Effect of Iron Deficiency in Offspring Rats: An Investigation of Fear and Anxiety-like Behavior Related to the Developing Hippocampus

Jannie Boss Nielsen

Aalborg University

Translational Medicine

Department of Health Science and Technology, Fredrik Bajers Vej 7, 9220 Aalborg Øst, Denmart

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Member:
Jannie Boss Nielsen
Student No: 20135758

Supervisor:
Torben Moos, Professor, Dr. Med, PhD
Aalborg University.

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Dansk Resume

Introduktion: Jernmangel er en udbredt mikro ernæringsforstyrrelse og påvirker omkring 2 milliarder mennesker på verdensplan. Behovet for jern forøges kraftigt under graviditet, fra 0.8 mg/dag i første trimester til > 6 mg/dag i tredje trimester, hvilket gør det svært at opretholde et tilstrækkeligt jern niveau uden brug af jerntilskud. Hippocampus spiller en vigtig rolle i bearbejdelsen af information og adfærd gennem en kompleks interaktion med amygdala og hypothalamus. Igennem oxidativ fosforylering bliver det høje energiforbrug fra hippocampus mødt. Cytochrome c oxidase er et jernholdig terminal enzyme som er involveret i sidste stadie af oxidativ fosforylering. Dette gør enzymet til en kvalificeret markør for neuronal metabolisk aktivitet. **Formål:** Formålet med studiet var at etablere et experimental model med gnaver for at undersøge jernmangel effekten på adfærd og den neurale udvikling i afkom fra rotter og hvorvidt tilskud med jern isomaltoside (Monofer) på to forskellige stadier (gestationsdag 1 og femte postnatale dag) af udviklingen kan forebygge hjerne abnormaliteter. **Metode:** 14 Wistar rotter blev inddelt i fire grupper; Kontrolgruppen (CN), gestationsdag 19 (G19), første postnatale dag (ID) og femte postnatale dag (P5). G19, ID, og P5 modtog alle jernmangel diæt og injektioner med Monofer på de tilsvarende dage. Afkommet blevet testet for frygt og angst-lignende adfærd med open field, elevated zero maze og light/dark boks, hvor udforskning var observeret i 10 minutter. Områder i hippocampus blev farvet for cytochrome c oxidase aktivitet og blev brugt til at understøtte observationerne i adfærdstestene. **Resultater:** I open field, viste stort set alle testene at CN var signifikant bedre sammenlignet med de tre andre grupperinger. Ved dag >28, viste G19 en forøgelse i distance sammenlignet med ID. Elevated zero maze viste en signifikant forøgelse i antallet af stræk mellem G19 og ID. I light/dark boksen viste ID og P5 sig at være mere signifikant end de resterende 2 grupper. Dot blot analyse viste ingen signifikant forskel mellem grupperne. Der blev dog observeret en tendens for CN, G19, og P5 til at have en forøget cytochrome c oxidase aktivitet sammenlignet med ID. **Konklusion:** Vægt og hæmoglobin niveau viste at jerntilskud havde en effekt sammenlignet med ID. Adfærdsstudiet viste svagt at G19 klarede sig bedre sammenlignet med ID. P5 havde en tendens til at være bedre sammenlignet med ID. CN præsterede bedre i open field sammenlignet med ID og G19. Der var ingen signifikante oberservering i de biokemiske analyse.

Abbreviations

ATP	Adenosine triphosphate
Hb	Hemoglobin
BBB	Blood brain barrier
CN	Control group
CNS	Central nervous system
COX	Cytochrome c oxidase
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
EZM	Elevated zero maze
Fe^{2+}	Ferrous
Fe^{3+}	Ferric
ID	Iron deficiency
IDA	Iron deficiency anemia
IR	Infrared
LD	Light/dark box
NDP	Nanozoomer digital pathology
O.D	Optical density
OF	Open field
PFA	Paraformaldehyde
WHO	World Health Organization

Contents

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Jannie Boss Nielsen

Aalborg University

Abstract

Introduction: Iron deficiency is a widespread micro nutritional disorder and affects approximately 2 billion people worldwide. Iron demand increases notably during pregnancy, from 0.8 mg/day in the first trimester to >6 mg/day in the third trimester, thus, making it difficult to maintain an acquired iron level without any iron supplementation. Hippocampus plays an important role in processing information and behavior through a complex interaction with amygdala and hypothalamus. Through oxidative phosphorylation, the high energy requirement from hippocampus is met. Cytochrome c oxidase is an iron-containing terminal enzyme involved in the last step of the oxidative phosphorylation reaction. Thus, making it a quantifiable marker for neuronal metabolic activity. **Aim:** The aim of this study was to establish a rodent experimental model to investigate how iron deficiency will affect behaviour and neurodevelopment in offspring rats and whether supplementation with iron isomaltoside (Monofor) at two various stages (gestation day 19 and postnatal day five) of development can prevent brain abnormalities. **Method**: 14 Wistar rats were divided into four groups; Control group (CN), gestation day 19 (G19), postnatal day one (ID) and postnatal day five (P5). G19, ID and P5 received all iron deficient diet and injection of Monofer at the corresponding days. Offsprings were tested for any fear and anxiety-like behavior with open field, elevated zero maze, and light/dark box, where exploration were assessed for 10 min. Areas in hippocampus were stained for cytochrome c oxidase activity and used to support the findings in the behavior tests. **Results:** In open field, nearly all tests showed CN was significant different compared to all three groups. At day >28 in distanced travelled, G19 showed a significant increase compared to ID. Elevated zero maze revealed a significant increase in number of stretches between G19 and ID. Light/dark box showed that ID and P5 performed better compared to CN and G19. Dot blot assays did not reveal any significant difference between groups, however, there was seen a tendency for CN, G19, and P5 to have an increased cytochrome c oxidase activity compared to ID. **Conclusion:** Growth and hemoglobin showed that iron supplementation had an effect compared to ID. The behavioral study did not revealed any significant difference between the two supplementation groups compared to ID. However, CN had a better performance in open field compared to ID. There were no significant findings in the biochemical assays

1. Introduction

Iron deficiency (ID) is among one of the most common and widespread micro nutritional disorders. The World Health Organization (WHO) estimates that approximately 2 billion people suffers from anemia, where the majority is caused by ID. [1, 2, 3, 4] ID affects a large number of both children and women in the developing countries and is the sole micro nutritional disorder with a significant prevalence in the industrialized countries, thus, making it a public health problem worldwide.[4] During menstrual bleeding and pregnancy, the risks of developing ID and iron deficiency anemia (IDA) increases, thereby making the women in reproductive age the most likely group to develop ID and IDA. [5, 6] Roughly, 30-50% of pregnancies worldwide are affected by ID. [2, 7, 4, 8] The iron demand increases notably during pregnancy, from 0.8 mg/day in the first trimester to >6 mg/day in the third trimester, thus, making it difficult to maintain an acquired iron level without any iron supplementation.[1, 9, 10, 11] The increased iron demand in pregnancy is caused by the expansion of maternal erythrocyte mass and growth and development of the fetus.[12] Several studies show that untreated ID in both human and rodent models have serious and both short term and long term consequences for the fetus if untreated.[13, 1, 14] Some of the consequences of IDA in mothers are fatigue, reduced physical performance, and reduced mental performance. Consequences for the fetus includes low birth weight, increased mortality, increased preterm birth, growth retardation, and delays in cognitive and behavioral responses, including learning deficits and increased fearfulness. [1, 5, 15, 6] Current treatment for pregnant women

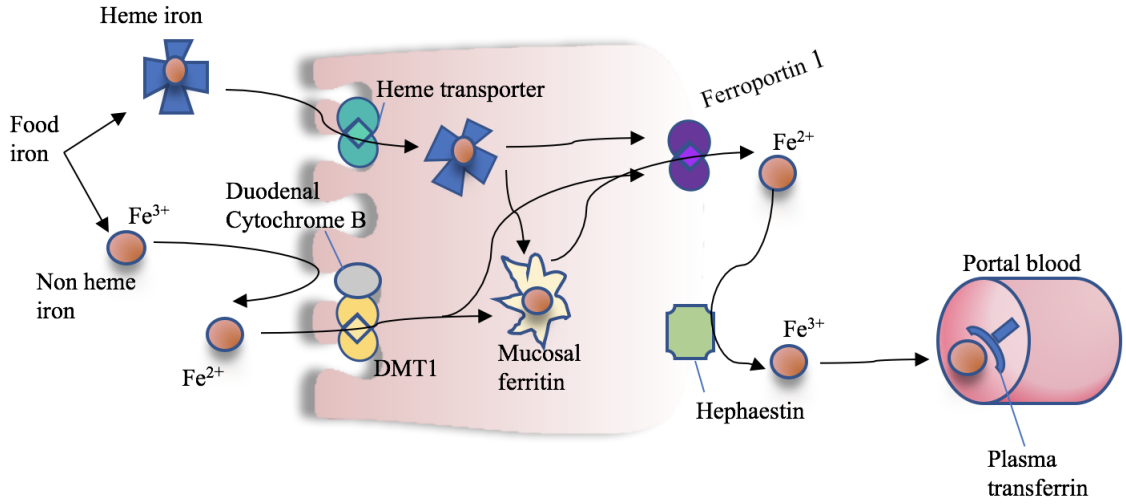

Figure 1: Digestion of food liberate iron as either heme or non heme. The absorption of non heme occurs by converting Fe^3 to Fe^2 by duodenal cytochrome B thus, being absorbed through the divalent metal transporter 1 (DMT1). Then Fe^2 can either be stored og exported to the basolateral membrane and exported out of the cell by ferroportin 1. When exported, Fe^2 is converted by hephaestin to Fe^3 and bound to plasma transferrin for systematic delivery. For Heme iron, the pathway is similar. However, heme is not converted but absorbed through a heme carrier protein 1 transporter, thus, enter a common pathway with non heme iron.

consist of oral iron in first and second trimester. Intravenous iron supplementation can be considered after 14 week's of gestation to those whom are unresponsive to oral iron.[1, 6] However, studies in human and rodent indicate that not all behavioral and neurochemical deficits associated with ID in early life can be corrected by maternal iron supplementation.[16, 17]

1.1. Iron

Iron is an essential and highly important micronutrient for all mammalian cells and is indispensable for almost all living organisms as it works as a cofactor for the most fundamental biochemical processes [9, 18, 19, 20] Human iron is incorporated into proteins as a component of heme (e.g. myoglobin, hemoglobin, cytochrome proteins), iron sulfur clusters (e.g. coenzyme Q_{10}, mitochondrial aconitase, DNA primase), or other functional groups. These iron-containing proteins play, among other things, a role in oxygen transport, nucleic acid replication and repair, deoxyribonucleic acid (DNA) synthesis, cell signalling, and development of central nervous system (CNS) [18, 20, 21, 22] The absorption of iron is low, ranging from 5% to 35% depending on the type of iron and circumstances [23]

1.2. Iron absorption

A tight regulation of iron homeostasis is crucial to avoid both iron overload and ID. As the body has no efficient excretion, the absorption of dietary iron through duodenum and upper jejunum are important. Absorption of dietary iron, found in both heme (10%) and non-heme (ionic, 90%), occur via different mechanisms at the apical surface of duodenal enterocytes.[24] Non heme is often presented in oxidized (fe^3) form, which is not bioavailable and thus, prior to intestinal uptake, must be reduced to Fe^2, as seen in Figure 1. The reduction of Fe^3 is mediated by a ferricreductases such as duodenal cytochrome B, on the apical cell membrane facilitated by ascorbic acid. Once reduced, Fe^2 is absorbed by the enterocytes through divalent metal transporter 1 (DMT1) where it can be used directly for intrinsic cellular metabolic processes, storage, or exported across the basolateral membrane for systemic delivery [23, 21, 24] In contrast to non heme, the absorption of heme by the enterocytes is greatest at the proximal intestine and is mediated by a not fully identified heme carrier protein 1. [24] As heme is internalized by the enterocytes, it is most likely that heme iron is liberated as ferrous iron by heme oxygenase and thus, enter a common pathway with non heme iron. [23, 21, 24] Once iron is inside the intestinal epithelial cell, it can either be used by the cell, stored, or be exported across the basolateral membrane of the enterocyte and released by ferroportin 1 into the circulation. [23, 24] After export of ferrous iron it is converted to ferric iron by a multi-copper oxidase protein called hephaestin and bound to plasma transferrin, which is the main plasma iron carrier.[23, 21, 24]

1.3. Role of iron in the brain

Acquisition of iron by the brain is obtained primarily through transferrin receptors expressed on endothelial cells forming the microvasculature of the brain.[25, 26] However, movement of

iron across the blood brain barrier (BBB) is not well understood. In excess of iron, the BBB, which constitutes an efficient regulator for movement of iron from the plasma pool to the cerebral spinal fluid, plays together with choroids plexus an important role of irons movement in and out of the brain. [25, 26] Presumably, iron from choroids plexus is distributed to glia and neurons for use and storage [26]

Different brain regions are vulnerable to ID in various stages of life. [26] As iron contributes to the normal neurodevelopment through enzymes processes as neurotransmitter synthesis, cell division, neuronal energy metabolism, and myelination these can be disrupted when ID occurs perinatal.[3, 27] Such disruption can be seen in a regionally specific manner, depending on which area of the brain are under rapidly development at the time of ID. As hippocampal development commence prenatally and continues postnatally in both humans and rats and due to its high energy requirement and rapid growth, hippocampus is one of the most vulnerable brain areas. [14, 8] Oxidative phosphorylation is a pathway which ensures the high energy demands of the brain and a disruption in this pathway may lead to metabolic abnormalities, which is known to occur in emotional disorders (65) Perinatal ID decreases the expression of cytochrome c oxidase (COX) [3], which is an iron-containing terminal enzyme involved in the last step of the oxidative phosphorylation reaction, thus, playing an important role in the generation of adenosine triphosphate (ATP). In addition, COX is an incorporated component of cellular oxidative metabolism by neurons and is fundamental for normal cellular function and its activity can thus, represents a quantifiable marker for neuronal metabolic activity, primarily in hippocampus and frontal cortex [18, 3, 27, 28] This makes it possible to investigate whether there is any alteration, influenced by iron, in hippocampus which might be associated with increased fear and anxiety.

