, 2010; Ghaedrahmati *et al.*, 2017; Silverman *et al.*, 2017), obstetric factors (Ghaedrahmati *et al.*, 2017; Robertson *et al.*, 2003; Silverman *et al.*, 2017), biological and hormonal risk factors (Brummelte *et al.*, 2016), social and lifestyle risk factors (Ghaedrahmati *et al.*, 2017).

#### *2.3.4.1 Psychological risk factors*

Previous history of depression and anxiety has been widely reported as increasing the likelihood of PPD (Davey *et al.*, 2011; Lee *et al.*, 2000; O'Hara *et al.*, 1984). The occurrence of other mood disorders such as depression during pregnancy (antenatal depression) is a powerful factor in predicting PPD (Lancaster *et al.*, 2010).

#### *2.3.4.2 Obstetric risk factors*

Pregnancy related complications such as preeclampsia, hyperemesis, premature contractions as well as delivery related complications, such as emergency / elective caesarean, instrumental delivery, premature delivery and excessive bleeding intrapartum are associated with triggering PPD. Complications during pregnancy that result in hospitalization or performing emergency caesarean section has been implicated as a strong risk factor for PPD by several authors (Ghaedrahmati *et al.*, 2017; Houston *et al.*, 2015). Even postpartum complications such as meconium passage, umbilical cord prolapse obstetric haemorrhages as well as low birth weight are indicated as risk factors for PPD (Gaillard *et al.*, 2014; Helle *et al.*, 2015; Leigh *et al.*, 2008; Mathisen *et al.*, 2013). Some studies have tried to associate postpartum depression with the number of births (Kheirabadi *et al.*, 2009; Mathisen *et al.*,

2013; Mayberry *et al.*, 2007). The differences between the results of these studies suggest that the number of childbirths alone is not an independent factor for developing PPD and that a multiplicity of several interacting factors account for PPD (Ghaedrahmati *et al.*, 2017).

#### *2.3.4.3 Social risk factors*

Social support is very important during pregnancy and the early postpartum stage in preventing PPD. Social support encompasses emotional support, financial support, intelligence support, and empathy relations (Feng *et al.*, 2015). Lack of social support, sexual violence and other forms of domestic violence during pregnancy are important factors in the onset of depression and anxiety disorders and by extension are seen as factors contributing to the incidence of postpartum depression (Landman-Peeters *et al.*, 2005 ; Ludermir *et al.*, 2010). Other social vices such as smoking, drinking of alcohol during the perinatal period has been associated with increased incidence of PPD (Jansen *et al.*, 2010).

Low- and middle-income countries are plagued with high rates of PPD which may be the reflection of the lack of protective factors against the onset of depression (Parsons *et al.*, 2012). For instance Husain *et al.* (2000) observed that well educated women are less likely to become depressed compared to poorly educated women.

#### *2.3.4.5 Biological and hormonal risk factors*

##### *2.3.4.5.1 Neurotransmitter dysfunction*

Monoamines have been the primary focus of the earlier theories of the neurobiology of postpartum depression. The main assumption of the monoamine hypothesis is that clinical depression is due to the dysregulation in function and/or amount of the monoamine neurotransmitters. This deficiency in the neurotransmission is mediated by serotonin (5-HT, 5-hydroxytryptamine), noradrenaline (NA) and/or dopamine (DA) (Kharade *et al.*, 2010).

The concentrations of these monoamines may be altered as a result of disrupted synthesis, storage or release of these neurotransmitters or in some instances the concentrations may be normal but the presynaptic receptors and/or the subcellular messenger activity may be impaired (Mirescu *et al.*, 2006).

*2.3.4.5.2 Hormonal changes*

The peripartum period is characterized by a lot of significant biological changes necessary to maintain pregnancy, support the development of the foetus, and promote labour, safe delivery and lactation. Immediately after the delivery of both baby and placenta, the maternal system undergoes drastic hormonal changes within the first postnatal days which may take some weeks to a few months before balance is restored. The endocrine system is one of the many systems that modulates some of these changes observed in the maternal body (Bloch *et al.*, 2000; Brummelte *et al.*, 2010; McCoy *et al.*, 2003). These endocrine changes as well as adjustments in other biological systems have an impact on maternal mental health (Yim *et al.*, 2015) resulting in mood disorders such as postpartum depression.

The role of reproductive hormones apart from orchestrating pregnancy, labour and birth, they have also been associated with mood disorders especially oestrogen and progesterone. Oestrogen withdrawal, fluctuations and sometimes sustained oestrogen deficiencies (Epperson *et al.*, 2006; Yim *et al.*, 2015) has been implicated in the pathophysiology of PPD. Bloch *et al.* (2000) in a landmark study on estradiol and progesterone withdrawal suggested that women with a history of PPD may be differentially sensitive to the mood-destabilizing effects of changes in gonadal steroids. They further indicated that assessment of estradiol and progesterone levels is not likely be an appropriate measure to adequately reflect the processes through which these hormones impact PPD development. Accordingly, several other authors have however found no association between the perinatal oestrogen levels or magnitude of

oestrogen drop with PPD (Chatzicharalampous *et al.*, 2011; Hohlagschwandtner *et al.*, 2001; Ingram *et al.*, 2003; Nappi *et al.*, 2001; O'Keane *et al.*, 2011; Okun *et al.*, 2011).

Progesterone is thought to be protective against depression because it exhibits anxiolytic and anaesthetic properties (Uphouse *et al.*, 2009) and has also been shown to modulate serotonergic receptors. Thus, changes in levels progesterone during pregnancy and postpartum may contribute to PPD (Bloch *et al.*, 2000). Just like oestrogen, few studies implicate progesterone withdrawal in PPD risk (Yim *et al.*, 2015). Three independent studies found no association between progesterone levels and PPD symptoms and furthermore found no evidence that the magnitude of the perinatal progesterone drop predicted PPD symptoms (Chatzicharalampous *et al.*, 2011; Ingram *et al.*, 2003; O'Keane *et al.*, 2011). There is paucity of evidence to support the hypothesis that progesterone is psycho-protective and lower levels in the third trimester or postpartum precipitates PPD symptoms (Yim *et al.*, 2015). It is noteworthy that some studies have also found associations between prolactin and PPD, oxytocin and PPD as well as testosterone and PPD.

*2.3.4.5.3 HPA axis dysregulation*

Stress hormones in particular those of the hypothalamic-pituitary-adrenal (HPA) axis have also been implicated in the aetiology of PPD. Mood changes, cognitive difficulties, and heightened anxiety are characteristic of depressive disorders and are hypothesized to involve dysregulation of the body's stress response systems (Ehlert *et al.*, 2001). Stress hormones follow a pattern similar to reproductive hormones, as they increase during pregnancy and then drop after delivery. However, the corticotrophin-releasing hormone (CRH) increases exponentially over the course of pregnancy (McLean *et al.*, 1995; Sandman *et al.*, 2006), reaching levels observed only under stressful conditions. The hypothalamus secretes corticotrophin-releasing factor (CRF) after the HPA axis is activated by stress. This

stimulates the synthesis and release of adrenocorticotrophin (ACTH) from the anterior pituitary, which, in turn, stimulates the synthesis and release of cortisol from the adrenal cortex. Under conditions of severe stress, sustained raised levels of glucocorticoids may damage hippocampal neurons, particularly CA3 pyramidal neurons, such that dendritic branching is reduced and dendritic spines where the neurons receive their glutamatergic synaptic inputs are lost (Nestler *et al.*, 2002). Enhanced CRF transmission in other areas such as the prefrontal cortex and hypothalamus contributes to symptoms of depression (O'Keane *et al.*, 2011; Yim *et al.*, 2009; Yim *et al.*, 2015).

Thyroid hormones have been proposed as a biomarker of PPD in large part because symptoms of PPD overlap with those of postpartum thyroiditis (Bunevicius *et al.*, 2009; Yim *et al.*, 2015). Depression accompanies thyroid pathologies and thyroid dysregulation (Berent *et al.*, 2014; Gulseren *et al.*, 2006; Nemeroff *et al.*, 1985; Placidi *et al.*, 1998) and the administration of thyroid hormones has been shown to augment antidepressant treatment (Cooper-Kazaz *et al.*, 2007; Cooper-Kazaz *et al.*, 2008). Oestrogen increases thyroxin-binding globulin (TBG) and causes increase in circulating thyroxin (T4) levels (Arafah, 2001; Schiller *et al.*, 2015 ) and may cause PPD in some women (Pedersen *et al.*, 2007; Pedersen *et al.*, 1993), previous studies have however failed to identify an association between thyroid hormone dysregulation and PPD in the majority of patients (Albacar *et al.*, 2010; Bloch, 2003; Kent *et al.*, 1999; Schiller *et al.*, 2015 ).

*2.3.4.5.4 Immune system dysregulation*

Dysregulation of the immune system has also been implicated in the development of PPD (Corwin *et al.*, 2008). Anti-inflammatory cytokines are elevated during pregnancy and are responsible for immunosuppression and maintenance of the pregnancy while proinflammatory cytokines are down regulated. After delivery, there is an abrupt shift in the

immune system into a proinflammatory state with higher levels of proinflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-6 (Schiller *et al.*, 2015 ) and IL-6 receptor (Maes *et al.*, 2000) which are characteristic of depressive states (Dowlati *et al.*, 2010; Schiller *et al.*, 2015 ). Cytokine administration has been shown to be associated with the onset of depressive symptoms (Raison *et al.*, 2006; Schiller *et al.*, 2015 ).

*2.3.4.5.5 Genetic factors*

Evidence of a genetic vulnerability to PPD has been established from family, candidate gene, genome-wide, and gene manipulation studies (Schiller *et al.*, 2015 ). These studies suggest that PPD aggregates in families (Forty *et al.*, 2006; Murphy-Eberenz *et al.*, 2006), is heritable (Schiller *et al.*, 2015 ; Treloar *et al.*, 1999), and may be genetically distinct from major depression (Treloar *et al.*, 1999). The role of specific genetic variations remains unclear although multiple genes have been implicated in PPD. Studies of candidate gene studies of PPD have identified several of the same polymorphisms implicated in major depression, including the Val66Met polymorphism of the brain-derived neurotrophic factor (BDNF) gene (Comasco *et al.*, 2011; Figueira *et al.*, 2010), the Val158Met polymorphism of the COMT gene (p-) (Alvim-Soares *et al.*, 2013 ; Comasco *et al.*, 2011), the BcII polymorphism of the glucocorticoid receptor and the rs242939 polymorphism of the CRH receptor-1 (Engineer *et al.*, 2013; Schiller *et al.*, 2015 ).

*2.3.4.5.6 Epigenetic factors*

Epigenetic processes have been implicated in the pathophysiology of major depression and is beginning to realign the understanding of depression in terms if epigenetic expressions of complex life experiences into research designs (Sun *et al.*, 2013). Even though this approach has not yet gained much attention in PPD research, it holds a valuable potential in this area. A study has found increased oestrogen-mediated DNA methylation changes observed in women

diagnosed with PPD within four weeks postpartum and point to two genes involved in oestrogen signalling (promoter regions of HP1BP3 and TTC9B) as significant biomarkers (Guintivano *et al.*, 2014).

*2.3.4.5.7 Nutrient deficiencies*

Nutritional deficiencies have been associated with mood disorders especially postpartum depression. Credible links between nutrition and mood has been reported for folate (Abou-Saleh *et al.*, 2006), vitamin B-12 (Bodnar *et al.*, 2005), calcium, selenium (Benton, 2002; Bodnar *et al.*, 2005) and iron (Bodnar *et al.*, 2005; Pick *et al.*, 2005). Iron has been implicated because of its role in synthesis of enzyme systems that regulate brain growth, myelination, and dopamine D2 and norepinephrine receptor synthesis, serotonin receptor and energy production cytochromes such as cytochrome c oxidase (CytOx).

## 2.3 IRON AND BRAIN FUNCTION

Iron is a ubiquitous metal that is essential for the function of all mammalian cells. Iron presents a significant risk to these cells and the central nervous system is no exception to the effects of iron deficiency and iron overload on the development and function of the brain (Georgieff, 2008 ). In all organisms, iron is involved in a series of very important biochemical functions including mitochondrial energy generation, oxygen transport, glucose metabolism and the synthesis of neurotransmitters, myelin, and DNA replication (Berg *et al.*, 2001; Piñero *et al.*, 2000b; Salvador, 2010). All these functions of iron are carried out by four main classes of iron compounds that include, iron-containing proteins, heme proteins, iron-sulphur enzymes, and iron-containing enzymes (Beard *et al.*, 1996). The alterations created by ID in pregnant mothers and their infants' results in several neuropsychiatric disorders including PPD in mother and neurobehavioural changes in the infants.

### 2.3.1 Brain uptake of iron

The absorption of iron at the intestinal level is regulated according to the iron status of the individual. Absorption of iron increases in iron deficiency and decreases when the iron stores augment (Piñero *et al.*, 2000b). The uptake pathway of brain iron starts in the intestines where dietary $Fe^{3+}$ (ferric iron) is reduced by duodenal cytochrome B to $Fe^{2+}$ (ferrous iron) which is then transported via the divalent metal transporter-1 (DMT-1) across the duodenal epithelium into the circulation. Ceruloplasmin or hephaestin regulates iron homeostasis in the blood, by oxidizing $Fe^{2+}$ to $Fe^{3+}$ and promoting it's binding to the predominant serum iron carrier, transferrin, (Connor *et al.*, 1995; Kell, 2009; Ponka, 2004; Roskams *et al.*, 1994; Salvador, 2010). Transferrin-iron complex circulating in the blood cannot directly cross the blood–brain barrier (BBB) into the central nervous system (CNS). The most common pathway for iron transference across the BBB is through transferrin receptors (Tfr) on brain endothelial cells, which bind iron circulating in the form of transferrin (Tf) that then enters the brain by endocytosis. Other transporter systems that deliver iron across the BBB include DMT and the lactoferrin receptor (Ponka, 2004). Ferritin is the most common iron-storage protein in the brain.

### 2.3.2 Iron deficiency and brain function

The role of iron in various brain functions has been highlighted in previous studies (Beard *et al.*, 1996; Kretchmer *et al.*, 1996). Iron is an essential element for the normal development of cognitive as well as neurobehavioral functions. Proper iron balance is essentially regulated at the uptake, storage, and release levels in relation to its availability and the iron status. When the iron stores are depleted and there is a decreased supply of iron to tissues and cells including the red blood cells, ID results. ID is the single most common micronutrient deficiency in the globally, affecting more than 2 billion individuals and 20 – 50 % of

pregnant women (Stoltzfus, 2001). Iron deficiency poses a lot of risks for the pregnant woman and the foetus. The effect of iron deficiency is a function of the timing of the deficiency and the relative nutrient requirement of the brain region or process (Georgieff, 2017; Kretchmer *et al.*, 1996) since the brain is not a homogenous organ with a single developmental pattern. The brain is rather made of multiple regions (e.g., hippocampus, frontal cortex, striatum and cerebellum) and processes (e.g., myelination, energy metabolism and neurotransmission) all of which have different trajectories of development (Georgieff, 2017; Thompson *et al.*, 2001). The late foetal stage and early postnatal stage through the first 3 years is a critical period for rapid development of brain regions such as prefrontal cortex, cerebellum, striatum, hippocampus, as well as the dopaminergic and glutamatergic neurotransmission systems (Georgieff, 2017; Rice *et al.*, 2000; Thompson *et al.*, 2001; Wachs *et al.*, 2014). Iron deficiency (ID) during later gestation and the early neonatal period in rodents has been shown to induce delay in the onset of development of the hippocampus (Georgieff, 2017), and caused neuronal structural abnormalities which persist in the adult animal despite iron treatment (Brunette *et al.*, 2010b; Fretham *et al.*, 2012).

The biological variables implicated in ID-induced behavioural changes include myelination (as the oligodendrocytes are major iron storage sites), neurotransmitter synthesis and regulation (particularly the monoamines), synaptogenesis and neurogenesis, and energy expenditure (Beard, 2007; Beard *et al.*, 2003; Georgieff, 2008; Lozoff, B. *et al.*, 2006; Lozoff, B. *et al.*, 2006; Wenger *et al.*, 2017). ID also significantly alters gene expression of important synaptic plasticity proteins such as BDNF (Tran *et al.*, 2015), postsynaptic density protein 95, and calcium or calmodulin-dependent kinase 2-$\alpha$ (Carlson *et al.*, 2007). The alterations in neurotransmission, myelination and adult gene expression are widespread and implicated in schizophrenia, mood disorders, and autism (Georgieff, 2017; Tran *et al.*, 2016). Neurotransmission impairments are implicated due to the several iron-containing enzymes

involved in neurotransmitter synthesis (Hidalgo *et al.*, 2007; Unger *et al.*, 2007; Wigglesworth *et al.*, 1988) which are invariably affected by ID. Tyrosine hydroxylase is required for dopamine and noradrenaline synthesis (Hidalgo *et al.*, 2007; Lehmann *et al.*, 1986), tryptophan hydroxylase, required for serotonin synthesis (Hasegawa *et al.*, 1999; Hidalgo *et al.*, 2007), glutamate decarboxylase and glutamate transaminase, involved in GABA and L-glutamate synthesis (Hidalgo *et al.*, 2007; Li, 1998; Shukla *et al.*, 1989) and the monoamine oxidases A and B (MAO-A/B) involved in dopamine catabolism (Ben-Shachar *et al.*, 1985; Hidalgo *et al.*, 2007; Yehuda, 1990 ), are all affected by ID.

Evidence from multiple mammalian species including mice, rats, nonhuman primates and humans shows that neonatal ID results in long-term neurobehavioural abnormalities including poorer attention, apathy, irritability, lethargy, increased anxiety and depression, and an increased risk of schizophrenia (Fretham *et al.*, 2012; Golub *et al.*, 2007; Insel *et al.*, 2008; Lukowski *et al.*, 2010; Piñero *et al.*, 2000a; Schmidt *et al.*, 2007). Cognitive impairment has also been related to iron deficiency (ID) (Gonzalez *et al.*, 2007; Grantham-McGregor *et al.*, 2001; Jáuregui-Lobera, 2014). Among the cognitive impairments caused by ID are those related to attention span, intelligence, and sensory perception functions (Jáuregui-Lobera, 2014).

Although ID would produce alterations in cognitive and behavioural functions, most of which are irreversible even with iron repletion, iron accumulation is also harmful since free iron interacts with oxygen to generate free radicals or reactive oxygen species (ROS) through Haber-Weiss and Fenton reactions (Halliwell, 1992; Halliwell, 1996; Salvador, 2010). Uncontrolled production of ROS causes the oxidation of lipid components of cellular membranes resulting in oxidative stress. Protein phosphatases, protein kinases, and transcription factors are all targets of ROS. Oxidative stress induces several changes in cells eventually resulting in impaired cell function and death (Keller *et al.*, 1997; Springer *et al.*,

1997). Since both ID and iron accumulation affect brain function, this study seeks to explore the role of gestational iron treatment and gestational ID on depressive-like symptoms in postpartum rats and their litter using depression models as well as examining the effects on neurons.

## 2.4 ANIMAL MODELS OF PPD

### 2.4.1 Forced Swim Test (FST)

The FST, as originally described by Porsolt and colleagues (Porsolt *et al.*, 1978; Porsolt , Le Pichon , *et al.*, 1977), has developed into the most widely used model for assessing antidepressant-like activity in rats and other species, including mice and rats (Cryan *et al.*, 2005; Porsolt *et al.*, 1978), Mongolian gerbils (Rupniak, 2001) and sand rats (a species of gerbil) (Einat *et al.*, 2006). Its widespread use is largely attributed to its high throughput, ease of use, interlaboratory reliability, strong predictive validity and specificity (Slattery *et al.*, 2012 ). The FST is based on the observation that when rats are exposed to water, after initial escape-directed behaviour they stop struggling and show passive immobile behaviour. The immobile behaviour is believed to reflect either a failure to persist in escape-directed behaviour after stress (i.e., behavioural despair) or the development of passive behaviour that disengages the animal from active forms of stress coping (Cryan *et al.*, 2002; Lucki, I. , 1997; Slattery *et al.*, 2005b).

The rat FST has proven highly valuable for assessing the antidepressant-like effects of the majority of currently available antidepressants (Borsini *et al.*, 1988; Cryan *et al.*, 2002; Cryan *et al.*, 2005). However, the major concern with the traditional FST initially developed by Porsolt *et al.* (1977) is its unreliable detection of antidepressant-like effects of SSRIs (Cryan *et al.*, 2002; Cryan *et al.*, 2005; Lucki, I. , 1997). This led Lucki and colleagues to alter specific parameters of the test in order to increase the reliable detection of SSRIs (Detke

*et al.*, 1996; Lucki, I. , 1997). The alterations further enabled investigators to distinguish specific behavioural components of active behaviours, namely; climbing and swimming (Cryan *et al.*, 2002; Lucki, I. , 1997). Moreover, several pharmacological and lesion studies have shown that the active behaviours are predominantly under the control of different neurotransmitter systems. Catecholaminergic antidepressants selectively increase climbing behaviour, whereas serotonergic agents selectively increase swimming behaviour (Cryan *et al.*, 2005; Detke *et al.*, 1996). The modified FST has an added benefit of determining whether a novel pharmacological agent predominantly activates either of these neurotransmitter systems (Slattery *et al.*, 2012 ).

### 2.4.2 Open Field Test (OFT)

OFT paradigm designed to inhibit behaviour such as exploratory activity or social investigation that is characteristic rodents against the aversive properties of an open, brightly lit area, new (novel) test environment (File, 1980; Prut *et al.*, 2003). Rodents taken from their home-cage and placed in a novel and aversive environment show anxiety and fear, by exhibiting changes in behavioural component such as decreased ambulation and exploration time in the centre of the open field with increased movement at the periphery (Bhattacharya, 1994; Bhattacharya *et al.*, 1991). These parameters can be altered by classical anxiolytics as well as anxiogenic agents. OFT represents a valid measure of anxiety-related behaviour in both pharmacological and genetically altered rodents (Choleris *et al.*, 2001; Prut *et al.*, 2003).

### 2.4.3 Novelty-Induced Hypophagia (NIH)

Reduced in feeding in response to a new environment has long been used as a measure of emotionality and anxiety in rats (Dulawa, 2009 ). NIH is an example of a conflict paradigm in which is based on the principle that when a rat is faced with a choice to approach and

consume a palatable meal in a new environment while trying to avoid the new environment at the same time (Dulawa *et al.*, 2005). Some hypophagia models incorporate overnight fasting of the animals, however, the use of a familiar and highly palatable meal makes food deprivations unnecessary (Merali *et al.*, 2004) in NIH. The same dependent measures are assessed in the home and novel cages to control the effects of the drug treatment on appetite (Dulawa *et al.*, 2005). The dependent variables widely used to assess anxiety-related behaviour are the latency to drink the milk and consumption of the palatable meal within the first five minutes of the test. Consumption measures are also used to assess anhedonia.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 STUDY DESIGN

The study design was experimental.

## 3.2 MATERIALS

### 3.2.1 Drugs

Fluoxetine hydrochloride (Prozac) was from Bristol laboratories Ltd. Berkhamsted, Hertfordshire, HP4 1 EG, UK. The iron supplement (crystalline ferrous sulphate) was obtained from Sigma-Aldrich Inc., St. Louis, MO, USA. Desferrioxamine methane sulphonate was purchased from Novartis Pharma Stein AG, Stein, Switzerland.

### 3.2.2 Animal preparation

Eighty female (180 – 250 g) and twenty male (200 – 250 g) young *Sprague-Dawley* (Harlan, Sprague-Dawley) rats at 7 weeks were initially obtained from the centre for plant medicine research (CPMR), Mampong and kept at the animal house of the Department of Microbiology, School of Biomedical and Allied Health Science, U.G. The rats were housed in standard cages (800 $cm^2$ x 14 cm) with a minimum floor area of 200 $cm^2$ per animal and soft wood shavings as bedding material. They were maintained at room temperature (25 ± 1℃), relative humidity between 45 - 65 % with a 12 h light/dark cycle. They acclimatized for 1 week prior to the experimental procedures and were fed standard rat chow and water ad libitum. All animals to be used in this study were handled according to the Guide for the Care and Use of Laboratory Animals and in accordance with the ethical procedures and policies approved by the ethical and protocol review committee of the college of health sciences, University of Ghana.

### 3.3 EXPERIMENTAL DESIGN

Eighty (80) young adult (7 months old) female rats were crossed. Five rats (1 male: 4 females) were initially housed in clear polyutherane cages under standard laboratory conditions for the crossing. The day of vaginal plug (male sperm deposits on the female) release was determined as gestational day (GD) 1. Pregnant rats (dams) were randomly allocated into 4 groups, n=10. Group I (VEH group): dams received saline orally as the negative control. Group II (Flx group): dams were treated with fluoxetine (3 mg/kg; 10 mg/kg; 30 mg/kg. *p.o*; n=10) as the positive control group. Group III (ID group): dam were treated with desferrioxamine (50 mg/kg, *s.c*) as the iron deficient group. Group IV (Fe group): dams were treated with iron (0.005 $mg$/kg; 0.8 $mg$/kg; 8 $mg$/kg. *p.o*; n=10) as the iron supplement group. Drug administration started on GD 1 and continued throughout the period of gestation (GD 21). During the period of gestation, the dams were subjected to mild stressors including cage tilting, damping of saw dust, changing of bedding. After parturition separation of the litter from the dams for a maximum of 2 h was included. Four (4) weeks after delivery, the litter were weaned. The litter together with the dams were taken through the open field test (OFT) for a day, the forced swim test (FST) for fourteen (14) consecutive days and then novelty-induced hypophagia (NIH) for five (5) consecutive days.

### 3.4 DRUG TREATMENTS

#### 3.4.1 Iron Treatment

Iron solutions of different concentrations (0.005 mg/ml/day, 0.8 mg/ml/day, 8 mg/ml/day. *p.o*) were prepared from 200 mg ferrous sulphate tablets. The solutions were administered to the dams via oral gavage for three (3) weeks from GD 1 to GD 21.

### 3.4.2 Fluoxetine Treatment

Fluoxetine solutions (3 mg/kg, 10 mg/kg and 30 mg/kg. *p.o*) was administered to the dam via oral gavage for three (3) weeks from GD 1 to GD 21.

### 3.4.3 Desferrioxamine Treatment

Desferrioxamine methane sulphonate (50 mg/kg) was administered subcutaneously (*s.c*) to the dams from GD 1 to GD 21. This mode of administration was chosen over intramuscular (*i.m*) because *s.c* administration has been shown to be more efficacious at improving iron excretion compared to *i.m* (Hussain *et al.*, 1976; Propper *et al.*, 1977).

## 3.5 ANIMAL MODELS

### 3.5.1 Novelty-Induced Hypophagia (NIH)

The procedure for the NIH test was based on that described by Dulawa (2009). Rats were divided in groups of ten (n=10) of four (4) main treatment groups and treated with vehicle (saline), iron, desferrioxamine or fluoxetine. Rats were singly housed several days before training began and then tested in two test environments: the home cage and a novel cage which is a standard home cage without bedding but rather a white surface with increased overhead illumination, which was provided by positioning overhead lamps close to the cages. Sweetened condensed milk was diluted (1:3; milk: water) and stored at 4 ℃ and drawn up into 10 ml serological pipettes immediately before use. After being housed singly for 24 h, rats were then trained to drink sweetened condensed milk for three consecutive days (days 1–3) in the home cage. For home-cage testing, rats were removed from their cages briefly to position the pipettes containing milk in wire lids and initial readings were taken and then quickly returned to their cages and a timer was started. The latency to drink and the volume consumed were recorded every 5 min for a 30 min period. Latency to drink is defined as the

time taken for the rat to first lick the sipper. To be counted as a lick, the tongue should make contact with the sipper; merely sniffing the sipper was not counted. The sipper was positioned such that a rat resting on the floor of the cage could drink comfortably. Home cage testing was conducted under relatively dim lighting. Rats that never drank during the 30 min of home cage testing were eliminated from the experiment. For novel-cage testing, pipettes containing the milk solution were positioned and any drops of milk solution on the floor of the novel cage during pipette positioning was cleaned thoroughly. Rats were then quickly placed into the novel cage and a timer started. The latency to drink and the volume consumed are recorded every 5 min for 30 min. The novel-cage testing was performed under bright lighting with white floor under the cages to increase aversiveness of the cage.

### 3.5.2 Forced Swim Test (FST)

The FST was based on that described by Porsolt *et al.* (1977) with modifications. The modified forced swim test procedure is more sensitive in detecting the antidepressant activity of new agents than the traditional forced swim test. The modifications to the traditional test employed in this study included increasing the water depth (Detke *et al.*, 1996), and using a time sampling technique to rate the predominant behaviour (swimming, climbing or immobility) over a 5-s interval (Cryan and Lucki, 2000; Cryan *et al*, 2002b). All the dam and selected litter were taken from each of the groupings described above and taken through the FST. One day after delivery (postnatal day, PND 2), dams were gently placed individually into transparent cylindrical plastic buckets (45 cm high, 20 cm internal diameter) containing water maintained at a temperature of 25 ± 1 ℃ up to a level of 30 cm and allowed to swim for 5 min. The water was replaced between each test session to avoid the effect of scent clues left by the previous animal. After each test session, the animals were removed from the bucket, dried with a towel and returned to their cage. Each test session was recorded by a video camera suspended approximately 250 cm above the buckets. This was done for

fourteen days (till PND 15). This process was repeated for the litter one week after weaning, PND 35. Each rat was said to be immobile when it ceased struggling and remained floating upright and motionless only making slight movements to keep its head above the water. Immobility was considered as a state of behavioural despair (Cryan *et al.*, 2000; Porsolt *et al.*, 1977; Slattery *et al.*, 2005a). The animal was considered to be swimming when it made active horizontal movements and climbing when it was involved in active vertical movements. A reduction in immobility score was an indication of antidepressant effect. An increase in climbing score without corresponding change in swimming behaviour is suggestive of adrenergic mechanisms while an increase in swimming score without change in climbing suggested serotoninergic activity.