1.4. Iron deficiency and Iron deficiency anemia

According to WHO, ID is defined as "*'a condition in which there are no mobilizable iron stores and in which signs of a compromised supply of iron to tissues, including erythron, are noted.*" [29] This occurs because of a long term negative iron balance. The delicate iron balance stabilizes the overall iron pool but can easily be broken due to the limited capability to absorb iron orally. Causes of ID could be due to low bioavailability of iron or, as mentioned, increased iron requirements such as rapid growth, menstruation, pregnancy, or blood loss due to pathologic infections. [24, 30, 29] ID can occur with og without anemia. Anemia is present when iron-deficient erythropoiesis occurs and the concentration of
hemoglobin (Hb) are reduced to <7 mmol/L for women and <8 mmol/L for men. [24, 30, 29] There are some functional changes in the presence of ID (thermoregulation, tiredness, and restless legs syndrom), but the most severe functional deficits occurs with the development of IDA.[23] Some of the adverse complication associated with IDA are functional impairments which affects the cognitive development and immunity mechanisms.[23]

1.5. Consequences of iron deficiency

During early development, timing, severity, and duration of ID are three very important factors when it comes to the ultimate impact on the fetus. [15] Several studies indicate that iron restriction during early periods of growth and development have more severe consequences for the fetus than ID during latter periods of life [2, 31, 32, 15] It is well-known that hippocampus has a role in processing certain forms of memory. However, it has become more acknowledge that hippocampus also plays an important role in processing of information and regulation of behavior. Evidences shows, in a complex interaction with amygdala and hypothalamus, that the ventral subregion of hippocampus in rodents (or anterior hippocampus in primates) are involved in fear, anxiety, and stress responses.[33, 34, 35] The behavioral effects of untreated ID can therefore results in cognitive deficits which account for decreased activity and exploration behavior and increased fear and anxiety-like behavior. [13, 31, 36]

Anxiety and fear are two defensive behavioral responses which enables the organism to avoid or reduce harm, thus ensuring its own survival.[37] In contrast to fear, which is evoked by a discrete and acutely threatening stimulus, anxiety is a generalized response to a internal conflict or unknown threat [37, 38] It has been suggested that underlying mechanisms of fear and anxiety are similar in both humans and animals and that the processes are partially mediated by overlapping of different brain areas. [37] Behavior tests as open-field, elevated zero maze, and light/dark box are widely applied for assessing fear and anxiety-like behavior in rodents. [37, 39] A major contributor to fear reaction is thigmotaxis which is a natural defence mechanism to which rats remain close to vertical surfaces thereby avoiding detection by predators. [40] With use of novel environments e.g the three-behavior tests, a conflict between exploration and fear/anxiety-like behavior are evoked in rodents. [41] Rodents perceive the dark and enclosed regions safer than bright and ex-

posed regions. Thus, the behavior tests are the most acceptable index of anxiety. [39, 41, 40] Decreased anxiety and fear are seen when time spend in an open and bright area is increased. In contrast, an increased fear and anxiety are correlated with a longer start latencies and search towards closed area. [41, 40]

1.6. Translational studies

Investigation of ID consequences in human neurodevelopment is difficult, if not impossible. The use of animal models enable the possibility to study the effect of ID while controlling environmental conditions and to induce ID at various stages of development.[3] Rodent models offer both advantages and limitation when used as an experimental model. A limitation with use of rodents, as a model, are that the neurodevelopment occurs primarily postnatally in rats. Postnatal, the efficient blood-placental-barrier to transfer iron to the fetus is replaced with the gastrointestinal barrier. The absorption of iron through duodenum and upper jejunum are limited, thus the amount of iron reaching the brain is fare less than what is fed to the dams. Since hippocampus is relatively large, matures in the late fetal period, and becomes functional in the early neonatal stages as in humans, rats have been proven to be a useful model. [18] Furthermore, an advantage of using rats to trace fear conditioning is that fear can be acquired rapidly, and it can be measured reliably at a relatively early age [42]. Furthermore, rodents as test animal have shown to produce similar outcomes as those observed in humans, thus, making it more reliable when translating. [31]

1.7. Aim

The present study aimed at establishing a rodent experimental model to investigate how ID will affect behaviour and neurodevelopment in offspring rats and whether supplementation with iron isomaltoside (Monofor) at two various stages (gestation day 19 and postnatal day five) of development can prevent brain abnormalities. The experiment seeks to investigate whether there is any coherence between behaviour and neurologic changes related to hippocampus. To fulfil the aim, behavior test as open field, light/dark box, and elevated zero maze are applied to investigate fear and anxiety-like behavior in offsprings. Furthermore, postmortem analysis as histochemistry and dot blot are made with focus on hippocampus.

2. Methods

2.1. Animal Studies

The present study was approved by the Danish Experimental Animal Inspectorate under the Ministry of Food and Agriculture (permission no. 2013-15-2934-00776) and conducted at the animal facility under the Department of Health Science and Technology (HST) Aalborg University (Fredrik bajers vej 7H, Aalborg, Denmark). The investigator has a certificate within Laboratory Animal Science (Reg. no: B100/32) (APPENDIX A). 26 six-weeks old female Wistar rats were purchased from Janvier (Le Genest-Saint-Isle, France) and housed two and two in individually ventilated cages under specific-pathogen-free classification. The rats had access to food and water *ad lib* and were maintained on a 12-h light:dark (06-18:18-06) cycle with room temperature at 19,5 ± 0,5 °C and humidity at 55%.

Study design: Figure 2 illustrates a timeline of the experiment. 7 days after arrival, ID groups were switched to iron deficient diet with an iron content of 5.2 mg/kg (C038, Altomin, Germany), while the control group remained on normal diet containing 158 mg/kg iron (C1000, Altromin, Germany). Male rats were maintained on a normal diet through the entire trial. Conceptions were carried out eight weeks after diet switch and one male was housed with two dams for two days. Three days before partus, the dams were separated to individual cages. Only 14 female rats were successfully fertilized during this experiments.

Four groups were included in this study and are presented in Figure 3. Control group (CN) received salt water injection at gestation day 19, postnatal day one, and postnatal day five. Gestation day 19 (G19) and postnatal day 5 (P5) represent two groups with iron supplementation, Monofer (Pharmacosmos A/S, Holbaek, Denmark; batch no. 155962-10). A group representing a 'no supplementation' group (ID) received $1/2''$ dose of Monofer at postanal day one. A group receiving $1/4''$ dose of Monofer was tested. However, all pups (n = 11) died before P15 and as seen in Figure 4, the difference between CN and the offspring treated with $1/4''$ dose, are distinctly in growth. All dams received injection subcutane.

Growth and hematology

Offsprings were each weighed at P1, P5, P10, P15, P20, P25, and P29, as seen in Figure 2. Blood samples were collected right after they were sacrificed and Hb was quickly measured.

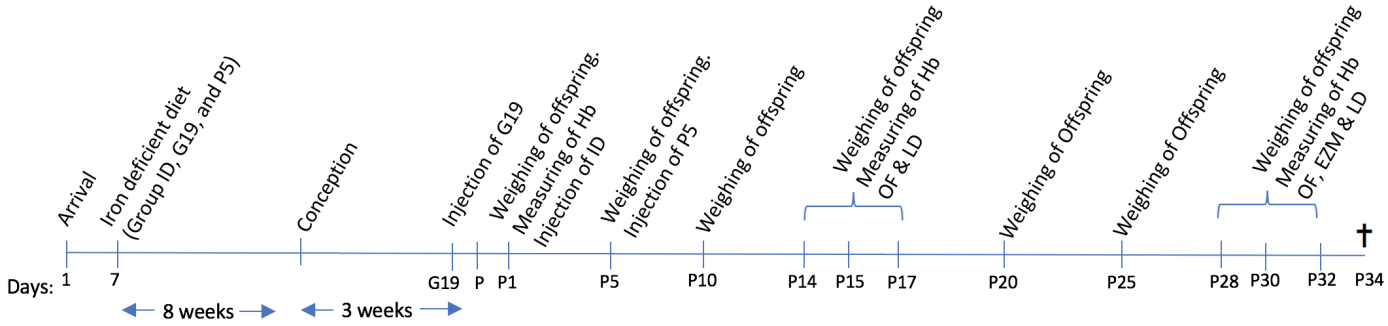

Figure 2: Schematic time line of the experiment. Groups; gestation day 19 (G19), 'no-supplementation (ID), and postnatal day five (P5)
P = parturition, Hb = hemoglobin, OF = open field, EZM = elevated zero maze, LD = light/dark box.

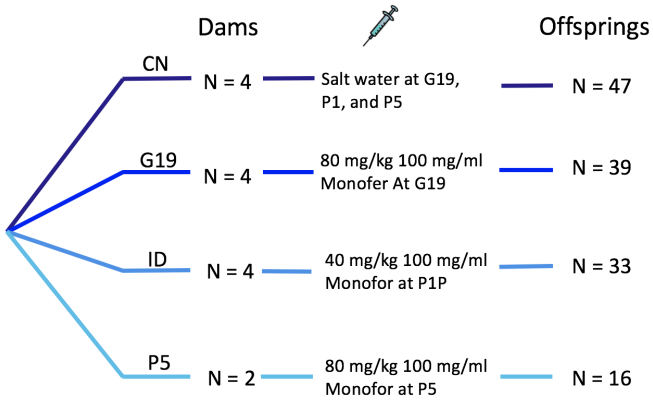

Figure 3: The four groups with belonging color codes. Control group (CN) received salt water injection at gestation day 19, postnatal day one, and postnatal day five to correspond to the supplementation groups, respectively. Groups: Control (CN), Gestation day 19 (G19), 'no supplementation' (ID), and postnatal day five (P5). Number of dams in each group and total number of offsprings in each group are presented. All dams received subcutaneous injection.

Figure 4: Picture of a control offspring and a offspring receiving 1/4″ dose of Monofer. It should be noticed, that the control was 15 days old whereas the poor offspring was 13 days old. Yet, the difference is notable.

2.2. *Behavior assessment*

All behavior tests were supervised using an infrared camera (Noldus, Wageningen, The Netherlands) mounted as an overhead camera attached to a computer with Noldus media recorder (version 4). The videos were blinded of an external investigator before analysed. For open field, videos were analysed with Behavior Cloud [43]. Videos from elevated zero maze and light/dark box were manually analysed.

Open field: The apparatus consisted of a 40*40*40 cm activity box (Noldus, Wageningen, The Netherlands) divided in a square and nine equal quadrants in Behavior Cloud, as seen in Figure 5. Offsprings were tested between day 14-18 (n = 45) and 28-32 (n = 37). offspring was placed in the center of open field and exploration was assessed for 10 min. After end trial, offsprings were moved to a new cage and the apparatus was cleaned with 70% ethanol immediately after. Seven offsprings were excluded as Behavior Cloud could not analysis the videos. All testing were performed between 8:00-12:00 h. (For a thorough protocol, see appendix B) The experimental set-up is seen in Figure 6.

Variables measured were:

- Total distance travelled (cm)
- Total center time (seconds)
- Center entered: (Number of entries in the center region in proportion to the eight other zones measured in percent (%)

Elevated zero maze: The apparatus was constructed of black acrylic in an annular track, 5 cm wide, 60 cm in diameter, and elevated 20 cm above the floor. (Stoelting, Wood Dale, IL, United States of America). The maze was divided in four equal quadrants with two opposing open quadrants and two opposing closed quadrants with dark grey outer walls and inner

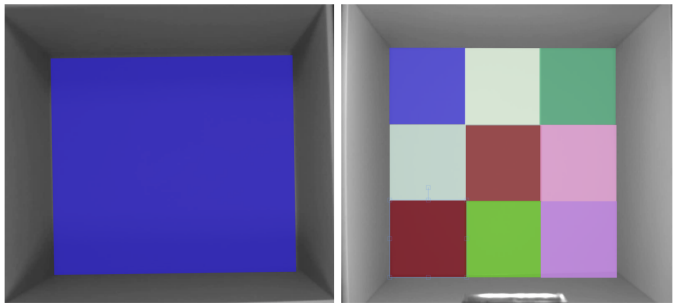

Figure 5: Open field activity box divided in a square and nine equal quadrants in Behavior Cloud.

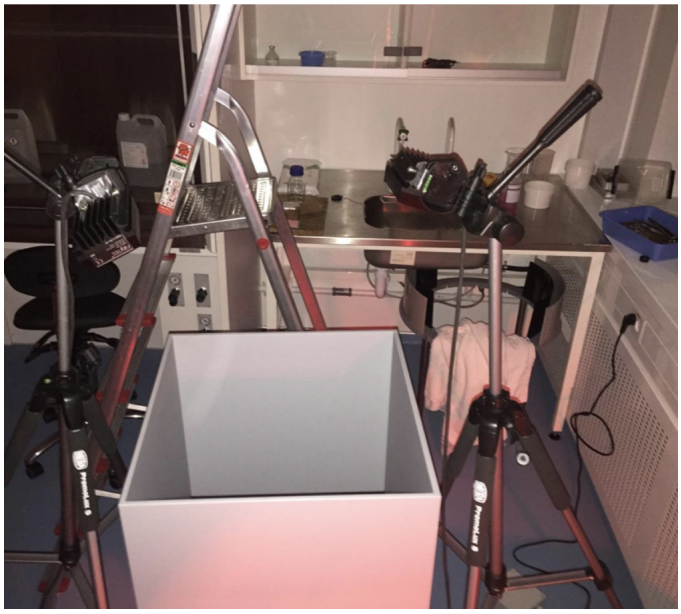

Figure 6: Experimental set-up of open field.

infrared (IR) translucent black walls, 15 cm in height. A dimly lit halogen lamp was placed in the center of the maze providing approximately 100 lux in each open quadrants. Offsprings were tested between day 28-32 (n = 30). Offsprings were additionally tested between day 14-18. However, due to their age, they were not able to stay on the maze, resulting in removal of this activity. Offsprings were placed in the center region in one of the open quadrants facing a closed quadrant and exploration was assessed for 10 min. After end trial, offsprings were moved to a new cage and the apparatus was cleaned with 70% ethanol immediately after. Two offsprings were excluded because they were unable to stay on the maze. All testing were performed between 09:00-14:00 h. (For a thorough protocol see appendix C) The experimental set-up is seen in Figure 7.
Variables measured were:

- *Transition:*Number of transition between the two closed quadrants.
- *Open area:* Duration of time spent in the open quadrants. (seconds)
- *Stretches:* Frequency of stretches (where at least a part of the head is outside the runway, but all four paws is still on the runway)
- *Latency:* Latency from released to enter the closed quadrants (seconds)

Figure 7: Experimental set-up of elevated zero maze

Light/dark box: The apparatus consisted of a 40*20*20 cm open compartment and a 20*19*25,5 cm enclosed compartment united with a 5*5 cm door, all made of IR translucent black (Noldus, Wageningen, The Netherlands). The open compartment was illuminated with 270-320 lux. Offsprings were tested between day 14-18 (n = 47) and 28-32 (n = 38). Testing began with placing the offspring gently in the light side of the box facing away from the door and exploration was assessed for 10 min. After end trial, offspring was moved to a new cage and the apparatus was cleaned with 70% ethanol immediately after. Four offsprings were excluded due to poor health and one was excluded because the light burst. All testing were performed between 8:00-13:00 h. (For a thorough protocol see appendix D). The experimental set-up is seen in Figure 8.
Variables measured were:

- *Transition:* Duration of time spent in the light chamber (seconds)
- *Transition:* Number of full body transition between the two chambers

- *Stretches:* Frequency of stretches (where at least a part of the head is outside the dark chamber, but not any of the paws)
- *Latency:* Latency from released in the light chamber to enter the dark chamber (seconds)

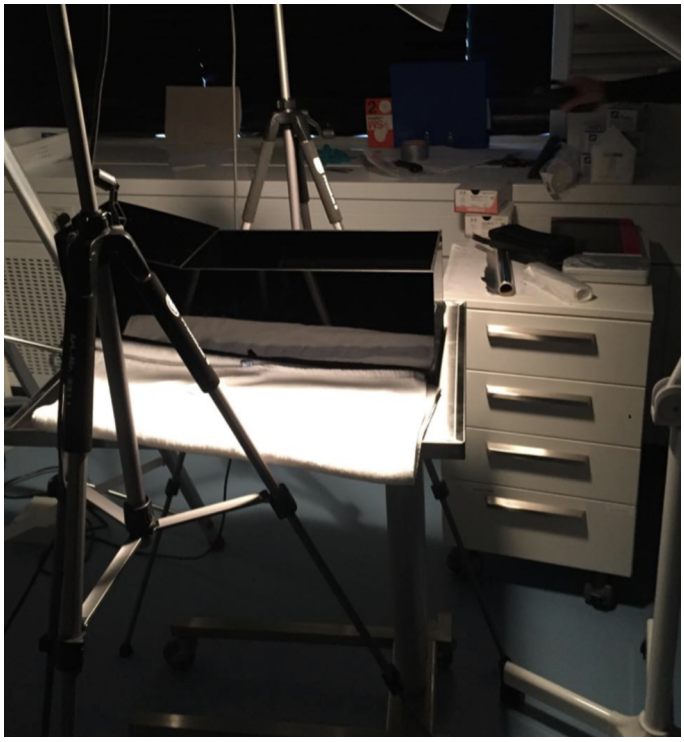

Figure 8: Experimental set-up of light/dark box.

2.3. Biochemical assays

Tissue preparation: A total of 10 and 14 offsprings at P14-18, and a total of 18 and 18 offsprings at day >28 were used for COX histochemistry and COX dot blot, respectively. For COX histochemistry the offsprings were deeply anesthetized subcutaneously with Hypnorm/Dormicum (Fentanyl/Fluanisone mixed with Midazolam and sterile water in ratio of 1:1:2) (0,1 ml/10 g) and perfused transcardially with KPBS followed by 4% paraformaldehyde (PFA) in 0.1 M PBS pH 7.4. Brain was removed and postfixed by immersion in 4% PFA for 24 h at 4 °C. After 24 h PFA was removed and the brain was stored in KBPS with 0.01% sodium azide. (For a thorough protocol, see appendix E) Three days prior to use, they were cryoprotected in 30% sucrose at 4 °C. [8]. For COX dot blot the offsprings were anesthetized with isoflurane and decapitation. Brain was quickly removed. Hippocampus and cerebellum were removed and immediately after put on ice and stored at -140 °C until use. (For a thorough protocol, see appendix F)

Histochemistry: Coronal sections were made by embedding the brain in Tissue-Tek (Sakura, Tokyo, Japan) and cut at 40-μm-thickness on a freezing cryostat and stored in antifreeze at -20 °C until use. (For a thorough protocol for histochemistry, see Appendix G). Briefly, coronal sections were washed three times for 5 mins in 0.1 M phosphate buffer and incubated with 2 ml of 0.1 M phosphate buffer, pH 7.4, containing 25 mg 3,3-diaminobenzidine (DAB; Sigma-Aldrich, st. Louis), 10 mg cytochrome c, (from equine heart; Cas: 9007-43-6; Sigma-Aldrich, st. Louis), and 2 g sucrose per 50 ml solution. Incubation was carried out at 36 ± 1 °C for 8 h in darkness. Sections were after incubation wash three times for 5 min in 0.1 M phosphate buffer and mounted on gelatin-coated slides. Hereafter, slides were air dried and coverslipped. A sample from each group at each time point (n =7) were used for negative control and was carried out with same procedure except cytochrome c was not added to the incubation buffer. Negative controls are seen in Appendix H. *This process has been optimized several times to find the best staining for the coronal sections. Time intervals of 3, 6, 8, 10, 12, and 16 h were tested. Various amount of incubation buffer and various amount of DAB were tested. Hydrogen peroxide was tried to be added to remove any endogenous substances, however this inactivated the enzyme. 9 coronal sections in this study did not stain or was poor stained. The same unstained 9 coronal sections were stained an additional time, but with same results. As the same sections were unstained or poor stained they were excluded, leaving 16 stained coronal sections for analysis.*

Dot blot: Extraction of proteins from hippocampus and cerebellum were carried out by homogenization 15 times while stored on ice. The mixture was incubated for 10 minutes on ice. The samples were centrifuged for 10 min on 10.000 G at 4 °C. Hereafter the pellet was saved in the freezer at -20 C, while the supernatant was stored in the freezer at -80 °C until use. The sample was diluted 1:1 with lysis buffer (N-Per; cat. No.: 87792 with one tablet protease inhibitor cat. No.:11836170001 pr 10 ml). Grid was drawn on a nitrocellulose transfer membrane (Whatman;Dassel, Germany; Ref.no: 10401180) to indicate the region of blotting. With a narrow-mouth pipette tip, 2 μl of sample was spotted on the nitrocellulose transfer membrane in the center of the grid. The sample was applied slowly to minimize the area, which the solution penetrates. Three dots were made for each sample. The membrane was air dried before it was incubated in TBST with 5% skin milk for 0.5-1 h.

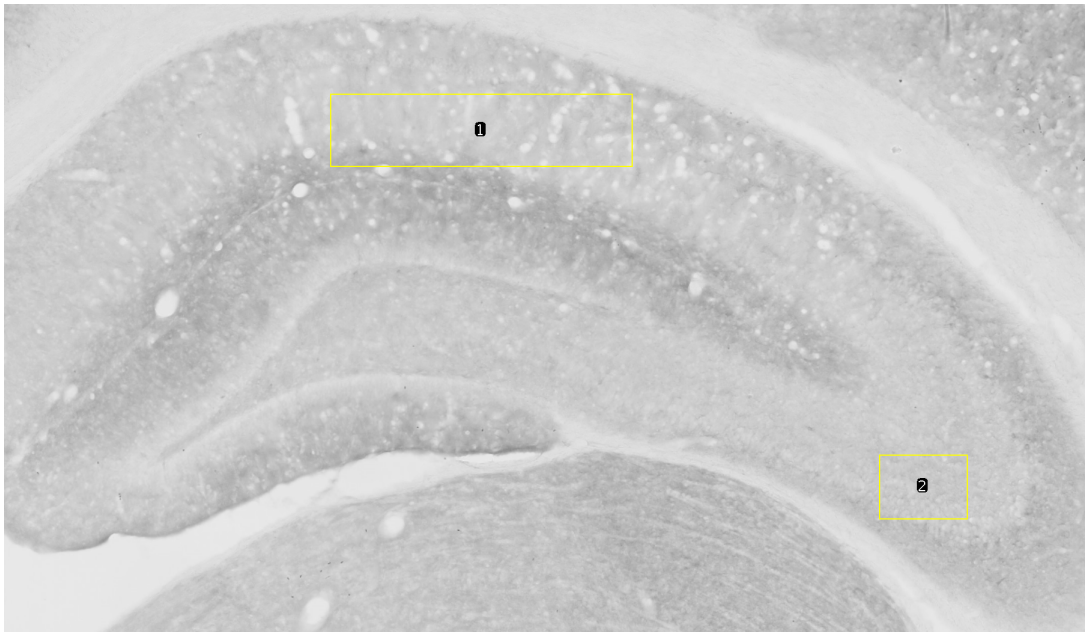

Figure 9: Image illustrating the chosen area of hippocampus. CA1 is marked with 1 and CA3 is marked with 2

After incubation, the membrane was wash three times for 5 min with 0.1 M phosphate buffer. The membrane was incubated in 36 ± 1 °Cwith for 5 h in darkness with 30 ml incubation buffer consisting of 0.1 M phosphate buffer, pH 7.4, containing 25 mg 3,3-diaminobenzidine (DAB; Sigma-Aldrich, st. Louis) 10 mg cytochrome c (from equine heart; Cas: 9007-43-6; Sigma) per 50 ml. The membrane was wash three times for 5 min with 0.1 M phosphate buffer and stored in the refrigerator . (For a thorough protocol of dot blot, see appendix I). A sample from each offspring were used as negative control and was carried out with same procedure except cytochrome c was not added to the incubation buffer. Negative controls are seen in Appendix H. Positive controls from cerebellum was used, which are seen in Appendix H. *This process has been optimized to achieve the best results. Time intervals of 3, 4, 5, and 8 h were tested. Different pipette methods were tested to make the best dot. Incubation with and without TBST with 5% skin milk were tested. Humid and dried nitrocellulose transfer membrane were also tested to see, which presented the best dot. Standards which should have been used to measure the amount of COX was prepared. However, this process was excluded.*

Quantification of histochemistry and dot blot: Images from coronal sections were acquired using a Nanozoomer digital pathology (NDP) scanner (Hamamatzu) and Nanozoomer digital pathology.view (version 2.5.88). Hippocampal areas (CA1 and CA3) were located according to the mouse brain atlas by Franklin & Paxinos (2008). For optical density analysis all images were saved as TIF files and imported into NIH Image J. (version 2.0) Prior to analysis, optical density calibration was made in NIH Image J. A step-tablet was imported and 19 measurement were made and calibrated according to the following website `https://imagej.nih.gov/ij/docs/examples/calibration/index.html` After calibration images were converted to 8-bit grayscale (Image>type>8-bit). Once converted into grayscale, one of the sides of hippocampus was chosen and CA1 and CA3 were located and measured, as seen in Figure 9. Mean gray value was measured which correspond to an average gray value which is the sum of the gray values of all pixels in the selected area divided by the number of pixels. The value will be reported in calibrated units; optical density (O.D). [44]. Background subtraction was made by measuring background of the image and minus the mean gray value for each sample. Images from dot blot was saved as a JPG file and converted to a TIFF file. The same measuring procedure as for histochemistry was used. Dot blot image was converted to a 8-bit gray scale and mean gray value for each dots were measured, as seen in Figure 10. The mean was found for three dots within each sample. Background subtraction was made by measuring the background.