### 3.5.3 Open Field Test (OFT)

The test was based on that described previously by Kasture *et al.* (2002). The open field apparatus was constructed with plywood and the walls painted white. It measured 72 cm x 72 cm x 36 cm (*l* x *b* x *h*). Black lines were drawn on the floor with a marker and were visible on the white floor. The lines divided the floor into sixteen 18 x 18 cm squares. A central square of (18 cm x 18 cm) was drawn in the middle of the open field. The set up was illuminated by a 100 W bulb placed about 150 cm directly above the centre of the apparatus floor. The test period was initiated by starting the video recorder and placing a single rat in the centre of the central square of the apparatus. The animal was allowed to move freely in the arena for 5 minutes. Each session was recorded by a video camera suspended approximately 100 cm above the arena. In order to evaluate the locomotor activity of the animal, the latency to leave the centre square and total line crossings (as all four paws crossing over the line) were recorded (Salari *et al.*, 2015).

## 3.6 HISTOLOGY OF THE FRONTAL CORTEX OF THE BRAIN

The histology was done as described by Zaqout *et al.* (2016) and Das *et al.* (2013) with modifications.

### 3.6.1 Preparation of Solutions

#### *3.6.1.1 Golgi-Cox Solution*

The Golgi-Cox solution has three components and was prepared as follows:

Solution A: 5 % w/v Potassium dichromate solution was prepared by dissolving 10.0 g of $K_2Cr_2O_7$ (Merck KGaA) in 200 ml doubled distilled water under fume hood.

Solution B: 5 % w/v Mercuric chloride (sublimate) solution was prepared by dissolving 10.0 g of $HgCl_2$ (Merck KGaA) in 200 doubled distilled on top of a hot plate under fume hood.

Solution C: 5 % w/v Potassium chromate solution was prepared by dissolving 8.0 g of $KCrO_4$ (Merck KGaA) in 160 ml doubled distilled water under fume hood.

Solution A and solution B were mixed in a 500 ml glass beaker while 400 ml distilled water was added to solution C in a 1000 ml glass beaker. Solution AB was poured into solution C while stirring continuously with a glass rod. The solution was then transferred into a 1.0 L reagent bottle and stored in the dark for seven (7) days. This is the Golgi-Cox solution.

#### *3.6.1.2 Sucrose Solution*

The 30 % w/v sucrose solution was prepared by dissolving 300 g of sucrose ($C_{12}H_{22}O_{11}$; 1.07687, Merck GaA, Germany) in 1000 ml distilled water on a hot plate while stirring. The solution was cooled in a refrigerator before use.

### 3.6.2 Gelatinization of Slides

The slides were coated with gelatine as described by Das *et al.* (2013). Five (5) grams of gelatine was dissolved in warm 500 ml of distilled water. Once the gelatine was dissolved, 0.5 g of Chromium Potassium Sulphate (CPS) and Thymol (preservative) were added to the gelatine and the solution stirred using a magnetic stirrer. The solution was then made to 1000 ml using distilled water and filtered with a Whatman filter paper before allowed to cool down to room temperature. The slides were dipped into the solution taking care to avoid bubble formation since this could result in a non-uniform coating. The slides were allowed to dry in an oven at 37 ℃ overnight before use.

### 3.6.3 Tissue collection

Brain tissues from both the control and treatment groups of the dams and litter were extracted immediately after the animals were perfused transcardially with 0.9 % saline. Brain tissues were post-fixed in a 40 ml bottle containing Golgi-Cox solution for twenty-four (24) hours (h). After 24 h, each brain sample was removed and the solution discarded. The sample bottle was washed and filled with new Golgi-Cox solution. The brain samples were placed back into the solution and stored in dark for fourteen (14) days. After which they were removed from the Golgi-Cox solution, slightly blotted with tissue paper and transferred into a 30 % w/v sucrose solution where they floated. The brain samples in the sucrose solution were then stored in the refrigerator until they sunk before sectioning was done at 50 µm using a microtome (Das *et al.*, 2013).

#### *3.6.3.1 Tissue processing*

Upon removal from the sucrose solution, each brain sample was divided into three coronal sections, placed in histological cassettes (Rotilabor embedding cassettes; K114.1, Carl Roth GmbH, Germany) and passed through an ethanol series of 70 % ethanol for 1 hour, 95 %

ethanol for 1.5 hours and100 % ethanol twice for 2 hours. The processed tissues were placed in molten paraffin wax for a total time of 3 hours after which they were embedded in molten paraffin wax and placed in the refrigerator at 4 ℃ until sectioning.

#### *3.6.3.2 Sectioning the brain tissues*

The microtome used for sectioning was a Leica RM 2235 manual microtome. The refrigerated tissue blocks of both the control and treatment groups were mounted on the microtome, sectioned at 50 $\mu m$ and placed on water. The floating sections were picked with a brush and mounted on the gelatine coated slides. The sections were blotted with tissue paper and direct, downward moderate pressure was applied with the heel of the palm (Gibb and Kolb, 1998) so that the sections were firmly glued to the gelatine slides. The slides with sections were transferred to racks and kept for drying in dark for 3 days.

#### *3.6.3.3 Colour development*

The racks with the slides were dewaxed by passing them through xylene twice for 2 minutes each. The racks were then passed through 100 % ethanol twice for 2 minute each and placed in a jar filled 50 % ethanol for 5 minutes before been placed in a 3:1 ammonia solution for 8 minutes in the dark at room temperature. The sections were washed with double distilled water twice for 5 minutes each. Next, the racks with the slides were immersed in 1 % sodium thiosulfate solution to fix the stain for 5 minutes at room temperature in the dark. The racks with the slides were washed in distilled water twice for 1 minute each. The sections were then incubated in 5 % Mallory stain C as a counter stain for 1 minute. The sections were then passed through an ethanol series of 70 %, 95 % and 100 % (twice) for 5 minutes each to completely dehydrate the sections. The racks with the slides were placed in fresh xylene for 2 hr in the dark. The slides were taken out carefully and mounted with DPX (mixture of

distyrene, a plasticizer, and xylene used as a synthetic resin mounting media) and allowed to dry under the fume hood for 3 days before examining under the microscope.

#### *3.6.3.4 Neuronal count and morphology*

The slides were observed under a light microscope (Leica Galen III-1154XV) at low (x100) and high (x400) magnifications. Images were captured using a coupled device (CD) eye piece (Lenovo Q350 USB PC Camera). The microscope stage was moved from around the tissue at 2 and 3 microscope stage unit intervals on the x and y axes respectively. Snapshots of the cell bodies within the field of view were captured (x100) onto a computer (HP Compaq dx2300 Micro tower) with the eyepiece. This was done until the whole area of tissue was covered. Using a minimum number of 3 animals per dose group, a total 3168 micrographs were randomly sampled per group from dams and their litter for stereological assessment to determine the volume of neurons using Cavalieri principle (Dezfoolian *et al.*, 2009). All images were enhanced using Adobe Photoshop CS6 version 13.0 × 64. Using ImageJ Software, a stereological grid consisting of uniformly spaced points, 1 cm x 1 cm was superimposed over each micrograph of the brain tissue to count the number of test points which intersected with the cell bodies (Figure 3.1).

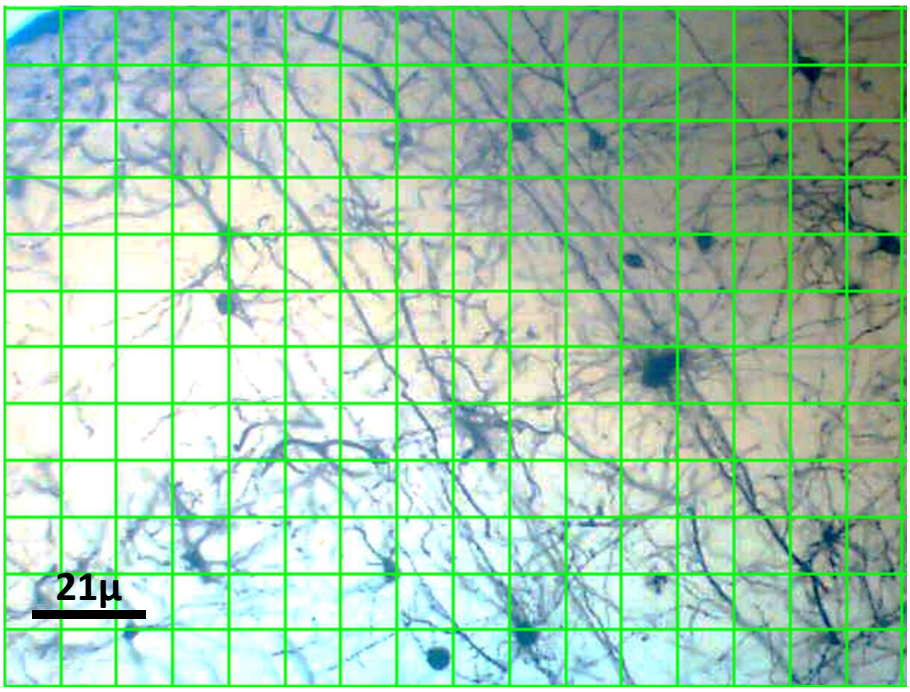



Figure 3. 1: A picture showing the stereological grid (1 cm x 1 cm) superimposed on a brain section of the cortex

The volume densities of the neurons were calculated using the equation (Heidari *et al.*, 2008):

$$V = \frac{\Sigma P \times \left(\frac{a}{p}\right) \times t}{M^2}$$

Where V is volume, 'ΣP' is the sum of all test points encountered, $\left(\frac{a}{p}\right)$ is the area per point of the stereological grid,'t' is the thickness of the section and 'M' is the linear magnification

### 3.7 STATISTICAL ANALYSIS

GraphPad prism for windows version 5.0 (GraphPad Software, San Diego, CA, USA) was used for all data and statistical analysis. $P<0.05$ was considered statistically significant. Differences in means was analysed by ANOVA followed by *post hoc* test. Doses for 50 % of the maximal effect ($ED_{50}$) for each drug was determined using an iterative computer least square method, with the following nonlinear regression (three-parameter logistic) equation:

$$Y = \frac{a+(b-a)}{1+10^{(\text{Log } ED_{50}-X)}}$$

Where, 'x' is the logarithm of dose and 'Y' is the response. Y starts at 'a' (the bottom) and goes to 'b' (the top) with a sigmoid shape.

# CHAPTER FOUR

# RESULTS

## 4.1 ANTIDEPRESSANT EFFECTS OF IRON

### 4.1.1 Forced swim test for dams

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on mean immobility score for dam***

From the time course curve, medium dose of iron 0.8 mg/kg representing normal iron dose given to humans during pregnancy caused a significant decrease in immobility from day three of the FST ($F_{4, 52} = 210.3$; $P < 0.0001$) (Figure 4.1a) and the effect was sustained throughout the 14 days of FST. Fluoxetine decreased immobility scores from the first day of FST and the effect was maintained throughout the fourteen days of FST ($F_{4, 52} = 222.8$; $P < 0.0001$) (Figure 4.1c). Both Iron ($F_{4,65} = 175.9$; $P < 0.0001$) and fluoxetine ($F_{4,65} = 396.0$; $P < 0.0001$) treatment during gestation decreased the immobility scores of the dams significantly in a dose dependent manner in FST as shown in the area under the curve (AUC) in figure 4.1b and figure 4.1d for iron and fluoxetine respectively. Perinatal desferrioxamine (DFx) treatment had no significant effect on the immobility score of the dam in FST relative to the control (saline). Though iron treatment caused no significant decrease in immobility score compared to DFx, both iron and fluoxetine treatment significantly decreased the total immobility score of the dams compared to DFx as shown in the AUC's in figure 4.3b and figure 4.3d.

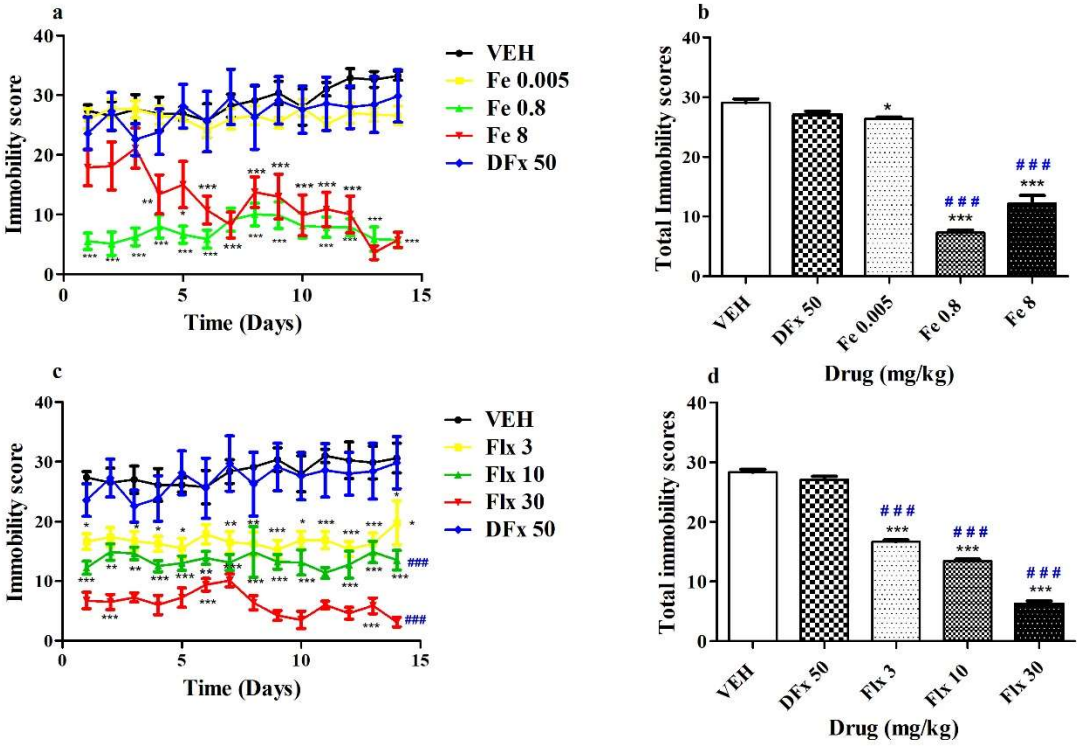



Figure 4. 1: Effects of Fe (0.005 - 8 mgkg$^{-1}$) and fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the duration of immobility in the forced swim test. Data are presented as both (a, c) time course curves and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Significantly different from vehicle-treated group: *P<0.05, **P<0.01, ***P<0.001; two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d). Comparison with DFx-treated group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001; two-way ANOVA followed by Bonferroni's (a, c) test and one-way ANOVA followed by Newman Keul's test (b, d).

### *Log dose-response curves of iron and fluoxetine for dams*

In FST, the order of antidepressant efficacy calculated from the dose-response curve (Figure 4.2) with regards to immobility was fluoxetine > iron ($E_{max}$ = 93.48, $ED_{50}$ = 6.468 > $E_{max}$ = 86.23, $ED_{50}$ ≅ 1.0 respectively). Iron was more potent than fluoxetine in reducing the immobility score in FST but Flx had a higher efficacy.

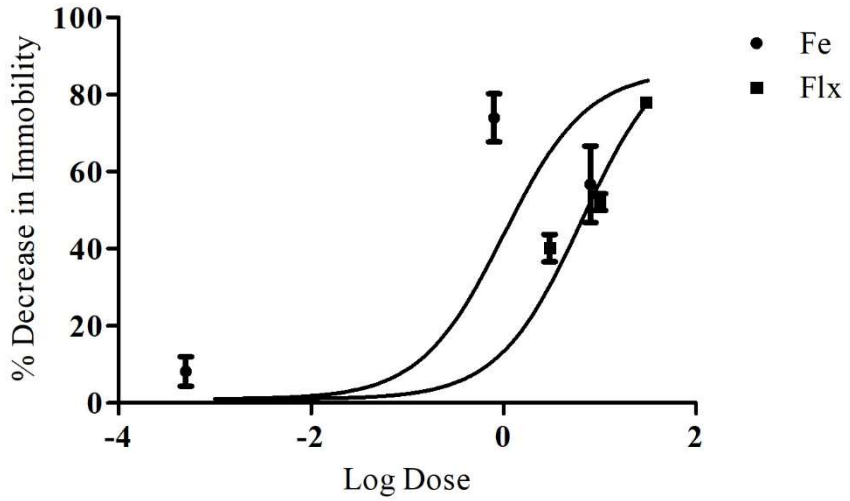



Figure 4. 2: Dose–response curves of iron and fluoxetine showing % decrease in immobility in the forced swimming test in rats. Each point is the mean ± S.E.M. of 7 animals.

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on mean swimming score for dams***

Iron increased the swimming score of the dams in FST ($F_{4,\ 52}$ = 91.36; $P$ < 0.0001). The effect of iron was observed on day three in the time course graph (Figure 4.3a) and was sustained throughout the duration of the FST. From the time-course graph of fluoxetine ($F_{4,52}$ = 1158.0, $P$ < 0.0001) (Figure 4.3c), there was a sustained effect for fluoxetine on the swimming score from day one with a slight increase on day eight which was sustained throughout the fourteen day period of the FST. Both iron ($F_{4,\ 65}$ =35.54; $P$ < 0.0001) and fluoxetine ($F_{4,\ 65}$ =388.6, $P$ < 0.0001) significantly increased the swimming score of the dam in a dose dependent manner in the FST. This is shown in the area under the curves (AUC) in figure 4.3b and figure 4.3d for iron and fluoxetine respectively. However, medium dose iron (0.8 mg/kg) gave a higher swimming score ($F_{4,\ 65}$ = 15.99; $P$ < 0.05) than the highest dose (8.0 mg/kg) ($F_{4,\ 65}$ = 10.09; $P$ < 0.05) as shown in figure 4.3b. Even though DFx significantly increased the swimming score of the dams in FST relative to the control, Comparing to DFx,

both iron and Flx significantly increased the total swimming score in a dose-independent and a dose-dependent manner for iron and fluoxetine respectively as shown in the AUC's in figure 4.3b and figure 4.3d.

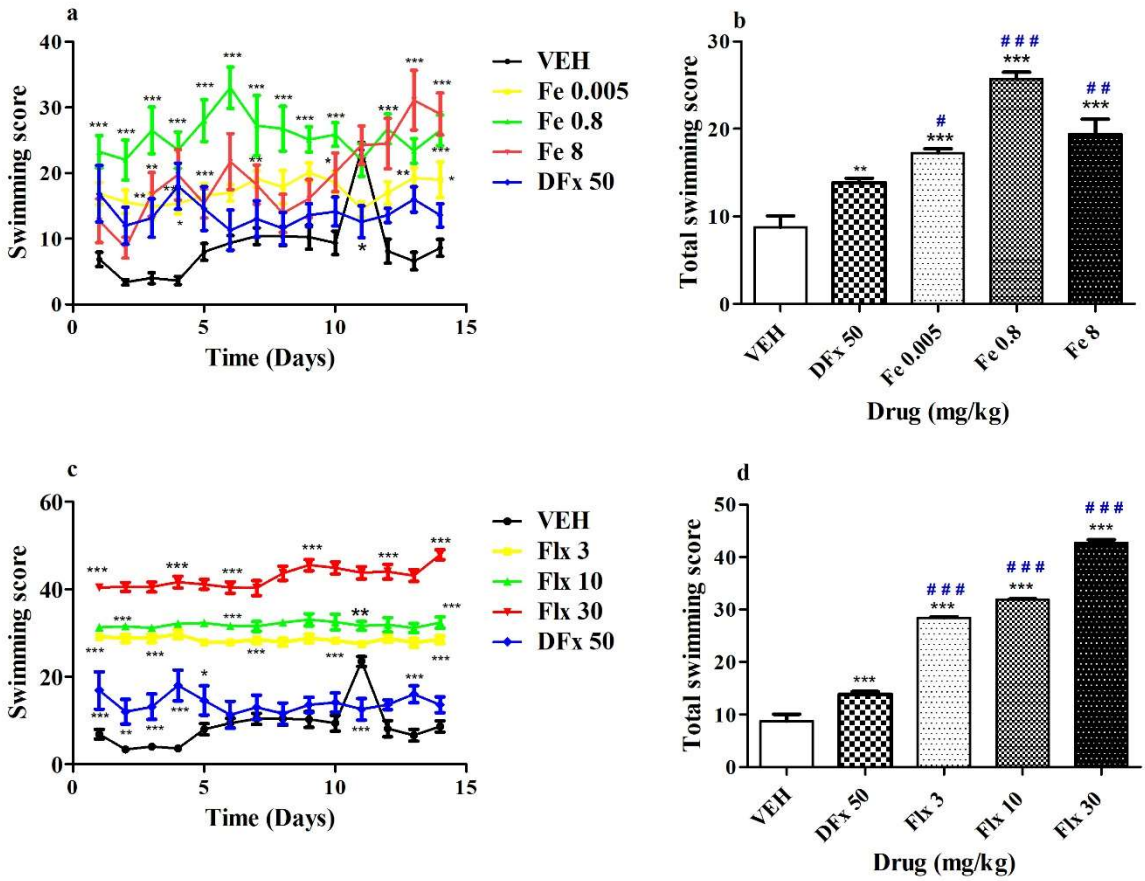



Figure 4. 3: Effects of Fe (0.005 - 8 mgkg$^{-1}$) and fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the swimming score in the forced swim test. Data are presented as both (a, c) a time course curves and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001; two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d). Comparison with DFx-treated group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001; two-way ANOVA followed by Bonferroni's (a, c) test and one-way ANOVA followed by Newman Keul's test (b, d).

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on mean climbing score for dams***

From the time course curve (Figure 4.4a), iron caused a significant increase in the climbing score in the FST ($F_{4, 52}$ = 35.28; $P$ < 0.0001). The effects was observed from around day four and was sustained up to day ten where the climbing score decreased till day fourteen of the FST. From the time course curve of fluoxetine (Figure 4.4c), the climbing scores decreased in FST ($F_{3, 55}$ = 79.86; $P$ < 0.0001). This effect of fluoxetine on the climbing score was observed throughout the fourteen day period of the FST. Dams treated with iron during gestation showed significant increase in climbing scores during the FST in a dose dependent manner at ($F_{4, 65}$ = 36.86, $P$ < 0.0001). Fluoxetine was able to decrease the climbing score of the dam significantly ($F_{4, 65}$= 69.5; $P$ < 0.0001) (Figure 4.4d) during the FST in a dose dependent manner. DFx decreased the climbing score of the dam significantly compared to vehicle, iron ($F_{4, 65}$ = 6.232; $P$ < 0.05) and fluoxetine ($F_{4, 65}$ = 8.905; $P$ < 0.05) as shown in the AUC's in figure 4.4b and figure 4.4d respectively. Perinatal iron treatment but not fluoxetine increased climbing score compared to DFx as shown in the AUC's in figure 4.4b and figure 4.4d.

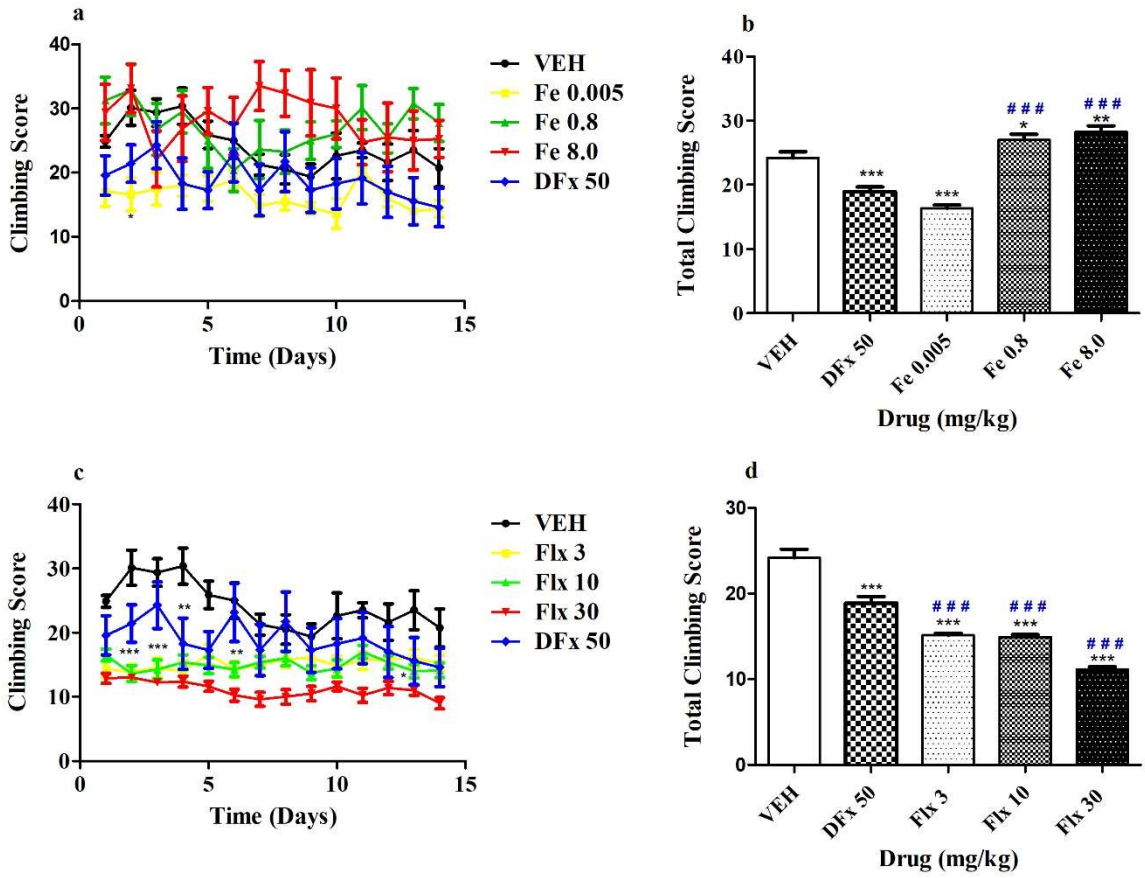



Figure 4. 4: Effects of Fe (0.005 - 8 mgkg$^{-1}$) and fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the climbing score in the forced swim test. Data are presented as both (a, c) a time course curve and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001; two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d). Comparison with DFx-treated group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001; two-way ANOVA followed by Bonferroni's (a, c) test and one-way ANOVA followed by Newman Keul's test (b, d).

### 4.1.2 Forced swim test for litter

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on mean immobility score for first generation litter***

Iron decreased the immobility score of the litter in FST ($F_{4, 52}$ = 207.6; $P$ < 0.0001) (Figure 4.5a). The effect of iron was observed on day one in the time course graph (Figure 4.3a) and was sustained throughout the duration of the FST. From the time-course graph of fluoxetine ($F_{4, 52}$ = 222.0; $P$ < 0.0001) (Figure 4.5c), there was a decrease in the immobility score and

this effect observed on day one and sustained throughout the fourteen day period of the FST. Both iron ($F_{4, 65}$ = 172.9; $P$ < 0.0001) and fluoxetine ($F_{4, 65}$ = 150.1; $P$ < 0.0001) significantly decreased the immobility score of the dam in a dose dependent manner in the FST. This is shown in the area under the curve (AUC) in figure 4.5b and figure 4.5d for iron and fluoxetine respectively. DFx exposure in-utero had no significant effect on the immobility score of the litter in FST compared to the vehicle but iron and fluoxetine exposure significantly decreased immobility scores of the litter in a dose-dependent manner as shown in the AUC's in figure 4.5b and figure 4.5d respectively.