2.4. Statistical analysis

A two-way Analysis of Variance (ANOVA) with Tukeys multiple comparison post hoc test was performed using GraphPad Prism (version 6.0) to analysis growth, Hb, elevated zero maze, and light/dark box. A one-way ANOVA ordinary with

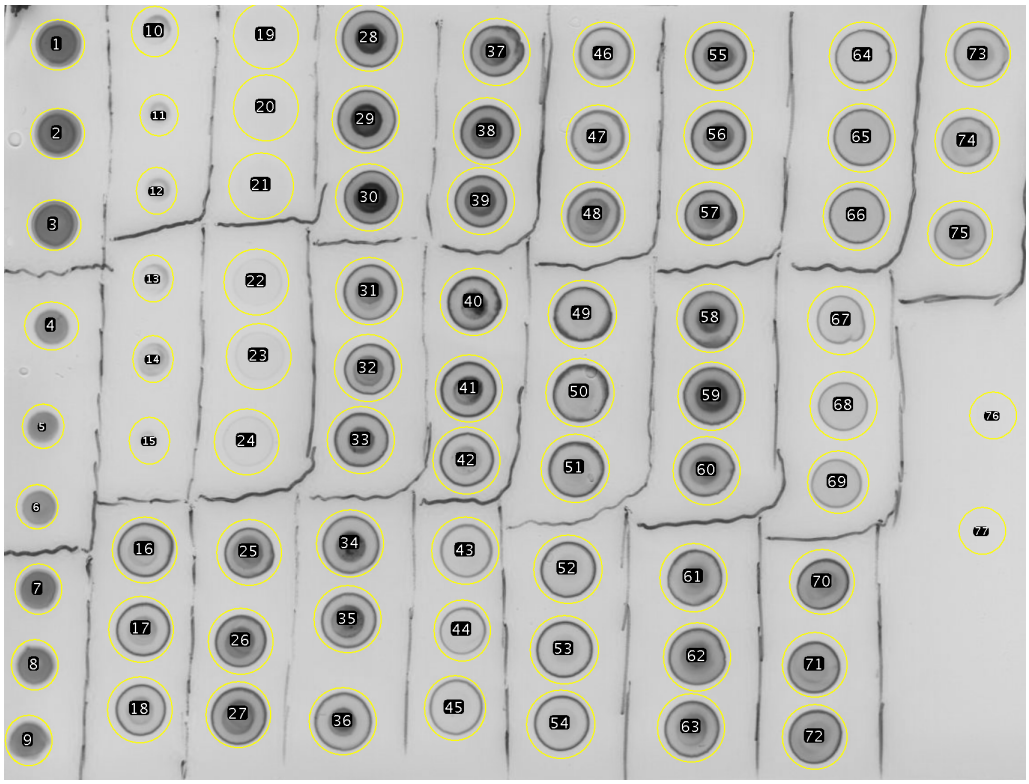

Figure 10: Image illustrates how dot blot was measured. 1-15 illustrate standards which should have been used to measure the amount of cytochrome c oxidase activity in the brain, but were excluded. 16-75 illustrate dots from offsprings. 76-77 are dots made for background subtraction.

a Tukeys multiple comparison post hoc test was performed to analysis dot blot, histochemistry, and open field. Values of $p < 0.05$ were considered statistically significant. All data are presented as mean ± standard error of the mean (SEM)

3. Results

In this study, Monofer was tested at two various stages (G19 and P5) to investigate whether consequences as fear and anxiety-like behavior could be corrected compared to CN. A ID group received $1/2''$ dose of Monofer and should be regarded as the sick group. Three behavior tests; open field, elevated zero maze, and light/dark box were used to test if any fear and anxiety-like behavior appeared. As these behaviors are related to amygdala-hippocampus, biochemical assays as histochemistry and dot blot were used to support the findings.

3.1. Growth and hematology

Offsprings were weighted at day 1, 5, 10, 15, 20, 25, and 29, as illustrated in Figure 11.A, to follow their growth. A two-way ANOVA with Tukeys multiple comparisons post hoc test revealed no significant differences until day 20. At day 20, there was seen a significant increase of weight between G19 and ID ($p< 0.05$) and a significant increase between CN and ID ($p< 0.05$)). At day 25, there was revealed a significant increase between CN and G19 ($p< 0.01$), G19 and ID ($p< 0.0001$), CN and ID ($p< 0.0001$), CN and P5 ($p< 0.01$), and P5 and ID ($p< 0.01$). At day 29, there was revealed a significant increase between CN and G19 ($p< 0.0001$), G19 and ID ($p< 0.001$), CN and ID ($p< 0.0001$), CN and P5 ($p< 0.001$), and between P5 and ID ($p< 0.0001$). This could indicate that iron supplementation has an effect on weight compared to ID. As there is no significant difference between G19 and P5, iron supplementation at both time points can rectify weight compared to ID. However, as a significant difference between G19 and ID occur at day 20, this could indicate that injection on gestation day 19 has an earlier effect.

Blood samples were collected between day 1-3, 14-18, and > 28, as illustrated in Figure 11.B. A two-way ANOVA with a post hoc test showed a significant elevation of Hb between CN and G19, ID, and P5 at all three time points ($p< 0.0001$), except day 14-18, where p-value was $p< 0.05$ between CN and P5. At day 14-18 and >28 there were seen a significant elevation of Hb between G19 and ID ($p< 0.01$ and $p< 0.05$, respectively).

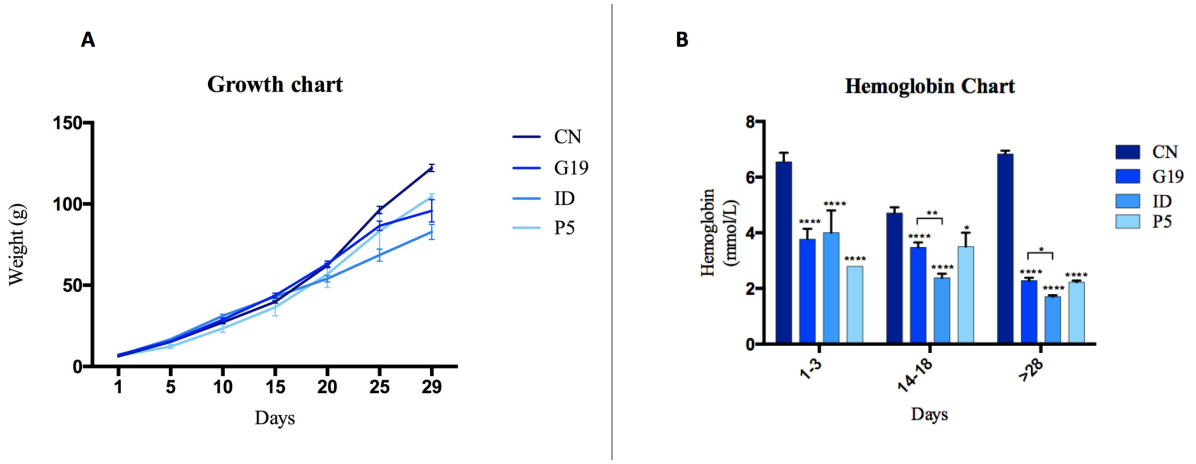

Figure 11: **A:** Growth chart of offsprings from day 1-29. There was no significant differences between the four groups until day 20. **B:** Overview of hemoglobin concentration (mmol/L) measured in the four different groups of offsprings at different time points (days). All groups were significant difference compared to the control groups (CN). A significant difference at day 14-18 between G19 and ID^{**} were observed. At day >28 there was revealed a significant difference between G19 and ID^{*}. Groups: Control (CN), gestation day 19 (G19), 'no supplementation' (ID), postnatal day five (P5). Data is presented as mean ± Standard Error of Mean.
p<0.05*, p<0.01**, p<0.001***, p<0.0001****

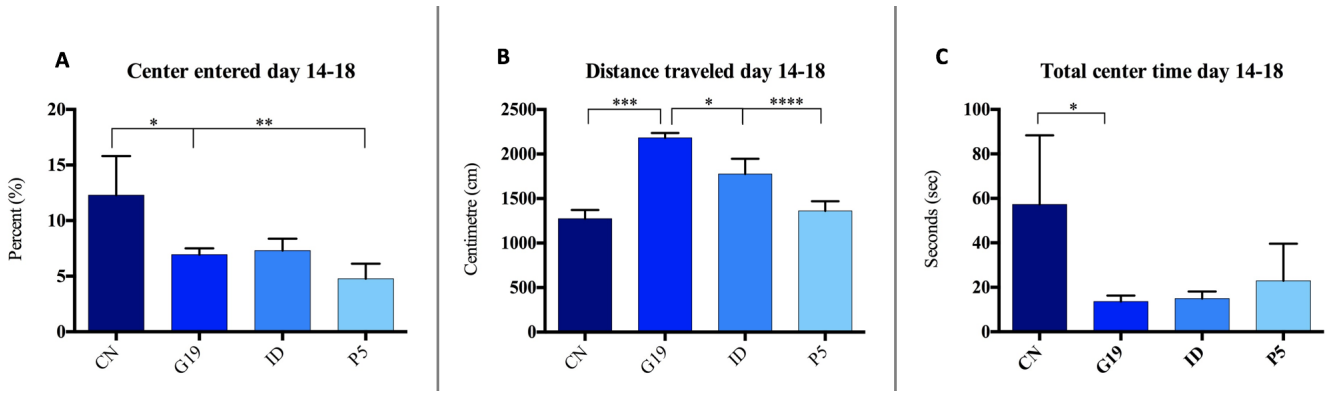

Figure 12: **A:** Center entered revealed a significant difference between CN and $G19^{*}$ and CN and $P5^{**}$. **B:** Distance showed a significant difference between G19 and CN^{***} , G19 and ID^{*}, and G19 and $P5^{****}$. **C:** In total center time there was a significant difference between CN and $G19^{*}$. Number for offsprings: CN = 4, G19 = 17, ID =9, P5 = 6. Groups: Control (CN), gestation day 19 (G19), 'no supplementation' (ID), postnatal day five (P5). Data is presented as mean ± Standard Error of Mean.
p<0.05*, p<0.01**, p<0.001***, p<0.0001****

Thus, showing that iron supplementation at G19 elevates Hb compared to ID. A tendency of elevated Hb was seen between P5 and ID on both days. However, this is not statistical significant.

3.2. Behavior assessment

Open field activity performed at day 14-18 and > 28

Center entered, distance travelled, and total center time were measured for each offspring. As illustrated in Figure 12.A, there was revealed a significant increase in percent of times, the center was entered between CN and G19 (p< 0.05) and CN and P5 (p< 0.01). This reveal that G19 and P5 have a higher degree of thigmotaxis compared to CN. Figure 12.B revealed an increase in distance between G19 and CN (p< 0.001), G19 and ID (p< 0.05), and G19 and P5 (p< 0.001). In total center time, as illustrated in Figure 12.C, only one significant difference were found between CN and G19 (p< 0.05). There is seen a tendency to which P5 has a higher total center time compared with ID. Yet, this is not significant.

Same tests were performed at day >28. As seen in Figure 13.A there was a significant increase between CN and G19 (p< 0.05) and CN and ID (p< 0.05). Additionally, a tendency showed an increase between P5 and ID and a slightly increase between G19 and ID. Figure 13.B illustrates a significant increase between CN and ID (p< 0.01) and G19 and ID (p< 0.01). Further-

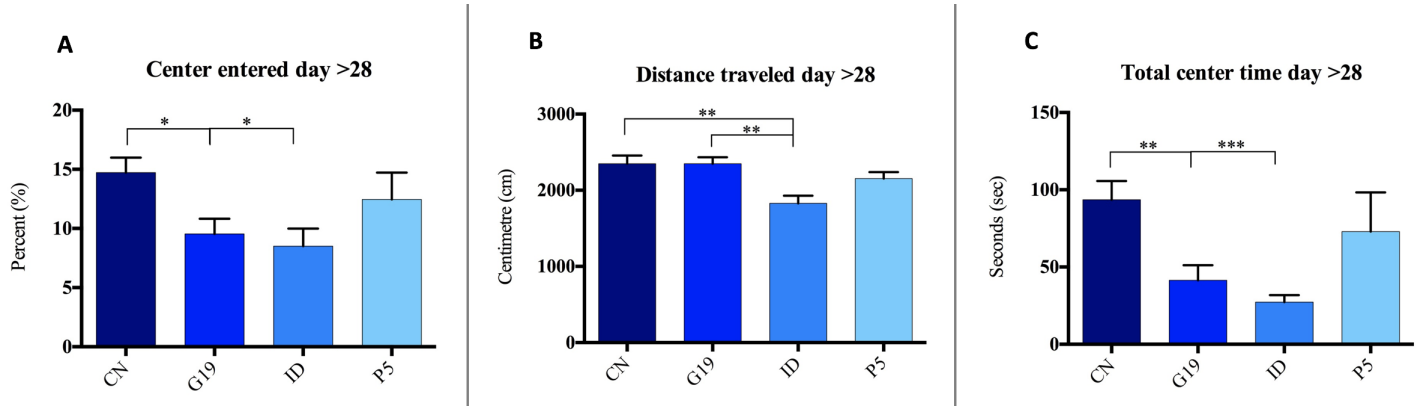

Figure 13: **A:** Center entered revealed a significant difference between CN and *ID*** and G19 and *ID*** . **B:** Distance showed a significant difference between CN and *G19** and CN and *ID**. **C:** In total center time there was a significant difference between CN and *G19*** and CN and *ID***** . Number of offsprings: CN=11, G19=12, ID=9, and P5=4. Groups: Control (CN), gestation day 19 (G19) 'no supplementation' (ID), postnatal day five (P5). Data is presented as mean ± Standard Error of Mean.
$p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****

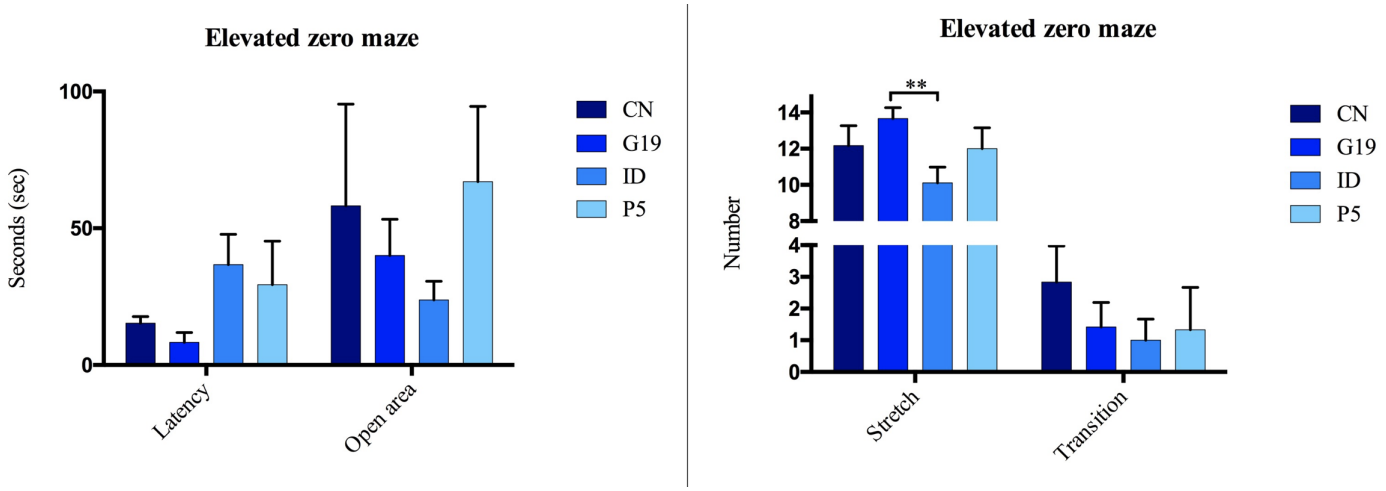

Figure 14: **A:** Center entered revealed a significant difference between CN and *ID*** and G19 and *ID*** . **B:** Distance showed a significant difference between CN and *G19** and CN and *ID**. **C:** In total center time there was a significant difference between CN and *G19*** and CN and *ID***** . Number of offsprings: CN = 11, G19 =12, ID = 9, and P5 = 4. Groups: Control (CN), gestation day 19 (G19), 'no supplementation' (ID), postnatal day five (P5). Data is presented as mean ± Standard Error of Mean.
$p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****

more, P5 had an increased distance tendency compared with ID, thus, indicating all three groups are more active compared to ID. In total time, CN had a significant increase of total center time compared to G19 ($p< 0.01$) and ID ($p< 0.001$)). This revealed that CN performed better than all three supplementation groups. However, both G19 and P5 showed tendency to performed better than ID in all three tests.

Elevated zero maze performed on offsprings at day > 28

As seen in Figure 14, there was found no significant difference between all four groups in latency, time spend in open area, and number of transition. However, in number of stretches there was found a significant increase between G19 and ID ($p< 0.01$). This could indicate that G19 have more exploration compared ID. Additionally, there was seen a tendency that both CN and P5 had a more exploration nature compared to ID. In both open area and number of transition, there are seen a tendency for CN, G19, and P5 to have a decreased fear and anxiety-like behavior compared to ID.

Light/dark activity performed at offsprings at day 14-18 and > 28

Same variables as for elevated zero maze were supervised in the light/dark box at day 14-18 and day <28. As seen in Figure

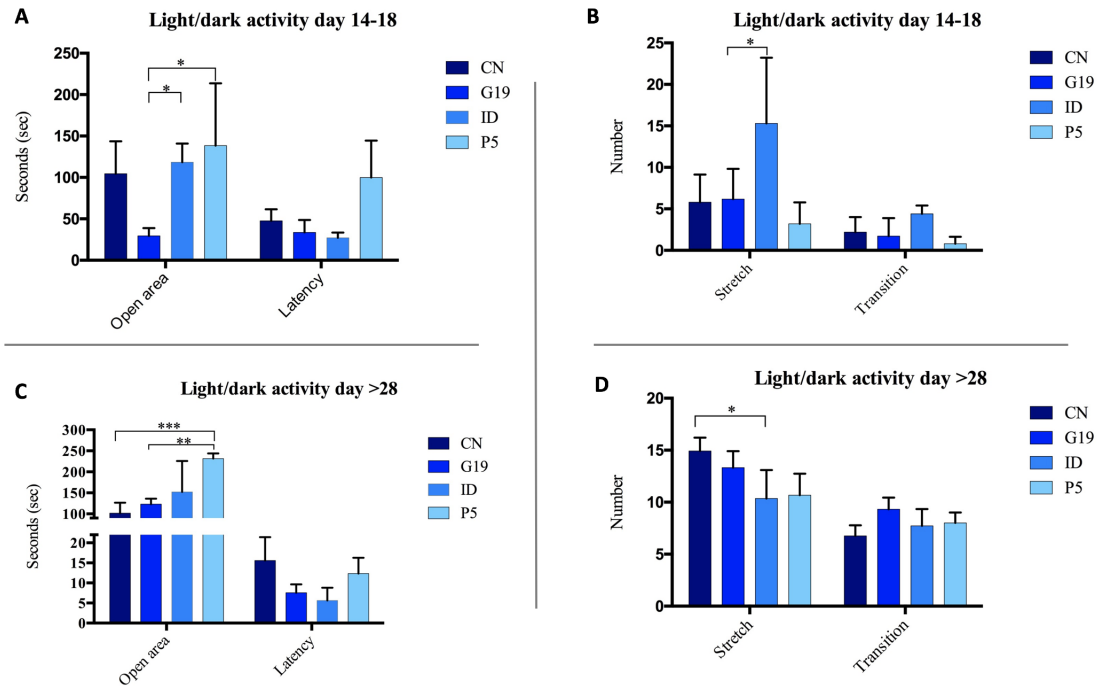

Figure 15: textbfA: A significant difference was revealed in open area between ID and $G19*$ and P5 and $G19^{**}$. **B:** In number of stretches, there was a significant difference between ID and $G19^{*}$. **C:** In open area there was found a significant difference between P5 and CN^{***} and P5 and $G19^{**}$. There was no significant difference between the groups in latency at day > 28. **D:** At day > 28, there was a significant increase in number of stretches between CN and $ID*$,but no significant difference in number of transition. Number of offsprings at day 14-18 CN=10, G19=22, ID=10, P5=5. Number of offsprings at day > 28 CN=12, G19=12, ID=11, P5=3. Groups: Control (CN), gestation day 19 (G19), 'no supplementation' (ID), postnatal day five (P5) Data is presented as mean ± Standard Error of Mean. $p<0.05*$, $p<0.01**$, $p<0.001***$, $p<0.0001****$

14.A-B, there was found no significant difference in both transition and latency. A significant increase of number of stretches was found between ID and G19 and ($p< 0.05$). In time spend in open area, there was found a significant increase between both ID and P5 compared to G19 ($p< 0.05$). However, at this time it was observed that both ID and P5 pups were more bewildered and have more difficulty to orientate in the light/dark box.

As seen in figure 14.C-D, there was no significant difference in transition and latency at day >28. A significant increase in number of stretches between CN and ID ($p< 0.05$) were found. In open area there was found a significant increase between P5 and G19 ($p< 0.05$) and ID and G19 ($p< 0.05$).

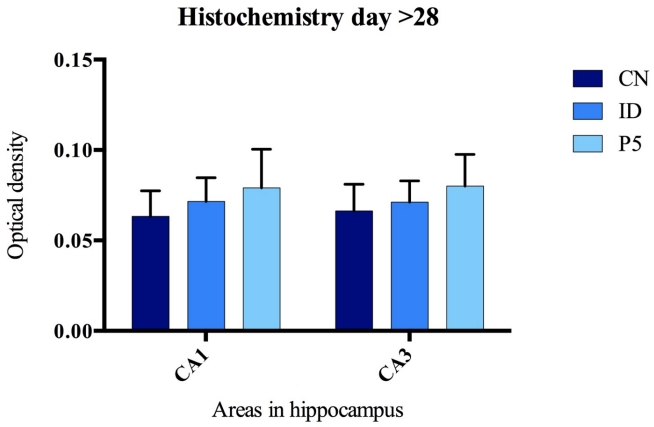

Figure 16: Histochemistry assay was performed to support the findings from the behavior test. Two areas in hippocampus, CA1 and CA3, were measured. However, there was found no statistic difference between the three groups. Number of offsprings: CN=3, ID=6, P5=3. Groups: Control (CN), gestation day 19 (G19), 'no supplementation' (ID), postnatal day five (P5).
Data is presented as mean ± Standard Error of Mean.

3.3. Biochemical assays

As behavior can be influenced by many external factors, biochemical assays were made to support the findings. Histochemistry and dot blot were used in this study and both were used to quantify the activity of COX between groups.

Histochemistry

Histochemistry assays were performed at day 14-18 and >28. However, as many of the coronal sections were unstained or poor stained, which made it impossible for the NDP scanner ti scan them, thus, sections from day 14-18 were excluded. Addi-

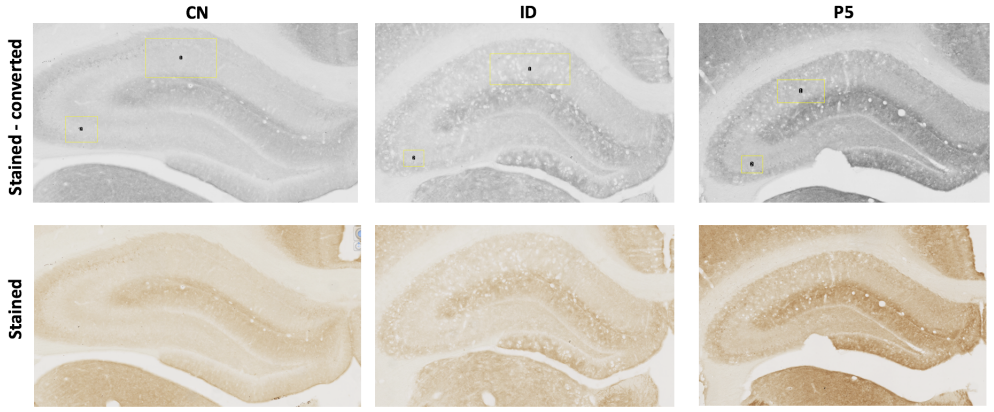

Figure 17: Illustration of coronal sections from each group. Top panel shows they those converted to 8-bits grayscale. Bottom panel shows those before they are converted. Groups: Control (CN), gestation day 19 (G19) 'no supplementation' (ID), postnatal day five (P5)

tionally, group G19 was excluded at day >28 due to unsuccessful staining. As seen in Figure 18, both areas of hippocampus (CA1 and CA3) revealed no statistical difference between the groups. Figure 17 illustrates the scanned section before they were converted to 8-bit grayscale and after they were converted to 8-bit grayscale. One sample from each group at day >28 are shown.