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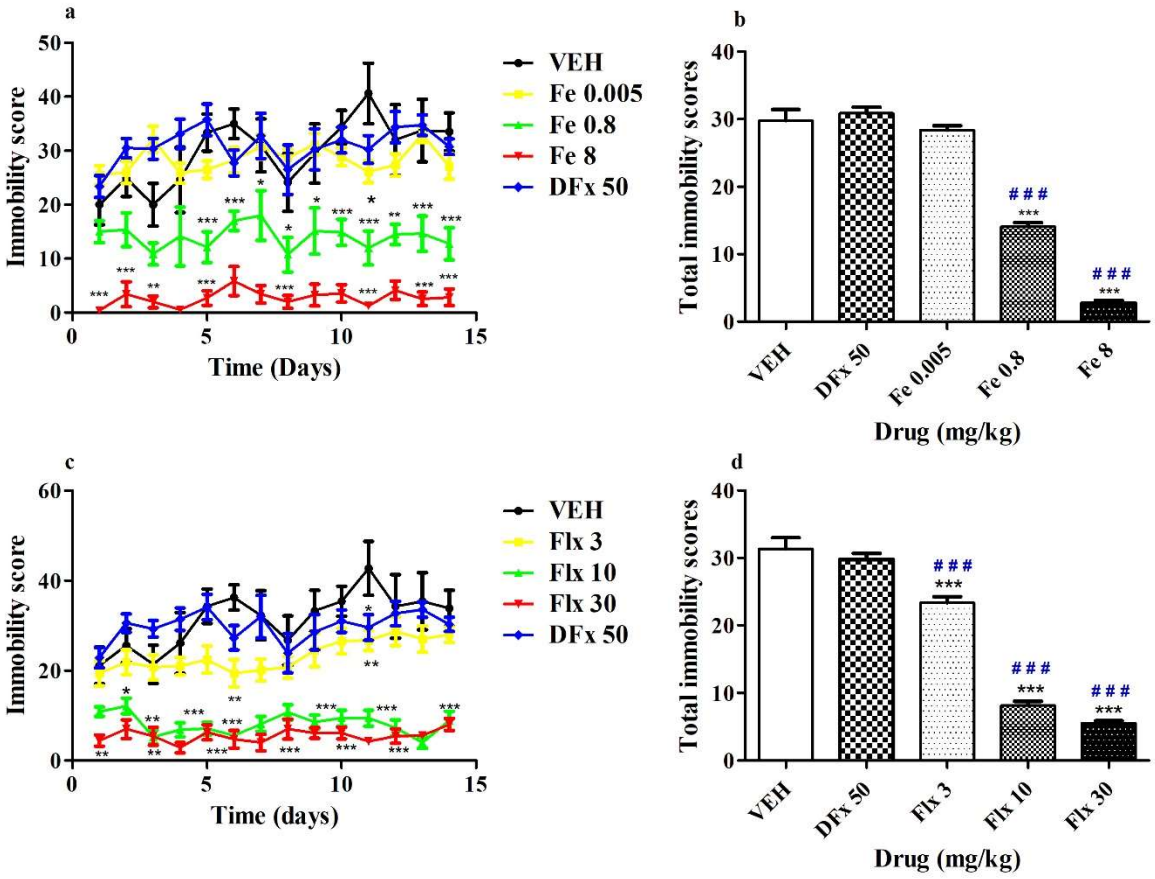


Figure 4. 5: Effects of Fe (0.005 - 8 mgkg$^{-1}$) and fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the duration of immobility for the first generation litter in the forced swim test. Data are presented as both (a, c) a time course curve and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 two-way ANOVA followed by Bonferroni's (a, c) test and one-way ANOVA followed by

Newman Keul's test (b, d); comparison with DFx-exposed group: #P<0.05, ##P<0.01, ###P<0.001; two-way ANOVA followed by Bonferroni's (a, c) test and one-way ANOVA followed by Newman Keul's test (b, d).

***Log dose-response curve for litter***

In litter FST, the order of antidepressant efficacy calculated from the dose-response curve (Figure 4.6) with regards to immobility was iron > fluoxetine ($E_{max}$ = 100 $ED_{50}$ = 0.7658; $E_{max}$ = 98.7 $ED_{50}$ = 4.783 respectively). Iron was more potent than fluoxetine in reducing the immobility score in FST.

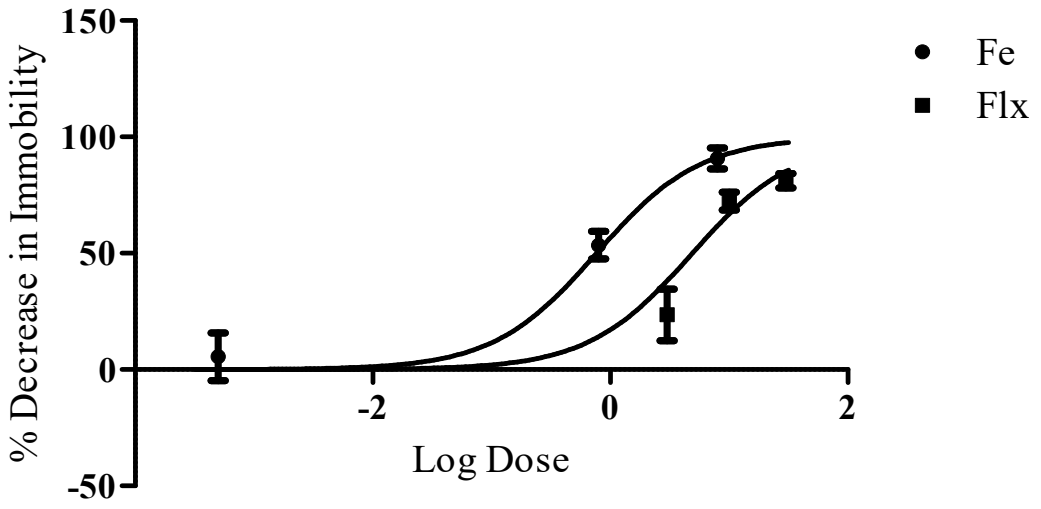


Figure 4. 6: Dose–response curves showing the effect of iron, Fe and fluoxetine, Flx on % decrease in immobility score in the forced swim test in rats. Each point is the mean ± S.E.M. of 7 animals.

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on mean swimming score for first generation litter***

Iron showed an increase in swimming score from day 2 of FST which was sustained throughout the 14 days of FST ($F_{4, 52}$ = 291.5; $P$ < 0.0001) (Figure 4.7a). All three doses of fluoxetine showed an increase in the swimming score from day 1 of FST and the effect was sustained throughout the 14 days of FST ($F_{4, 52}$ = 130.8; $P$ < 0.0001) (Figure 4.7c). Both iron

($F_{4, 65}$ = 229.3: $P$ < 0.0001) and fluoxetine ($F_{4, 65}$ = 128.8; $P$ < 0.0001) significantly increased the swimming score of the litter in FST in a dose dependent manner. This is shown in the area under the curve (AUC) in figure 4.7b and figure 4.7d for iron and fluoxetine respectively. DFx significantly decreased the swimming score of the litter in FST compared to vehicle. However, perinatal iron and fluoxetine treatment increased the swimming score of the litter in a dose-dependent manner as shown in the AUC's in figure 4.7b and figure 4.7d respectively.

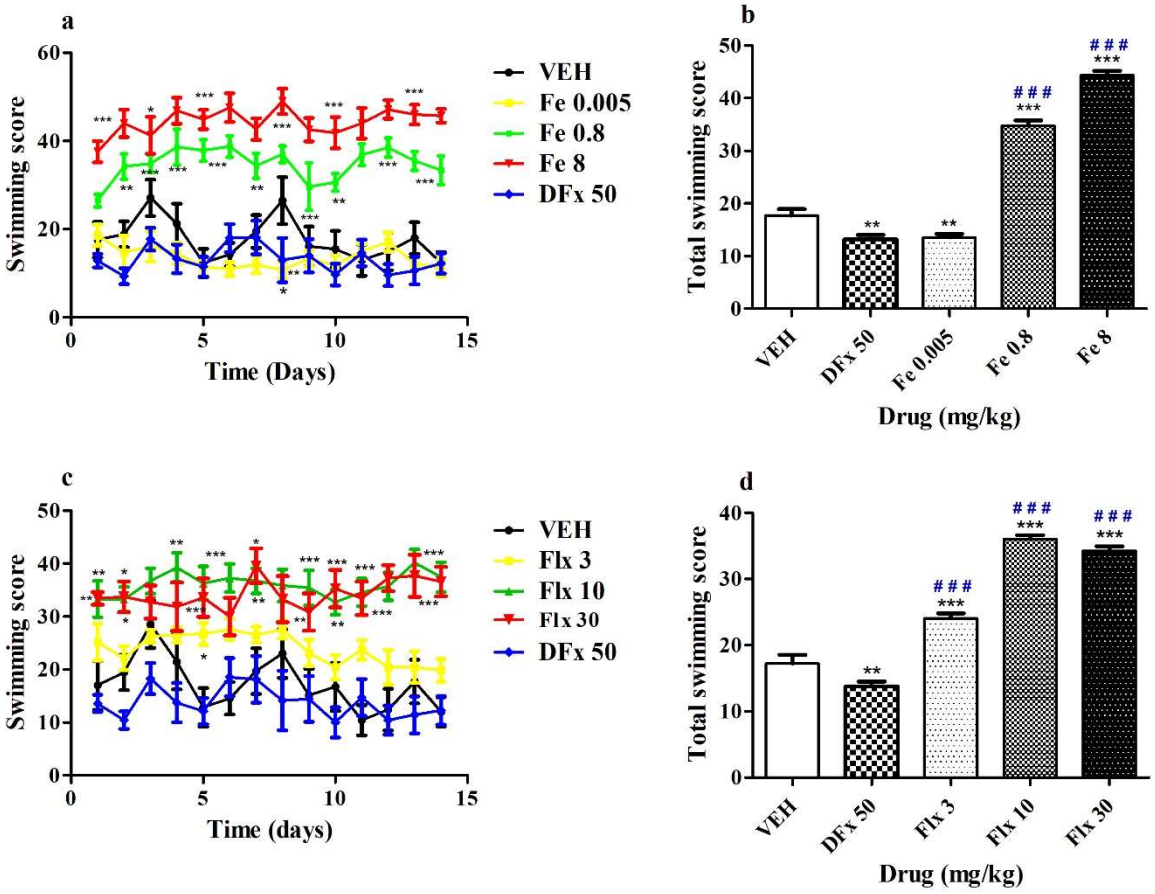


Figure 4. 7: Effects of Fe (0.0005 - 8 mgkg$^{-1}$) and fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the swimming score for the first generation litter in the forced swim test. Data are presented as both (a, c) a time course curve and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d); comparison with DFx-exposed group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001; two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d).

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on mean climbing score for first generation litter***

Unexpectedly, low dose iron (0.005 mg/kg) showed a significant increase in climbing score. This effect was seen on day 4 of FST and was sustained throughout the 14 days of FST ($F_{4, 52}$ = 11.13; $P < 0.0001$) (Figure 4.8a). Fluoxetine showed a decrease in climbing behaviour after day 4 of FST and the effect was sustained up to day 11 and decreased till day 14 of FST ($F_{4,65}$ = 15.22; $P < 0.0001$) (Figure 4.8c). Iron had a significant effect on the litter climbing score in FST ($F_{4, 65}$ = 7.834; $P < 0.0001$) (Figure 4.8b). Fluoxetine significantly decreased the climbing score in FST in a dose dependent manner ($F_{4, 65}$ = 18.51; $P < 0.0001$) (Figure 4.8b). DFx significantly increased the climbing score of the litter compared to vehicle and iron but not fluoxetine as shown in the AUC's in figure 4.8b and figure 4.8d respectively..

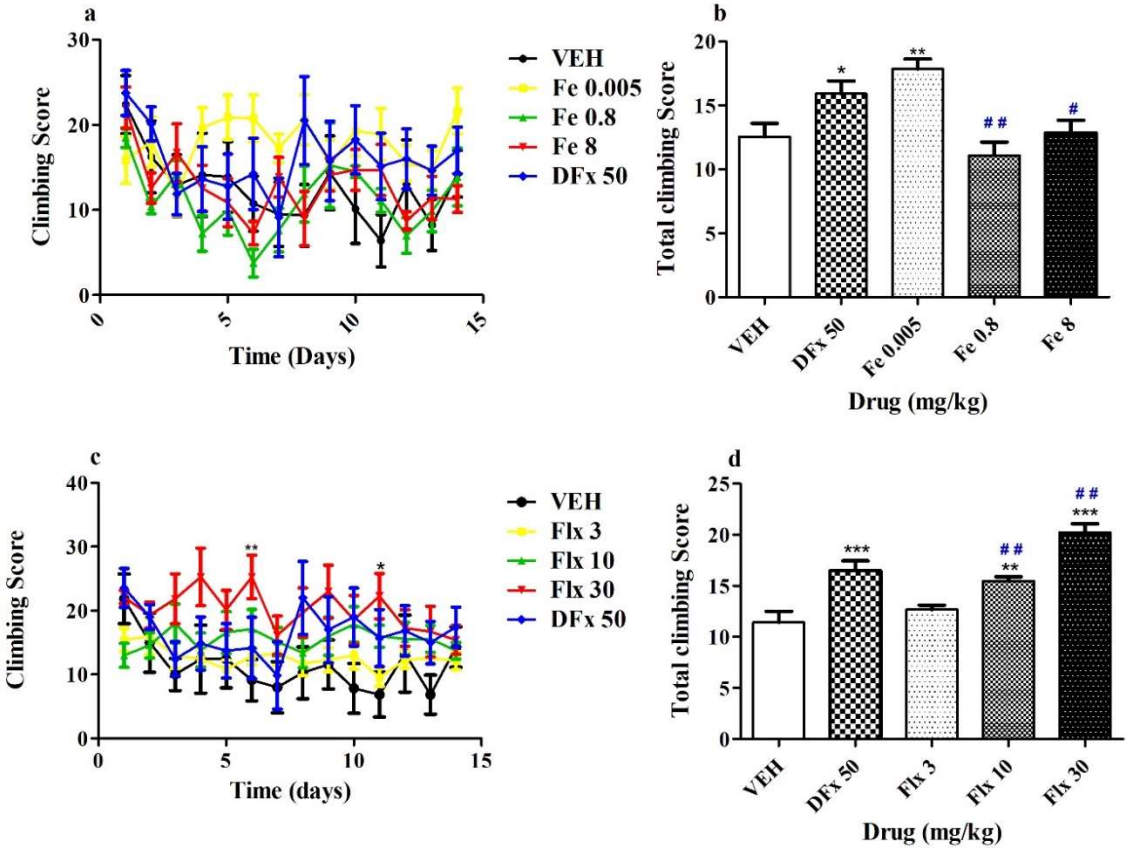


Figure 4. 8: Effects of Fe (0.0005 - 8 mgkg$^{-1}$) and fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the climbing score for the first generation litter in the forced swim test. Data are presented as

both (a, c) a time course curve and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001; two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d); comparison with DFx-exposed group: #P<0.05, ##P<0.01, ###P<0.001; two-way ANOVA followed by Bonferroni's (a, c) test and one-way ANOVA followed by Newman Keul's test (b, d).

## 4.2 OPEN FIELD TEST (OFT)

### 4.2.1 Dams OFT

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on locomotor activity for dam in the OFT.***

Both the dams and their litter were taken through the open field test to assess locomotor activity and some anxiety measures. One way ANOVA revealed that perinatal iron treatment of dams caused no significant change in locomotor activity ($F_{4, 10}$ = 0.8026; $P$ = 0.5332) in OFT. Perinatal fluoxetine treatment also caused no significant change in the locomotor activity of the rat dams in OFT ($F_{4, 10}$ = 0.9728; $P$ = 0.4370). There was no significant difference in the locomotor activity of the DFx treated dams relative to the control group and drug treatment groups.

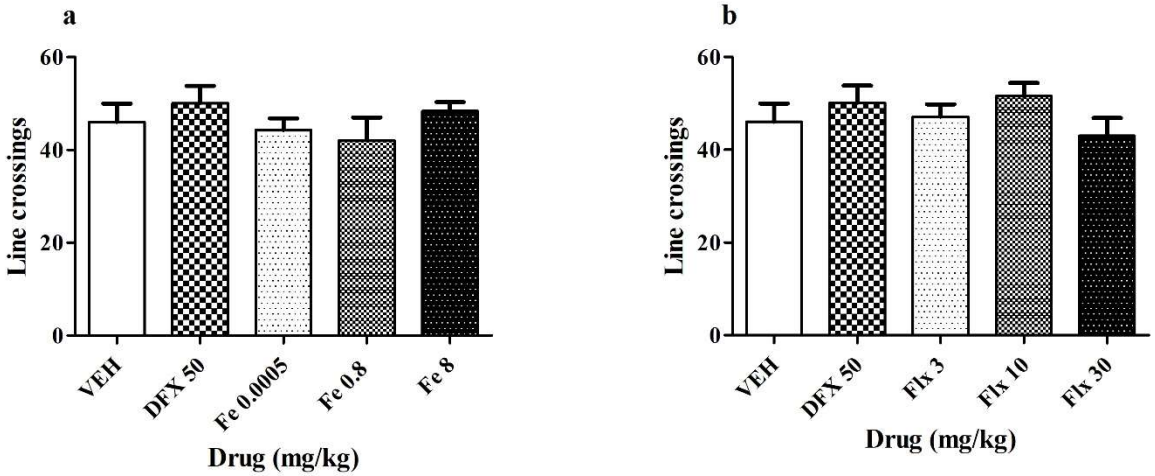


Figure 4. 9: Effect of (a) iron, Fe (0.005 - 8 mg $kg^{-1}$) and (b) fluoxetine, Flx (3 – 30.0 mg $kg^{-1}$) treatment on number of line crossings respectively in the open field test. Data is presented

as Mean ± SEM of their number of line crossings. Significantly different from control: P<0.05 One-way ANOVA followed by Newman Keul's test.

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the latency time for the dam to leave the central square.***

Iron treated dams spent a significantly shorter time in the central square compared to controls ($F_{4, 10}$ = 11.02; $P$ = 0.0011) (Figure 4.10a). The effect of iron on the latency of the dams to leave the central square was dose dependent manner. For the latency scores of fluoxetine, dams treated with 3 mg/kg and 30 mg/kg fluoxetine showed significantly lower times required to leave the central square compared to the controls ($F_{4, 10}$ = 17.28; $P$ = 0.0002) (Figure 4.10b). The effect of fluoxetine on latency was not dose dependent. Both iron and fluoxetine treatment significantly decreased the latency to consume compared to DFx group as shown in Figure 4.10a and Figure 4.10b respectively.

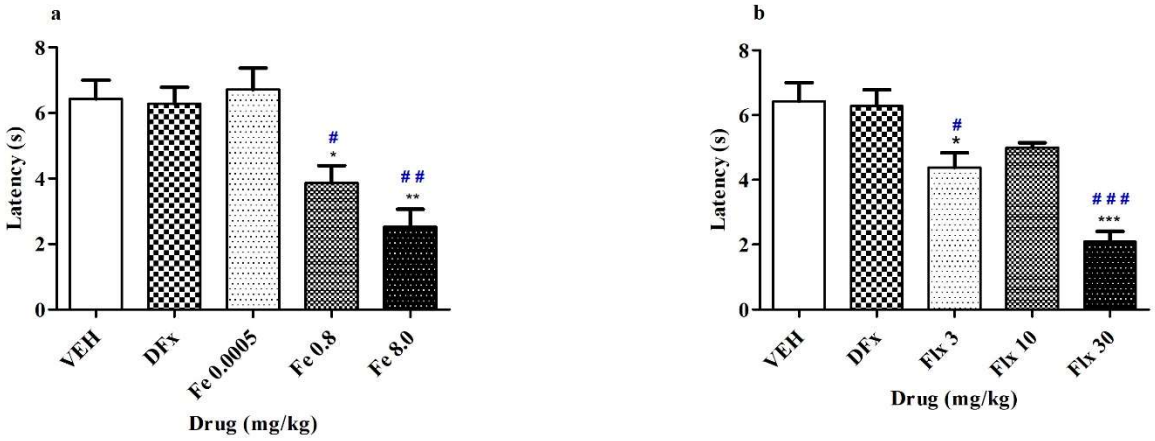


Figure 4. 10: Effect of (a) iron, Fe (10 - 100 mg $kg^{-1}$) and (b) fluoxetine, Flx (3 – 30.0 mg $kg^{-1}$) treatment on latency to leave the central square in the open field test. ***P<0.001; **P<0.01; *P < 0.05 compared to vehicle-treated group; One-way ANOVA followed by Newman Keul's test; comparison with DFx-treated group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 one-way ANOVA followed by Newman Keul's test.

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the percentage of centre time for the dams***

Iron treated dams spent a significantly higher amount of time exploring the central square compared to controls ($F_{4, 10}$ = 109.3; $P$ < 0.0001). Fluoxetine on the other hand also caused a significant increase in the amount time spent in the central square in a dose dependant manner compared to controls ($F_{4, 10}$ = 47.65; $P$ < 0.0001).

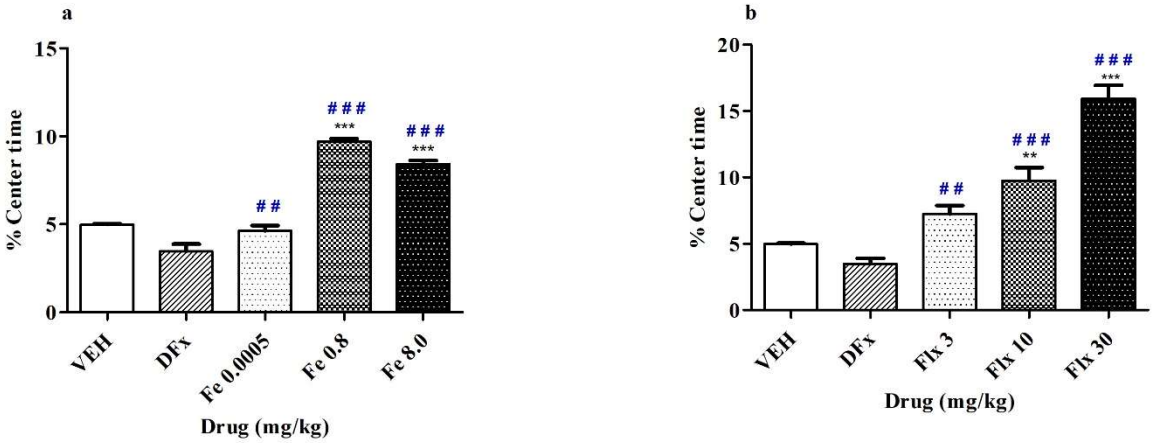


Figure 4. 11: Effect of (a) iron, Fe (10 - 100 mg $kg^{-1}$) and (b) fluoxetine, Flx (3 – 30.0 mg $kg^{-1}$) treatment on % centre entries in the open field test. ***P<0.001; **P<0.01; compared to vehicle-treated group; One-way ANOVA followed by Newman Keul's test. Comparison with DFx-treated group: #P<0.05, ##P<0.01, ###P<0.001 one-way ANOVA followed by Newman Keul's test.

***4.2.1.4 Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the time spent in the outer perimeter for the dam***

The iron treated dams showed a significant decrease in the time spent in the outer perimeter of the open field in a dose dependent manner compared to controls ($F_{4, 10}$ = 109.3; $P$ < 0.0001). Fluoxetine also caused a significant decrease in the time spent in the outer perimeter of the open field in a dose dependent manner ($F_{4, 10}$ = 47.35; $P$ < 0.0001).

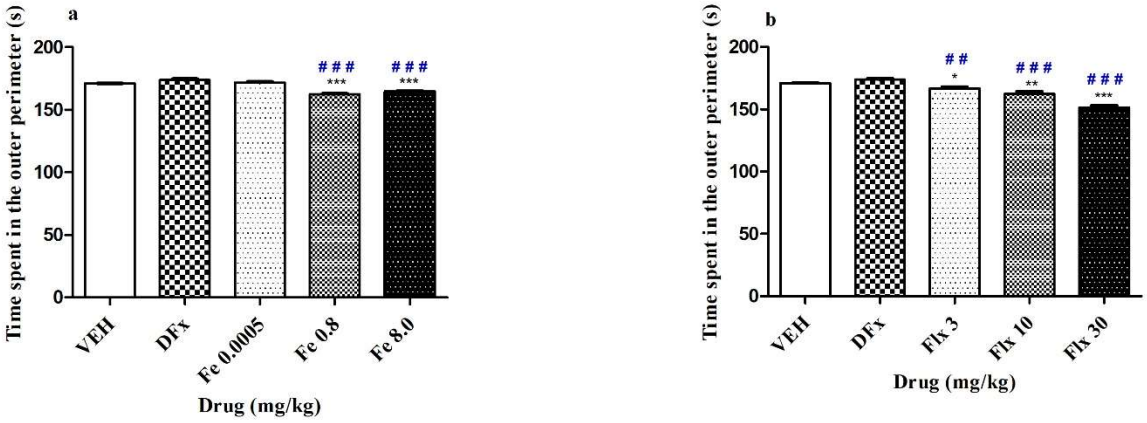


Figure 4. 12: Effect of (a) iron, Fe (10 - 100 mg $kg^{-1}$) and (b) fluoxetine, Flx (3 – 30.0 mg $kg^{-1}$) treatment on the time spent in the outer perimeter in the open field test. ***P<0.001; **P<0.01; *P < 0.5 compared to vehicle-treated group; One-way ANOVA followed by Newman Keul's test; comparison with DFx-treated group: ##P<0.01, ###P<0.001 one-way ANOVA followed by Newman Keul's test.

### 4.2.2 Litter OFT

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on number of line crossings for litter in the OFT.***

Perinatal iron treatment caused no significant change in litter locomotor activity ($F_{4,\ 10}$ = 5.519; $P$ = 0.0019) in OFT after one way ANOVA followed by Newman Keul's test. Perinatal fluoxetine treatment also caused no significant change in the locomotor activity of the rat litter in OFT ($F_{4,\ 10}$ = 6.234; $P$ = 0.0009). DFx-exposed litter exhibited no significant change in the locomotor activity in OFT.

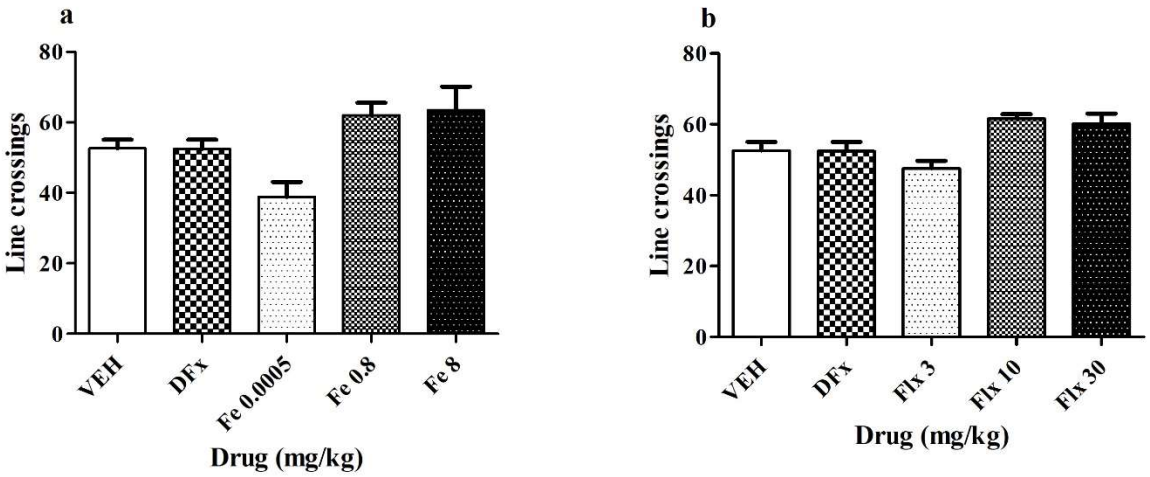


Figure 4. 13: Effect of (a) iron, Fe (0.005 - 8 mg $kg^{-1}$) and (b) fluoxetine, Flx (3 – 30.0 mg $kg^{-1}$) treatment on number of line crossings in the open field test. Data is presented as Mean ± SEM of their number of line crossings. Significantly different from control: $P<0.05$ One-way ANOVA followed by Newman Keul's test.

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the latency time for the litter to leave the central square.***

Perinatal iron treatment was able to significantly reduce the latency required for the litter to leave the central square ($F_{4,10} = 7.422$; $P = 0.0048$) in OFT after one way Anova followed by Newman Keul's test. The litter of the fluoxetine treated dams also exhibited a significant reduction ($F_{4,10} = 18.33$; $P < 0.0001$) in the latency time required to exit the central square in OFT after one way ANOVA followed by Newman Keul's test. DFx-exposed litter exhibited no significant change in the locomotor activity in OFT compared to the vehicle group. Iron and fluoxetine exposure caused a significant decrease in locomotor activity relative to the DFx-exposed group.

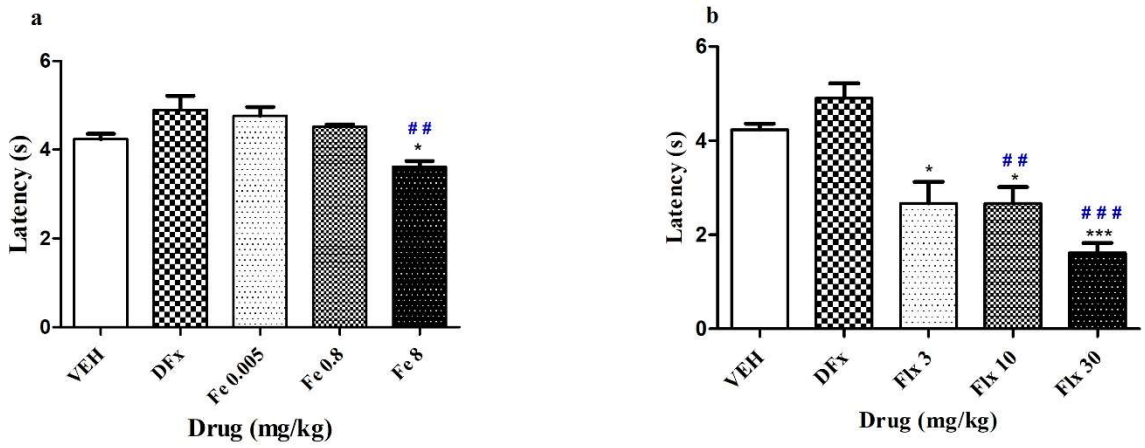


Figure 4. 14: Effect of (a) iron, Fe (0.005 - 8 mg $kg^{-1}$) and (b) fluoxetine, Flx (3 – 30.0 mg $kg^{-1}$) treatment on latency to leave the central square in the open field test. Data is presented as Mean ± SEM of their number of line crossings. Significantly different from control: ***P<0.001, *P<0.05; one-way ANOVA followed by Newman Keul's test; comparison with DFx-exposed group: $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001; one-way ANOVA followed by Newman Keul's test.

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the percentage of centre time for the litter in OFT.***

The litter of iron treated dams spent a significant amount of time exploring the central square compared to controls ($F_{4,\ 10}$ = 8.934; $P$ < 0.0001). DFx treatment caused no significant decrease in the % centre time of the litter in OFT. Fluoxetine on the other hand also caused a significant increase in the amount time the litter spent in the central square in a dose independent manner compared to controls ($F_{4,\ 10}$ = 11.69; $P$ < 0.0001).

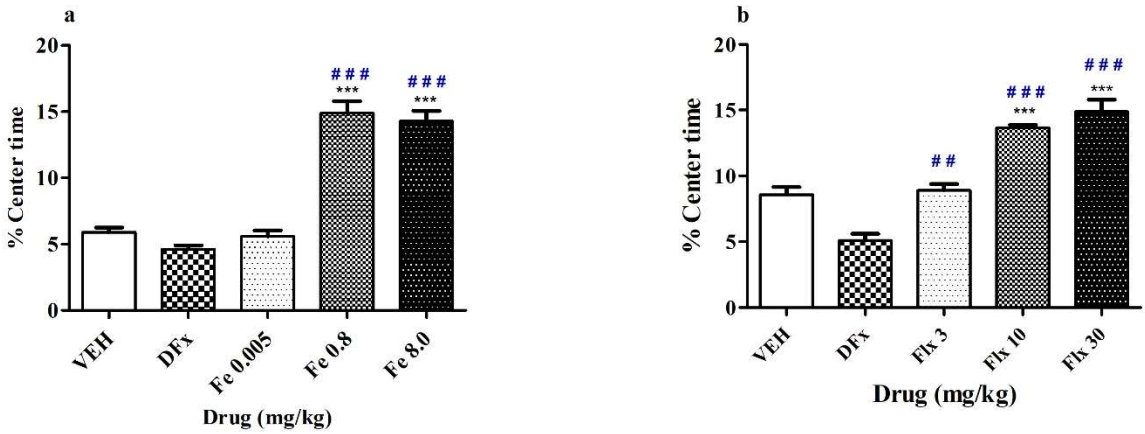


Figure 4. 15: Effect of (a) iron, Fe (0.005 - 8 mg $kg^{-1}$) and (b) fluoxetine, Flx (3 – 30.0 mg $kg^{-1}$) treatment on % centre time in the open field test. Data is presented as Mean ± SEM of their number of line crossings. Significantly different from control: ***P<0.001. One-way ANOVA followed by Newman Keul's test; comparison with DFx-exposed group: ##P<0.01, ###P<0.001; one-way ANOVA followed by Newman Keul's test.

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the time spent in the outer perimeter for the litter in OFT.***

Perinatal iron treatment showed a significant decrease in the time spent in the outer perimeter of the open field in a dose dependent manner compared to controls ($F_{4, 10}$ = 11.68; $P$ < 0.0001). DFx had no significant effect on the time spent in the outer perimeter of the OFT by the litter. Fluoxetine also caused a significant decrease ($F_{4, 10}$ = 16.70; $P$ < 0.0001) in the time spent in the outer perimeter of the open field in a dose dependent manner. Iron and fluoxetine treatment decreased the time spent in the outer perimeter of the open field in a dose dependent manner during the postpartum period.

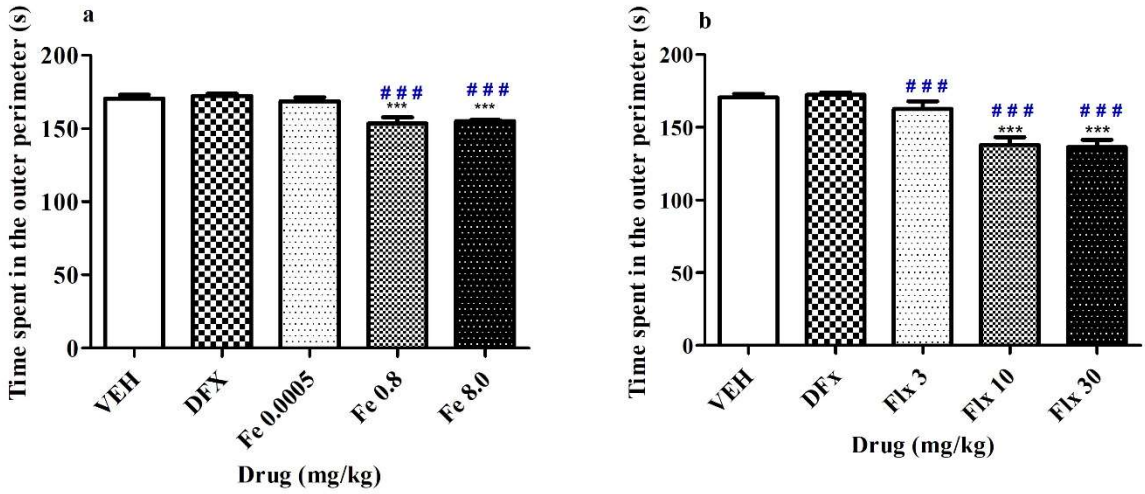


Figure 4. 16: Effect of (a) iron, Fe (0.005 - 8 mg $kg^{-1}$) and (b) fluoxetine, Flx (3 – 30.0 mg $kg^{-1}$) treatment on the time spent in the outer perimeter of the open field test. Data is presented as Mean ± SEM of their number of line crossings. Significantly different from control: ***P<0.001; one-way ANOVA followed by Newman Keul's test; comparison with DFx-exposed group: ###P<0.001; one-way ANOVA followed by Newman Keul's test.

## 4.3 NOVELTY-INDUCED HYPOPHAGIA (NIH)

### 4.3.1 Dams NIH

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the latency for the dam to consume a palatable meal in the home cage and novel cage***

The overall latency to drink was increased in the novel cage for both iron and fluoxetine. Iron treatment during the perinatal period significantly decreased the latency to drink diluted condensed milk in the novel cage as well as the home cage. Iron reduced the latency in the home cage ($F_{4, 30}$ = 6.988; $P$ = 0.0004) (Figure 4.17a) in a dose dependent manner. In the novel cage, all three doses of iron reduced the latency to drink relative to control in a dose dependent manner ($F_{4, 30}$ = 10.14; $P$ < 0.0001) (Figure 4.17b). The effect of fluoxetine on the latency was similar to that of iron. Fluoxetine had a significant effect on the latency in the home cage ($F_{4, 35}$ = 3.696; $P$ = 0.0130) (Figure 4.17c). All three doses of fluoxetine however

significantly reduced the latency to consume the milk in the novel cage in a dose dependent manner ($F_{4, 35}$ = 18.02; $P$ < 0.0001) (Figure 4.17d). Perinatal DFx treatment did cause a significant change in the latency of the dams to drink the milk in both the home and novel cages but showed increased latency compared to iron and fluoxetine in both the home and novel cages.

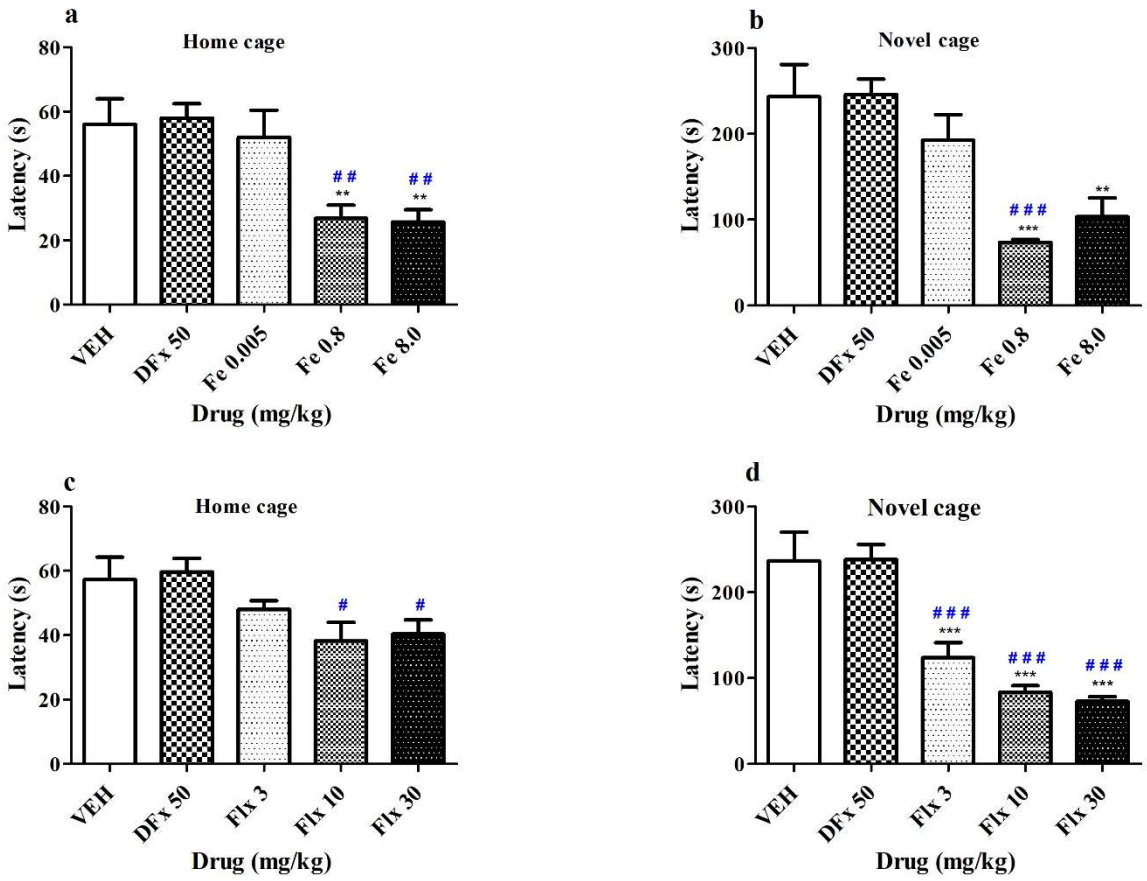


Figure 4. 17: The effects of perinatal (a, b) iron (0.005 - 8 mgkg$^{-1}$) and (c, d) fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the latency to consume a palatable meal in the home cage and novel cage is shown for dams. Values are means ± SEM. ***P < 0.0001;**P<0.001 vs control group with one-way ANOVA followed by Newman Keul's test; comparison with DFx-treated group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001; one-way ANOVA followed by Newman Keul's test.

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the latency difference for the dam to consume a palatable meal in the home cage and novel cage***

For latency difference scores (novel cage minus home cage), iron treated dams exhibited significantly larger latency difference scores ($F_{4, 34}$ = 4.406; $P$ = 0.0138) (Figure 4.18a). All three doses of fluoxetine showed a larger difference in latencies relative to the control ($F_{4, 34}$ = 12.49; $P$ < 0.0001) (Figure 4.18b) in a dose dependent manner. DFx treatment caused comparable latency difference scores with vehicle treatment but exhibited significantly high latency difference scores compared to iron and fluoxetine treatment as shown in (Figure 4.18a) and (Figure 4.18b) respectively.

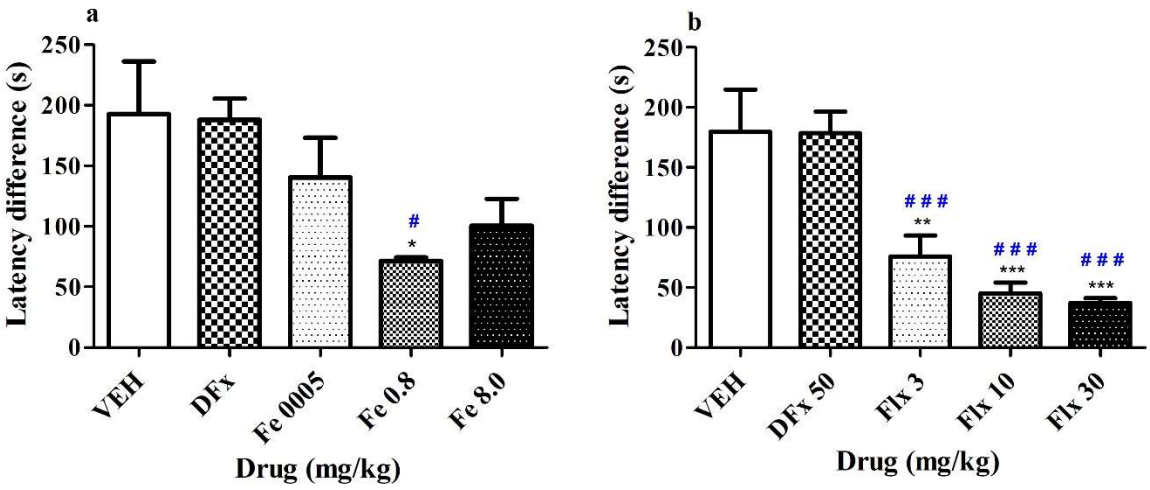


Figure 4. 18: The effects of perinatal (a) iron (0.005 - 8 mgkg$^{-1}$) and (b) fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the latency difference for dams. Values are means ± SEM. ***P<0.0001; **P<0.001; *P < 0.05 vs control group with one-way ANOVA followed by Newman Keul's test; comparison with DFx-treated group: $^{\#}$P<0.05, $^{\#\#\#}$P<0.001; one-way ANOVA followed by Newman Keul's test.

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the consumption of a palatable meal for dam in the home cage***

Consumption for the dams in the iron treatment group and fluoxetine treatment group was high in the first five minutes of the test but generally decreased till the end of the test. Iron had no significant effect on the consumption for 30 minutes duration of the test in the home cage ($F_{4,\ 24}$ = 0.9223; $P$ = 0.4519) (Figure 4.19a). Fluoxetine treatment also showed no significant change in the consumption of the dams ($F_{4,\ 24}$ = 0.6587; $P$ = 0.6213) (Figure 4.19c) in the home cage. There was no significant effect of the different doses of both iron ($F_{4,\ 24}$ = 0.07793, $P$ = 0.9885) and fluoxetine ($F_{4,\ 30}$ = 0.04728; $P$ = 0.9956) on the consumption in the home cage relative to the control as shown by the area under the curve (AUC) in figures 4.19b and 4.19d for iron and fluoxetine respectively. DFx treatment did not cause any significant difference in consumption.

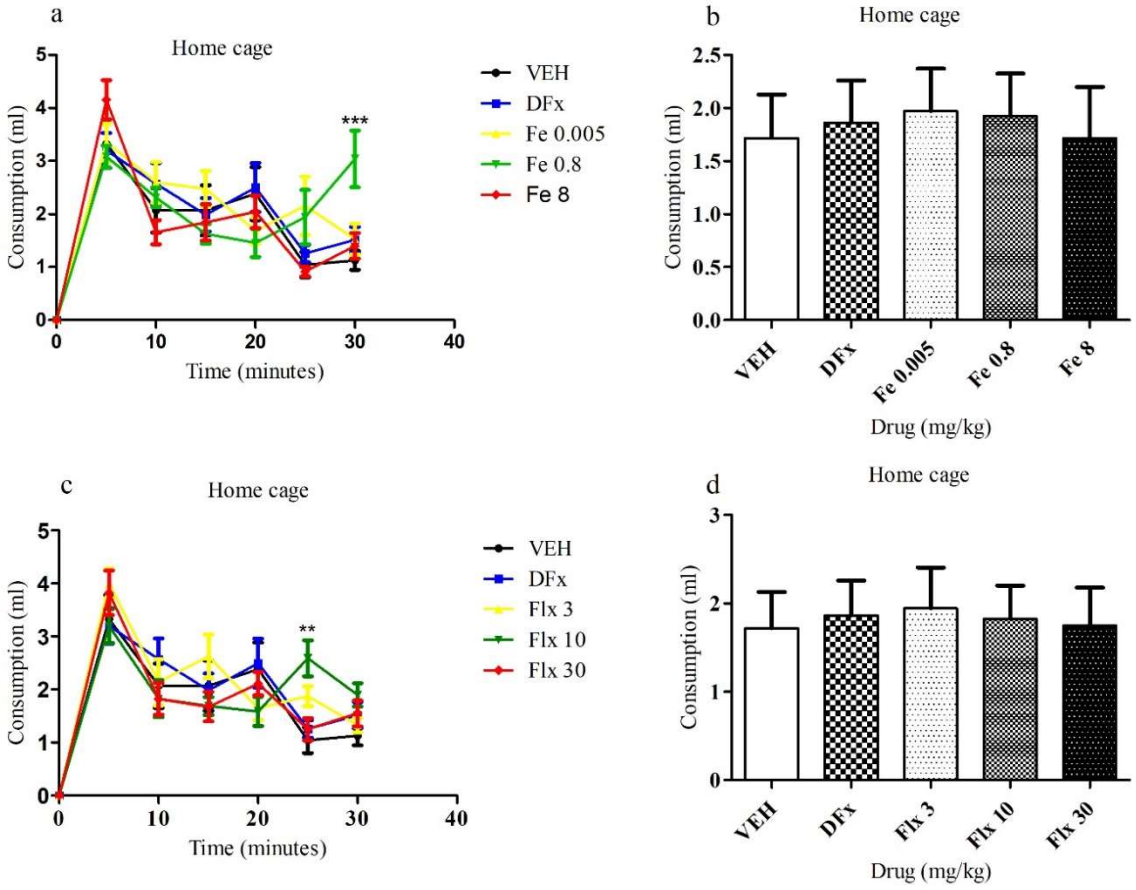


Figure 4. 19: Effects of perinatal iron (0.005 - 8 mgkg$^{-1}$) and fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the amount consumed of a palatable meal in the novelty-induced hypophagia test. Data are presented as both (a, c) a time course curve and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA Newman Keul's test (c, d).

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the consumption of a palatable meal for dam in the novel cage***

Milk consumption was reduced overall in the novel cage compared to the home cage during the 30 minute period of the test. The consumption was high in the first five minutes in the novel cage in all the treatment groups and decreased considerably for the rest of the time. Both iron ($F_{4,\ 24}$ = 2.778; $P$ = 0.0279) and fluoxetine ($F_{4,\ 24}$ = 4.948; $P$ = 0.0008) had a significant effect on the consumption during the period of the test in the novel cage. However, there was no significant difference in consumption between the various doses of

iron ($F_{4, 30}$ = 0.4747; $P$ = 0.7540) and fluoxetine ($F_{4, 30}$ = 0.7544; $P$ = 0.5631) treatment relative to the control. This is shown in the AUCs for iron and fluoxetine in fig 4.20b and 4.20d respectively.

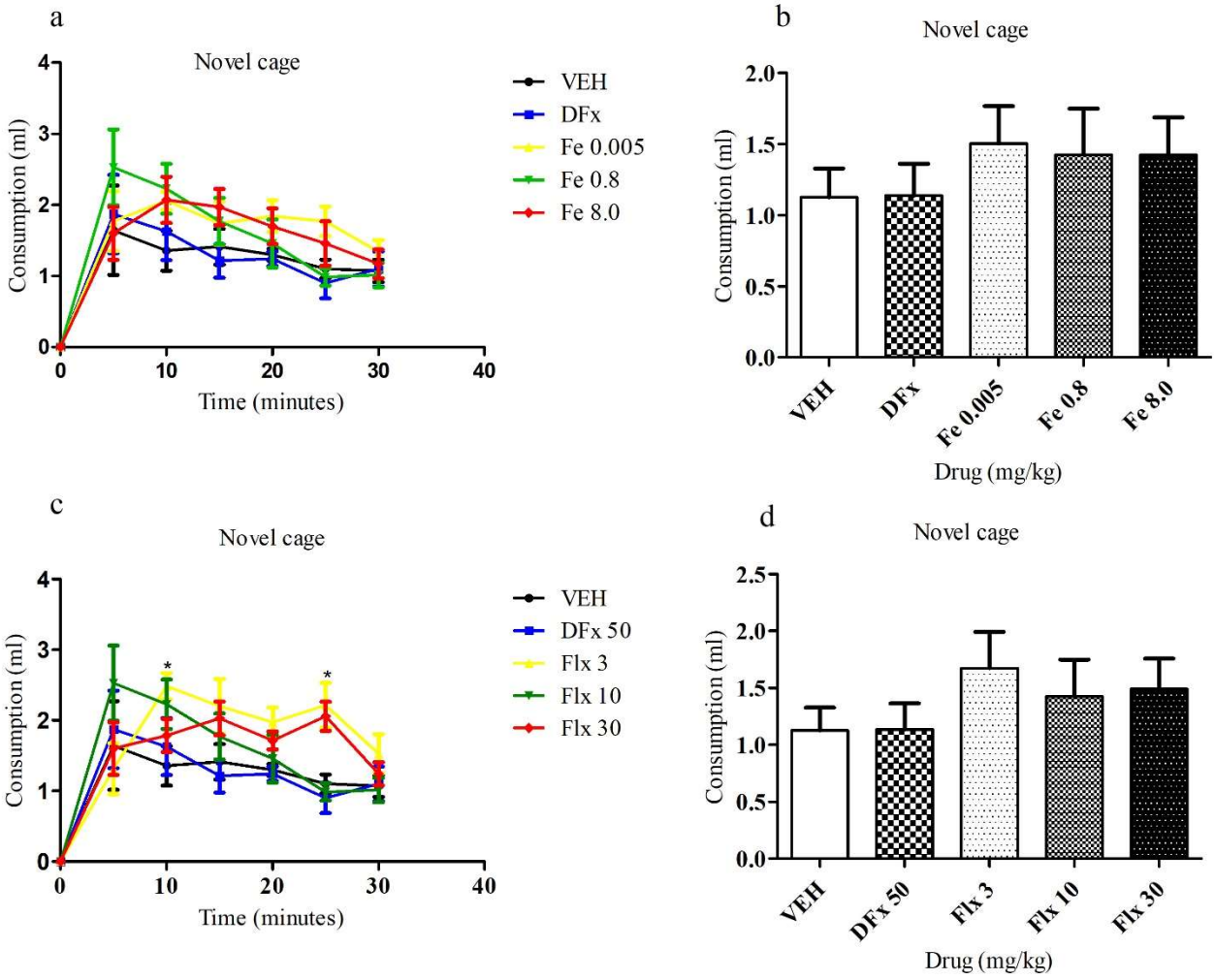


Figure 4. 20: Effects of perinatal iron (0.005 - 8 mgkg$^{-1}$) and fluoxetine (3 – 30 mgkg$^{-1}$) treatment on the amount consumed of a palatable meal in the novelty-induced hypophagia test. Data are presented as both (a, c) a time course curve and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001; two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d); comparison with DFx-treated group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001; two-way ANOVA followed by Bonferroni's (a, c) test and one-way ANOVA followed by Newman Keul's test (b, d).

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the consumption of a palatable meal for the first five (5) minutes in the home cage and novel cage***

Consumption during the first 5 minutes is an anxiety-related measure. Overall, milk consumption was reduced in the novel cage compared to the home cage for the first five minutes of the test. There was a significant effect of perinatal iron treatment on the milk consumption of the dams in the home cage ($F_{4, 30} = 3.908$ $P < 0.0114$) (Figure 4.21a) but not in novel cage ($F_{4, 34} = 0.5477$ $P = 0.7021$) (Figure 4.21b). Perinatal fluoxetine treatment of dams also had a significant increase in the consumption in the home cage ($F_{4, 34} = 3.792$; $P = 0.0121$) (Figure 4.21c) as well as the novel cage ($F_{4, 34} = 3.732$; $P < 0.0140$) (Figure 4.21d) for the first 5 minutes of the test. Iron increased consumption in the first 5 min in the home cage compared to DFx.

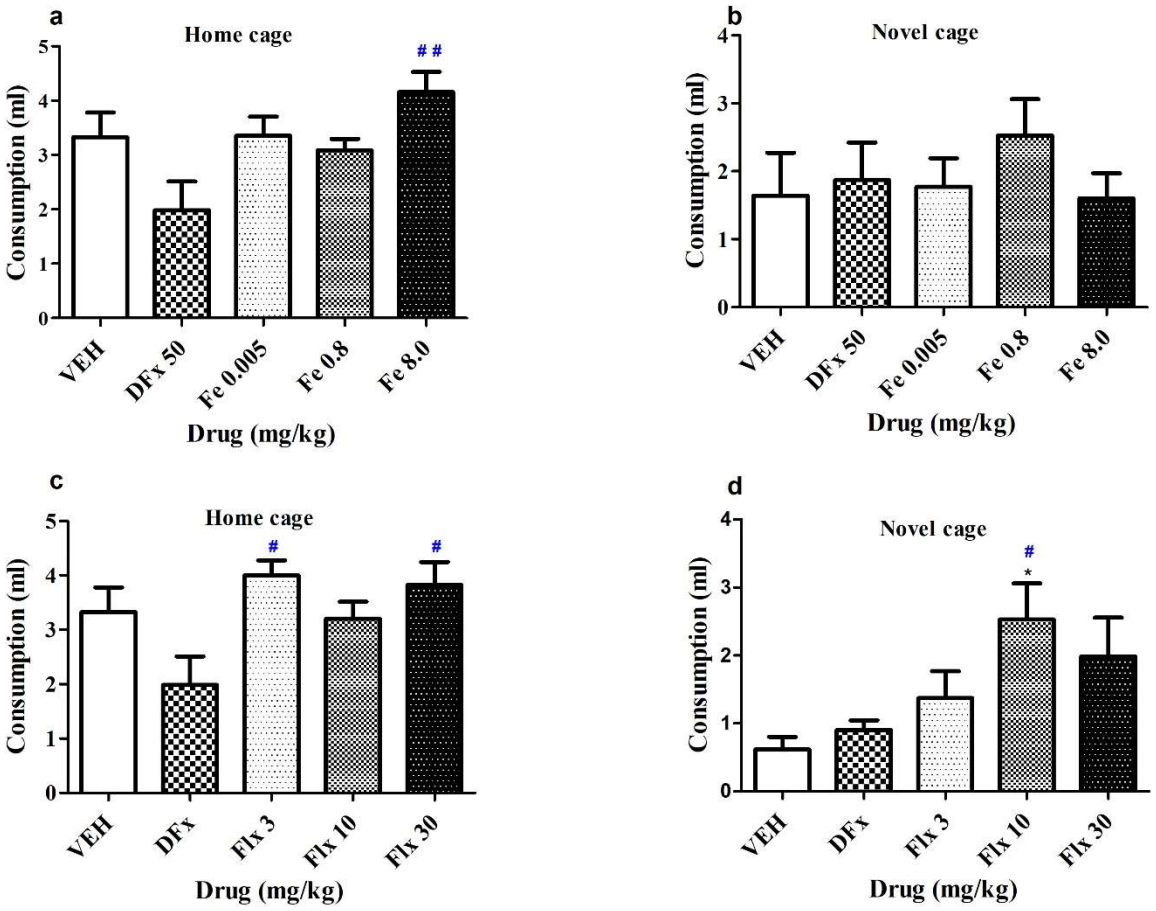


Figure 4. 21: Effects of perinatal (a, b) iron (0.005 - 8 mgkg-1) and (c, d) fluoxetine (3 – 30 mgkg-1) treatment on the amount of milk consumed in the first five minutes of the novelty-induced hypophagia test. Data are presented as Mean ± SEM of their consumption within the first five minutes. Significantly different from control: *P<0.05, **P<0.01, ***P<0.001(one-way ANOVA followed by Newman Keul's test); comparison with DFx-treated group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001(one-way ANOVA followed by Newman Keul's test).

### 4.3.2 Litter NIH

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the latency for the litter to consume a palatable meal in the home cage and novel cage***

The latency to consume the milk was higher in the novel cage compared to the home cage for the litter of both iron treated and fluoxetine treated dams. The litter from the iron group showed a significant decrease in latency relative to the control in a dose dependent manner in the home cage ($F_{4, 30}$ = 14.32; $P$ < 0.0001) (Figure 4.22a) as well as the novel cage ($F_{4, 30}$ =

6.451; $P$ = 0.0007) (Figure 4.22b). Litter from the fluoxetine treated dams also showed a significant decrease in the latency to consume the milk in a dose dependent manner in both the home cage ($F_{4, 34}$ = 4.768; $P$ = 0.0043) (Figure 4.22c) and the novel cage ($F_{4, 34}$ = 27.46; $P$ < 0.0001) (Figure 4.22d). Iron and fluoxetine decreased immobility in both home and novel cages compared to the DFx group (Figure 4.22 a, b, c, d).