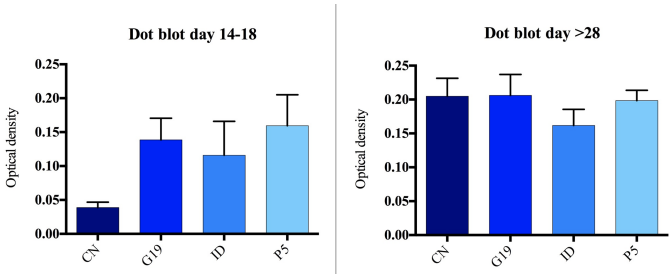

Figure 18: Dot blot assay from day 14-18 and >28. There was found no significant difference between the groups at the two time points. However, there was seen a tendency, that G19 and P5 had an increased optical density compared to ID at day 14-18, and CN, G19, and P5 had an increased optical density compared to ID at day >28. Number of offsprings at day 14-18: CN = 5, G19 =5, ID = 4, and P5 =2. Number of offsprings at day >28: CN = 10, G19 =5, ID=8, and P5 =3. Groups: Control (CN), gestation day 19 (G19) 'no supplementation' (ID), postnatal day five (P5) Data is presented as mean ± Standard Error of Mean.

Dot blot

Dot blot assays were performed at day 14-18 and >28 to support findings from behavior tests. There was found no significant difference between the groups at both time points as seen in Figure 19. At day 14-18, there was found a tendency to which both G19 and P5 had a higher O.D compared to ID. Same tendency was seen at day >28, where CN had an increased tendency as well. Thus indicating there could be a difference between the three groups and ID, if the sample size were large enough. Figure 20 illustrates dot blot assays, where three dots are made for each samples. Standards were prepared and are showed as the five first columns in both images, however, these were excluded.

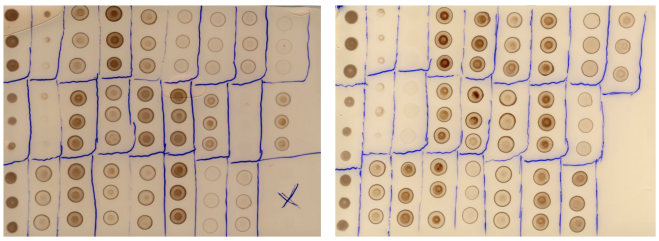

Figure 19: Dot blot assay unstained, where sample 1-5 depict the standards which was run with the samples. However, these were excluded from this study.

4. Discussion

Currently, there is an ongoing exertion to identify critical periods of which iron supplementation can prevent long-term deficits caused by ID. [32] Literature found that iron supplementation at P21 is too late to adjust for behavioral effects of earlier IDA in rats. [32] However, rats treated at P4 only showed minor deficits in monoamine metabolism and no alteration in exploratory behaviors [32, 15] The aim of this study was to investigate whether iron supplementation at G19 and P5 could prevent brain abnormalities and rectify fear and anxiety-like behavior. It should be taken into consideration, to the author's knowledge, that no other studies performed such an extreme ID model.

4.1. Growth and hematology

Unger et al (2012 and 2006) [32, 15] found a significant increase in weight between CN and ID at P8 and P15, respectively. This is contradictory to findings in this study, which demonstrated the first significant difference at P20 with an increase of weight between CN and ID and G19 and ID. However, *Unger et al* found a difference at P21, which supports the findings from this study. As seen in figure 11.A, injection at G19 could increase weight compared to ID earlier than P5. From day 25 and forward both injection at G19 and P5 increase weight compared to ID, but none of the groups did reach same weight curve as CN. The findings of this study are additionally supported by a study from *Gewirtz et. al* (2008) [13] which found a difference between CN and ID at P28, P35, and P56. As significant difference between the groups first occurs at day 20 and the curve for G19 starts to descend after day 25, it could be relevant to follow the curve for an extended period of time to follow the further development of the offsprings.

Hb was measured at day 1-3, 14-18, and >28 and revealed that CN had a significant elevated level of Hb compared to the three remaining groups. Several studies did all find a significant elevation of Hb between CN and ID. [31, 15] *Unger et. al* (2006) demonstrated that CN and ID at P4, P9, P15, and P21 had a significant difference in Hb, which support the findings in this study.[31] At day 14-18 and >28, G19 demonstrated a significant elevation of Hb compared to ID, while P5 showed a tendency of elevated Hb compared to ID. This is supported by a study from *Gambling et. al* (2009), which showed that fetal hematocrit was raised after iron supplementation compared to ID, but did not reach the level of their control.[45] The study had a second iron supplementation, resulting in an additionally raise of hematocrit level reaching the level of CN. It could be discussed whether Hb will raise additionally after a second supplementation of Monofer, thus, rectify CN. [45] However, this study revealed promising results, that iron supplementation at G19 improves Hb compared to ID and P5 shows to have a tendency to elevated Hb compared to ID.

4.2. Behavior assessment

Open field

Open field is a widely and popular used test to investigate fear and anxiety-like behavior in rodents. [42] *Unger et al* (2012) performed open filed on offsprings at P21 divided into 4 different groups; control (CN), formerly ID, moderate iron treatment (FID-40), formerly ID, high iron treatment (FID-400), and total ID (TID). They found that there was no different between the four groups in sectors entered. However, they did found that time to leave the center was greater in TID rats compared with FID-40, FID-400 and CN. [32] Another study by *Unger et al* (2007) found that their ID group had a 30% reduction in distance travelled compared to their control group. Two additionally groups; iron deficiency group switched to iron adequate diet (IDCN) and iron adequate switched to iron deficient diet (CNID) did not differ from CN.[31] *Beard et. al* (2002) did find that their iron deficiency groups did spend less time in the center compared with CN at P21. [36] An additionally study by *Beard et. al* (2006) did test the difference between ID and CN in sectors entered at P25, where they did find a reduction in ID compared to CN. [46] This is consistent with findings in this study, where all three variables revealed a significant increase between CN and ID at day >28. Furthermore, a significant increase between G19 and ID in distance travelled and an increased tendency between P5 and ID was found. *Beard et. al* (2002) did additionally find a difference in time spend i center. However, this study did not find any significant difference. Center entered and total time in center revealed no significant difference between G19, P5 and ID. However, there is seen a tendency of increased activity for both G19 and P5 compared to ID. A study by *Beard et. al* (2007) [15] investigated thrigomaxis with offsprings at P60-63 divided in three groups; control (CN) iron deficient diet (ID), and an iron deficient group from G15-P4, which were switched to iron adequate diet after P4 (IDCN). The study found that ID rats had 30-50% reduction in distance travelled compared to CN, but no difference between CN and IDCN. Furthermore, they found that ID spend less time in the center of the open field compared to CN. [15] This support the findings, that CN and G19 performed better in distance travelled compared to ID, and CN performed better than ID in center time. In distance travelled, it shows that injection with Monofer at G19 have an effect compared to ID. Furthermore, there is seen a tendency of both G19 and P5 to perform better than ID, which can indicate a reduction in anxiety-like behavior compared to ID at day >28.

Unger et al (2007) and *Beard et al*(2006) did also perform the test on offsprings at P15. [46, 31] They did not find any difference between groups at P15 in distance travelled, center time and sector entered. This is contradictory to the findings in this study, where CN in center time performed better than G19, and in center entered performed better than G19 and P5. G19 performed better than CN, ID, and P5 in distance travelled. This shows that CN has a reduction in fear and anxiety-like behavior compared to G19 and P5. However, it could be indicated that measuring fear and anxiety will be more reliable and optimal when offsprings are above P21, as both studies first revealed a

significant differences in the same test at >P21. [46, 31] It was observed that offsprings at day 14-18 were more fragile and bewildered in the tests.

Elevated zero maze

To the author's knowledge, there has only been a few studies with ID offsprings performing on an elevated zero maze, but no one with similar experimental model as this study, making it difficult to compare results. However, in this study, there was found no significant difference in the four variables except of an increase of stretches between G19 and ID. This indicates that G19 has a higher exploration compared to ID. A study by *Amos-Kroohs et al* (2015) used the elevated zero maze on offsprings at P29 with low ID. [47] Their findings revealed a significant difference between the two groups; low ID offsprings (FeD) and iron sufficient offsprings (FeS) at P29. [47] The study found a significant difference between FeD and FeS, where FeD used a significant less time in open area and had a decreased number of open entries, which could support findings in this study. A tendency for CN, G19, and P5 to perform better than ID are seen in number of transition and time spend in open area. Several studies using elevated zero maze, regardless of the measured variables, all have 1 cm curbs around the open area to prevent rats slipping over the edge, which might explain the difficulty to investigate behavior with our elevated zero maze. [47, 48, 49, 39] Furthermore, rodents can become immobile or freeze in open areas due to disturbance as loud noises. [40] It was observed that offsprings freezed and became immobile in the start of the test, as they were placed in open area. Elevated zero maze is great for behavioral testing [42] however, it could be interesting to test if disturbance could be avoided and place the offsprings in the closed quadrant.

Light/dark box

As for elevated zero maze, published studies regarding ID, light/dark box, and offsprings are limited. This study found a significant difference in increase of stretches between ID and G19 and a difference in time spend in open area between P5 and G19 and ID and G19 at day 14-18. To the authors knowledge, no one has performed light/dark activity at this day, thus, comparing results are difficult. An explanation, as mentioned under open field, could be due to their age and that they are more fragile and bewildered. A study by *Beard et al* (2002) [36] performed this activity at offsprings P21 and revealed that ID had a quicker movement into the dark compartment compared to CN. This supports the findings in this study at day >28. It should be taken into consideration, that latency only revealed a tendency for CN, G19, and P5 to perform better compared to ID. It is observed that P5 had a significant increase of time in the open area compared to G19 and CN. This might be due to the large standard error of mean for P5 compared to the rest of the groups, which could be reduced by adding more animals to this study.

Limitation of behavioral tests

Interpretation of behavior shall always be made with precaution. Many external factors can influence behavior and mislead the interpretation. A summary of such factors applicable for all three behavioral tests will be discussed and should be taking into consideration when interpreting the results. For this study, one of the major factors are the sample size [40] The missing validity in the variables could with high probability be avoided by adding more animals to the studies, where especially P5 suffers. At each time point (day 1-3, day 14-18) offsprings were sacrificed leaving maximal three offsprings left per dam at day > 28. This could explain why most of the results shows a tendency and not a significant difference. If the study should be performed additionally time, it could be interesting to increase the sample size. Another consideration which can influence this study is age. As seen in other studies, it is more difficult to get a significant difference at P15, which might explain the variation in the tests and lack of significant difference at day 14-18. [31, 46] It has been reported that offsprings due to neuronal maturation have difficulty in expressing long-term habituation under P30 [40] This could explain why the findings at day >28 is more tally compared to other studies. [31, 46] Therefore, it could be interesting to test offsprings later than day >28 to assess their behavior. One major interpretation of the results regards experience of stress-related changes on the offsprings. The stress-related changes could be caused by movement to another cage, separation from cage mates and/or disturbance as odor or loud noises [49] It has been difficult to determine the probably used lu x in elevated zero maze and light/dark box, as several of studies use a wide range of lux, ranging between 12-1600 lux. [40, 41, 39, 49] Therefore, it would be an advantage to find the most optical value of lux to ensure the right amount of light in the two activity boxes.

4.3. Biochemical assays

Interpretation of fear and anxiety-like behavior in rodents can be difficult. Even with reliable and highly qualified test appa-

ratus, many external factors can influence the outcome. Thus, analysis with more quantified methods can be relevant to support the findings. [42] Activity of COX in the brain is directly proportional to ATP production and is for instance used to compare activity within different regions of the brain in both animal models and human postmortem studies. [42, 28] Hippocampus is a structure critical for many processes as memory and learning and is particularly sensitive to prenatal and neonatal ID. [13]

Several studies have applied histochemical staining for cytochrome c histochemistry in neural tissue and in 1968 a major improvement of this method came, when DAB was introduced as an electron donor to cytochrome c in neural, heart, liver and kidney tissue.[50] Thus, making cytochrome c a brilliant endogenous metabolic marker for neuronal activity [50, 30] Studies from *DeUngria et al* (2000) [27] found that perinatal ID decrease the expression of cytochrome c in the hippocampal subareas CA1 and CA3. [3, 51] Furthermore,*DeUngria et al* (2000) found a loss of >90% of iron in the hippocampal subareas between CN and ID pups. [51] This is not in line to the findings of this study, yet, several factors could influence the results. As seen in Figure 15, there is no significant difference between the groups. G19 had to be removed from this study, as none of the coronal sections were stained sufficient to be used for further analysis. As seen in Figure 16, P5 had a sufficient staining in contrary to CN and ID which might be solved, if this method is optimized. As found in several other studies, CN should have been the most stained sections [3, 51, 30]. However, this is not the case.

In contrary to the histochemistry assays, dot blot at day >28 could reveal some more promising results, which might support the findings from the behavioral tests. None of the groups were significant difference from each other, however, there is found a tendency, that CN, G19 and P5 have a higher O.D compared to ID. This could support the findings that both G19 and P5 performed better than ID in most of the behavioral test. At day 14-18 and >28, there is a tendency that both G19 and P5 have a higher O.D compared to ID, indicating iron supplementation at the biochemical level improve ID alteration.

5. Conclusion

The aim of this study was to establish a rodent experimental model and investigate how ID will affect the neurodevelopment and fear and anxiety-like behavior in offsprings rats. Furthermore, it was investigated whether iron supplementation at G19 and P5 could prevent brain abnormalities.