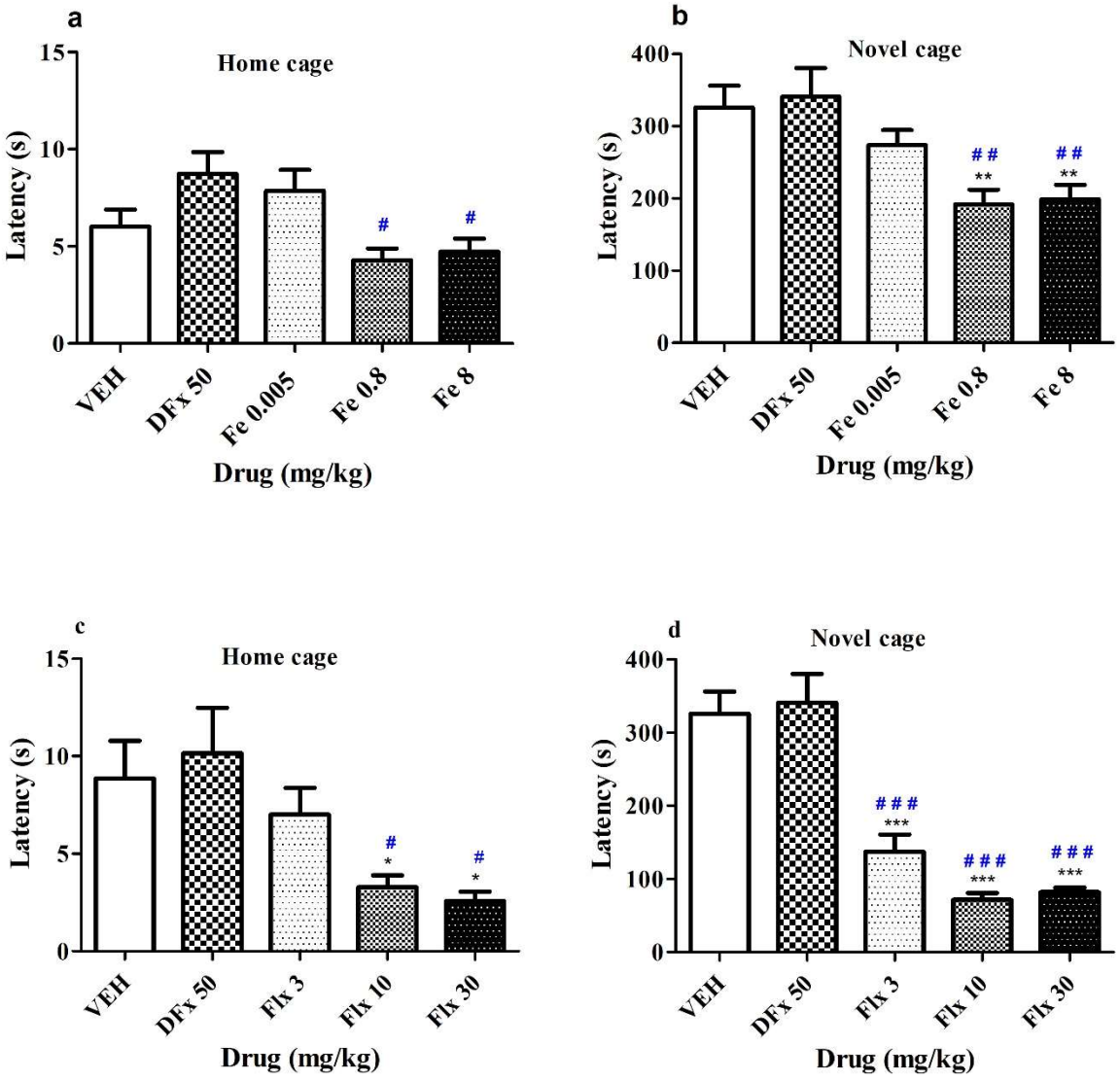


Figure 4. 22: The effects of perinatal iron (0.005 - 8 mgkg-1) (a, b) and fluoxetine (3 – 30 mgkg-1) (c, d) treatment on the latency to consume a palatable meal in the home cage and novel cage is shown for litter. Values are means ± SEM. ***P < 0.0001; **P<0.001; *P<0.05 vs control group with one-way ANOVA followed by Newman Keul's test; comparison with DFx-exposed group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 (one-way ANOVA followed by Newman Keul's test).

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the latency difference for the litter to consume a palatable meal in the home cage and novel cage***

For the latency difference, iron ($F_{4, 30}$ = 5.888; $P$ = 0.0013) (Fig. 4.23 a) showed a significant difference in latencies between the home cage and novel cage. Fluoxetine ($F_{4, 30}$ = 25.69; $P$ < 0.0001) (Fig. 4.23 b) also showed a significant difference in the latencies between the home cage and novel cage. Both iron and fluoxetine did not exhibit a dose dependent effect in the litter. Iron and fluoxetine exposure decreased latency difference in both home and novel cages (Fig. 4.23 a, b).

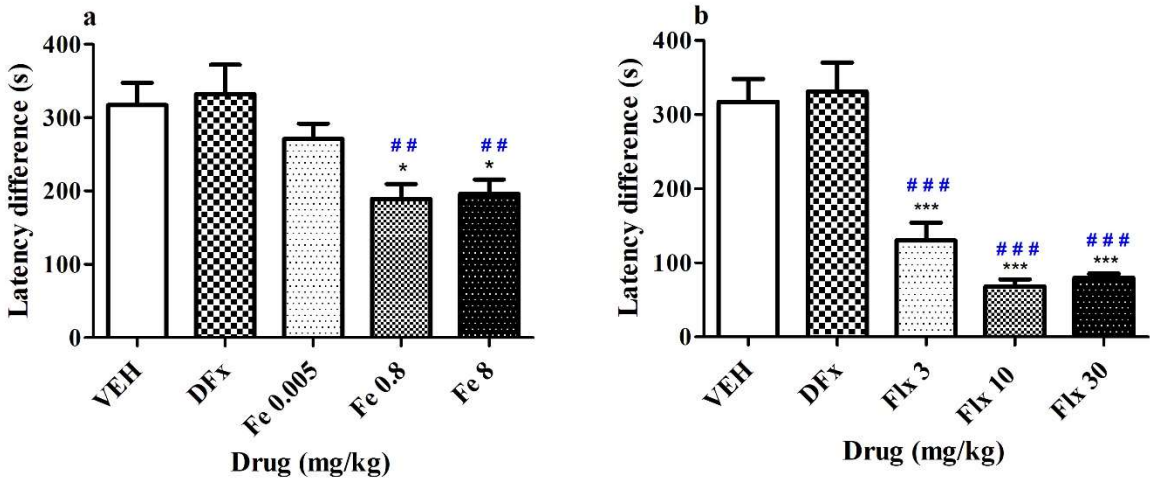


Figure 4. 23: The effects of perinatal (a) iron (0.005 - 8 $mgkg^{-1}$) and (b) fluoxetine (3 – 30 $mgkg^{-1}$) treatment on the latency difference for litter. Values are means ± SEM. ***P<0.0001; **P<0.001; *P < 0.05 vs control group with one-way ANOVA followed by Newman Keul's test; comparison with DFx-exposed group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 (one-way ANOVA followed by Newman Keul's test).

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the consumption of a palatable meal for litter in the home cage***

Consumption for the litter in the iron and fluoxetine treatment groups was high in the first five minutes of the test but generally decreased afterwards until the end of the test. The vehicle group consumed a high amount of milk within the first 5 minutes of the test and lower amounts till the end of the test. Iron ($F_{4, 24}$ = 20.66; $P$ < 0.0001) exhibited a significant

effect on the consumption of the litter in the time course curve (Figure 4.24a). Perinatal fluoxetine treatment ($F_{4, 24}$ = 12.91; $P$ < 0.0001) also had a significant effect on the milk consumption of the litter (Figure 4.24c). Different doses of iron ($F_{4,30}$ = 0.7801; P < 0.5470) and fluoxetine ($F_{4,30}$ = 0.6969; P < 0.6001) treatments had no significant effect on the consumption of the litter in the home cage as shown by the area under the curve (AUC) in figure 4.24c and figure 4.24d for iron and fluoxetine respectively compared to vehicle and DFx groups.

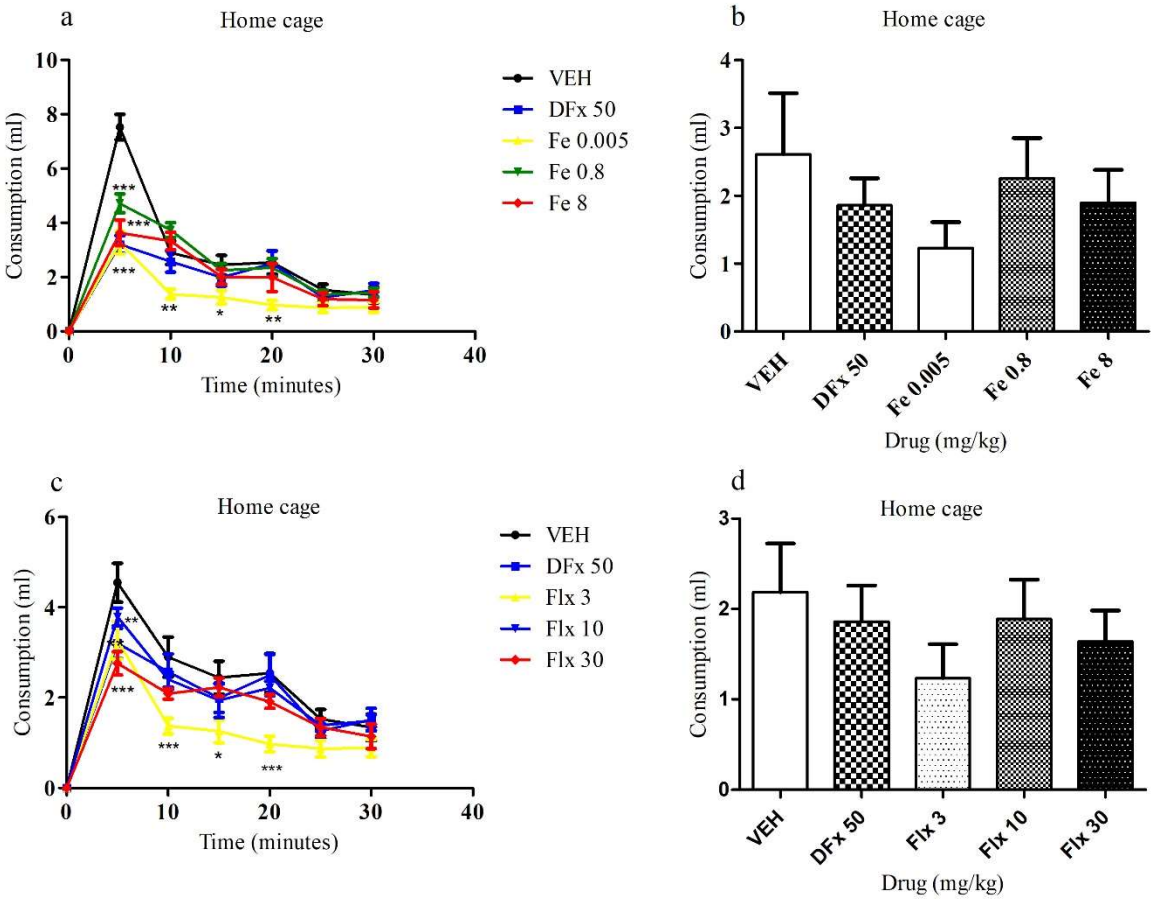


Figure 4. 24: Effects of perinatal iron (0.005 - 8 mgkg$^{-1}$) (a, b) and fluoxetine (3 – 30 mgkg$^{-1}$) (c, d) exposure on the amount consumed by the litter of a palatable meal in the novelty-induced hypophagia test. Data are presented as both (a, c) a time course curve and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001; two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d); comparison with DFx-exposed

group: $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$; two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d).

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the consumption of a palatable meal for litter in the novel cage***

The overall litter consumption in the novel cage was relatively lower for both treatment groups compared to the home cage. The litter for the three dose groups of iron ($F_{4, 24} = 12.44$; $P< 0.0001$) (Figure 4.25a) consumed significantly more milk in the first five minutes of the test. Consumption decreased afterwards until the end of the test. The litter of the fluoxetine group also consumed significantly more milk during the first 5 minutes of the test compared to the vehicle group within the same time period. Consumption was significantly high throughout the test duration ($F_{4, 24} = 12.58$; $P < 0.0001$) (Figure 4.25c). Different doses of both iron ($F_{4,30} = 1.311$; $P = 0.2883$) and fluoxetine ($F_{4,30} = 1.441$; $P = 0.2447$) treatments had no significant effect on the consumption of the litter in the novel cage as shown by the area under the curve (AUC) in figure 4.25c and figure 4.25d for iron and fluoxetine respectively.

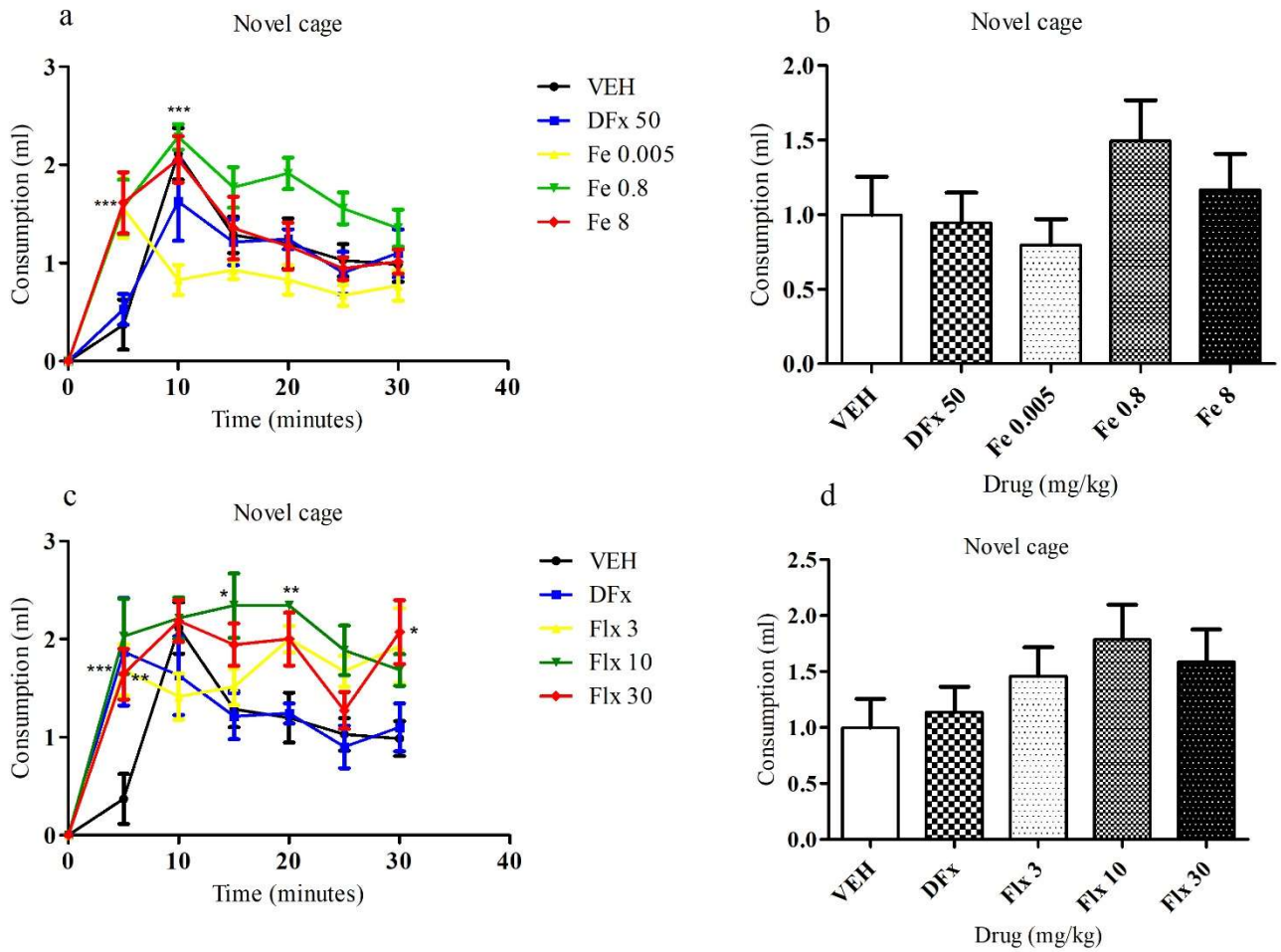


Figure 4. 25: Effects of perinatal iron (0.005 - 8 mgkg$^{-1}$) (a, b) and fluoxetine (3 – 30 mgkg$^{-1}$) (a, b) exposure on the amount consumed by the litter of a palatable meal in the novelty-induced hypophagia test. Data are presented as both (a, c) a time course curve and the (b, d) Mean ± SEM of their areas under the curves (AUCs). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d); comparison with DFx-exposed group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001; two-way ANOVA followed by Bonferroni's test (a, c) and one-way ANOVA followed by Newman Keul's test (b, d).

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the consumption of a palatable meal for litter for the first (5) minutes in the home cage and novel cage***

Overall, milk consumption was reduced in the novel cage compared to the home cage for the first five minutes of the test in all treatment groups. There was a significant dose independent effect of perinatal iron treatment on the milk consumption of the litter in the home cage ($F_{4, 30}$ = 16.08; $P$ < 0.0001) (Figure 4.26a) and novel cage ($F_{4, 30}$ = 6.759; $P$ = 0.0005) (Figure

4.26b). Perinatal fluoxetine treatment of dams had a significant effect on litter consumption in the home cage ($F_{4, 30} = 4.045$; $P = 0.0097$) (Figure 4.26c) and novel cage during the first 5 minutes of the test ($F_{4, 30} = 6.756$; $P= 0.0005$) (Figure 4.26d) in a dose independent manner. DFx-exposure increased consumption in the home cage but decreased consumption compared to iron and fluoxetine treatment in the novel cage.

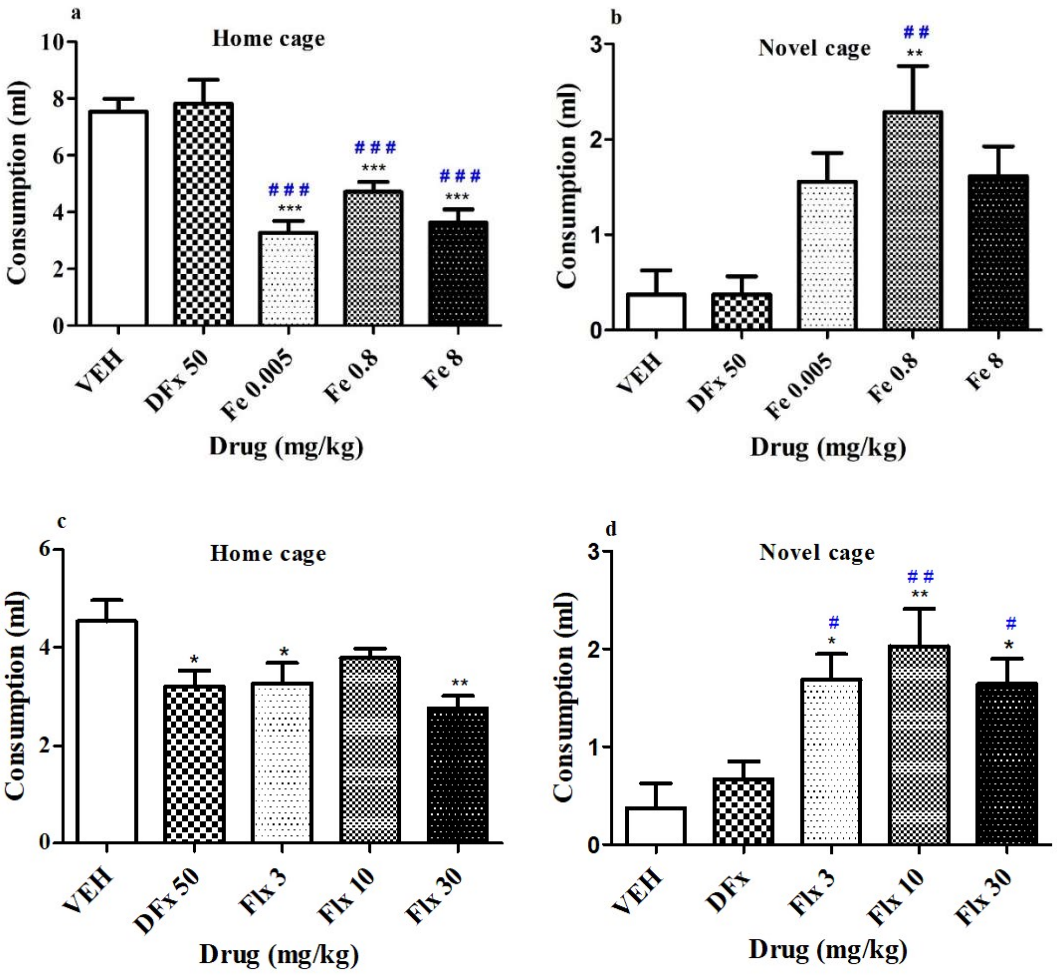


Figure 4. 26: Effects of perinatal iron (0.005 - 8 mgkg$^{-1}$) (a, b) and fluoxetine (3 – 30 mgkg$^{-1}$) (c, d) treatment on the amount consumed of a palatable meal in the novelty-induced hypophagia test. Data are presented as Mean ± SEM of their areas under the curves (AUCs). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA followed by Newman Keul's test); comparison with DFx-treated group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 (one-way ANOVA followed by Newman Keul's test).

***Effect of perinatal iron, fluoxetine and desferrioxamine treatment on the weight of the dams during FST.***

There was a significant difference in the relative weight of the dams throughout the fourteen days of FST for both iron ($F_{4, 34} = 9.213$; $P < 0.0001$) (Figure 4.27a) and fluoxetine ($F_{4, 34} = 8.221$; $P < 0.0001$) (Figure 4.27b) compared to the desferrioxamine (iron deficient) group but not the vehicle (saline) group.

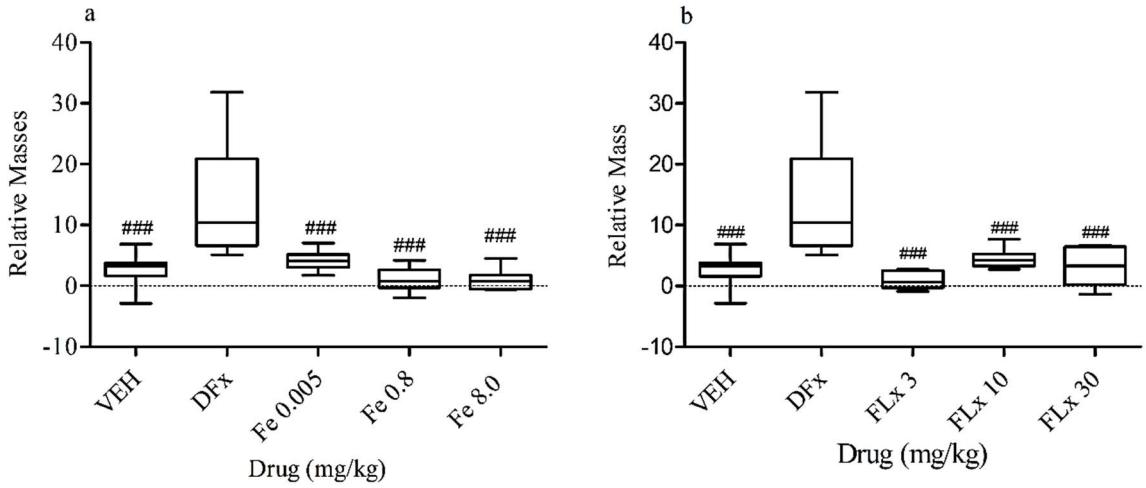


Figure 4. 27: Effects of perinatal iron (0.005 - 8 mgkg-1) (a) and fluoxetine (3 – 30 mgkg-1) (b) treatment on the weight of the dams during FST. Data are presented as relative masses. Significantly different from DFx-treated group: $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ (one-way ANOVA followed by Newman Keul's test).

***Effect of perinatal iron, fluoxetine and desferrioxamine exposure on the weight of the litter during FST.***

There was a significant difference in the relative weight of the litter throughout the fourteen days of FST for both iron ($F_{4, 34} = 3.969$; $P < 0.0001$) (Figure 4.28a) and fluoxetine ($F_{4, 34} = 4.332$; $P < 0.0070$) (Figure 4.28b) compared to the desferrioxamine (iron deficient) group but not the vehicle (saline) group.

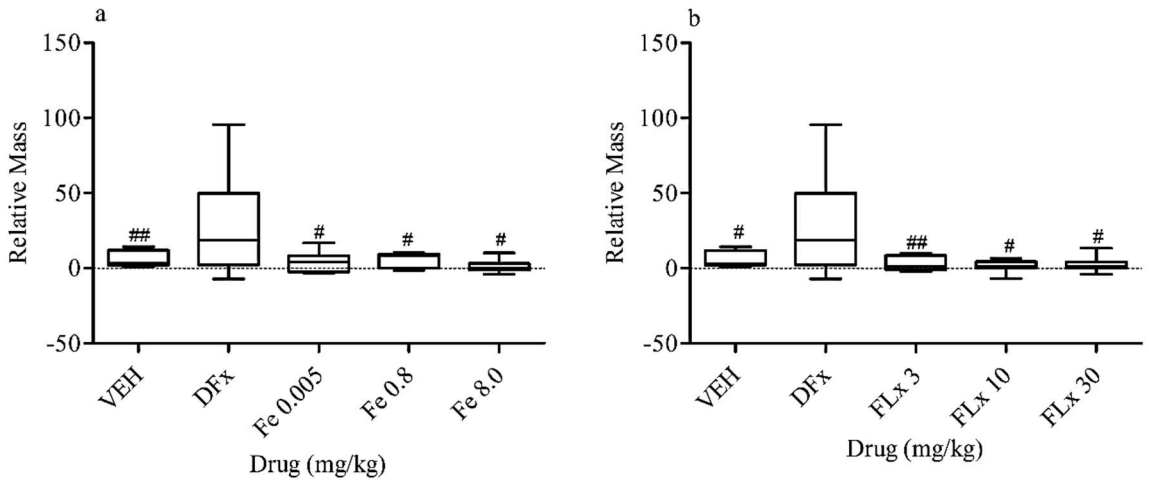


Figure 4. 28: Effects of perinatal iron (0.005 - 8 $mgkg^{-1}$) (a) and fluoxetine (3 – 30 $mgkg^{-1}$) (b) treatment on the weight of the litter during FST. Data are presented as relative masses. Significantly different from DFx-exposed group: $^{\#}P<0.05$, $^{\#\#\#}P<0.01$, $^{\#\#\#}P<0.001$ (one-way ANOVA followed by Newman Keul's test).

## 4.4 HISTOLOGICAL FEATURES OF BRAIN SECTIONS OF RATS FROM THE VARIOUS EXPERIMENTAL GROUPS

### 4.4.1 General Features

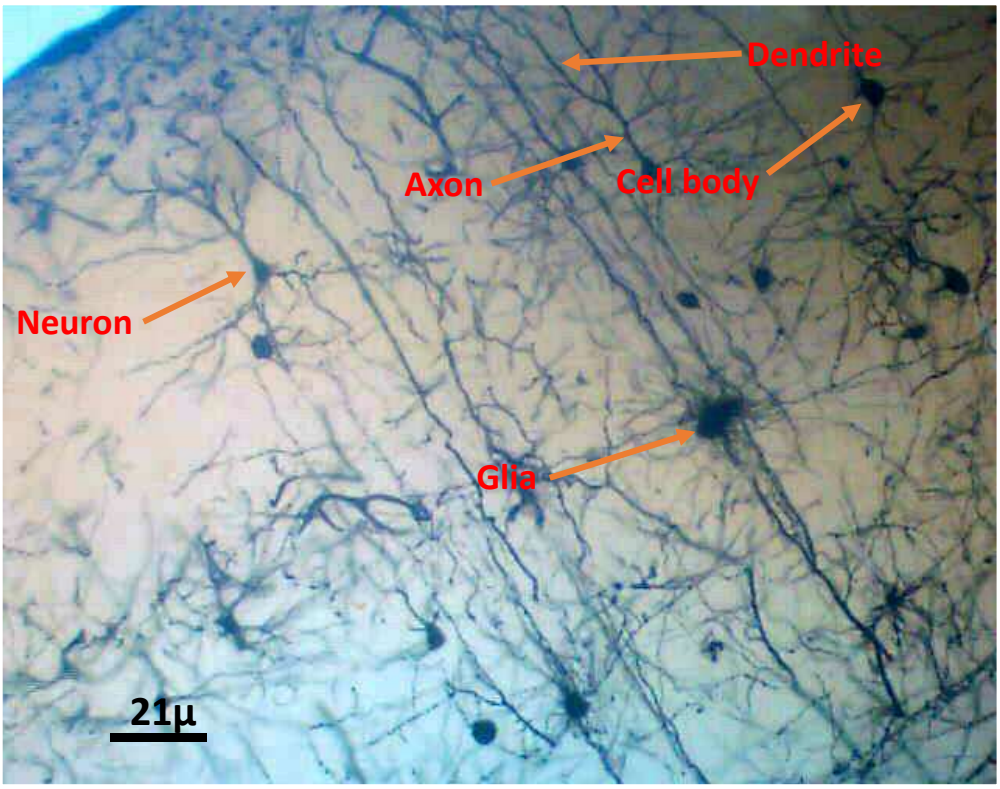


Figure 4. 29: Photomicrograph of Golgi-Cox stained brain cortex section showing neurons and glia (100×).

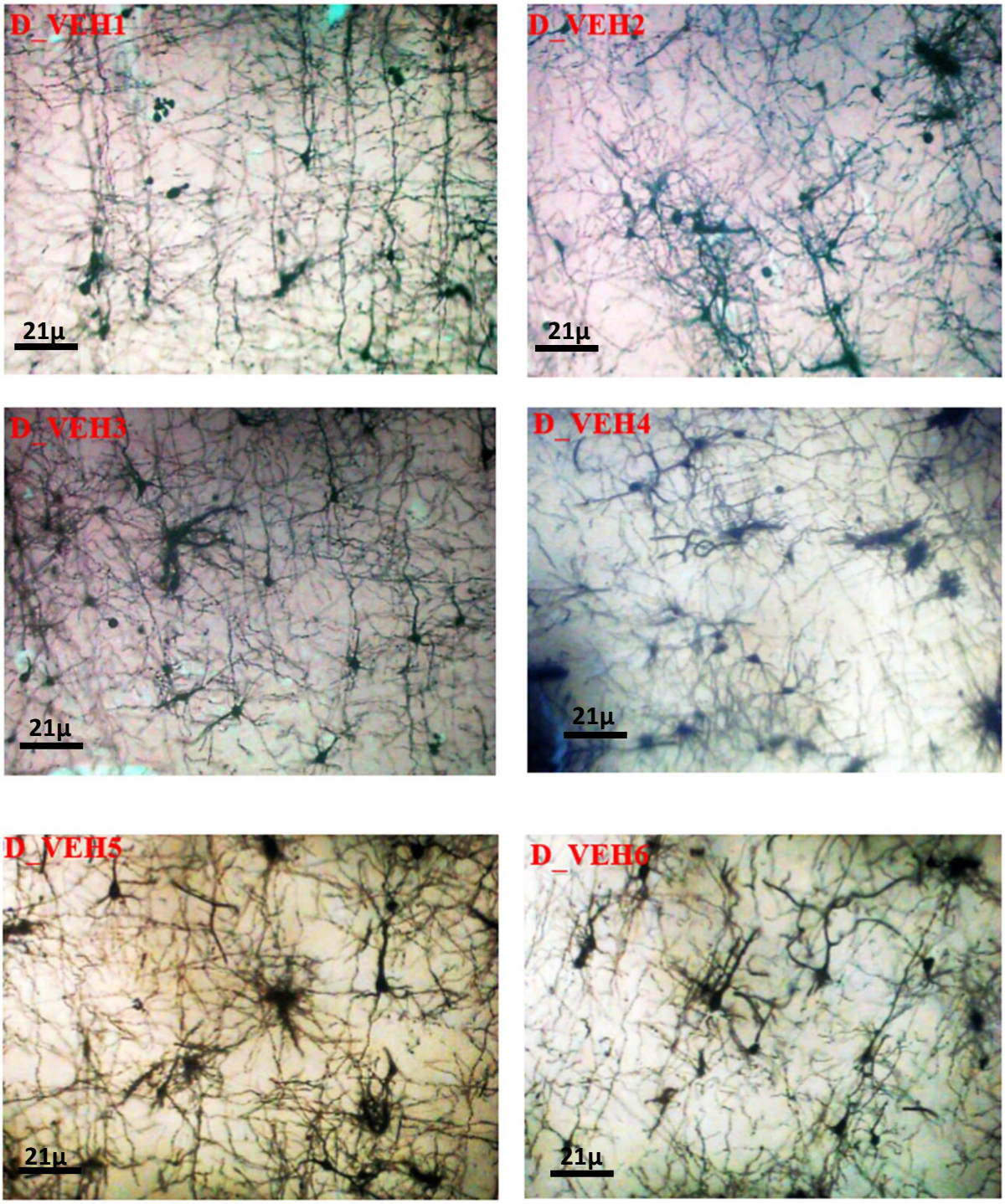


Figure 4. 30: (D_VEH1 – D_VEH6): Photomicrographs of Golgi-Cox stained brain cortex sections of the dams of the vehicle (saline) group (100×).

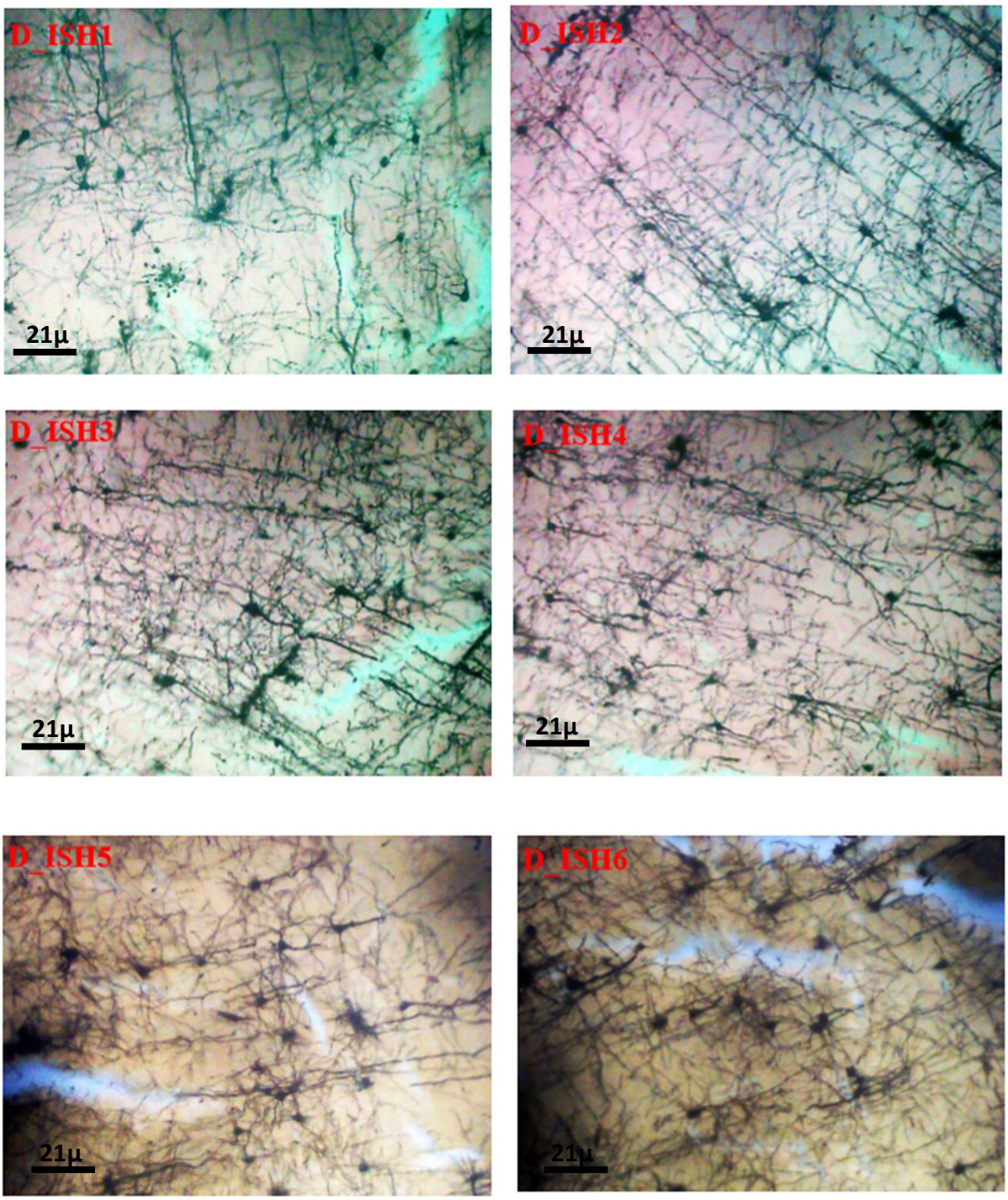


Figure 4. 31: (D_ISH1 – D_ISH6): Photomicrographs of Golgi-Cox stained brain cortex sections of the dams of the high dose iron treatment group (100×).

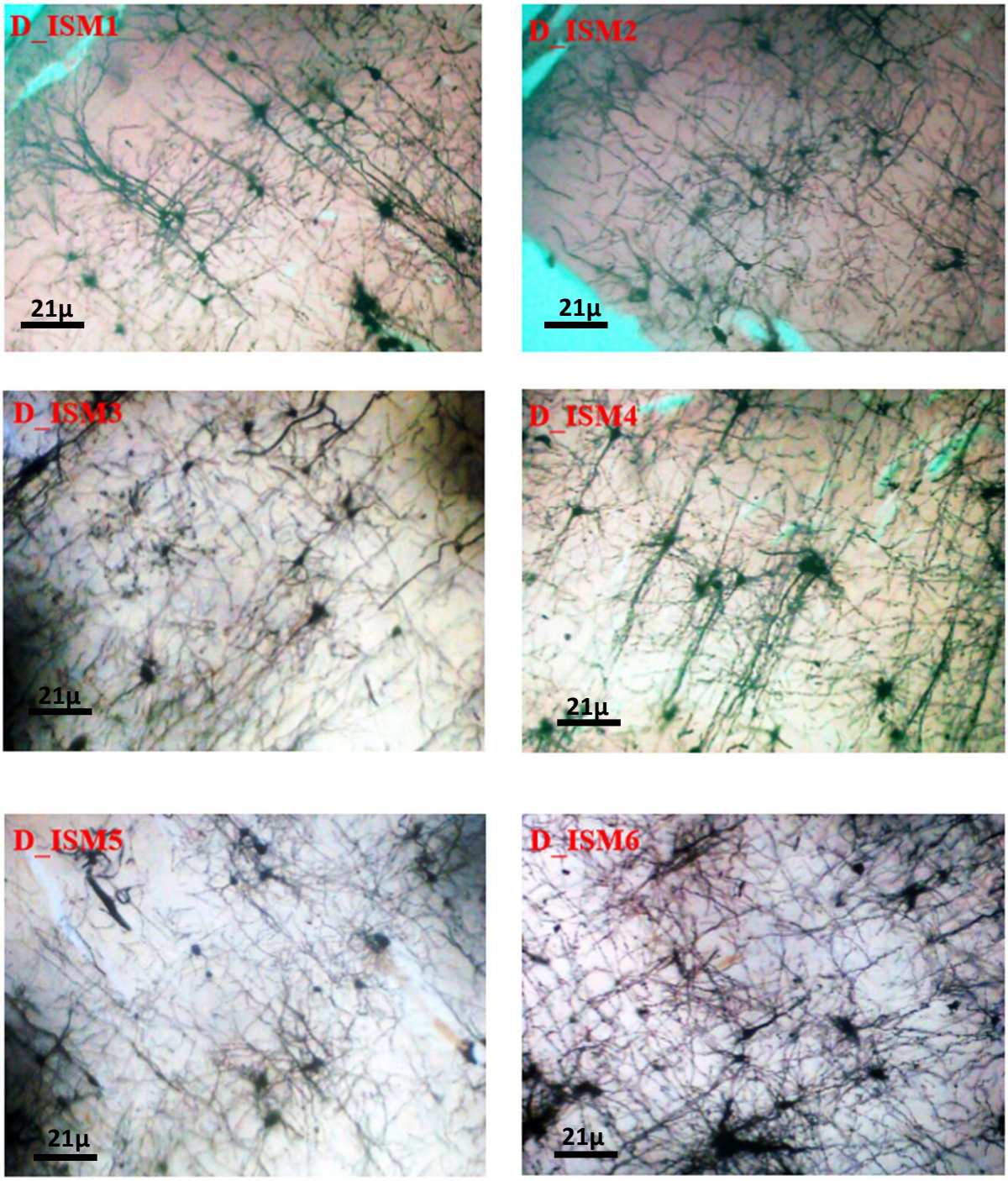


Figure 4. 32: (D_ISM1 – D_ISM6): Photomicrographs of Golgi-Cox stained brain cortex sections of the dams of the medium dose iron treatment group (100×).

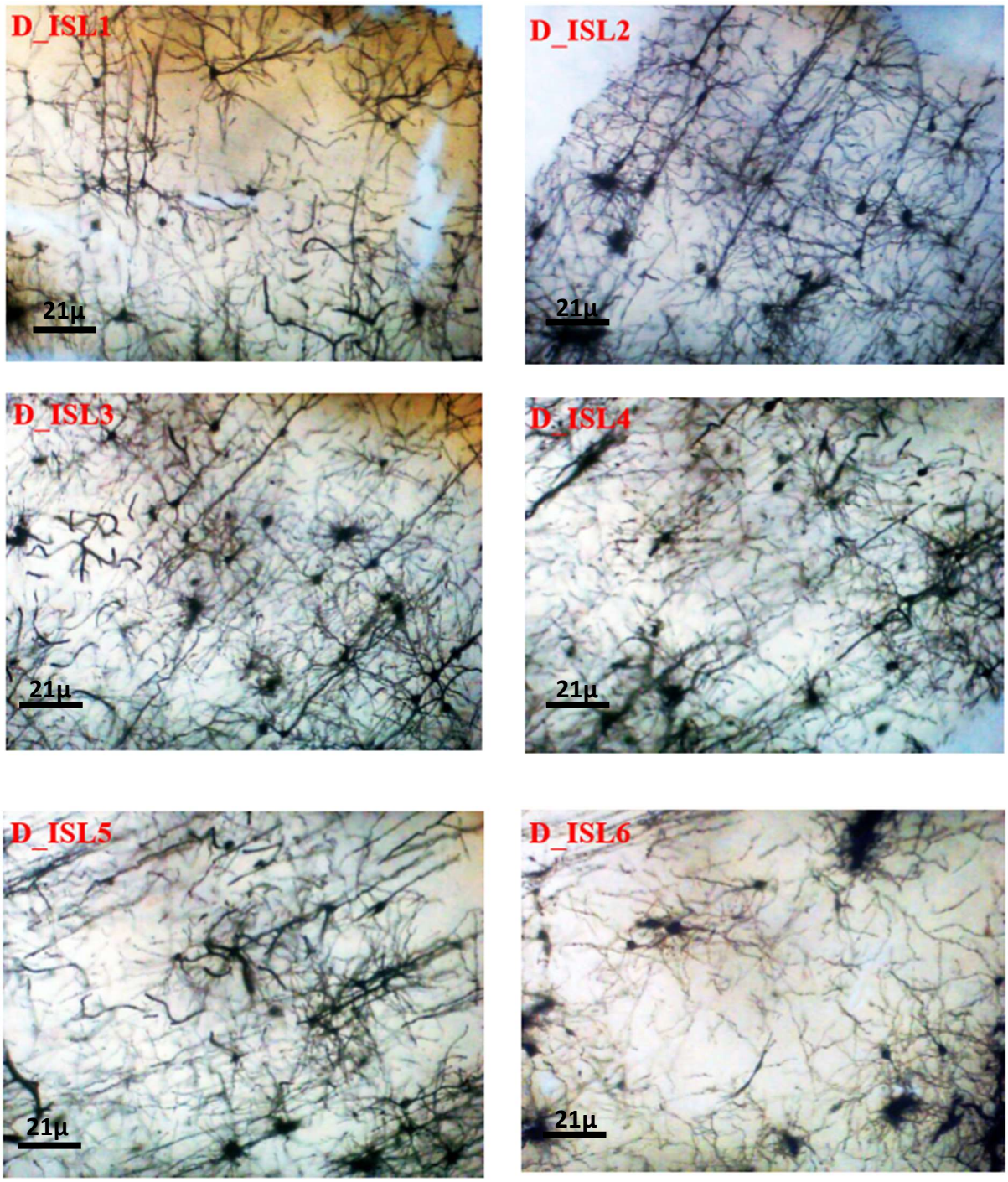


Figure 4. 33: (D_ISL1 – D_ISL6): Photomicrographs of Golgi-Cox stained brain cortex sections of the dams of the low dose iron treatment group (100×).

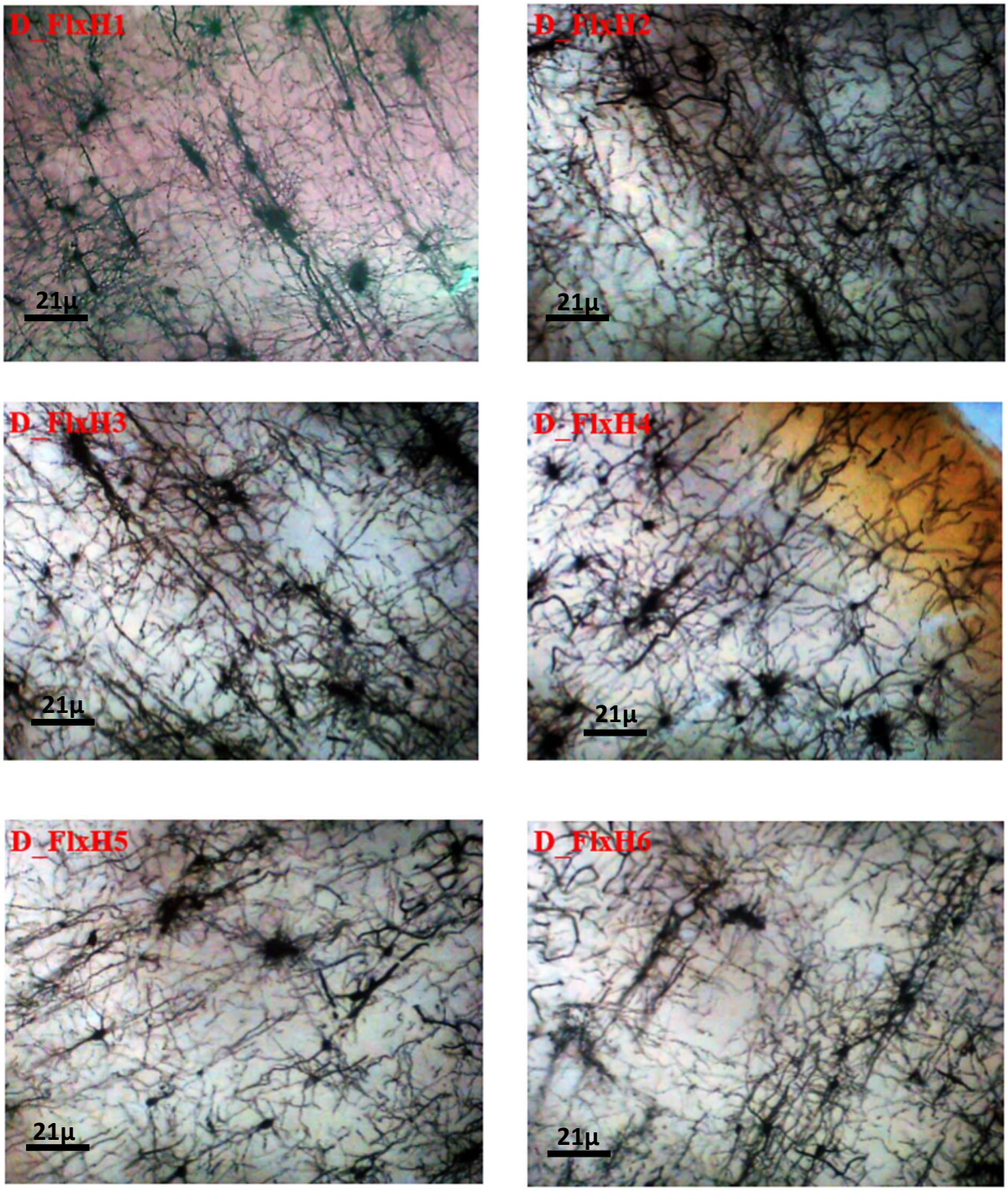


Figure 4. 34: (D_FlxH1 – D_ FlxH6): Photomicrographs of Golgi-Cox stained brain cortex sections of the dams of the high dose fluoxetine treatment group (100×).

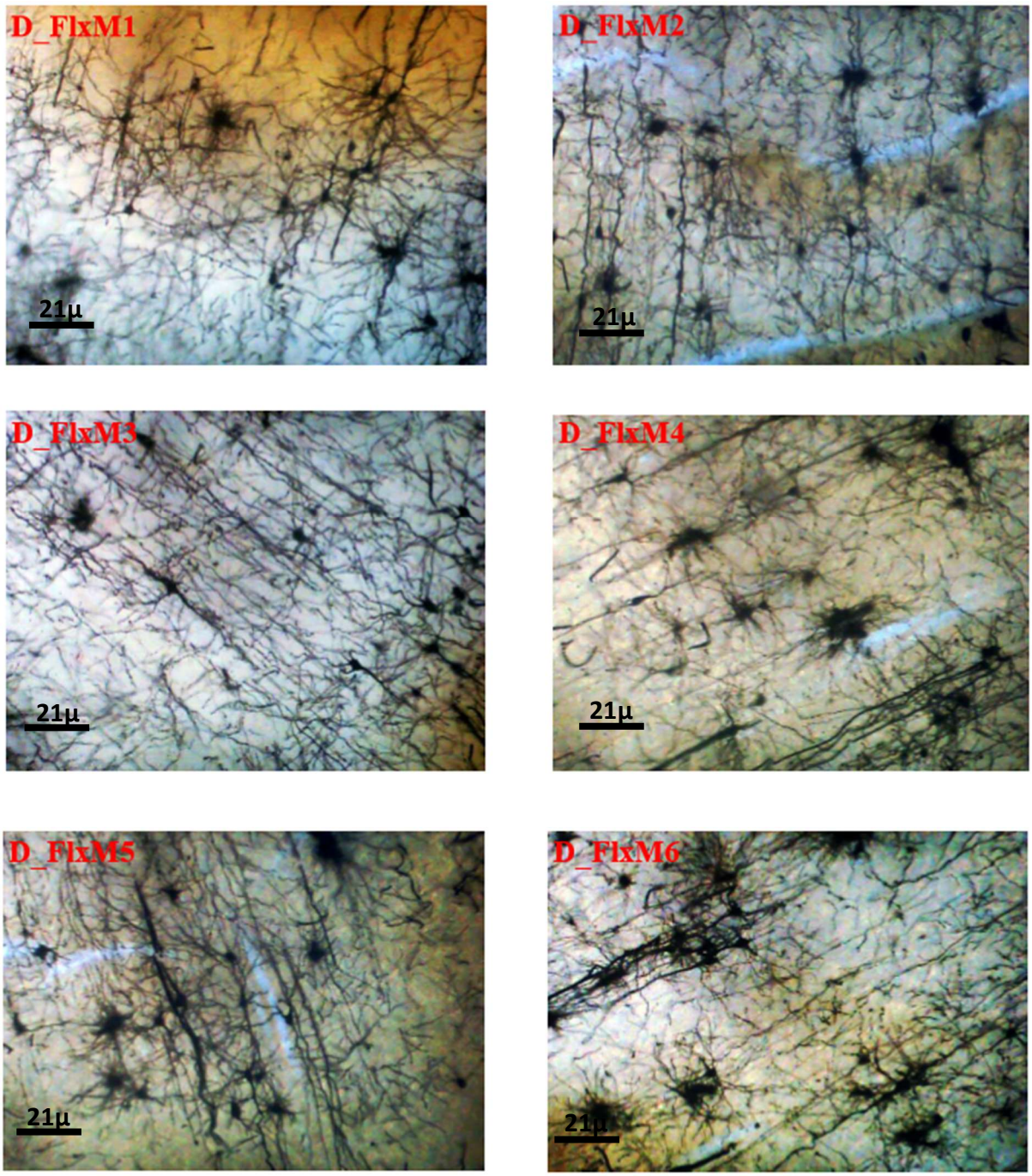


Figure 4. 35: (D_FlxM1 – D_ FlxM6): Photomicrographs of Golgi-Cox stained brain cortex sections of the dams of the fluoxetine treatment group (100×).

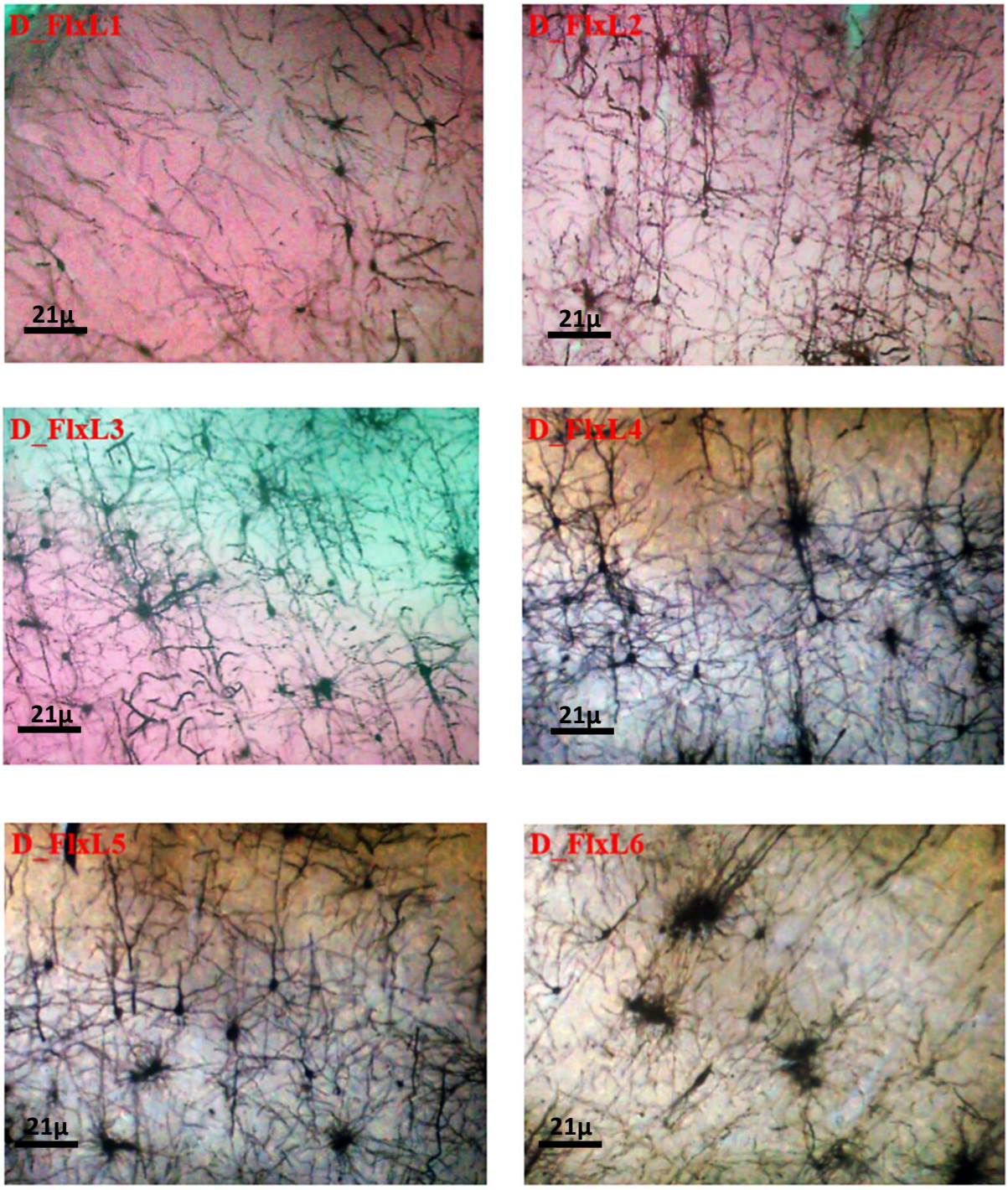


Figure 4. 36: (D_FlxL1 – D_ FlxL6): Photomicrographs of Golgi-Cox stained brain cortex sections of the dams of the low dose fluoxetine treatment group (100×).