Taking only growth and Hb into consideration, it can be concluded that iron supplementation with Monofer can improve growth at both stages compared to ID. It is seen that G19 had an earlier effect compared to P5 in growth. However, none of the supplementation rectifies growth compared to CN. Blood sample shows that iron supplementation at G19 improves Hb, but do not rectifies compared to CN.

The behavioral study did not elucidate any clear conclusion. In open field, CN performed overall better compared to G19 and ID, yet, there was seen an overall tendency for G19 and P5 to perform better compared to ID. No definitive conclusion can be made from elevated zero maze and light/dark box. Further experiments needs to be performed to elucidate the effect of iron supplementation in the behavioral studies. However, a tendency shows that G19 and P5 in some sort perform better than ID and thereby having an effect on decreasing fear and anxiety-like behavior in offsprings compared to ID.

The biochemical assays did not revealed any significant difference. However, dot blot at day >28 did show a tendency for CN, G19 and P5 to have an increased COX activity compared to ID. This indicates a coherence between the behavior tests and the biochemical findings. However, further experiments and optimization needs to performed before the activity of cytochrome c oxidase can be elucidated.

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Appendices

A. Certificate

HEALTH
AARHUS UNIVERSITY

Jannie Boss Nielsen
Ditlev Bergs vej 31.1.28
9000 Aalborg

CERTIFICATE

You have satisfactorily completed the PhD course held at Health, Aarhus University on the August 28 - September 1, 2017

Laboratory Animal Science

Helene Nørrelund
Head of Graduate School of Health

Course leader: Frederik Dagnæs-Hansen

Reg. No.: B100/32

ECTS: 3.80

Contents: To give the participants understanding of the basic principles laboratory animal science. The course is obligatory for persons, who wish to obtain their own permission for performing research using laboratory animals. The basic course in Laboratory Animal Science, gives an admission to experiments with animals. This means that you get the admission to assist at experiments with laboratory animals. This corresponds to FELASA B license.

Graduate School of Health
Aarhus University
Katrinebjergvej 89F, bld. 5132
DK-8200 AarhusN

Phone: 87150000
E-mail: graduateschoolhealth@au.dk
Web: http://talent.au.dk/phd/health

B. Standard Operating Procedure - Open field test

Purpose: This test relies on rats innate conflict between approaching and exploring versus avoiding potentially dangerous areas. (46)

1. **Preparation**

   1.1. Materials: Light meter (Goobay No: 5873574), lamp with adjustable dimmer, video tracking system (https://www.behaviorcloud.c camera (hvilken specifik camera det er) (Noldus, Wageningen, The Netherlands), open field box (40*40*40 cm) (Noldus, Wageningen, The Netherlands), gloves, paper, 70% ethanol, timer.

   1.2. Make sure the room is soundproof and blacked out with curtains.

   1.3. Any stress situation as transfer to new cages and exposure to new novelty are reduced

   1.4. Offsprings with dam are moved into the test room at least one hour prior to testing.

2. **Measuring parameter**

   2.1. Total distance traveled (cm)

   2.2. Total center time (seconds)

   2.3. Center entered: (Number of entries in the center region in proportion to the eight other zones (%)

3. **Procedure**

   3.1. The room is blacked out with curtains before the test offsprings enters the room.

   3.2. Open field is cleaned with 70% ethanol and dries to remove any odor which can influence the experiment.

   3.3. Turn on the camera.

   3.4. Put on gloves

   3.5. Pick up the pup from the cage and stay with it for a while, to calm the pup down.

   3.6. Place the test pup gently in the center.

   3.7. Start timer.

   3.8. The investigator leaves the room or place oneself as far away from the open field and field of view of the offspring.

   3.9. After 10 minutes the trial is ended.

   3.10. Move the pup to a new cage.

   3.11. Clean open field with 70% ethanol and let dry.

   3.12. Upload the video to behavioral cloud.

END

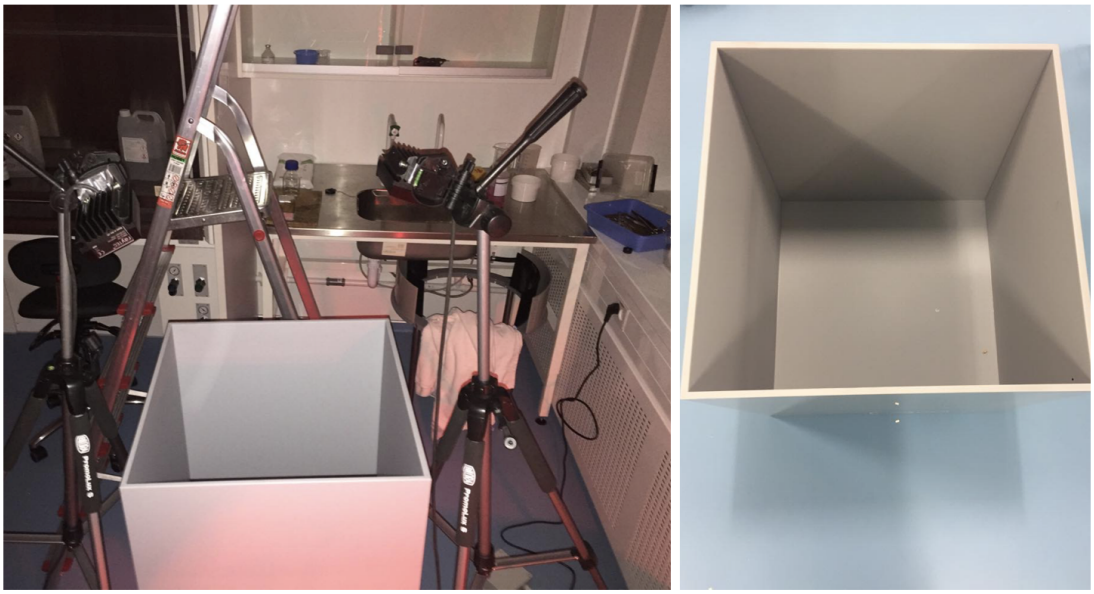

Figure .20: Experimental set-up of open field and the open field box

C. Standard Operating Procedure - Elevated zero maze

Purpose: This test relies on rats innate conflict between approaching and exploring versus the avoiding potentially dangerous areas. In the elevated zero maze, the bright and exposed regions (open quadrants) represent the dangerous areas whereas darkness and enclosed regions (closed quadrants) perceives as safe. (46) (46)

1. **Preparation**

   1.1. Materials: Light meter (Goobay No: 5873574), lamp with adjustable dimmer, camera (hvilken specifik camera det er) (Noldus, Wageningen, The Netherlands), elevated zero maze (annular platform 60 cm diameter with 5 cm footpath, elevated 20 cm above ground level. The inner walls consist of IR black) (Stoelting, Wood Dale, IL, United States of America), gloves, paper, 70% ethanol, timer.

   1.2. White light with a lighting of 100 lux is used in the open quadrants.

   1.3. Make sure the room is soundproof and blacked out with curtains.

   1.4. The offsprings are placed in the room at least one hour prior to testing with dam.

   1.5. Any stress situation as transfer to new cages and exposure to new novelty are reduced 24 hours prior to testing.

2. **Preparation**

   2.1. *Transition:* number of transition between the two closed quadrants.

   2.2. *Open area:t*duration of time spent in the open quadrants. (seconds)

   2.3. *Stretches:* Frequency of stretches (where at least a part of the head is outside the runway, but all four paws is still on the runway)

   2.4. *Latency:* from released to enter the closed quadrants (seconds)

3. **Procedure**

   3.1. The room is blacked out with curtains before offsprings enters the room.

   3.2. Elevated zero maze is cleaned with 70% ethanol and dries to remove any odor which can influence the experiment.

   3.3. Turn the light on with 100 lux.

   3.4. Turn on the camera.

   3.5. Put on gloves.

   3.6. Pick up the offspring from the cage and stay with the offsprings for a while to calm it down.

   3.7. Place the offspring gently in the middle of one of the two open quadrants.

   3.8. Start timer.

   3.9. The investigator leaves room or place oneself as far away from the elevated zero maze and field of view the offspring.

   3.10. After 10 minutes the trial is ended.

   3.11. Move the offspring to a new cage.

   3.12. Clean elevated zero maze with 70% ethanol and let dry

END

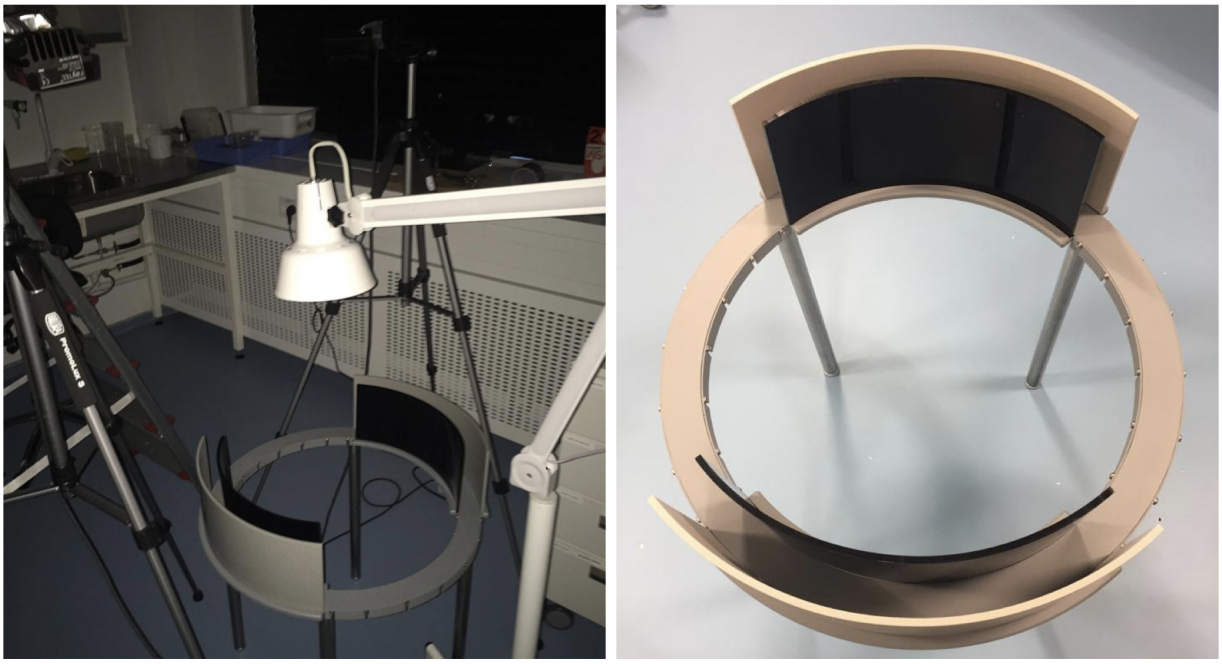

Figure .21: Experimental set-up of elevated zero maze and the elevated zero maze

D. Standard Operating Procedure - Light/dark box

Purpose: This test relies on rats innate conflict between approaching and exploring versus avoiding potentially dangerous areas. In the light/dark box, the bright and exposed chamber represent the dangerous area whereas the dark chamber perceives as safer. (46)

1. **Preparation**

   1.1. Materials: Light meter (Goobay No: 5873574), lamp with adjustable dimmer, infrared camera (hvilken specifik camera det er) (Noldus, Wageningen, The Netherlands), light/dark box (Consist of IR black: 60*20*20 cm with two chambers united with a 5*5 cm door (Noldus, Wageningen, The Netherlands), gloves, paper, 70% ethanol, timer.