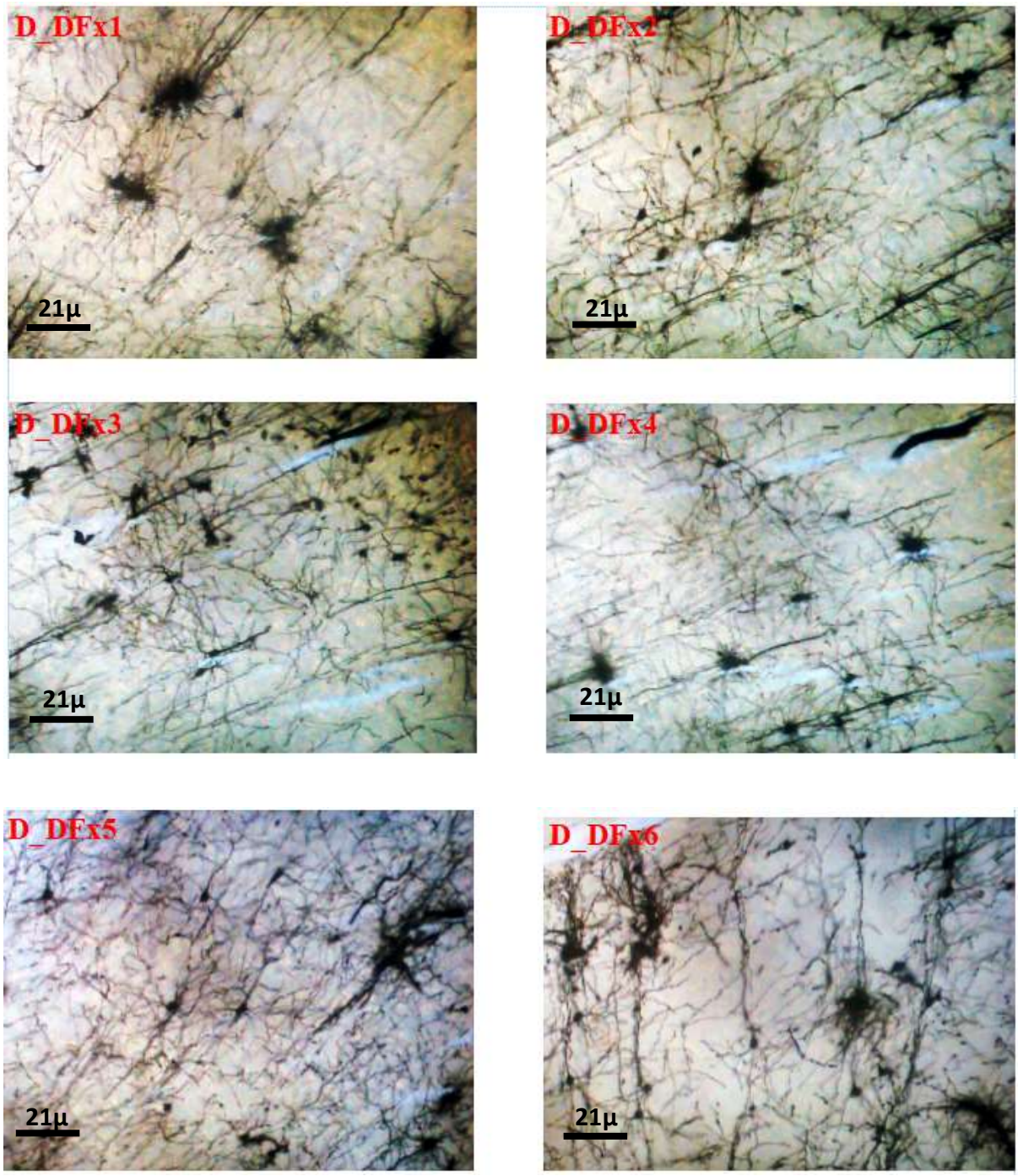


Figure 4. 37: (D_DFx1 – D_ DFx6): Photomicrographs of Golgi-Cox stained brain cortex sections of the dams of desferrioxamine treatment group (100×).

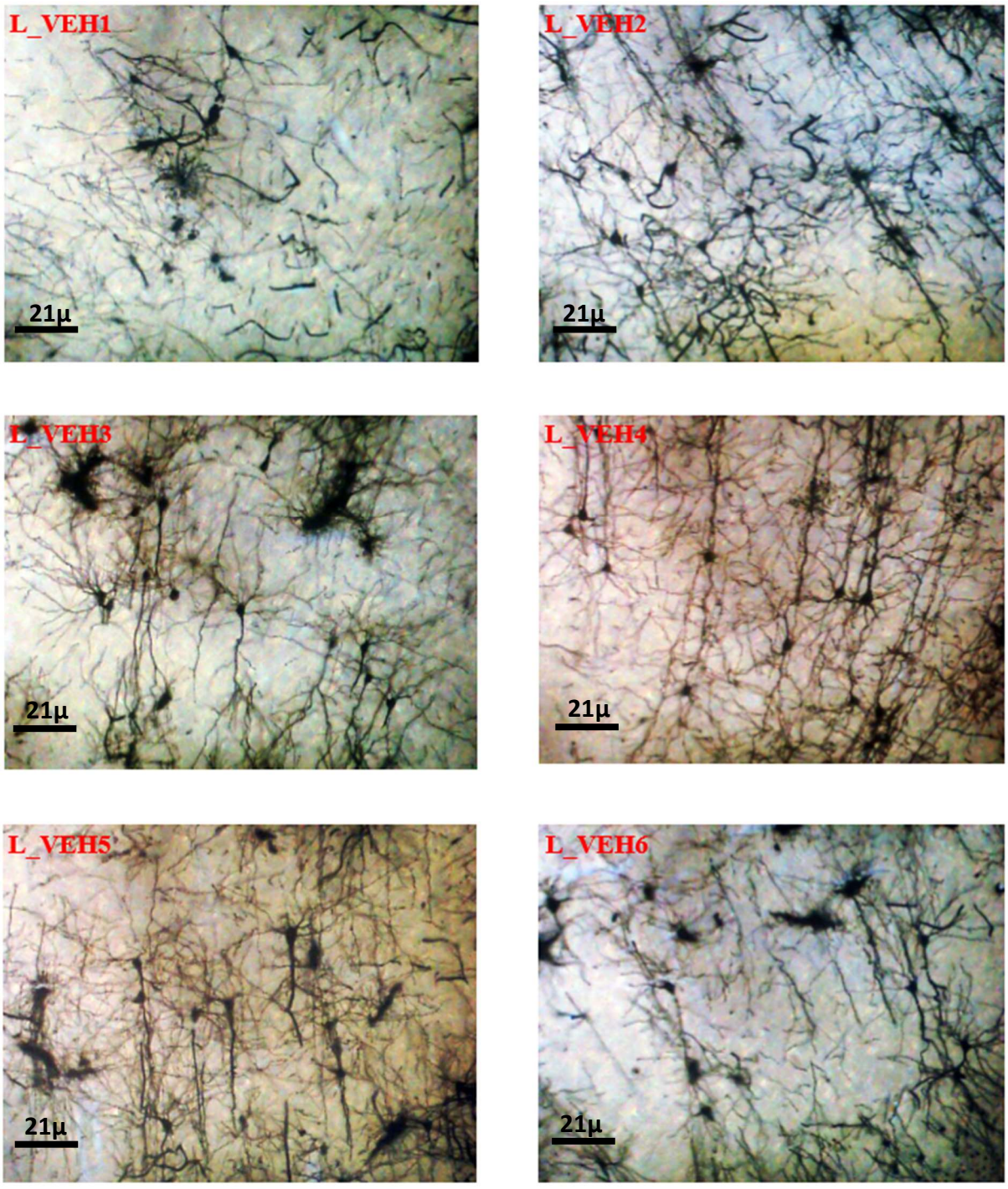


Figure 4. 38: (L_VEH1 – L_ VEH6): Photomicrographs of Golgi-Cox stained brain cortex sections of the litter of the control group (100×).

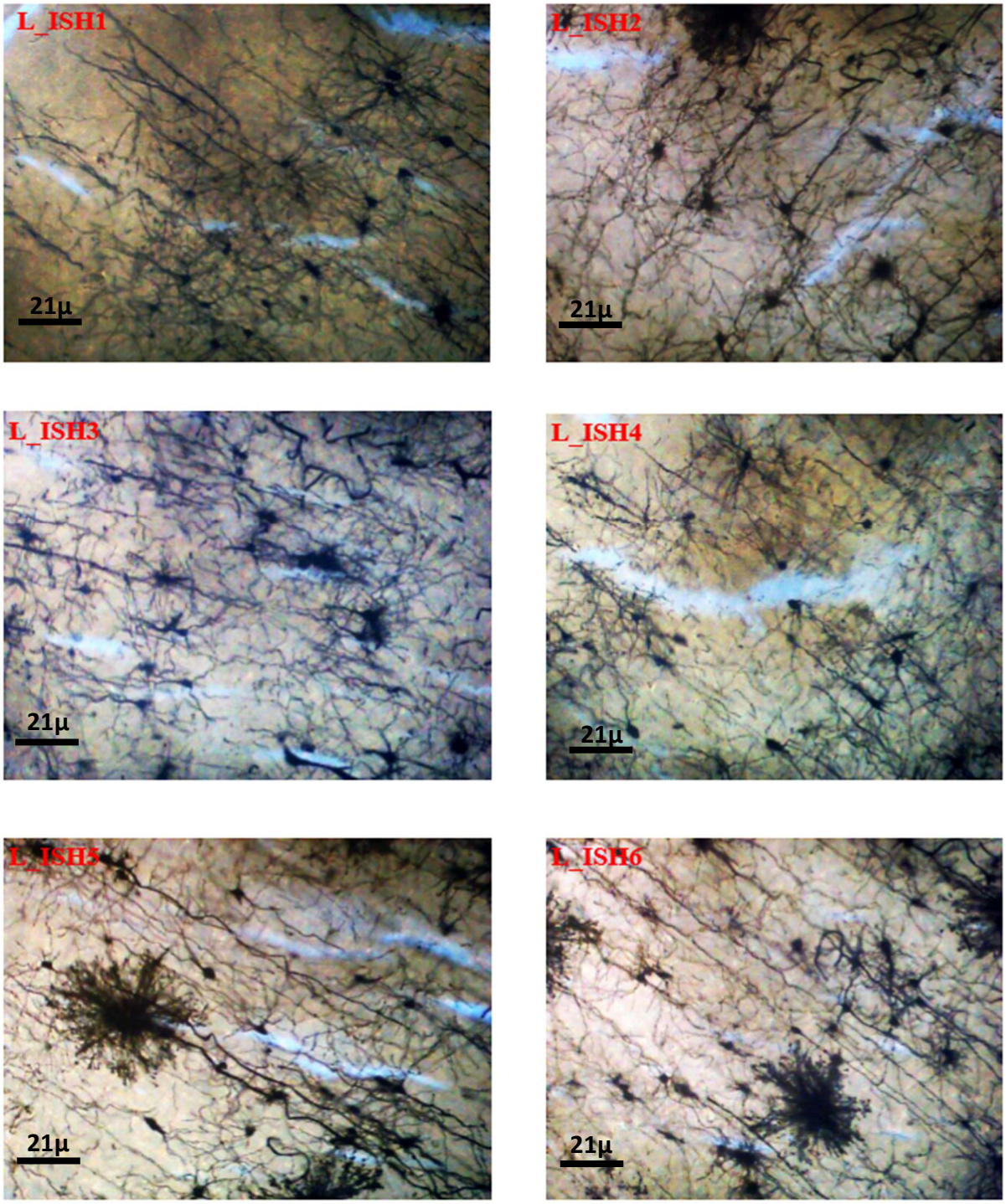


Figure 4. 39: (L_ISH1 – L_ISH6): Photomicrographs of Golgi-Cox stained brain cortex sections of the litter of the high dose iron treatment group (100×).

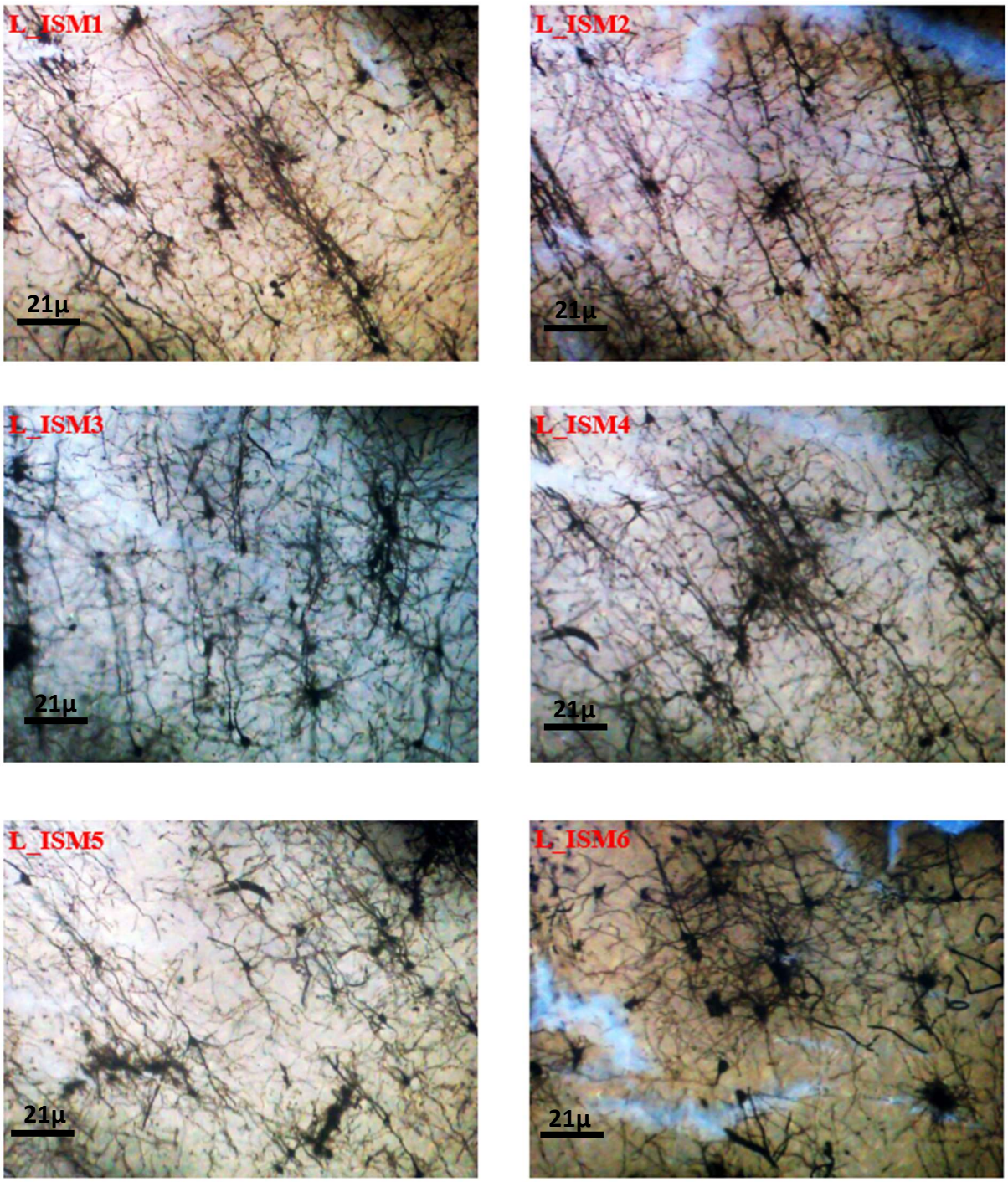


Figure 4. 40: (L_ISM1 – L_ISM6): Photomicrographs of Golgi-Cox stained brain cortex sections of the litter of the medium dose iron treatment group (100×).

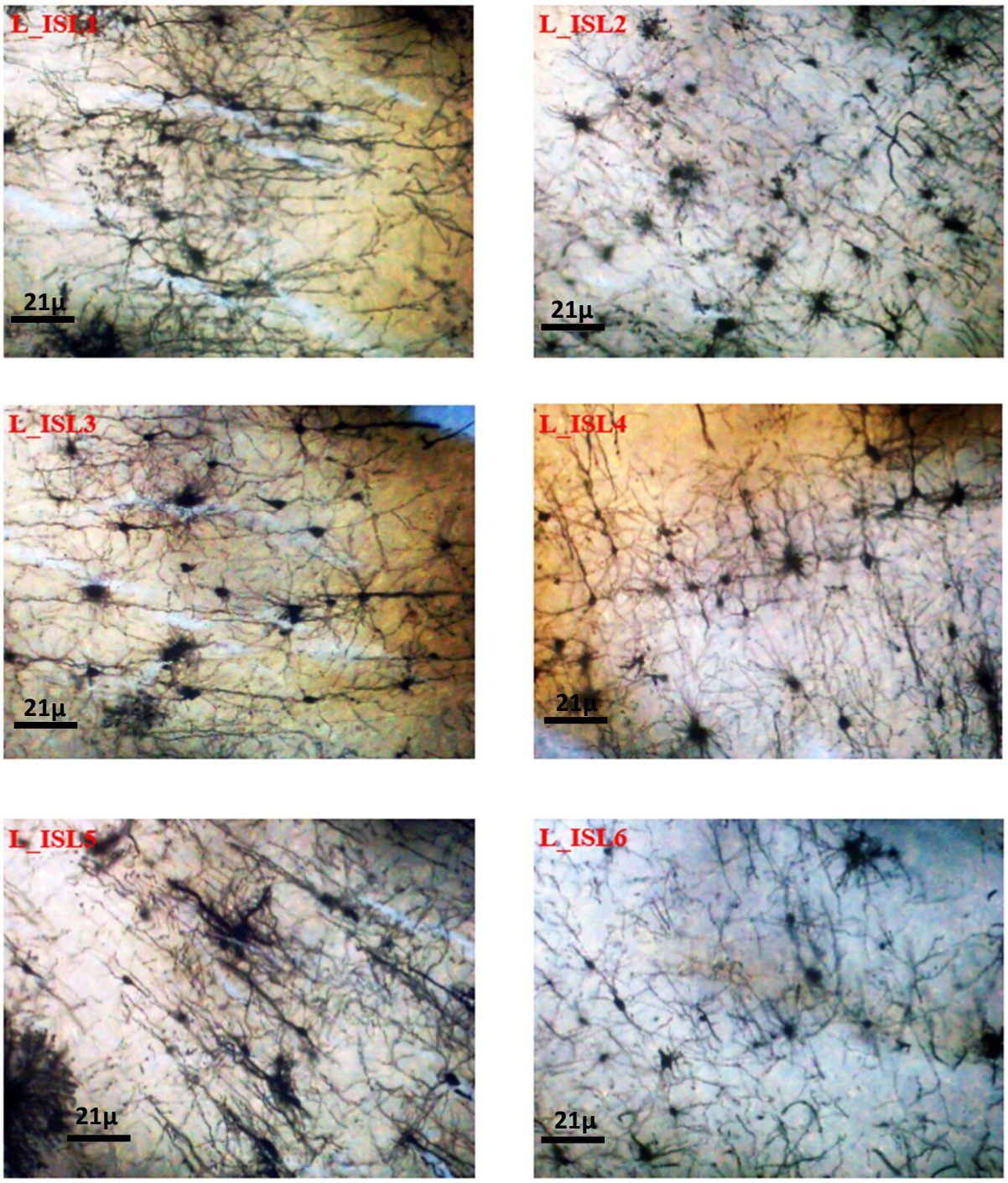


Figure 4. 41: (L_ISL1 – L_ISL6): Photomicrographs of Golgi-Cox stained brain cortex sections of the litter of the low dose iron treatment group (100×).

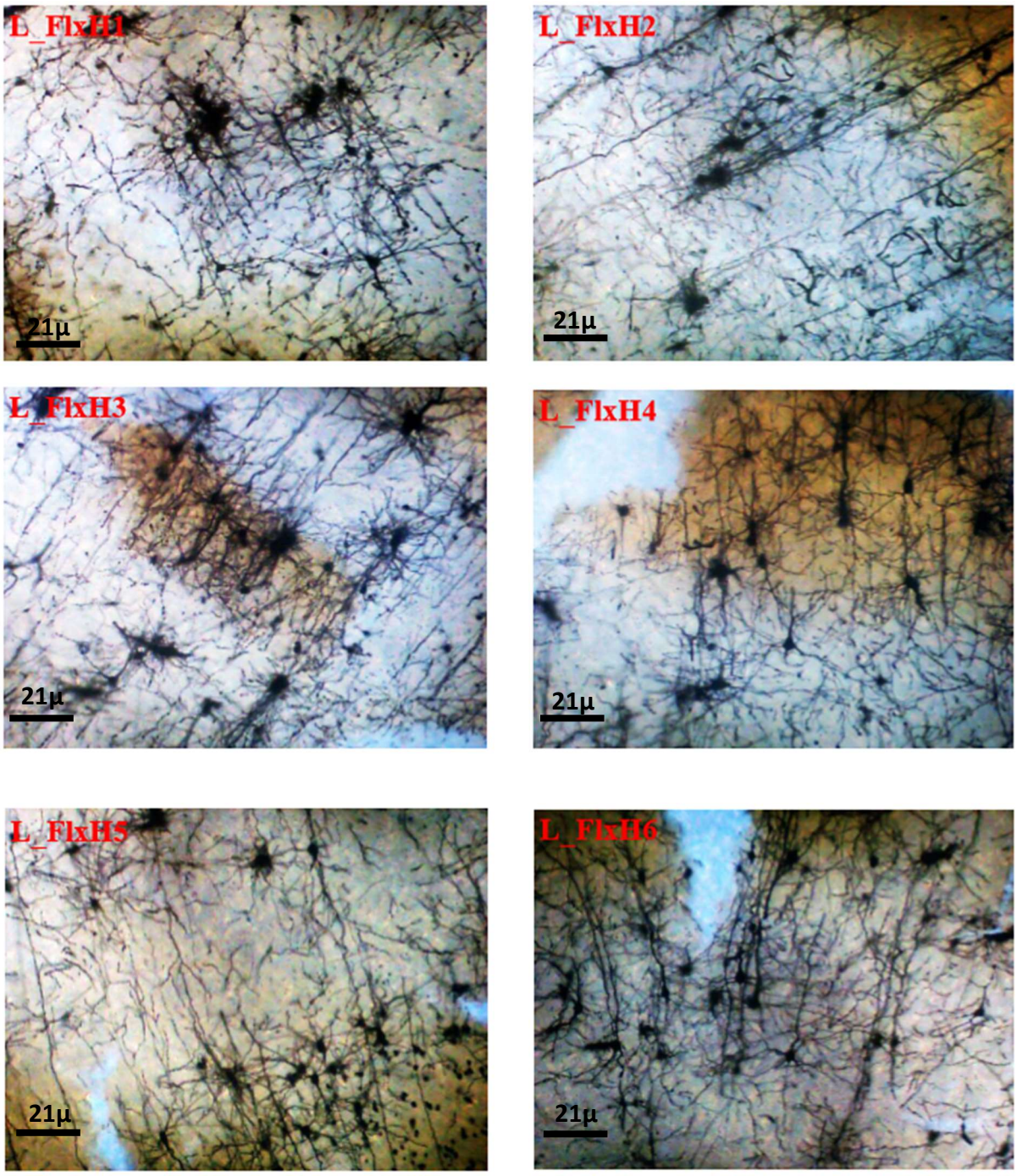


Figure 4. 42: (L_FlxH1 – L_FlxH6): Photomicrographs of Golgi-Cox stained brain cortex sections of the litter of the high dose fluoxetine treatment group (100×).

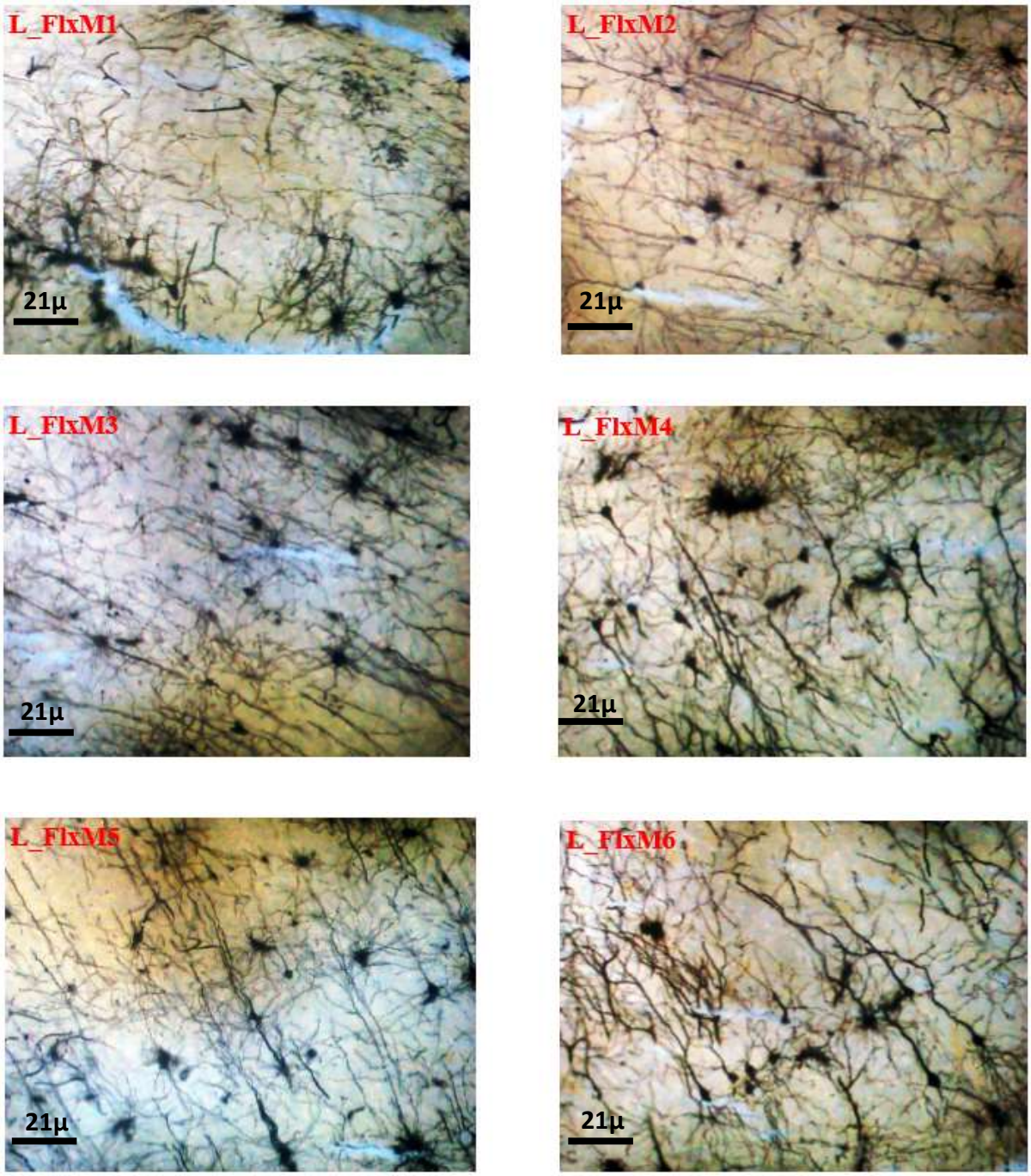


Figure 4. 43: (L_FlxM1 – L_FlxM6): Photomicrographs of Golgi-Cox stained brain cortex sections of the litter of the medium dose fluoxetine treatment group (100×).

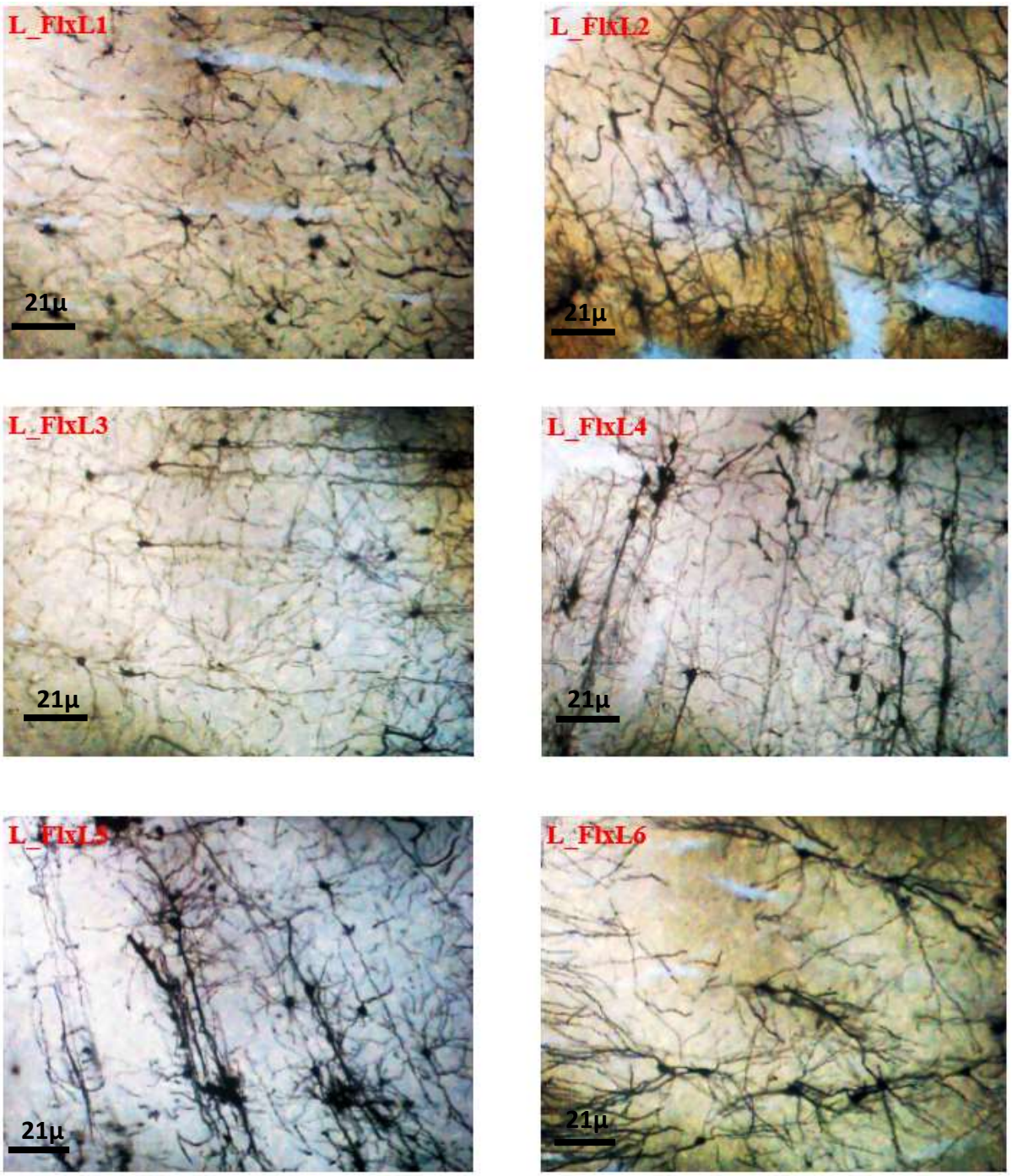


Figure 4. 44: L_FlxL1 – L_FlxL6): Photomicrographs of Golgi-Cox stained brain cortex sections of the litter of the low dose fluoxetine treatment group (100×).

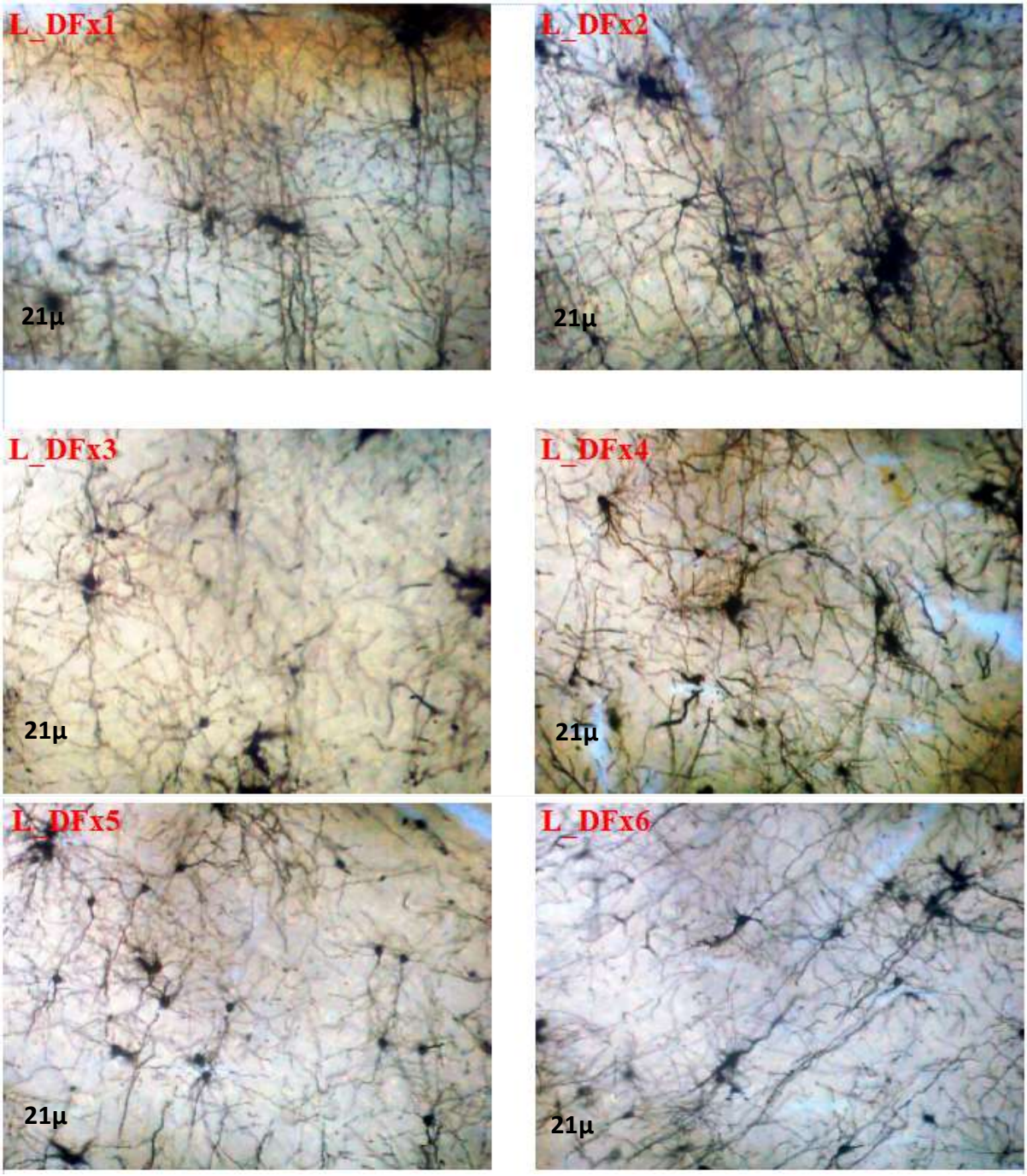


Figure 4. 45: (L_DFxL1 – L_DFxL6): Photomicrographs of Golgi-Cox stained brain cortex sections of the litter of the desferrioxamine treatment group (100×).

## 4.5 VOLUME OF NEURONS FOR DAMS AND LITTER

One-way ANOVA for the dams showed that there was a significant increase in the absolute volume of neurons in the frontal cortex of the rat brain for both iron ($F_{4, 14}$ = 11.02, P=0.0011) and fluoxetine ($F_{4, 14}$ = 12.82, P=0.0006) treatment group compared to the vehicle group. In the litter as well there was a significant increase in the absolute volume of neurons for both iron ($F_{4, 14}$ = 10.7, P=0.0012) and fluoxetine ($F_{4, 14}$ = 36.72, P<0.0001) treated groups compared to the vehicle.

Table 4. 1: Summary of the absolute volume of neurons in the treatment groups

| TREATMENT | GROUP | DAMS (x$10^3$ $\mu m^3$) | LITTER (x$10^3$ $\mu m^3$) |
|---|---|---|---|
| **SALINE (VEHICLE)** | **VEH** | 167.3±3.095 | 138.2±4.055 |
| **DESFERRIOXAMINE (DFx, 50 mg/kg)** | **DFx** | 110.3±0.6219$^{***}$ | 77.9±3.68$^{**}$ |
| **IRON (Fe, mg/kg)** | **Fe 0.005** | 186.1±5.154$^{\#\#\#}$ | 154.7±11.55$^{\#\#\#}$ |
| | **Fe 0.8** | 203.0±9.680$^{***\ \#\#\#}$ | 134.8±5.134$^{\#\#}$ |
| | **Fe 8.0** | 196.0±5.530$^{\#\#\#}$ | 140.7±15.35$^{\#\#}$ |
| **FLUOXETINE (FLx, mg/kg)** | **FLx 3** | 188.7±15.45$^{\#\#}$ | 142.3±7.120 |
| | **FLx 10** | 207.2±20.51$^{\#\#}$ | 176.5±9.674$^{**\ \#\#\#}$ |
| | **FLx 30** | 235.3±12.29$^{*\ \#\#\#}$ | 176.8±7.839$^{**\ \#\#\#}$ |

Effects of perinatal iron (0.005 - 8 $mgkg^{-1}$), fluoxetine (3 – 30 $mgkg^{-1}$) and desferrioxamine (50 mg/kg) treatment on the volume of neurons in the prefrontal cortex of postpartum dams and their litter. Data are presented as Mean ± SEM of the number of neurons. Significantly different from control: *P<0.05, **P<0.01, ***P<0.001; one-way ANOVA followed by Newman Keul's test; compared with DFx group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 one-way ANOVA followed by Newman Keul's test

# CHAPTER FIVE

# DISCUSSION AND CONCLUSION

## 5.1 DISCUSSION

Depression during the postpartum period is an area of research that is gaining global attention even though it is rarely investigated in certain jurisdictions such as Ghana. Patients with PPD often have several associated physical disorders, suicidal as well as infanticidal tendencies. The effects of PPD are far reaching since it has been implicated in cognitive and neurobehavioral abnormalities in the offspring of PPD mothers (Beard, 2003; Beard *et al.*, 2005; Georgieff, 2011; Lozoff, B. *et al.*, 2006). Current antidepressants used in the treatment of PPD have been associated with side effects on breast fed infants as well as prolonged improvement of symptomatology and may cause an upsurge in mortality if drugs with rapid onset, continued effect, accessible and multiple therapeutic targets that better addresses the heterogeneous nature of the disorder are not sought. It is therefore imperative to seek for agents that can minimize or at best prevent the symptomatology associated with PPD as well as avoid the cognitive and neurobehavioral deficits and lower side effects for both mothers and their infants. The use of mineral elements such as iron during pregnancy is reported in previous studies to have a significant positive effect on neuronal functions and processes in the mother and the developing foetus (Georgieff, 2006; Georgieff, 2008; Lozoff, B. *et al.*, 2006; Rao *et al.*, 2001 ). Iron has also been proven in both experimental and observational research to lessen the burden of mood disorders of postpartum mothers and their infants (Georgieff, 2011; Grantham-McGregor *et al.*, 2001). The goal of the present study was to ascertain the effects of perinatal iron and desferrioxamine treatment on depressive behaviour in postpartum Sprague-Dawley rats and their litter.

The results of the current study demonstrated that iron has significant antidepressant effect just like fluoxetine in FST which is the most widely used pharmacological model for assessing antidepressant activity. In this model iron decreased in a dose dependent manner the duration of immobility by increasing behavioural components like swimming and climbing. This observation is common with all antidepressants in clinical practice (Cryan *et al.*, 2005; Kukuia *et al.*, 2014; Slattery *et al.*, 2011). Thus, there was a significant reduction in immobility behaviour in the iron treated dams which is suggestive of an antidepressant effect in FST.

Modifications of the traditional FST by Lucki and Cryan demonstrated that specific behavioural components of active behaviours differentiate neurochemically unique antidepressants (Cryan *et al.*, 2002; Lucki, 1997). Drugs that decrease immobility by causing an upsurge in the swimming behaviour without significantly altering the climbing behaviour are purported to be sensitive to the serotoninergic pathway. Drugs with specific effects on catecholamine neurotransmission selectively increase climbing behaviour (Cryan *et al.*, 2005; Detke *et al.*, 1996; Page *et al.*, 1999; Rénéric *et al.*, 2001; Slattery *et al.*, 2012 ). In this study, iron exhibited significant increase in both swimming and climbing scores in the dams suggesting that it might be acting via serotoninergic and noradrenergic pathways just like the serotonin noradrenaline re-uptake inhibitors (SNRIs) such as venlafaxine. The antidepressant-like effect exhibited by iron in the dams is possibly through the enhancement of both serotonergic and noradrenergic neurotransmission.

The dams treated with fluoxetine, a classical antidepressant in clinical use, demonstrated a significant decrease in immobility score in FST which is indicative of antidepressant-like effect. The decrease in immobility was as a result of increased swimming score suggestive of enhanced serotonergic neurotransmission. This is commensurate with its mode of action as an established selective serotonin reuptake inhibitor (SSRI).

The dose-response curve showed that iron was more potent than fluoxetine in reducing the immobility score in FST but Fluoxetine had a higher efficacy. The effect of iron on potency is possibly due to its affinity for the large number of receptors modulating the synthesis of 5-HT and NA, the major neurotransmitters implicated in PPD (Salvador, 2010). Fluoxetine preferentially enhances only 5-HT neurotransmission (Cabrera-Vera *et al.*, 1998; Peroutka *et al.*, 1981; Wong *et al.*, 1994) in its antidepressant action.

For the dams treated with desferrioxamine, there was no significant increase in the immobility scores of the dams in FST relative to the vehicle group even though the immobility scores were as high as the vehicle scores. This suggests that desferrioxamine did not cause significant iron deficiency beyond the levels of the control group. Desferrioxamine is a BBB permeable iron chelator designed for its potential use in the treatment of neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Salvador, 2010). It was administered subcutaneously during gestation to induce gestational iron deficiency and iron deficiency during the postpartum period in the dams and their litter. ID been the most common single nutrient deficiency globally affects most women in the reproductive age (Etebary *et al.*, 2010; Sheikh *et al.*, 2015). Maternal ID has been linked with disruptive effects on mental health of women and their infants including deficits in cognitive function, mood, short term memory, verbal learning, attention span/concentration, intelligence (Beard *et al.*, 2005; Georgieff, 2008; Georgieff, 2017). According to Etebary *et al.* (2010), ID can lead to depression and also negatively affect the oxidative capacity of tissues. Severe ID results in reduced haemoglobin concentration and eventually disrupts the oxygen carrying capacity of blood (Haas *et al.*, 2001). Several researches have shown a positive association between iron deficiency anaemia (IDA) and depressive disorders (Beard *et al.*, 2003; Corwin *et al.*, 2003; Georgieff, 2008 ; Radlowski *et al.*, 2013). Other studies have shown that cognitive impairment and postpartum depression in women can be correlated

to iron deficiency anaemia and that depressive disorders responds to iron therapy (Beard *et al.*, 2005; Etebary *et al.*, 2010). These facts suggest the possible role iron plays in brain function and the establishment of mood disorders as a result of ID (Etebary *et al.*, 2010; Shariatpanaahi *et al.*, 2006). The role of iron in the brain is very diverse in that it acts at the molecular level as a cofactor for the synthesis of enzymes involved in neurotransmitter synthesis, including tyrosine hydroxylase and tryptophan hydroxylase, as well as catabolism of these neurotransmitters (Etebary *et al.*, 2010; Fretham *et al.*, 2011; Georgieff, 2006; Salvador, 2010). ID has been reported by Shariatpanaahi *et al.* (2006) to affect dopamine synthesis by causing decreased levels of this neurotransmitter which plays a vital role in mood disorders, in depressed patients. Apart from that, in the rat model, iron deficiency has been postulated to affect the neuronal surface protein Thy1 which alters the release of neurotransmitters and the synaptic efficacy which could contribute to a variety of abnormal neuron-neuron communications in the iron-deficient rat (Erikson *et al.*, 2000; Lozoff, B. *et al.*, 2006; Wang *et al.*, 2004). Hence, the effect of ID-induced alterations in neurotransmission could cause depressive-like traits in the postpartum period and other neurobehavioral disorders because impaired neurotransmission has been implicated in the pathophysiology of mood disorders including PPD. In this study, desferrioxamine-induced gestational ID could impair neurotransmission in the dams and cause high immobility scores which is suggestive of depressive-like behaviour as reported for this group.

For the first generation litter of iron treated dams, iron exposure in-utero decreased the immobility scores compared to the vehicle group which is suggestive of antidepressant-like effect. The process of iron transfer from the mother to the foetus through the placenta appears to be the main mechanism through which the foetus gets iron (McArdle *et al.*, 2008). Studies in rodents have demonstrated that iron transport increases steadily during pregnancy and is mediated by proteins and strict processes that have not been fully elucidated (Cetin *et al.*,

2011; McArdle *et al.*, 2008). There is no iron transfer to the infant in human breast milk after birth due to poor bioavailability causing infant brain iron concentration to decrease during the first 6 months (Radlowski *et al.*, 2013). In-utero iron-exposure is a requirement for the development of neurological structures and processes in the infant since sufficient iron levels are required early in pregnancy for neurogenesis, dendritogenesis, synaptogenesis, brain energy production, myelination and neurotransmitter synthesis (Fretham *et al.*, 2011; Radlowski *et al.*, 2013; Salvador, 2010) from late gestation to early infancy. Iron serves as a co-factor in various enzymes that regulate these processes (Etebary *et al.*, 2010). The role of gestational iron supplementation in all of these processes has been associated with improvements in behavioural disorders associated with the above brain processes (Beard *et al.*, 2003). The antidepressant-like effect exhibited by the iron-exposed progeny in FST is possibly due to the action of iron on these brain processes and functions.

Prenatal iron-exposed progeny demonstrated a significant increase in the swimming score in a dose-related manner during the FST. Early-life iron exposure enhances the synthesis of 5-HT neurotransmitter systems. Enhanced serotonin neurotransmission prevents depressive-like symptoms in rodents by increasing swimming score in FST. The high swimming scores of the iron-exposed progeny is suggestive of enhancement of serotonergic neurotransmission by iron in this group which could be due to the effect of iron on 5-HT neurotransmission.

The fluoxetine-exposed litter also had significantly decreased immobility scores in FST in a dose related fashion which is indicative of antidepressant-like effect. The group also exhibited significant dose dependent increase in swimming scores suggestive of enhanced serotonergic neurotransmission. These findings are consistent with Karpova *et al.* (2009) and Mendes-da-Silva *et al.* (2002) who reported less depressive behaviour in the litter of dams treated with fluoxetine in the perinatal period. This was attributed to the fact that, in rats, fluoxetine readily crosses the placenta, with foetal blood levels reaching 83% of the

mother's blood (Olivier *et al.*, 2011; Rampono *et al.*, 2009), allowing it to subsequently bind to 5-HT transporters present and functional in the foetus (Hendrick *et al.*, 2003; Ivgy-May *et al.*, 1994; Koren, 2014; Mercado *et al.*, 1992), where transporter occupancy was reported by Capello *et al.* (2011) to be greater than 80%. In lactating litter, fluoxetine and its active metabolite norfluoxetine which is also found in high concentration in the lactating dam's milk, causes a 5-HT transporter occupancy rates of up to 57% in the nursing litter (Capello *et al.*, 2011). A basic tenet of the monoamine hypothesis of depressive disorders is that synaptic serotonin is reduced during depressive episodes which is caused partly by the reuptake of serotonin by 5-HT transporter (Willeit *et al.*, 2008) into the presynaptic neuron. Given the role of serotonin transporter in the development of PPD and the fact that fluoxetine-exposure causes SERT occupancy in the litter, the antidepressant activity exhibited by this group is possibly a result of the inhibition of the reuptake of 5-HT at the synapses by fluoxetine. Inhibition of 5-HT levels at the synapses causes increased 5-HT levels at the resulting an upsurge in the swimming behaviour (Cryan *et al.*, 2005; Detke *et al.*, 1996; Page *et al.*, 1999; Rénéric *et al.*, 2001; Slattery *et al.*, 2012 ) in FST.

Desferrioxamine-exposed litter showed high immobility scores similar to the vehicle group but with significantly lower swimming score and significantly higher climbing scores. Desferrioxamine was administered during gestation to induce ID in the dams-litter dyad similar to ID during pregnancy in humans caused by maternal IDA, preterm birth, and gestational complications such as maternal diabetes mellitus, intrauterine growth restriction, maternal smoking, maternal obesity and inflammation (Cusick *et al.*, 2018). The risks associated with ID is greatest in late foetal through infancy where iron needs for growth is high (Cusick *et al.*, 2018) and competes with the requirements for erythropoiesis. During periods of low iron balance in early life, available iron is prioritized to the red blood cells (RBCs) over all other organs, including the brain (Cusick *et al.*, 2018; Georgieff *et al.*, 1995;

Guiang *et al.*, 1997) to prevent IDA. However, studies in human infants (Cusick *et al.*, 2018; Georgieff *et al.*, 1990) and rats (Rao *et al.*, 2003) demonstrate reduced brain iron levels prior to the occurrence of IDA. Late prenatal and neonatal iron deficiency has been linked with altered temperament (Wachs *et al.*, 2005), abnormal recognition memory (Cusick *et al.*, 2018; Geng *et al.*, 2015; Siddappa *et al.*, 2004) and mental and psychomotor deficits in full-term infants (Tamura *et al.*, 2002) as well as abnormal neurological reflexes (Armony-Sivan *et al.*, 2004) and auditory brain-stem response in preterm infants (Amin *et al.*, 2010). An association between foetal iron deficiency and schizophrenia has been reported (Insel *et al.*, 2008). Postnatal ID in human infants has been associated with lower IQ, slower processing speed, Attention Deficit Hyperactivity Disorder (ADHD) as well as deficits in motor, cognitive and behavioural functions (Lahat *et al.*, 2011; Lozoff, B. *et al.*, 2006; Piñero *et al.*, 2000b) which are all correlates of depressive symptoms. While early treatment has proven to improve motor performance, behavioural deficits often persist into adulthood (Cusick *et al.*, 2018; Lozoff *et al.*, 2013). Desferrioxamine-exposed litter demonstrated depressive behaviour in FST which could possibly be due to desferrioxamine-induced ID in the litter following maternal treatment.

Changes in weight is a major neurovegetative symptom of depressive disorders and is very prominent during PPD. During this study, there was no significant variation in the weight of the dams and their litter throughout the period of FST compared to the vehicle. Desferrioxamine-exposed litter which exhibited depressive-like behaviour during FST had significantly higher weights compared to iron and fluoxetine-exposed litter confirming depressive-like behaviour.

The open field test (OFT) was used to assess anxiety related behaviour as well as motor activity of gestational iron supplementation in postpartum Sprague-Dawley rats and their litter. OFT by design tends to inhibit characteristic behaviours such as exploration in rodents

against the unfamiliar properties of a brightly lit and open new test environment (Bailey *et al.*, 2009; File, 1980; Prut *et al.*, 2003). Rodents taken from their home environment and placed in a novel and unfamiliar environment express anxiety and fear, by showing changes in behavioural parameters such as decrease in ambulation and exploration time in the centre of the open field with increased peripheral movement (Bhattacharya, 1994; Bhattacharya *et al.*, 1991). These parameters can be altered by classical anxiolytics as well as anxiogenic agents. OFT represents a valid measure of anxiety-related behaviour in both pharmacological and genetically altered rodents (Choleris *et al.*, 2001; Prut *et al.*, 2003). Generally, agents that cause increased locomotor activity in OFT can as well cause a decrease in the immobility scores in FST and result in false positive outcomes. Compounds such as stimulants, convulsants and anticonvulsants can cause increased locomotor activity and decreased immobility in FST meanwhile they are not antidepressants (Arbabi *et al.*, 2014; Butterweck, 2003; Slattery *et al.*, 2012 ). The open field test (OFT) was therefore used to rule out any likely effect of iron on locomotor activity that can contribute to bias in the FST results. The OFT results for the postpartum dams showed that, there was no significant differences between the iron treatment groups and the vehicle group in terms of the number of line crossings. These findings indicate that iron supplementation during gestation, which was observed to reduce immobility score in FST, did not have any significant effect on locomotor activity. The dams treated with fluoxetine, a classical antidepressant which caused a significant decrease in immobility score in FST also had no significant modification on the locomotor activity in the OFT. Postpartum behavioural assessment of the litter in OFT also showed no significant differences in their locomotor activities. In this present study therefore, the reductions in immobility score following gestational iron and fluoxetine treatments was due specifically to their antidepressant effect and not attributed to locomotor activity. Desferrioxamine which is an iron chelator used for treatment during gestation also had no

effect on the locomotor activity of the dams and their litter following postpartum OFT assessment. In measuring the anxiolytic effects of the treatments in the OFT, latency and the percentage of centre time was employed. Perinatal iron treatment caused a significant decrease in the latency to leave the centre square and increased the percentage of the centre time for both postpartum dams and their litter just like perinatal fluoxetine treatment. The results demonstrate the anxiolytic effect of perinatal iron treatment in the postpartum period. The OFT demonstrated that perinatal iron treatment exhibited anxiolytic effects in mothers and their litter but that perinatal drug treatment caused no motor impairments in the postpartum rats and litter.

A third model, the novelty induced hypophagia (NIH) test, was utilized to further clarify the possible effects of iron. The NIH test is used to assess the emotional state of animals. NIH is another conflict test in which animals are confronted with a choice to approach and consume a palatable meal in a new environment while avoiding the new environment (Dulawa *et al.*, 2005). Unlike some hyponeophagia models that incorporate overnight fasting of the animals, the use of a familiar and highly palatable milk makes food deprivations unnecessary (Merali *et al.*, 2004) in this NIH model. The same parameters were assessed in the home and novel cages to control the effects of drug treatment on appetite. The dependent variables used to assess anxiety-related behaviour in this study were the latency to drink the milk and consumption of the palatable milk within the first five minutes of the test. The latency to consume and consumption measures were taken in the home cage and novel cage for analysis. (Bilkei-Gorzo *et al.*, 2008; Bodnoff *et al.*, 1988; Merali *et al.*, 2004; Santarelli *et al.*, 2003). In the present study, the novel cage used was anxiety-provoking, since the latency to drink was generally increased while the consumption of milk in the first 5 min of the test was decreased in the novel cage relative to the home cage. In the novel cage, two doses of iron 0.8 mg/kg and 8.0 mg/kg reduced the latency unlike for fluoxetine where all three doses

reduced the latency to consume milk in a dose dependent manner relative to control in the dams. Additionally, only 0.8 mg/kg decreased difference scores for latency significantly (home vs novel) relative to control, and an insignificant increase in the first 5 min of milk consumption in the novel environment. All three doses of fluoxetine used for perinatal treatment decreased difference scores for latency significantly (home vs novel) relative to control and only the 10 mg/kg dose caused a significant increase in the first 5 min of milk consumption in the novel environment. Even though the novel cage was anxiogenic, perinatal iron treatment was able to significantly show anxiolytic effects in the novel cage for both postpartum rats and their litter. The NIH paradigm can also be used to assess iron's effects on hedonic processes in addition to anxiety since the intake of palatable fluids, including sucrose and saccharin solutions has been used to measure reward sensitivity (Le Pen *et al.*, 2002; Willner, 1997). These paradigms interpret reduced drinking to reflect anhedonia, which is a core symptom of depression according to the Diagnostic and Statistical Manual of Mental Disorders, fifth Edition (APA, 2013). Perinatal iron treatment was able to reduce anhedonia by increasing the consumption in the new cage compared to the control in both the dams and litter. This clearly reflects the antidepressant effect of effect of iron just like fluoxetine as reported by Dulawa *et al.* (2004). Perinatal iron treatment was able to exert anxiolytic effects in the postpartum period on mothers and litter as well as decrease anhedonia in litter but not mother in NIH.

### 5.1.1 Histology

Human post-mortem brain imaging studies have reported a reduction in the volume of the hippocampus in patients with depression or post-traumatic stress disorder (PTSD) (Sheline *et al.*, 2000). Alterations such as decrease in the volume of the subgenual prefrontal cortex and a decrease in the number of neurons and glia (Drevets, 2000; Duman, 2002; Rajkowska, 2000) in the cerebral cortex of patients with major depressive disorder or bipolar disorder has been

observed. Since the symptoms of depressive episodes occurring at any time is not different from those of PPD, similar changes are likely to be observed in the brains of PPD patients. Neuronal atrophy and neuronal loss in the hippocampus, as well as cerebral cortex, could result from a number of factors including glutamatergic excitoxicity, hyperactivation of the HPA axis, excitoxins, viral or bacterial infections, hypoxia-ischemia, or vulnerability to stress or other insults of genetic background (Duman *et al.*, 1997; Duman *et al.*, 2000) as well as nutritional deficiencies such as ID (Salvador, 2010; Yien *et al.*, 2016). Although acute exposure to ID and any of the above factors alone may not be enough to cause structural and behavioural alterations, the cumulative effects over time could have devastating consequences on neuronal morphology and number. ID has been associated with several structural and functional changes in the brain including but not limited to diminished BDNF expression affecting synaptic remodelling, reduction in dendritic length in pyramidal neurons (Tran *et al.*, 2015), decrease in branching complexity of cortical neurons (Greminger *et al.*, 2014) and eventual loss of neurons (Salvador, 2010; Yien *et al.*, 2016). These changes are similar to those observed in the brain during depressive states (Miguel-Hidalgo *et al.*, 2002). In this study perinatal iron treatment in Sprague-Dawley rats was able to protect the mothers against depressive-like traits in 3 behavioural models of PPD as well as prevent gross alterations in the neuronal architecture of the prefrontal cortex. The maintenance of neuronal architecture through perinatal iron treatment coupled with the role of iron in neurotransmission, myelination and brain energy metabolism could be the reason for the antidepressant-like effects of iron in the postpartum period.

Perinatal iron exposure was able to protect the litter against loss of cell bodies and dendrites in the prefrontal cortex of the brain compared to the vehicle and desferrioxamine treated groups. This seemed to play a role in the positive behavioural changes observed during FST, OFT and NIH unlike the desferrioxamine treated group. Several studies in animal models and

humans indicate that late gestational and early neonatal ID is associated with impaired maturation and function of neurons (Brunette *et al.*, 2010a; Dallman, 1986; Georgieff, 2008 ). The cognitive impairments resulting from ID persist into adulthood in spite of iron treatment, suggesting that ID-induced changes in structure and function of neurons are permanent.

This study has demonstrated that gestational perinatal iron treatment in Sprague-Dawley rats exerted an antidepressant-like effect in the postpartum mothers and their litter as well as maintain the neuronal architecture of mothers and offspring compared to the vehicle and iron deficient (Desferrioxamine) groups.

## 5.2 SUMMARY OF KEY FINDINGS

- ✓ Perinatal Fe treatment did not affect locomotor activity of both dams and litter.
- ✓ Perinatal Fe treatment exerted antidepressant-like effect in both dams and litter after FST mediated possibly through enhancement of 5-HT and NA neurotransmission.
- ✓ Perinatal iron treatment had no significant effect on anhedonia in dams after NIH but significantly decreased it in litter.
- ✓ Perinatal iron treatment increased neuronal density in both dams and litter compared to vehicle and desferrioxamine.

## 5.3 CONCLUSION

Iron supplementation during gestation ameliorates depression in postpartum rats and their litter and prevents neuronal loss.

## LIMITATIONS OF STUDY

- ✓ The study did conduct blood analysis for ferritin and transferrin due to logistical constrains.
- ✓ A microtome was used for the sectioning instead of a vibratome due its absence in the facilities contacted.
- ✓ Due to limited time, neurotransmitter levels could not be measured.

## RECOMMENDATIONS

- ✓ The exact mechanism of iron in ameliorating PPD should be elucidated.
- ✓ Subcortical changes in neuronal morphology and number should be characterized.
- ✓ The antidepressant-like effects of iron in postpartum mothers and their litter should be investigated clinically.
- ✓ The resilience to depression conferred on the litter should be investigated through genetic studies.
- ✓ Changes in spine density and morphology should be characterized using Sholl analysis.
- ✓ Iron status of the dams prior to gestation and after parturition should be determined.

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