   1.2. White light with a lighting of 270-320 lux.

   1.3. Make sure the room is soundproof and blacked out with curtains.

   1.4. The offsprings are placed in the room at least one hour prior to testing with dam.

   1.5. Any stress situation as transfer to new cages and exposure to new novelty are reduced 24 hours prior to testing.

2. **Preparation**

   2.1. *Transition:* Number of full body transition between the two chambers

   2.2. *Open area:t*Duration of time spent in the light chamber (seconds)

   2.3. *Stretches:*Frequency of stretches (where at least a part of the head is outside the dark chamber, but not any of the paws)

   2.4. *Latency:* from released in the light chamber to enter the dark chamber (seconds)

3. **Procedure**

   3.1. The room is blacked out with curtains before offsprings enters the room.

   3.2. Elevated zero maze is cleaned with 70% ethanol and dries to remove any odor which can influence the experiment.

   3.3. Turn the light on with lux between 270-320 measured with the light meter

   3.4. Turn on the camera.

   3.5. Put on gloves.

   3.6. Pick up the offspring from the cage and stay with the offsprings for a while to calm it down.

   3.7. Place the offspring gently in the middle of one of the two open quadrants.

   3.8. Start timer.

   3.9. The investigator leaves room or place oneself as far away from the light/dark box and field of view the offspring.

   3.10. After 10 minutes the trial is ended.

   3.11. Move the offspring to a new cage.

   3.12. Clean elevated zero maze with 70% ethanol and let dry

END

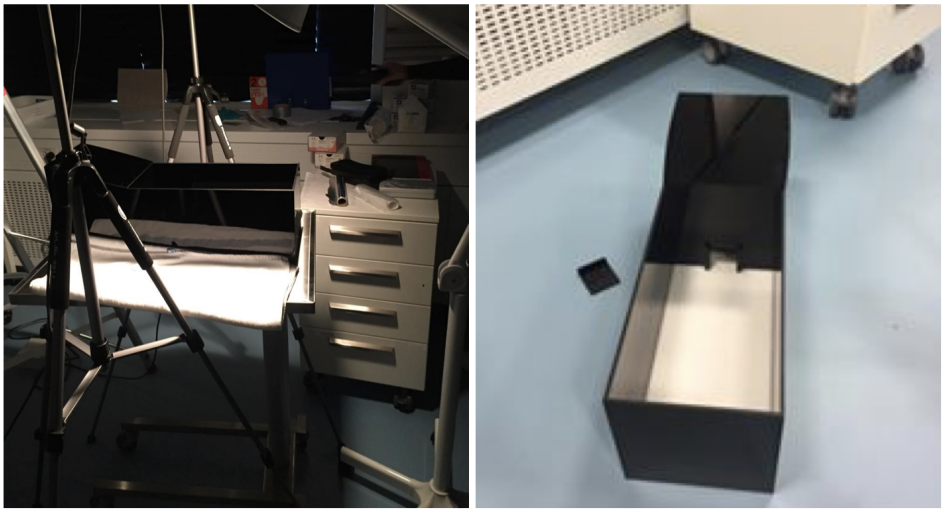

Figure .22: Experimental set-up of light/dark box and the light/dark box

E. Standard Operating Procedure - Perfusion Fixation

1. **Preparation**

   1.1. Dissolutions: 4% Paraformaldehyde (PFA).

   1.2. Instruments: plastic tray, cork tray, surgical forceps, tweezers, curved scissors, straight suture scissors, oval spoon, needles, and H-dustbin.

   1.3. Hypnorm and dormicum with sterile water in ratio 1:1:2. There is given 0.1 ml per 10g weight

   1.4. Pink hypodermic needles. 10-20 ml syringes.

   1.5. Test tubes

   1.6. Blood sample sticks

2. **Procedure: Fixation**

   2.1. Place the instruments readily accessible in the LAF bench and place the cork tray in the plastic tray.

   2.2. Anaesthetize the rat with hypnorm/dormicum ratio 1:1.

   2.3. Two syringes (10-20 ml) is filled with PFA and KPBS, respectively.

   2.4. After 7-10 min, the reflexes are tested by squeezing a tweezers towards the tail and bones in the paws.

   2.5. If reaction, check the reflexes again after 2 minutes. If no reaction, the procedure can commence.

   2.6. When the reflexes are tested, the rat is placed with the spine towards the cork tray and paws are fasten with needles.

   2.7. The skin around processus xiphoideus is lifted with the tweezers.

   2.8. The skin is penetrated with the curved scissors and clipped through costa on both sides.

   2.9. Diaphragm is punctured and removed. The heart is now devoid.

   2.10. With the curved scissors, clip gently along the sides and with the surgical forceps, fixate the skin, so the heart is total devoid.

      2.10.1. **NB:** If the heart is stocked to the skin, remove gently the heart with the point of the curved scissors.

   2.11. Puncture the right atrium

   2.12. Blood sample is taken.

   2.13. With the angle tweezers, the heart is lifted.

   2.14. Then the syringe with KPBS is injected into the left ventricle and flush around 10-20 ml KPBS slowly.

   2.15. Quickly, the syringe with 10 ml paraformaldehyde is injected in the left ventricle to fixate the rat.

3. **Procedure: Removal of brain**

   3.1. The fixated rat is turned around and fasten with needles.

   3.2. Cut the head off

   3.3. Remove as much skin from the head as possible so the scalp is devoid.

   3.4. When the scalp is exposed, take the angle tweezers and gently rip off the scalp to reach the brain.

   3.5. When the brain is cleared of the scalp, take the oval spoon and gently slide it under the brain.

   3.6. The brain is loosen and lifted slowly to make sure, that there is no resistance.

   3.7. Place the brain in 50 ml test tube with PFA

   3.8. Store the brain in the refridger at 4 overnight

   3.9. Next day, changes the medium to KBPS and 0.01

END

F. Standard Operating Procedure - Decapitation

1. **Preparation:**

   1.1. Gas chamber, Isoflurane, Scissors, a small straight scissor, liquid nitrogen, ice, scalpel, eppendorf tubes, tweezers,

2. **Procedure: Removal of brain tissue**

   2.1. Drops of isoflurane is poured into the gas chamber.

   2.2. Place the offspring in the chamber and leave for 1-2 mins after it has passed out.

   2.3. Lift the offspring and make a neck pull.

   2.4. Cut off the head and take a blood sample.

   2.5. Remove the brain

   2.6. Gently separate the hemisphere

   2.7. Hippocampus will easily be pulled out.

   2.8. Then a peace of cerebellum is taken and used as a positive control for dot blot.

   2.9. The sample is stored in an eppendorf tube and quickly put in liquid nitrogen.

   2.10. Store on ice and place in a -140 freezer.

END

G. Standard Operating Procedure - Histochemistry

1. **Preparation: From brain to coronal sections**

   1.1. After removal, the brain is stored in the refrigerated at 4 in KPBS + 0.01% sodium azide. (50 m + 1 ml)

   1.2. 30% sucrose dissolution is made by mixing 30 g sucrose with 100 mL KPBS in a measuring cup.

   1.3. The dissolution stirs for 10-15 min with a magnet until dissolved.

   1.4. Sodium azide from the vial with the brain is gently poured off.

   1.5. 2 ml sucrose solution is filled up, mixed and poured off again.

   1.6. The vial is then filled with sucrose dissolution and stored in the refrigerated at 4 for three days or until the brain reach the bottom of the vial.

2. **Cryostat**

   2.1. Turn on the Cryostats 1 h prior to use and set the temperature to -21

   2.2. Put a blob of Tissue-Tek (Sakura, Japan) on a plate and place the brain with cerebellum towards the plate.

   2.3. Let freeze in the cryostat.

   2.4. Slowly cover the brain with a thin layer of Tissue-Tek and make sure to avoid any air bubbles.

   2.5. The brain is placed in the cryostat while gently holding the brain in an upright position until the brain can be kept stable by Tissue-Tek

   2.6. Proceed this procedure until the whole brain is covered with a thin layer of Tissue-Tek.

   2.7. Let the brain stay in the cryostat for 10-15 min until completely frozen.

   2.8. The coronal sections of the brain are split in six piles.

   2.9. When the piles are larger enough, place the piles in six vials.

   2.10. The sections i placed in antifreeze.

3. **Procedure: Histochemistry**

   3.1. Coronal sections are poured in vials and washed 3*5 min in 0.1 M sodium phosphate buffer.

   3.2. The incubation buffer per 50 mL is made in darkness:

      i. 0.1 M sodium phosphate buffer, pH 7.4, 25 mg 3,3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, 10 mg cytochrome C from equine heart (Sigma-Aldrich, St. Louis), and 2 g sucrose

   3.3. Empty the vials with coronal sections completely by pipetting the remaining solution off.

   3.4. Cover the vials with aluminum foil and place the samples in the laf-bench.

   3.5. Add 2 ml of incubation buffer to the vials and cover it completely with aluminum foil for darkness

      i. NB: For negative control, proceed same steps. However, the incubation buffer is without cytochrome C.

   3.6. The samples are placed in a heating chamber at 36 on a shaker for 8 h

   3.7. After the incubation time, the vials are filled with 0.M sodium phosphate buffer

   3.8. Wash 3*5 min with 0.1 M sodium phosphate buffer

   3.9. They are stored in the refrigerator at 4 for next day, where they are mounted on gelatin-coated slides.

   3.10. Let air dry and cover with perotex

END

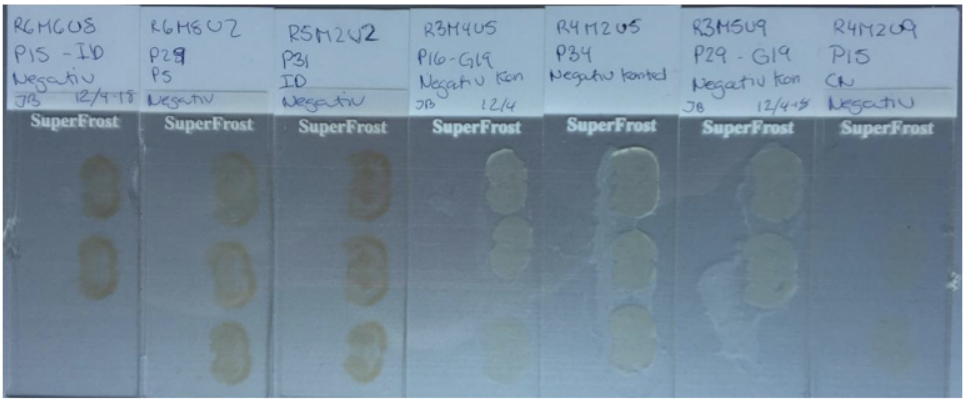

Figure .23: Negative control for histochemistry

H. Negative and positive controls

Figure .24: Negative results from dot blot

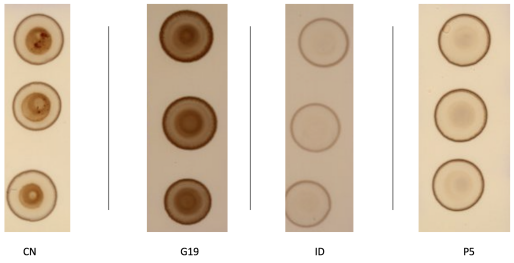

Figure .25: Positive results from dot blot - Cerebellum

END

I. Standard Operating Procedure - Dot blot

1. **Preparation**

1.1. To obtain a mean weight of a 2 ml eppendorf tube, five empty eppendorf tube are weighted on styrofoam

1.2. The tissue samples are then weighed on the styrofoam

1.3. The samples are kept on ice.

1.4. Lysis buffer is made by using N-Per (cat. No.: 87792) with one tablet protease inhibitor (cat. No.:11836170001) pr 10 ml.

1.5. Lysis buffer is placed on a mini-shaker until dissolved.

1.6. Add 10 times more lysis buffer to the sample in the eppendorf tube. (For an example, see below)

i. Empty eppendorf tube on styrofoam = 4.7704 g
Eppendorf tube with sample on styrofoam = 4. 8163 g
Difference = 0,0759 g
Add 760 μl lysis buffer to the sample.

1.7. Extract the desired amount of lysis buffer and add to the eppendorf tube with the tissue.

1.8. With a pipette, remove tissue and lysis buffer to a dounce homogenizer.

1.9. The eppendorf tube is centrifuged and and the rest is removed to the homogenizer.

1.10. Keep the homogenizer on ice while squeeze the homogenizer up and down for 15 times.

1.11. Remove the mixture to a new eppendorf tube.

1.12. Incubate for 10 minutes on ice. Use the mini-shaker once a while.

1.13. The sample is put in the centrifuge for 10 min on 10.000 G at 4

1.14. After the centrifuge, the supernatant is removed to a new tube and the pellet is saved in the freezer at -20

1.15. The supernatant is saved in -80 freezer until use.

2. **Dot Blot**

2.1. Following reagents is made:

i. **x10 TBS** (1 L):
65 g Tris-base
87.5 g NaCl
Dissolve in Milliq water and adjust pH to 7.6 using NaOH

ii. **TBS-T with 5% skim milk** (250 mL)
Dilute 100 mL x10 TBS to 1 L with Milliq water and add 1 ml Tween20
Weight 12.5 g skim milk powder and dissolve in TBS-T.
When completely dissolve - adjust pH to 7.6

iii. Incubation buffer per 50 mL is made in darkness
Mix 0.1 M sodium phosphate buffer, pH 7.4, 25 mg 3,3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis), 10 mg cytochrome C from equine heart (cas: 9007-43-6; Sigma-Aldrich, St. Louis), and 2 g sucrose and stir until dissolved.

iv. NB: For negative control, the same solution is made, but without cytochrome c

2.2. Supernatant is thawed and the finale solution is made in concentration 1:1 (supernatant; lysis buffer)

2.3. Nitrocellulose transfer membrane (Whatman, Dassel, Germany; ref. No: 10401180) is prepared by drawing grids with a pen to indicate the regions which is going to be blotted.

2.4. For each samples, there is made three identical blots by using a narrow-mouth pipette tip.

2.5. Spot 2 μL of sample onto the nitrocellulose membrane by slowly pipetting 1 L out before touching the nitrocellulose membrane. Then, the rest is slowly applied to minimize the area, which the solution penetrates.

2.6. Block with TBS-T 5% skim milk for 40 min.

2.7. Wash 3*5 min in 0.1 M sodium phosphate buffer.

2.8. Empty the reaction chamber as much as possible and add 30 ml incubation buffer.

2.9. Place the reaction chamber on a shaker in 36 for 5 h in darkness.

2.10. Wash 3*5 min in 0.1 M sodium phosphate buffer.

END